QATAR UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

EFFECTS OF ACTIVE PHYTOCHEMICALS OF *PROSOPIS JULIFLORA* AS ANTI-SPOILING AGENTS OF POSTHARVEST FRUITS

BY

IMANE SALEH

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COMMITTEE PAGE

The members of the Committee approve the Thesis of
Imane Saleh defended on 29/11/2021.

Professor Mohammed Abu-Dieyeh
Thesis/Dissertation Supervisor

Professor Samir Jaoua
Committee Member

Professor Serhiy Souchelnytskyi
Committee Member

Professor Yousef Mohammad Hijji
Committee Member

Approved:

Ahmed Elzatahry, Dean, College of Arts and Sciences

ABSTRACT

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Title: Effects of active phytochemicals of *Prosopis juliflora* as anti-spoiling agents of postharvest fruits.

Supervisor of Thesis: Prof. Mohammed Abu-Dieyeh.

Global increase in food demand has made the fresh produce spoilage a worldwide concern. Adverse effects of commercial spoilage control agents on human health and on the environment are major public concern. In this study, *Prosopis juliflora* fruit and leaf extracts were studied for their antimicrobial activities against the growth of selected bacteria (*Escherichia. coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Proteus mirabilis,* and *Pseudomonas aeruginosa*), yeast (*Candida albicans*), and fungi (*Aspergillus niger*, *Penicillium chrysogenum*, *Botrytis cinerea*, *Fusarium oxysporum*, *Alternaria alternata*, *Penicillium citrinum*, *Colletotrichum gloeosporioides*, *Cladosporium cladosporioides,* and *Geotrichum candidum*). The novel *P. juliflora* water-soluble leaf ethanolic (PJ-WS-LE) extract showed the highest antibacterial activity. The potential effect of PJ-WS-LE extract coating on the extension of fruits' shelf-lives and in maintaining storage parameters were evaluated in addition to the genetic variation of the invasive *P. juliflora* species in Qatar. PJ-WS-LE extract was fractionated using FPLC in order to identify active phytochemicals. Antimicrobial tests showed total inhibition of *B. cinerea*, *A. alternata*, *B. subtilis*, *S. aureus* and *C. albicans* with MICs ranging between 0.125 mg/ml and 1mg /ml. Even fungal strains that were the least affected showed alterations in their hyphae and spores exposed to PJ-WS-LE extract when observed using scanning electron microscope (SEM). PJ-WS-LE extract showed high efficacy in protecting inoculated cherry tomato samples from *A. alternata* and *B. cinerea* infections. PJ-WS-

LE extract alone extended strawberries shelf-life at 4°C by 2.32 folds and maintained the best storage quality-parameters when embedded in 1% chitosan. On mangoes, 8 mg/ml of PJ-WS-LE extract has 80% efficacy in controlling *A. alternata* infection and it lowers *C. gloeosporioides* disease severity by 53.4%. PJ-WS-LE extract at 8mg/ml showed also efficacy in increasing cucumber shelf-life at 22°C by 77%. *P. juliflora* did not show genetic variation in Qatar and extracts of leaves collected from various locations showed the same antimicrobial activities. Fractionation purification method allowed the successful identification of one biologically active fraction that has high thermostability. In conclusion, PJ-WS-LE extract is a natural antifungal agent that can replace common anti-spoiling chemicals in field and protect vegetables and fruits at post-harvest stage.

DEDICATION

This work is dedicated to the soul of my aunt Jamile, God bless her, she had always believed that things will go for the best for me, even though she was not here, her words were always a motive.

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CHAPTER 1: INTRODUCTION

The food industry is a booming sector in Qatar, however, the country is importing around 90% of its food needs, nowadays. Food security remains one of the major challenges and is one of the pillars in Qatar National Vision 2030. Postharvest microbial infections of fruits and vegetables are mainly caused by fungal and bacterial pathogens that can be harmful to both human and animals, and it can lead to high economic losses to both manufacturers and consumers and a negative impact on easily spoiling brand names (Balali *et al.*, 2020). Food losses are a worldwide major concern with the growing world population, food demand increases, and therefore losses limitation and spoilage control become crucial. Chemical antibiotics and fungicides with their environmental pollution footprint are not a recommended option to extend produces shelf life, many countries around the world are setting a maximum residue limits (MRL) of chemicals on the skin of their exported fruits and vegetables (Mostafidi *et al.*, 2020). In addition, spoiling microorganisms are getting resistance to the commonly used antimicrobial agents, exerting stress on exploring natural novel products to replace synthetic chemical applications in field and at post-harvest stage (Bill *et al.*, 2014). A successful product should control pre-existing infections and leave residues that prevent subsequent infections and retard sporulation of the existing spores and therefore reduces economical losses (Sayago *et al.*, 2012; Leyva Salas *et al.*, 2017; Solanki *et al.*, 2018). In the case of fungi, applying an affordable natural non-hazardous formulation would prevent visible spoilage and would inhibit the growth of the fungi early before the formation of mycotoxins that are a burden on human health even after the decontamination of food products from the main contaminants (Saleh & Goktepe, 2019).

Spoilage control methods currently available are either very costly or polluting, and for some microorganisms' control, it is expensive and polluting at the same time. A natural anti-spoiling agent would not only solve a food security issue but it will also protect the environment and the human health from the risk of pollutants used. Fresh plants are being spoiled by bacteria, yeast and mold all over the world, yet developing countries that cannot afford to use available preventive measure are the ones that are most affected. Therefore, the problem of fresh produces spoilage affect mostly the poorer countries that are most in need of protecting their resources (Bhuvaneshwari *et al.*, 2019). Coming up with an affordable plant based formulation that help in fighting roots rot in field and preventing postharvest spoilage would support the agricultural domain not only globally but also in the countries that are most in need of support.

Thorough literature review about spoilage bio-controllers reveals various promising results, bacteriocins, plants extracts' phytochemicals, natural oils and other biological products have been screened for their antimicrobial properties. Yet very few of the tested agents has been patented and made available in the market to replace the currently used chemicals. Available knowledge is promising yet a structured work is needed to come up with additional affordable formulations that can lessen food losses around the world. Higher plants extracts are being explored in many researches as a natural bio-controller for food spoiling organisms, phytochemicals in higher plants are valuable drugs known to cure several diseases (Halonen *et al.*, 2020). Various studies are demonstrating recently the antifungal effect of higher plants extracts that serves as fungi-toxic agents against spores germination and/or growth of mycelia (Satish *et al.*, 2009). Similar studies are conducted on pathogenic and spoiling bacteria and promising results are proven (Bisi-Johnson *et al.*, 2017).

Among the tested plants, *Prosopis species* in general and *Prosopis juliflora* in particular has been screened for its antioxidant, anti-inflammatory, antibacterial, antifungal and anti-tumor effects (Aziz *et al.*, 2018). The various studies explored the extracts of different parts of the plant or even of purified phytochemicals, yet the studies are not interconnected, thorough literature review would reveal more of a general screening, results are encouraging yet they require further investigation before considering a possible formulation that can replace commonly used chemicals. It is important to evaluate the safety of the plants' extracts, to use different solvents in crude extract preparations, evaluate antimicrobial mechanisms, determine active concentrations, evaluate *in-vivo* protective and curative effects and determine active components in promising crude extracts.

Prosopis juliflora is an invasive species in the state of Qatar, chemical and mechanical invasion control are expensive and not recommended because of their possible adverse effects on the environment, nowadays the idea of control through utilization is recommended (Wei *et al.*, 2018). Using the various parts of this plant to prepare extracts that can serve as bio-controller or natural anti-spoiling agents would help in utilizing the plant and would solve a major food security problem.

Fruits and vegetables spoilage occurs mainly during three stages: in field, post-harvesting or during processing. The aim of this research is to find a safe and non-toxic natural product that can be active at the first two stages of fresh food productions, the aimed formula would have curative effect and long term preventive effect. If crude extracts would work fine, then farmers in poorer places would be capable of protecting their crops easily and safely.

This research will provide solution to a worldwide problem that is very relevant to Qatar. Although agricultural projects are evolving in Qatar, yet the climate conditions

and the arid land nature of the country impose limitations on plant productivity. Qatar is and will keep on importing most of its fresh vegetables and fruits. Local agriculture, even if it covers a part of the local consumption, will be costly. A large number of the imported fruits are being spoiled in stores, and many of the locally produced vegetables have short shelf life. Coming up with a naturally based anti-spoilage formulation will boost the Qatari economy, enhance food security measures and solve a worldwide agricultural problem. The natural extract, when validated, will also protect the environment by lessening the usage of polluting chemicals such as chemical fungicides and antibiotics.

The PhD project is divided into four main chapters; each chapter is divided into many specific aims:

1. Screening the antimicrobial effectiveness of the fresh juice and of the aqueous and ethanolic extracts of the leaves and pericarps of *P. juliflora*. The sub-aims of this objective include:
    1.1. Screening for the crude extracts antifungal, antibacterial and anti-yeast effects on a variety of previously identified microorganisms, and determining their modes of action and comparing their activities to commonly used antibiotics and fungicides.
    1.2. Determination of the minimum inhibitory concentration (MIC) of the crude extracts to various microorganisms.
    1.3. Determination of the extracts' anti-sporulation effect as a possible anti-fungal mechanism.
    1.4. Determination of the effect of the effective crude extract(s) on the microscopic morphology of the studied fungal species using a scanning electron microscope (SEM).

1.5. Checking the cytotoxicity of the effective crude extract(s).

2. Studying the *in-vivo* antimicrobial and quality enhancing activities of the effective chosen crude extract. The sub-aims of this objective include:

   2.1. Testing the preventive and curative activity of the crude extract on tomato and mango wounded and healthy samples using a randomized block design.

   2.2. Evaluating the effectiveness of the *in-vitro* active crude extract on extending fresh produce shelf-life. Cucumber and strawberry were chosen as models.

   2.3. Evaluating the long-term protective value of the effective extract as simple coating material or as a coating material embedded in chitosan on chosen fresh produces with timely evaluation of storage parameters. Chosen fruits models are: strawberry, cucumber, mango and lemon.

   2.4. Testing the crude extract efficacy, when used as priming material and as watering solution, on chickpeas germination.

3. Evaluating the genetic variation of *P. juliflora* in the state of Qatar (Doha municipality) and testing the antimicrobial effectiveness of crude extracts prepared from samples taken from different locations to compare efficacies. The sub-aims of this objective include:

   3.1. Evaluating the genetic variation of *P. juliflora* by Ribotyping.

   3.2. Running a comparative MICs evaluation of crude extract prepared using plant samples from various locations against some chosen fungi, bacteria and yeast.

4. Fractionating the active crude extract into groups of phytochemicals and testing the effectiveness of various fractions as antimicrobial agents. The sub-aims of this objective include:

   4.1. Crude extract fractionation.

4.2. Screening for different fractions' antifungal, antibacterial and anti-yeast effectiveness.

4.3. Evaluating the purified active components combined effectiveness to investigate their possible synergistic effects and evaluating their thermostability.

CHAPTER 2: LITERATURE REVIEW

2.1. FRUITS AND VEGETABLES SPOILAGE

Plants based raw material are prone to microbial spoilage by fungi and bacteria. Fruits become more susceptible to fungal contamination during the ripening process when they get rich with carbohydrates and they change their pH to become suitable and attractive to the fungal spoiling agents (Lorenzo *et al.*, 2018). Advanced fungal contamination cause visible symptoms on the plants and they mainly affect citrus fruits, berries, pome fruits, stone fruits, and tropical and solanaceous fruits. Table 1 shows the most important fungal spoiling agents of various fruits (Barth *et al.*, 2010; Mailafia *et al.*, 2017).

Table 1. Important postharvest fungal diseases of various fruits (Ansari & Tuteja, 2014).

Fungal and Bacterial Pathogen	Host Fruit	Disease
Penicillium spp., Botrytis cinerea	Grapes	Gray mold
Rhizopus stolonifera	Peach pulms	Rhizopus mold
Colletotrichum musae	Banana	Crown rot
Botrytis cinerea	Apple	Gray mold
Penicillium expansum	Peach	Blue mold
Phomopsis caricae-papayae	Papaya	Phomopsis rot
Monilinia fructicola	Peach	Brown rot
Alternaria alternata	Apple	Core rot
Alternaria citri	Lemon	Leaf spot
Colletotrichum gloeosporioides	Mango	Anthracnose

Fungal and Bacterial Pathogen	Host Fruit	Disease
Dothiorella dominicana, Lasiodiplodia theobromae	Mango	Sterm-end rot
Alternarta citrt	Citrus	Black center rot
Erwinia spp.	Potato	Bacterial soft rot
Pseudomonas syringae	Avocado	Dry rot

Vegetables are more affected by bacterial spoiling agents rather than fungi because of their composition and pH. Vegetables that are mainly spoiled by bacteria includes bulbs, crucifiers, cucurbits and legumes (Leyva Salas *et al.*, 2017). Table 2 shows the most important bacterial spoiling agents of various vegetables (Barth *et al.*, 2010). Susceptibility of different fruits and vegetables to microorganisms depends not only on their management during harvesting, their processing and their storage methods but also on their composition in terms of nutrients content, matrix type, and physical and chemical composition such as pH and water content (Leyva Salas *et al.*, 2017).

Table 2. Important postharvest bacterial diseases in vegetables (Barth *et al.*, 2010).

	Pseudomonas	*Erwinia*	*Xanthomonas*	*Bacillus*	*Clostridium*	Lactic acid bacteria
Broccoli	+	+	+			
Cabbage	+	+	+			
Carrots	+	+		+		
Cucumber		+		+		

	Pseudomonas	*Erwinia*	*Xanthomonas*	*Bacillus*	*Clostridium*	Lactic acid bacteria
Lettuce	+	+	+			
Onions		+		+		
Potatoes	+	+		+	+	
Tomatoes	+	+	+	+		+

2.1.1. Fruits and vegetables contamination routes

Fresh produce microbial contamination could originate from contaminated irrigation water and/or from soil. Certain microorganisms can cause pre-harvesting infections by penetrating inside the fruits through their calyx or along the stem (Song *et al.*, 2019). Nevertheless, fruits contamination might occur at harvesting and post-harvesting stages including: storage, packaging, transportation, and processing of fruits (Saleh & Goktepe, 2019). Mechanical skin damages of fruits or damages caused by insects or animal bites, or even by chilling injuries, cause the fruit to become more prone for microbial spoilage which gives bacteria and fungi access to their soft tissue. Animals like birds, rodents, and insects must always be kept far from fresh fruits as they can carry infectious microorganisms. Some spoilage microorganisms can, on the other hand, infect undamaged fruits and cause lesions to allow their own penetrations, in addition to the penetration of other possible microorganisms (Barth *et al.*, 2010). Post-harvesting contamination starts during transportation of goods. Food products might be transported in contaminated container or even get contamination from the vehicle used for transportation. Contamination can occur during packaging and storage, this include contaminated packaging material, contaminated stores, contaminated fridges and others. Inappropriate display of food products in the market would also lead

to cross contamination among goods, therefore, spoiled fruits and vegetables should be discarded and separated from healthy plants. Contamination during the displaying and the final marketing processes can also be caused by workers poor hygienic practices. Finally, any bad manufacturing practices is considered a possible contamination pathway (Tournas, 2005; Mailafia *et al.*, 2017).

Microorganisms including bacteria and fungi cause high economical losses by spoiling not only harvested fruits and vegetables but also crops in their fields. The identification of such spoiling microorganisms is a crucial step toward their control, some of the fruits pathogenic strains are pathogenic to humans as well, especially those that produce toxins (Mailafia *et al*., 2017). Many of the microorganisms metabolites are heat stable which indicates that they remain in the food after heat processing and continue to cause toxicity (Tournas, 2005). Therefore, spoiling agent growth control is of major importance not only to stop visible food spoilage but also to stop metabolites production early during the food production process.

2.1.2. Microorganisms invasion mechanisms

Weather, insects, birds, rodents, and/ or any harsh practices during harvesting or processing wound the plants and therefore, facilitate the penetration of microorganisms to the nutritious part of the fruit to proliferate and lead to fruits spoilage (Lorenzo *et al.*, 2018). However, microbiological spoilage of intact fruits is possible if the microorganisms in charge can change the physicochemical properties of the plants tissue (Ragaert *et al.*, 2007). Even the smallest cells damage in the fruits skin triggers further damage as it leads to the secretion of enzymes that can degrade the cell wall polysaccharides including pectin (Airianah *et al.*, 2016).

The outer layer of the plants' skin or the cuticle is a waterproof protective layer made of lipids, hydrocarbon polymers, wax and polyester cutin (Yeats & Rose, 2013).

However, many fungal spores are adapted to attach themselves to the dry or wet cuticle (Magyar *et al.*, 2016). Some fungal spore produce a slime or a matrix on the cuticle that allows their settlement. Certain *Colletotrichum* species for example were shown to produce a matrix within 20 to 30 minutes after infection, while *Botrytis species* in addition to some *Colletotrichum* species make a layer of glycoprotein to help in their settlement. The amount of water of the surface of the plant will affect its interaction with the microbial contaminant (Mendgen, 1996).

Fungal spores germination on plants is induced by a cascade of chemical signals (Akiyama & Hayashi, 2006). After the formation of the germination tube, fungi might develop appressorium which is a specialized cell typical of many fungal plant pathogens. Appressorium is a flattened hyphae that enters the host, using turgor pressure that enables it to push through (Ryder & Talbot, 2015). The next step of fungal plants invasion is the growth of the penetration hyphae. Degradation enzymes play an important role in the appressorium and hyphae penetration. Different isoforms of cutinase have been identified in different species of pathogenic fungi such as *B. cinerea*, *Alternaria* and *Colletotrichum* (Davies et al., 2000). Besides cutinase, polysaccharides are also enzymatically degraded by fungi, this include the secretion of cellulase involved in many fungal types invasion (Kubicek *et al.*, 2014). In addition to enzymes facilitated penetration, some fungal appressorium penetrate into the plant by the force of turgor pressure only, such fungi build up a high pressure (force) in their penetrating hyphae through melanization, among the spoiling fungi that adopt this contamination mechanism are: *Colletotrichum*, *Magnaporthe*, and *Cochliobolus* (Deising *et al.*, 2000). It is worth noting that many plants respond to fungal contamination by developing defensive mechanisms including thickening of the cuticle and cell wall alteration (Mendgen, 1996).

2.1.3. General overview of spoiling microorganisms

2.1.3.1.Mold

Fungi, especially their airborne spores, could contaminate fresh produces at any stage of their processing, in field, post-harvesting, during processing, in stores, at the wholesale, retailer and at consumer levels (Leyva Salas *et al.*, 2017). Overall, three main stages are the most crucial during which fungi can spoil foods. The first stage is in the field where irrigation water, soil and air are natural niches of fungi. The second stage is post-harvesting stage where plants can get fungal contamination during their collection, transportation, storage and packaging. Finally, fruits and vegetables can get contaminated during their processing stage into the final sold product (juices, jams, and others) (Saleh & Goktepe, 2019). Fungal growth in fruits and vegetables lead to visible deterioration of the product with noticeable smell, texture and flavor changes (Leyva Salas *et al.*, 2017). Fungal contamination can have bad impacts on human health even before the development of spoilage signs on the fresh produces by secreting mycotoxins (Agriopoulou *et al.*, 2020). The main postharvest diseases are caused by fungi belonging to the following genera: *Penicillium, Botrytis, Monilinia, Rhizopus, Alternaria, Aspergillus, Fusarium, Geotrichum, Gloeosporium,* and *Mucor* (Leyva Salas *et al.*, 2017). Accumulative mycotoxins in the body can be toxic, carcinogenic, and mutagenic which makes mycotoxins producing fungi a major public health concern. Adverse effects might reach various systems in the body. The most concerning mycotoxins are: ochratoxin A, aflatoxins, patulin, zearalenone, fumonisins, T-2 and HT-2 toxins, and deoxynivalenol (Agriopoulou *et al.*, 2020). Most mycotoxins' health risk assessment studies are based on individual mycotoxins evaluation analysis, however, multiple mycotoxins might occur at the same time in food which increases the level of concern. It is estimated that 25% of the raw food products produced in the

agricultural world are contaminated by fungal mycotoxins (van Egmond *et al.*, 2007; Leyva Salas *et al.*, 2017).

Black mold is a common fruits disease caused by *Aspergillus spp.* and it can affect not only fruits but also human health by causing aspergillosis in immunocompromised patients, a disease that is known by causing: fever, cough, chest pain and short breath. *Aspergillus spp*. might produce 145 secondary metabolites several of which are toxic, among the human poisonous metabolites are mycotoxins known as ochratoxin A (OTA) and fumonisin B_2 (FB_2). These two mycotoxins are serious health hazards as they are carcinogenic, neurotoxic and immunotoxic (Mailafia *et al.*, 2017). The section Flavi of *Aspergillus* can produce toxic, carcinogenic compounds known as aflatoxins (AFs) (Tournas *et al.*, 2015). Being heat stable, it is very difficult to manage mycotoxins amount in food once they are secreted. Mycotoxins are stable in storage and very insensitive to physical and chemical treatments. Therefore, lessening mycotoxins formation in the first place is the best way to limit exposure to them (Copetti *et al.*, 2014).

Penicillium are the most common spoiling agents of citrus fruits. *P. digitatum* cause green mold while *P. italicum* cause blue mold. Conidia of these fungi can be easily transferred during storage. When refrigerated, blue mold is more dangerous than green mold as it can still be spread to affect healthy fruits (Askarne *et al.*, 2012). Among *Fusarium species*, some can cause ear blighting and root rotting of cereals, in addition to post-harvesting rotting of various crops. *Rhizopus stolonifer* grows commonly on many fruits and food products, they depend on sugar and starch and affect mostly soft fruits like grapes and strawberries (Mailafia *et al.*, 2017).

Grey mold, a disease that affects more than 200 types of plants around the word is caused by the necrotrophic ascomycete known as *Botrytis*. In humid climate

Botrytis cause serious losses in crop yields especially in fruits and flowers. *Alternaria* is also among the fungus that causes large economic losses in the tomatoes market, it might affect the fruits in their field or after harvesting, the disease caused stars with brown to black spots and ended up with an inedible fruit. *Alternaria* genus can produce different mycotoxins among which alternariol, alternariol monomethyl ether and the mutagenic tenuazonic acid (Pane *et al.*, 2016; Hessel-Pras *et al.*, 2018).

Colletotrichum gloeosporioides is a common post-harvest infectious fungi that causes anthracnose disease, this species causes the spoilage of expensive and highly nutritious fruits, such as avocado and mango, fungicides are nowadays used to increase the fruits shelf life (Zakaria, 2021). Mango fruits are also found to get spoiled by species of *Aspergillus* and *Penicillium* (Al-Najada Ahmed & Al-Suabeyl Mohammed, 2014; Bill *et al.*, 2014). *Penicillium expansum* is the main spoiling agent of apples and it secretes patulin which is an immunotoxin and neurotoxic mycotoxin that can be found in apples-based foods such as apple juice and apples-based sauce and baby food (Zhong *et al.*, 2018).

Beside the actual spoilage of fruits and vegetables that cause losses in the agricultural domain, the secondary metabolites of fungi are a public health concern due to their severe chronic adverse effects. Fungal growth must be controlled early during the development of fruits and vegetables to avoid mycotoxins accumulation (Huang *et al.*, 2021). Several mycotoxins occur in nature, the most occurring ones in fruits are aflatoxin, ochratoxin A, patulin, and *Alternaria* toxins. Mycotoxins might diffuse in the intact tissue of the infected fruits, they are stable to physical treatments so they remain in fruits' juices and fruits-based foods produced from contaminated fruits (Drusch & Ragab, 2003). Ochratoxin A is secreted by different species of *Aspergillus* and by *Penicillium verrucosum*, ochratoxins are mainly encountered in

grapes, fig and tomatoes. Main adverse effects caused by the presence of ochratoxins are: nephrotoxicity, immunological, teratogenicity, and carcinogencity (Duarte *et al.*, 2010). Patulin is another mycotoxin produced by various fungi, in particular, *Penicillium, Aspergillus,* and *Byssochlamys*. The main food contaminant known to produce patulin is *Penicillium expansum*, this fungus shows preference to pome fruits such as apples in addition to stone fruits such as peach and cherry. Patulin is known to be linked with neurological, gastrointestinal, and immunological adverse effects and it is very common in apple juice and apple-based food products (Tannous *et al.*, 2018). *Alternaria* mycotoxins are mainly found in tomatoes and tomatoes-based products. *Alternaria* is the main spoiling agent of tomatoes. *Alternaria* mycotoxins include: alternariol, alternariol methyl ether, tenuazonic acid and others, they are known to cause gastrointestinal hemorrhages and teratogenic effects (Escrivá *et al.*, 2017). In addition, aflatoxins mainly secreted by *Aspergillus spp*. were often found in citrus fruits and figs. Possible adverse effects of aflatoxins include: toxicity, mutagenicity, immunological problems, and carcinogenicity. Aflatoxicosis, is the term used to describe the acute exposure to large amount of aflatoxins, symptoms are: liver damage (jaundice), hemorrhage, edema and probable death (Marchese *et al.*, 2018). It is worth noting, that levels of mycotoxins such as patulin were shown to be higher in juices made from organic apples, therefore, the usage of fungicides in the conventional apples farms has led to the elimination of fungi and therefore lessened the levels of the toxicant metabolic byproduct (Beretta *et al.*, 2000). These results indicates the urgent need for natural biological agent that provide human with toxicant free fresh produces and protect the environment and the human health as well from the accumulation of chemical pesticides.

Mycotoxins surveillance studies in Qatar are at infancy, yet they have shown alarming results. A study conducted in 2004, on samples of cereal and cereal products, nuts and nut products, spices, dry fruits and beverages showed various levels of mycotoxins. Twenty-eight samples showed aflatoxin levels between 0.14 and 81.64µg/kg. Eleven samples showed ochratoxin levels between 0.20 and 4.91µg/kg, and 13 samples showed zearalenone levels between 0.18–6.81 µg/kg (Abdulkadar *et al.*, 2004). Knowing that the maximum permissible level (MPL) of aflatoxin has been set by the European Union (EU) to 50ng/Kg, which means that all Qatari samples in the previous analysis are of unacceptable quality (Rahmani *et al.*, 2018). A more recent analysis in Qatar on apples, apple juice and apple-based baby food samples showed that patulin was detected in 100% of apple juice samples at levels ranging from 5.27 to 82.21µg/kg. Five of the tested samples showed levels higher than the EU recommended limit (50µg/kg) (Hammami *et al.*, 2017). Although a health risk assessment study on the levels of patulin in those apple-based food products did not show a risk on the Qatari population, yet when the additive effects of various mycotoxins will be considered as an uncertainty, human health could be at risk (Saleh & Goktepe, 2019).

2.1.3.2.Yeast

Beside mold, food products and raw material contamination by yeast is very common. After contamination, yeast play a major role in food spoilage via several mechanisms including production of lytic enzymes (Hernandez *et al.*, 2018). Humans have always used yeast in food production, bakery and alcoholic beverage productions have been recorded as early as the 7000 BCE (Sicard & Legras, 2011). Yeast have always been underestimated as spoiling agent, as mold and bacteria spoilage usually overcome their effect, yeast spoilage affect mainly food with high sugar content and low pH (Hernandez *et al.*, 2018). The density and diversity of yeast in fruits and

vegetables depends on the weather, agricultural conditions and quality of the plants. Ascomycetous yeast types such as *Candida* and *Pichia* are dominant in fruits (Molnárová *et al.*, 2014).

Various yeast species are involved in grapes spoilage, i.e. grape yards grown under organic conditions were found to be contaminated with *Kluyveromyces thermotolerans, Hanseniaspora guilliermondii* and *Saccharomyces cerevisiae*. As for yards where conventional conditions were used, the main yeast species were found to be *Grenache musts* and *Candida stellate* (Cordero-Bueso *et al.*, 2011). Juice industry is highly affected by the presence of thermophilic, osmotolerant and acid-tolerant yeasts that might ferment sugars in juices and lead to juice spoilage and explosions of packages, the origin of yeasts in juices is mainly associated with their presence in the raw material used in juices production (Wang *et al.*, 2016).

Yeasts are responsible of the spoilage of fermented vegetables, for example *Pichia manshurica* and *Issatchenkia occidentalis* cause spoilage in fermented cucumber (pickles) tanks (Franco & Pérez-Díaz, 2013). As for olives, the main yeast species isolated from the raw fruits are: *S. cerevisiae, Pichia guilliermondii* and *Candida guilliermondii*, those might play a role in the spoilage of olives during olives processing and fermentation (Pereira *et al.*, 2015). Various yeast species play a major role in milk, dairy products and meat spoilage. Yeast identification in the environment in general and in the food products in particular becomes much more accurate with the latest molecular based technologies. Table 3 shows few examples of spoiling yeasts in various food products (Hernandez *et al.*, 2018).

Table 3. Yeast species affecting different foods products and caused symptoms (Hernandez *et al.*, 2018).

Yeast Species	Foodstuff	Spoilage's symptoms	References
Saccharomyces cerevisiae, P. manshurica, Candida boidinii	Green olive	Deep softening (pectinolytic activity)	(Golomb *et al.*, 2013)
Pichia manshurica, Issatchenkia occidentalis	Fermented cucumber	Increase of pH and absorption of organic acids	(Franco & Pérez-Díaz, 2013)
Zygosaccharomyces balii, Zygosaccharomyces roxii	Fresh Fruits juices	Fermentation and yeast growth on the surface	(Wang *et al.*, 2016)
Pichia membranifaciens, Pichia anomala, Candida spp.	Wine	Biofilm formation	(Ocón *et al.*, 2013)

2.1.3.3. *Bacteria*

Fresh fruits and vegetables contain their own normal microbiota originated from the soil, water and air, the bacterial count on fresh produces ranges from 10^2 to 10^7 CFU/g (Erkmen & Bozoglu, 2016). Storage conditions (temperature, humidity etc.) affect microbial growth and therefore determine if spoilage occurs or not (Shori, 2017). Bacterial spoilage is characterized by slimy and watery appearance, although some fungal spoiling agents show similar appearance yet they are distinguished by the growth of mycelia (Tournas, 2005). The bacteria that cause soft-rot belong to six genera: *Erwinia, Xanthomonas*, *Pseudomonas*, *Clostridium*, *Cytophaga,* and *Bacillus* (Barth *et al.*, 2010). Among vegetables that get spoiled by bacteria, celery can get pink and watery soft rot when exposed to *Sclerotinia*

sclerotiorum which can also harm the skin of the workers in the field (Tournas, 2005). Bacterial soft rot caused in field are usually induced by coliforms, *Erwinia carotovora* and *Pseudomonas spp*. Many *Erwinia* species grow at low temperatures and cause spoilage of fresh refrigerated produces (Barth *et al.*, 2010). Lactic acid bacteria, spore formers, *Corynebacterium* and *Micrococcus* may cause postharvest spoilage in fruits and vegetables (Erkmen & Bozoglu, 2016). Table 4 summarizes a variety of diseases that affect different types of fruits and vegetables, with a list of bacteria causing the spoilage type (Erkmen & Bozoglu, 2016).

Spore formers bacteria might affect heat treated food products as those can resist heat, the mesophilic spore formers can cause different types of spoilage in vegetables, some *Bacillus spp*. cause the production of butyric acid in canned vegetables and therefore their wastage (Rawat, 2015). Lactic acid bacteria are beneficial bacteria used in food production (yogurt and pickles), yet when they increase in number above healthy levels they cause spoilage, example: bloating of pickled vegetables, spoiling of canned vegetables and fruits juices such as orange and tomato juice. Gram-positive lactic acid bacteria spoilage affects also cheese, milk, wine and canned meat (Kalschne *et al.*, 2015).

Table 4**.** Different fruits and vegetables spoiling bacteria and the diseases they cause (Erkmen & Bozoglu, 2016).

Product	Bacteria responsible	Type of spoilage
Cabbage	*Xanthomonas campestris*	Black rot
Lettuce, spinach	*Pseudomonas marginalis*	Slime
Peaches, cherries	*Clostridium herbarum*	Clostridium rot

Product	Bacteria responsible	Type of spoilage
Pears	*Erwinia*	Erwinia rot
Potatoes	*Erwinia carotovora*	Black rot
	Corynebacterium sepedonicum	Vascular ring and discoloration
	Streptomyces scabies	Common scab
Tomatoes	*Corynebacterium michiganense*	Bacterial spot
	Xanthomonas vesicatoria	Leaf blight
	Pseudomonas syringae	Bacterial speck
	Pseudomonas tomato	Soft rot
Various vegetables	*Bacillus, Clostridium, Erwinia, Pseudomonas marginali*	Bacterial soft rot

Pseudomonas spp. are Gram-negative aerobic bacteria that originated from the soil, they require high water activity and pH that is not less than 5.4 to grow. *Pseudomonas fluorescens* and *P. viridiflava* can secrete enzymes that might degrade pectin in plant cells and therefore cause soft-rot in fruits and vegetables. These two species cause postharvest spoilage in fresh fruits and vegetables stored at low temperatures (Rawat, 2015). In addition, *Propionibacterium cyclohexanicum* is a none spore-forming bacteria that can tolerate acidity and high temperature and spoils fruits juices (Walker & Phillips, 2007).

2.1.4. Spoiling agents studied

The overall aim of the project is to prepare an antimicrobial agent using *P. juliflora* extract. To screen the antimicrobial activity of the prepared extracts, a group

of food originated bacteria and fungi were used. Although it is known that fruits and vegetables postharvest spoilage can be caused by several microorganisms, yet fungal decay is the most important. Fungi can sporulate and remain on fresh produces in dormant forms that could resist postharvest treatment and cause spoilage at later stages. This is why antifungal activity was the main focus of this study. Fungal strains used in the screening include one *Candida* strain and nine molds strains: *Aspergillus niger*, *Botrytis cinerea*, *Fusarium oxysporum*, *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Cladosporium cladosporioides*, *Geotrichum candidum*, *Penicillium chrysogenum* and *Penicillium citrinum*.

Two strains of *Penicillium* were tested for their susceptibility to *P. juliflora* extracts. *Penicillium* belongs to the phylum of Ascomycota and it is known by its abundance in the environment. Being one of the most common fungi, *Penicillium* has a broad range of habitats including air and soil. The various species of this fungi can survive extreme conditions (temperature, salinity, water deficiency and pH) and they have been isolated from a variety of food products (Yadav *et al.*, 2018). *Penicillium citrinum* is a commonly isolated filamentous fungi that can secrete a nephrotoxin mycotoxin named citrinin. *Penicillium citrinum* has been demonstrated to secrete a variety of other extrolites including: tanzowaic acid A, quinolactacins, quinocitrinines, asteric acid and compactin (Houbraken *et al.*, 2010). Citrinin (CTN or CIT) is a mycotoxin secreted by some *Penicillium* and *Aspergillus* species including *P. citrinum*. This mycotoxin is a common food contaminant that occurs in combination with ochratoxin (OTA). CTN and OTA are rapidly absorbed and their poisoning symptoms are very similar, they affect many organs including kidney and liver. In cattle, CTN plays an important role in serious disease known as pyrexia-pruritus-hemorrhagic

syndrome, several outbreaks of this disease has been reported with a significant death rate among animals seriously affected (Hamuel, 2015).

Among the 350 *Penicillium* species around the word, *Penicillium chrysogenum* is one of the most studied as a commercial β-lactam antibiotic producer. This penicillin producer strain has been discovered first by Alexander Fleming and since then it has been the subject of many analysis (Guzmán-Chávez *et al.*, 2018). However, in nature *P. chrysogenum* has been known to cause postharvest decay of many perishable fruits and vegetables and it has been controlled together with other *Penicillium* strains using antifungal agents (Phillips *et al.*, 2012).

The most notorious plants' pathogens belongs to the genera of *Penicillium* and *Aspergillus*. Despite the fact that most of the species of those two genera cannot invade living plant tissue, yet they can cause a wide range of plant diseases as postharvest contaminant (Mousavi *et al.*, 2016). *Aspergillus* and *Penicillium* species are known to contaminate fruits and vegetables at any stage of from in field harvesting to processing and displaying, the diseases make the fresh produce inedible (Varga *et al.*, 2008). *Aspergillus* species can have their conidia suspended in the environment for a long time which disseminate the fungi in the soil, air, plants, human body and decaying organic matters. *Aspergillus* come in a large number of species with various pathogenicity, some of them are opportunistic pathogens, toxins producers that account for a wide range of agricultural and environmental pollution. Many of this genus species can tolerate extreme conditions (Plascencia-Jatomea *et al.*, 2014).

Aspergillus niger is among the most reported food contaminant around the world, it is known as black mold. *Aspergillus niger* spread more in warmer conditions both in field and in stores, it grows on plant surfaces and it causes huge losses of seeds in storage (Mousavi *et al.*, 2016). Due to their dark color and composition, the black

spores of this fungus are protected against sun light (UV light) which gives them a competitive advantage against other molds. *Aspergillus niger* is one of the most encountered postharvest spoiling agents on vegetables, crops and fresh and dried fruits. Beside the economic losses caused by black mold, the fungi threaten directly human health by secreting a variety of mycotoxins including: ochratoxin A, fumonisin B2 and fumonisin B4 (Ráduly *et al.*, 2020). Although it spreads faster in warm environment, *Aspergillus* can grow at a temperature ranging from 10˚C to 50˚C, they can also tolerate a wide pH range from 2 to 11 and high salinity up to 34%. Once they grow to the filamentous form, *Aspergillus* can secrete enzymes that allow them to grow in various samples. This wide range of environmental conditions tolerance increases the genus abundance, both abundance and rapid growth make black mold the cause of high economical losses in the agricultural domain (Plascencia-Jatomea *et al.*, 2014). Table 5 shows a list of fresh produces that are susceptible to *Aspergillus* and the main species that can contaminate them.

Table 5. *Aspergillus species* involved in plant postharvest pathogenesis (Varga *et al.*, 2008).

Plant	Disease	Species involved
Almond	Kernel decay	*A. niger, A. flavus,* and *A. parasiticus*
	Chlorosis	*A. niger*
Apples	Fruit rot	*A. sclerotiorum* and *A. terreus*
Apricot	peach Ripe fruit rot	*A. niger*
Carrot	Sooty rot	*A. niger*

Plant	Disease	Species involved
Citrus (Citrus spp.)	Albinism	*A. flavus*
	Black mold rot	*A. niger*
	Minor ear rots	*A. niger* and others
	Fruit and root rot	*A. flavus*
Date Palm	Fruit rots	*Aspergillus sp.*
Fig	Fig smut	*A. niger*
Grape	Vine canker	*A. niger*
	Bunch rot (sour rot)	*A. niger*
	Berry rots	*A. aculeatus* and *Aspergillus sp*
Mango	Black mold rot	*A. niger*
Onion and garlic	Black rot	*A. niger* and *A. alliaceus*
Peanut	Crown rot	*A. niger*
Pineapple	*Aspergillus* rot	*A. flavus*
Pistachio	*Aspergillus* fruit rot	*A. niger*
Strawberry	Fruit rots	*A. niger*

Among the food poisoning fungi, this study focused on *A. alternata*, a fungus associated with food spoilage and directly linked to asthma and fungal keratitis in patients. *Alternaria alternata* secretes a list of health concerning mycotoxins including alternariol, altenuene, alternariol monomethyl ether, altertoxins and L-tenuazonic acid. Among the most dangerous mycotoxins, alternariol and alternariol methyl ester are known to directly affect human DNA (Fraeyman *et al.*, 2017). *Alternaria alternata* reduces the postharvest shelf-life of different agricultural products around the world by directly affecting the fruits and it can also affect plants yield by impairing the plants

photosynthesis in field by seeds, stems and roots infections. Table 6 summarizes a list of plants diseases caused by *A. alternata* (Troncoso-Rojas & Tiznado-Hernández, 2014).

Table 6**.** Plants affected by *A. alternata* spoilage and their affected parts (Troncoso-Rojas & Tiznado-Hernández, 2014).

Plant	Disease	Plant's part(s) affected
Mango	Stem-end rot	Stem
Cherry tomatoes	Black rot	Fruits
Fuji apples	Core browning and moldy core	Fruits
Apples	Fruit spot	Fruits
Mandarins	Black rot and brown spots	Fruits
Kiwi	Black rot	Fruits
Immature cucumbers	Fruit rot	Fruits
Melon	Black spot	Fruits
Peaches	Moldy heart	Fruits
Tangerines	Brown spots	Fruits
Pepper	Fruit rot	Fruits
Fig	*Alternaria* rot	Fruits
Litchi	Pericarp browning	Pericarp
Pingguoli pear	*Alternaria* rot	Fruits
Citrus fruits	*Alternaria* brown spot	Fruits
Persimmon	Black spots	Fruits
Persian walnuts	Brown apical necrosis	Pericarp

Plant	Disease	Plant's part(s) affected
Pistachios	*Alternaria* late blight	Leaves
Grapefruits	Brown spots	Fruits
Sunflowers	Seeds and leaves blight	Leaves and seeds
Various species	Leaf spot	leaves

Alternaria species can usually survive harsh conditions, spores or mycelia can survive for long time in the soil, infected crop debris, and old leaves or barks to infect new plants when conditions are convenient (Panth *et al.*, 2020). Although some *Alternaria species* require stimuli to initiate sporulation, *A. alternata* can simply initiate conidiophores formation without any trigger, this species can also remain in a quiescent state on the cuticles of fruits such as mango and avocado until fruits ripen and become convenient for infection. *A. alternata* infect fruits mainly via natural opening or wounds that have occurred during harvesting or processing. The optimum temperature of *A. alternata* germination varies depending on the infected host, yet infection can still occur in a wide range of temperatures from below 10˚C to above 35˚C (Troncoso-Rojas & Tiznado-Hernández, 2014). To lessen economical losses and to protect human health, various pre-harvest and post-harvest measures have been implemented to control *A. alternata* contamination, fungicides have played a major role in this control. However, the increase demand on chemicals free fruits and vegetables, and the awareness about pesticides adverse effects urge the search for alternative fungicides to control postharvest spoiling agents such as *A. alternata* (Pimentel & Burgess, 2013).

Among the widespread pre and post-harvest pathogens, *B. cinerea* has a wide range of hosts and spoil many fruits and vegetables around the world yearly. *Botrytis cinerea* is one of the top ten pathogens that affect fresh produce shelf-life with more

than 200 dicotyledonous crop species hosts around the world (De Simone *et al.*, 2020). With a global effort to control *B. cinerea* known as the gray mold, countries around the world spend a lot of money to follow good agricultural measures and to use botryticides, wide spectrum fungicides and bio-controller to stop *B. cinerea* rot. Despite all the measures, fresh produce spoilage and quality deterioration during retail chain are costing much more, *B. cinerea* related economic losses range from $10 billion to $100 billion worldwide (Hua *et al.*, 2018). Vegetables and fruits that are most susceptible to gray mold after harvesting include: tomato, lettuce, lemon, pumpkin, blueberry, artichoke, apple, asparagus, bean, beet, blackberry, broccoli, cabbage, carrot, cauliflower, celery, cucumber, currant, eggplant, endive, grape, kale, kaki, kiwi, leek, lentil, onion, pea, peanut, pepper, pear, peach, plum, pomegranate, potato, raspberry, strawberry, sweet cherry, and many others (Romanazzi & Feliziani, 2014).

Botrytis cinerea can infect fresh produce at any stage of their processing and can remain latent until conditions are favorable (De Simone *et al.*, 2020). Infection starts at natural openings of fruits and vegetables or at wounded zones, it starts with a black spot followed by sporulation and hyphae development intro a white or gray wide zone. This pathogen progress easily from infected fruits to healthy ones even at low storage temperatures which can lead to entire lots spoilage in few weeks (Droby & Lichter, 2007). *Botrytis cinerea* exists in different forms that can be transferred by different methods. Mycelia, chlamydospores, sclerotia, apothecia, ascospores, microspores and macrospores can all serve as initial inoculum for the progress of the disease. In addition to the sclerotia that can resist harsh conditions to sporulate again with every new fresh produce, *B. cinerea* mycelium can also survive within previously infected dead host tissues (crop debris) and inside seeds to serve as new inoculum when conditions are convenient (Romanazzi & Feliziani, 2014).

Over ripe fruits are more prone to gray mold rot than less ripe ones, therefore, fruits maturity is among the important factors considered to control *B. cinerea* rot. *Botrytis cinerea* can occur in fired and remain latent until storage. Different storage conditions have been controlled around the world to lessen progress of this fungi. Careful handling to avoid injuries is one of the control measures applied together with the traditional fungicides application. Less polluting bio-controller of *B. cinerea* are a hot research topic for many scientists around the world (Yusoff *et al.*, 2020).

Among the fungi of focus is a filamentous yeast-like fungi known as *G. candidum*. This ubiquitous fungi survives in various environments including water, air, soil and some food products mainly dairy products (Pottier *et al.*, 2007). Although it is one of the human digestive tract normal microbiota yet it has been linked with infections in immune-compromised patients (Keene *et al.*, 2019). *Geotrichum candidum* is considered also as plant pathogen causing sour-rot in many fruits and vegetables including citrus fruits, tomatoes, carrots and others (Thornton *et al.*, 2010). Over ripe fresh produce kept in moisture conditions are more prone to *G. candidum* rot especially those with injured tissues, the disease spread fast and turn the plant into a sour-smelling watery mass. Although higher temperature and humidity are preferred by *G. candidum*, yet it remains active at low storage temperature, which becomes a significant postharvest storage pathogen. Furthermore, *G. candidum* is a major contaminant of tomatoes processing machinery and it is used an indicator microorganism to assess equipment hygiene in the tomato processing industry (Thornton *et al.*, 2010). It is finally worth noting that *G. candidum* can cause an opportunistic fungal infection in animals and human known as *Geotrichosis*, this infection can be serious in patients with low immune system (Haque, 2018).

Cladosporium are common fungi in the environment that are characterized by olive green colonies, *Cladosporium* have slow growth but abundant sporulation with a dusty appearance, spores can be easily transferred by air, conidia can be single cells or double or triple celled (Bensch *et al.*, 2012). Among *Cladosporium species*, *Cladosporium cladosporioide*s and *Cladosporium herbarum* are known to cause diseases in fruits, *C. cladosporioide*s in particular is one of the main cause of grapes rot (Mengal *et al.*, 2019). The two common food fungal species *C. cladosporioide*s and *C. herbarum* can grow at low storage temperatures and cause black spots on food, *Cladosporium species* have been isolated from a variety of food products including cereal grains, peanuts, fruits, vegetables and refrigerated meet causing decolonization and food spoilage (Bullerman, 2003).

Among the chosen fungal species, *C. gloeosporioides* was evaluated for its sensitivity to the prepared plant extracts. *Colletotrichum* is a large fungal genus that include many important pathogenic species that cause diseases mainly among tropical and sub-tropical fruits and vegetables (Zakaria, 2021). *Colletotrichum spp.* are widely encountered is all types of plants, they are among the most important pathogenic fungi in the world and they lead to large economical losses by spoiling cereals, legumes, vegetables and perennial crops including fruit trees (Siddiqui & Ali, 2014). Anthracnose disease caused by *C. gloeosporioides* is a threat on the global agriculture, fruits contamination can occur in field or during handling, yet onset of the disease is usually delayed to cause severe symptoms and huge postharvest losses, the main fruits affected are: papaya, mango, avocado, dragon fruit and others. In non-tropical countries, those fruits are costly imported fruits which shelf-life extension is highly important (Siddiqui & Ali, 2014).

Anthracnose can affect plants in field when *C. gloeosporioides* invade their fruits, leaves and/or stems (Zakaria, 2021). On the fruits, the first signs of the disease are depressed watery lesions with translucent brown margins known as chocolate spots, as the disease progress, pink to orange conidia appear, the fruit internal parts start showing grayish to white decolonization that becomes brown later. In papaya and mango, the infection can occur early, but the fungi remain dormant in the shape of appressorium or subcuticular hyphae that remain quiescent until the fruit ripen. Once ripen, the disease symptoms start to develop fast, the first stages of the infection affect only the peel but at later stages, conidia can penetrate to the pulp. In avocado, *C. gloeosporioides* infect immature fruits wounded by insects, the source of fungi can be dry dead leaves from the previous season (Siddiqui & Ali, 2014).

Colletotrichum gloeosporioides sporulates best at temperature between 25 and 30˚C with high relative humidity. Mango fruits displayed or stored at room temperature are much more prone to anthracnose that fruits stored at 10˚C and at low humidity as the fungi fail to grow at these conditions. Various measures are being evaluated to control anthracnose, conventional recommendations such as appropriate handling and fungicides application have their cost and health effect, which makes the development of a biological control method of crucial importance (Siddiqui & Ali, 2014).

Finally, *F. oxysporum* is an economically important fungus characterized by a large species complex known as *F. oxysporum* species complex (FOSC) that is abundant in different environments including air, soil and water (Srinivas *et al.*, 2019). *Fusarium oxysporum* cause vascular diseases in over 100 plants species and lead to their spoilage, it can also cause infectious diseases in immunocompromised patients and human eyes infections even in healthy individuals (Summerell *et al.*, 2011). Therefore, the species threatens both public health and food safety. Fungal fusariosis is

the second most occurring opportunistic fungal infection in human after aspergillosis, in addition, the fungal keratitis causes by this fungi lead to numerous cases of blindness annually around the world (Walther *et al.*, 2021). *Fusarium oxysporum* is among the top ten economically threatening fungi that cause losses in banana, cotton, canola, tomato and other important crops (Dean *et al.*, 2012). Among the devastated plants diseases caused by *F. oxysporum* is Panama disease in banana that cause huge losses in the banana agricultural domain. Many species of *Fusarium* produce mycotoxins that remain in processed food even when infected fruits are discarded. The control of this fungi using broad spectrum fungicides is polluting the environment and leading to resistance. Safe bio-controllers are the best alternatives that should be explored in all postharvest fungal infections cases (Zhang & Ma, 2017).

2.1.5. Economical losses caused by food spoilage

Globally, it has been reported that 20% the harvested vegetables and fruits are wasted every year by spoilage and around one third of the overall produced food is either wasted or lost (Leyva Salas *et al.*, 2017; Mailafia *et al.*, 2017). Food and Agriculture Organization of the United Nations stated that around 1.3 billion tons of food are wasted every year (FAO, 2017). Food losses affect 40 to 50% of root crops, fruits and vegetables and 30% of cereals. In developing countries, food's losses occur mainly at the post-harvesting and processing stage and 30 to 40% of the food products are affected. As in developed countries, 30% of the produced food is lost mainly at the retail and consumer levels (FAO, 2017; Leyva Salas *et al.*, 2017). Massive global food loss has many reasons, yet microbial spoilage play a major role. The number and the types of microorganisms on fruits differ among locations and it reflects the growth environment, the harvesting conditions, and the handling and storage processes. Even though freshly harvested vegetables and fruits are contaminated with a variety of

bacteria and fungi and other microorganisms, molds are the main causative agent of spoilage especially in products refrigerated in open boxes (Tournas, 2005; Mailafia *et al.*, 2017).

Literature reports that 25 to 40% of mango in India is subject to post-harvest loss, while this number can go as high as 69% in Pakistan. In overall Asia the post-harvest loss of mango due to microbial decay is between 17 and 26.9% of the total production (Al-Najada Ahmed & Al-Suabeyl Mohammed, 2014). As for avocado, if the fruit is not treated with spoilage control agents, 80% of the harvested fruits will be lost for anthracnose (Bill *et al.*, 2014). In the USA, an estimation study showed that fruits loss at the supermarkets level was 12.3% in 2012, the percentage changes among different fruits categories, with the highest percentage loss for papaya fruits with 43.1%, and the lowest for bananas with 4.1% loss. A total of 21.1% of mangos were lost every year during the study on USA supermarkets, in addition to 19.2% of apples, 14.8% of oranges, and 19% of avocado fruits. The same estimation analysis, reported total vegetable loss in the USA's supermarkets by 11.6%, with green leafy plants at the top highest percentage loss and onion, garlic and corn at the lowest. Annual losses of tomatoes and cucumbers were estimated at 14.5% and 12.2% respectively (Buzby *et al.*, 2015). Kitinoja and Kader (2015) have searched the literature thoroughly until 2015, postharvest losses of fruits and vegetables in some of the Middle East and North Africa countries are summarized in table 7, percentage loss of fresh produce varies between 13% and 23% depending on the climate conditions and diseases incidences. Data was collected either through surveying or through sampling.

Table 7. Fruits and vegetables postharvest loss in selected countries (Kitinoja & Kader, 2015).

Country	Commodity	Loss percentage	Reference
Egypt	Oranges	14%	(El-Shazly, 2009)
	Tomatoes	15%	
	Pomegranate	23%	(Kitinoja & Kader,
	Onion	19%	2015)
Jordan	Tomato	18%	(El-Assi, 2002)
	Eggplant	19.4%	
	Pepper	23%	
	Squash	21.9%	
KSA	Tomato	17%	(Kitinoja & Kader,
	Cucumber	21.3%	2015)
	Figs	19.8%	
	Grapes	15.9%-22.8%	
	Dates	15%	

2.1.6. Spoilage control

Protecting humans health require adherence to good manufacturing practices before, during and after harvesting, which is the key to protect fruits and vegetables from contamination, this includes good agricultural practices, irrigation with non-contaminated water, appropriate handling and transportation and storage, clean equipment throughout the process, appropriate display in the market and proper

workers hygienic practices. Hazard analysis critical control point (HACCP) system and good agricultural practices (GAP) applications would improve food protection at all stages and therefore protects human health. Finally, it is important to separate damaged fruits from healthy ones to avoid spreading of contaminations (Tournas *et al.*, 2015).

Under field conditions, some spores can survive for long time in the soil and contaminate crops every season, in such cases, fields should be treated and decontaminated (Alegbeleye *et al.*, 2018).

Post-harvested fruits and vegetables should be stored at low temperatures (0-5˚C) to protect them from microorganisms' growth; however, it is very important to store sensitive produces such as cucumbers at higher temperatures (7-10˚C) to avoid chill bites. Microorganisms' growth is also affected by the CO_2/O_2 concentrations, therefore modifying these gases concentrations might help in controlling some specific types of fungi such as *Botrytis, Penicillium*, and *Mucor* that are sensitive to the increase of CO_2 (Tournas, 2005).

Many technologies can help in proper fruits handling, among these are vacuum packaging, high-pressure food preservation, disinfection with ozone, chlorine treatment, hydrogen peroxide treatment, pH modification, heat treatment and bio-preservation. However, fruits texture and composition must be studied in order to choose the appropriate techniques for each type. Some of those techniques might be costly; therefore, further research is required to enhance contamination preventive methods, and to develop affordable microbial control techniques (Leyva Salas *et al.*, 2017; Mailafia *et al.*, 2017).

Plants extracts are being explored nowadays as natural method to control spoiling agents and many have shown wide spectrum antimicrobial activity. Various parts of plants contain various nutraceutical compounds; many plants phytochemicals

are associated with human health benefits including diseases' prevention through their antimicrobial activities (Sofowora *et al.*, 2013). Plants secondary metabolites belong to different classes including: alkaloids, amines, cyanogenic glycosides, diterpenes, flavonoids, polyketides, sesquiterpenes, tetraterpenes, triterpenes, glucosinolates, monoterpenes, non-protein amino acids, phenylpropanes, polyacetylenes, saponins and steroids. Many of these components such as alkaloids are water soluble while other organic molecules require an organic solvent such as alcohol to be dissolved (Karim & Azlan, 2012). Among the mechanisms that plants have developed to protect themselves against fungal infections is the production of low-molecular-weight active protein and peptides. Those proteins have various modes of action such as fungal cell wall degradation, ribosomal restriction and others, and they could be explored as safe natural antimicrobial agents to be adopted in the agricultural field (Solanki *et al.*, 2018).

Conventionally, pesticides are the most used agents to control any undesirable creature that affect the agricultural productivity. We divide pesticides based on their target organism as shown in table 8 (Fishel, 2014). According to the Food and Agricultural Organization (FAO), a pesticide is any formulation that is used to prevent or to kill existing pests that could harm agricultural commodities during their production, processing, storage or marketing. This include a list of antibiotics and other chemicals that are usually administered to animals or applied on their bodies to control pests' infections (Li & Jennings, 2017).

Table 8. Different classes of pesticides and their target pests (Fishel, 2014).

Pesticide Class	Primary Target/Action	Example(s)
Acaricide	Mites	Bifenazate

Pesticide Class	Primary Target/Action	Example(s)
Algaecide	Algae	Copper sulfate
Attractant	Attracts wide range of pests	Pheromones
Avicide	Birds	Avitrol (aminopyridine)
Bactericide	Bacteria	Copper complexes, streptomycin
Bait	Wide range of organisms	Anticoagulants
Biopesticide	Wide range of organisms	*Bacillus thuringiensis*
Defoliant	Removes plant foliage	Tribufos
Desiccant	Removes water	Boric acid
Fumigant	Wide range of organisms	Aluminum phosphide
Fungicide	Fungi	Azoxystrobin, chlorothalonil
Herbicide	Weeds	Atrazine, glyphosate, 2,4-D
Insect growth regulator	Insects	Diflubenzuron
Insecticide	Insects	Carbaryl, imidacloprid
Molluscicides	Snails, slugs	Metaldehyde
Nematicide	Nematodes	Ethoprop
Piscicide	Fish	Rotenone
Plant growth regulator	Regulates plant growth	Gibberellic acid, 2,4-D
Predacide	Mammal predators	Strychnine
Repellent	Vertebrates and invertebrates	DEET, methiocarb
Rodenticide	Rodents	Warfarin
Silvicide	Trees	Tebuthiuron

Pesticides are classified based on their chemical composition into: carbamates, neonicotinoids, pyrethroids, organochlorine compounds and organophosphorus compounds (Mao *et al.*, 2012). Fungicides and antibiotics are widely used to control microorganisms' growth in field and in stored fruits and vegetables as well as to extant shelf life. Fungicides and bactericides formed 46% of the sold pesticides by the European Union (EU) countries in 2016 (Vela *et al.*, 2019). Fungicides are agrochemicals belonging to various chemical groups with different modes of actions. Among the largest groups are Triazoles with a half-life of 100 days, which makes them highly persistent in the soil. Other fungicides groups include: organophosphates (e.g. edifenphos, iprobenfos, triamiphos), phenylamides (e.g. metalaxyl, furalaxyl, benalaxyl), and benzimidazoles (e.g. benomyl, carbendazim, thiabendazole), among others (Murillo-Zamora *et al.*, 2017). Nowadays, food security is an increasing consumers and public authorities concern, awareness regarding the health adverse effects of pesticides is increasing. Hence, the market demands for chemical free as well as diseases free fresh produces is increasing, and therefore, it is crucial to come up with natural eco-friendly formulation to control diseases and spoilage of fruits and vegetables (Bill *et al.*, 2014).

2.1.7. History of pesticides

According to Gavrilescu (2005) The usage of pesticides dates decades back in the history, with its first signs with the Chinese during the 1000BC in Samaria when they have used sulfur as fumigant. Other pests including body lice were controlled by the Chinese using mercury and arsenic. Greeks and Romans have used other controllers to protect themselves and their crops such as oil, ash and sulfur. In general, various cultures have used salts and spices in addition to insects-repelling plants to preserve their foods. Japanese have sprayed their rice paddies with a mixture of whale oil and

vinegar to prevent the development of insect larvae in the 16th and 17th century, they have also made an insect killer from the water extract of tobacco leaves. In the 19th century farmers started using insecticides extracted from plants. They have used also arsenic tioxide, copper arsenite, and a mixture of copper sulfate and lime water to prevent weeds, beetles, and vine downy mildew, respectively. With the development of the organic chemicals industry around the World War II, chemicals pest controllers were introduced to the agriculture (Gavrilescu, 2005). Organochlorines were the first synthetic pesticides used and commercially produced. Organic pesticides production has developed rapidly to reach around 500 different formulations of pesticides nowadays. Despite our knowledge about the risk that these chemicals represent on both environment and human health, they are an agricultural necessity that help nations to fulfill food demands, pesticides are our way to control the growth of any organism that compete with humans for food and nutrients (Goodwin *et al.*, 2017).

2.1.8. Environmental chemical fingerprint of food spoilage controller agents

When pesticides are applied only 0.1% of the applied amounts reached the target pests, the remaining (99.9%) are drifted to affect non-target population and/or remain in the environment to cause losses in the biodiversity and key species eliminations, water pollution, and soil contamination, beside long term human health adverse effects, and pest acquired resistance. This latter results either in the increase of the effective pesticide dose or in the requirement of alternative formulation (Pimentel, 1995; Mahmood *et al.*, 2016). As most of the pesticides are directly applied to fresh produces, human of any age groups (infants, children and adults) can get exposed by ingesting contaminated foods (Saleh & Goktepe, 2019).

Pesticides adverse effects on human is divided into acute and chronic effects. Upon acute exposure, the adverse effects might be seen in different body systems, this include oral effects such as burned mouth and sore throat, upset stomach, inhalation effects like pain and tightness in the chest, dermal effects such as itching, blister and rash, and ocular effects such as irritation and temporary or permanent blindness (Mahmood *et al.*, 2016). Some pesticides are more toxic than others with very low lethal dose 50 (LD50), in such cases exposure might even cause death, especially if the pesticide is ingested (Damalas & Koutroubas, 2016).

When exposed to low doses of pesticides for a long period of time, chronic adverse effects starts appearing, this include: tumors, genes mutations, miscarriage, birth defects, infertility, nervous system disorders and others (Mahmood *et al.*, 2016). Chronic pesticides toxicity affect not only human health but also the entire environment including animal, plants, water quality and may reach even the underground water (Damalas & Koutroubas, 2016). Fungicides are pesticides used against fungal infections of plants or seeds. Fungicides have usually a moderate toxicity, yet, many are of concern to public health as they are known to cause developmental toxicity and oncogenesis. Among the fungicides of concern are: alkyldithiocarbamic acid (manganese, zinc, and ammonium salts), halogenated substituted monocyclic aromatics (dinocap), carbamic acid derivatives, ferbam, mancozeb and maneb metabolites, benzimidazoles, and tridemorph. Many studies showed that fungicides may disrupt the endocrine system and lead to reproductive and developmental abnormalities. Several fungicides including cycloheximide were banned in different parts of the world due to their teratogenicity, however, poorer region are still using them (Gupta, 2018). In Qatar, the amounts of pesticides imported increased from 88,172 kg to 174,463 kg between 2009 and 2012, which reflects an increase in the use and therefore, an increase in the

environmental fingerprint (Nagy *et al.*, 2014). These numbers must have increased largely in recent years with the noticeable increase in investments in local agricultural farms.

Pesticides maximum residue limits (MRLs) are the maximum tolerable concentration of a certain pesticide that can exist on a specific agricultural product. MRLs are regulated by many nations. However, exposure levels vary according to the consumption rates, geological location, economical status, cultural habits and availability of the produces. Therefore, evaluations of pesticides with MRLs levels in highly consumed foods is an important monitoring measure (Horváth *et al.*, 2014). A recent study was conducted in Qatar on organochlorine pesticides (OCPs) levels in 127 fruits and vegetables samples collected from the Qatari markets including: leafy vegetables, cucumber, tomatoes, potatoes, lemons and strawberries. Collected samples were of local origins or imported, results of the two groups (washed and unwashed samples) were discussed (Al-Shamary *et al.*, 2016). Organochlorine pesticides are of environmental concern as they can accumulate in the human adipose tissue, this category of pesticides has been banned in many countries around the world (Genuis *et al.*, 2016). Around 90% of the tested imported samples showed OCPs level above the maximum residue levels (MRLs), while only 30% of the local samples had levels above the MRLs. Interestingly, no significant difference was reported between the OCPs levels in washed and unwashed samples, which indicates that washing the fruits and vegetables is not enough to get rid of the toxic chemical pesticides (Al-Shamary *et al.*, 2016).

2.2. PROSOPIS SPECIES

2.2.1. *Prosopis* as invasive species: general information

Many plants' species have been transferred in the last few decades from their native environment to different places around the world due to the increased and facilitated human movement. New crops can be introduced either accidentally or intentionally if believed to be beneficial to human. *Prosopis spp.* (mesquite; Fabaceae) are found in hot and dry areas around the world and in semi-arid places as well, either as native species or introduced (Shackleton *et al.*, 2014). *Prosopis* occurs nowadays in around 129 countries and territories, it is very common in Australia and known as major invasive species in South Africa, India, Ethiopia, Kenya, Sudan and other places (Low, 2012). Invasive plants have usually ecological, economical and social impacts on the areas of invasion, *Prosopis spp.* are native in some places and invasive in others, they have a wide range of usages and benefits yet they have negative impacts on the biodiversity, ecosystem services, and the economy of the countries where they are heavily grown (Qasem, 2007; Al-Assaf *et al.*, 2020). Factors that make *Prosopis* an invasive species are the high number of seeds produced per tree, the long viability of the seeds, the fast growth, their roots systems that can go as deep as 50m underground, their allelopathic and allelochemical interaction with other plants (Shackleton *et al.*, 2014). Many publications addresses *Prosopis* secondary metabolites synthesized by roots, leaves and fruits as allelochemicals. Allelopathic potentials is disseminated by fallen leaves, plant leachates and roots exudates In addition, the plant triggers the secretion of allelochemicals by their rhizospheric microorganisms (de Brito Damasceno *et al.*, 2018).

Prosopis species are the subject of many researches; some scientists call it wonder plant while biodiversity ecologists call for its eradication. The genus *Prosopis*

include 44 species, among which *P. chilensis, P. glandulosa*, *P. juliflora, P. pallida* and *P. velutina* are the most studied ones (Rejmánek & Richardson, 2013). *Prosopis spp*. provide the word with a variety of goods and services including woods for construction and fuel productions, windbreaker, soil stabilizer, source of food for animals and human and source of pharmaceutical products (Qasem, 2020). Those plants are a part of the economical income in many countries including India, Brazil and Kenya (Wise *et al.*, 2012). The main negative impacts of those invasive species are related to the decrease in biodiversity, and the alteration of ecosystem services including animal grazing blockage, soil quality and water supply modifications (Kaur *et al.*, 2012). Invasive species control and management is a complex issue (Qasem, 2007). Every method has its advantages and disadvantages, invasive species can be managed using mechanical or chemical control methods, which can be expensive and might harm the environment. Nowadays, control through utilization is encourage, as it allow the usage of the species and the control of its spread (Handayani & Hidayati, 2020).

Prosopis juliflora (Mimosaceae), the focus of this study, is a small tree, native to Mexico, South America and Caribbean, it is an increasingly spreading invasive species in Asia, Austria and in other places around the world. Among the globally distributed plants, *P. juliflora* is being considered as one of the word's 100 most invasive species (Raghavendra *et al.*, 2009; de Brito Damasceno *et al.*, 2018). It is an evergreen tree with wide canopy, its height is between 5 and 10 m. The stem of *P. juliflora* is greenish brown with rough, red and dull bark. The roots can go very deep in the soil and the leaves have a dark green color and they come in 13 to 25 pairs of leaflets. The flowers of this plant are yellowish green made of five petals each. Fruits are pods full of seeds, of dark brown color, pods are rich with protein and sugars

and they are grazed by some animals (Sathiya & Muthuchelian, 2008). The pericarp of the pod is made of three layers: the epicarp or exocarp, the middle layer known as mesocarp and the layer that encloses the seed known as endocarp. The pericarp is known to be rich with nutraceutical substances (Karim & Azlan, 2012). The plant can well adapt itself not only to dry and hot climate but also to extreme conditions such as rocky and saline soil (Henciya *et al.*, 2017). *P. juliflora* is an invasive species in Qatar as well, and it is affects the biodiversity of the areas where it heavily grows.

2.2.2. *Prosopis juliflora* direct utilization around the world

Many countries use different parts of *P. juliflora* for different purposes mainly for pharmaceutical usages in curing diseases and in preventing infections. In India, the plant is used in colds treatment, food poisoning, dysentery, it is also used for its antimicrobial effect as antiseptic for wound and in the treatment of sexually transmitted diseases (Choudhary & Nagori, 2013). Other studies reveals also that *P. juliflora* is used as animal food, to restore green areas after deforestation, to make charcoal and firewood, and for houses and furniture construction (Raghavendra *et al.*, 2009). Some types of bees have preference to *Prosopis* flowers, *Prosopis* honey has a very good quality compared to other types (Sathiya & Muthuchelian, 2008). *Prosopis spp*. are nitrogen fixator plants that are utilized sometimes in the recovery of degraded soil. The pods of the plants are rich with sucrose, which makes it the subject of several studies that examine the possibilities of its usage as raw material for food production like syrups and other products (da Silva *et al.*, 2018).

In Monte desert area in Argentina, *Prosopis* plants are of major importance. The fresh fruits are collected by local inhabitants and the exo-mesocarp (pulp) is consumed directly. Dry pods' pulp is also milled into a gluten free flour used to make traditional bread known in the region as Patay or Algarobbo bread, *Prosopis* flour is as rich in

protein as wheat flour. The flour is also traditionally mixed with water to prepare a drink, or fermented into an alcoholic beverage known as Chicha. In addition, *Prosopis* is the favorite source of nutrients for bees in Monte Desert, *Prosopis* honey has very high quality and a distinct taste (Vilela *et al.*, 2009). In Kenya, the widespread of *Prosopis* species has led to the development of policies to educate people on how to use it, this including a cook book that shows how to adapt its use as flour (de Brito Damasceno *et al.*, 2018).

In Mexico, in the Tehuacan Valley, people have always consumed *Prosopis* pods and have used its wood for fuel. Until our days, children and adults in rural areas consume the fresh sweet pods. The pods are also dried and ground either all together or using the mesocarp alone to produce traditional flour known elsewhere in the world (Pasiecznik *et al.*, 2001). A team in Brazil in 2009 tried to prepare a honey like product from the pods of *P. juliflora*, the syrup quality parameters were checked together with the mineral and nitrogen contents. The syrup obtained have superior nutritional value and could be a low-cost replacement of natural honey, the quality parameters obtained were in agreement with the regulations of honeys' parameters except of moister content (Guilherme *et al.*, 2009).

2.2.3. *Prosopis* plant's extracts effectiveness "experimental work"

According to the Indian council of Forestry Research and Education (ICFRE) *Prosopis spp*. extracts have valuable pharmaceutical values, the flowers extracts have been used in miscarriage prevention, while their barks' extracts have shown efficacy in treating asthma, bronchitis, leukoderma, rheumatism, leprosy and dysentery. Traditionally, eyes infections used to be treated by the smoke of *Prosopis* leaves, and leaf extracts were used against scorpion and snake bites (Henciya *et al.*, 2017). In South

Africa a local pharmaceutical company has used *P. juliflora* pods to develop a medicine to control human blood sugar levels (Wise *et al.*, 2012).

Prosopis juliflora extracts has been explored in the last two decades, an old study have shown that the alkaloid julifloricine of *P. juliflora* had an antimicrobial effect against Gram-positive bacteria at different concentrations (Aqeel *et al.*, 1989). More recent literature showed that *P. juliflora* extracts have been explored and they have exhibited promising results as anti-inflammatories, anti-tumor, and antifungal against *Fusarium*, *Drechslera* and *Alternaria* (Raghavendra *et al.*, 2009). Phenolic compounds of *P. juliflora* have also shown anti-HIV and anti-ulcerogenic analgesic effects, they were effective as vasodilator and immune-stimulator. The alkaloids of the same plants were effective as anti-malaria and as analgesics (Henciya *et al.*, 2017).

Mazzuca *et al*. (2003) have tested the extracts of four different species of *Prosopis* plants against bacterial and fungal isolates. Dichloromethane extract of *Prosopis alpataco* revealed antimicrobial and antifungal effects while the methanolic and aqueous extracts of *Prosopis denudans* showed good antifungal activity. *Prosopis chilensis* ethanolic bark's extracts that is rich with Tannins showed antimicrobial activity against *Micrococcus luteus*, *B. Subtilis*, *S. aureus*, *S. pneumonia*, and *Cryptococcus albidus* (Singh et al., 2010). The methanolic extract of *P. cineraria* pods have shown an antioxidant activity and efficacy against *Candida albicans* (Salar & Dhall, 2010). Solanki *et al*. (2018) have discovered recently a thermostable novel protein extracted from *P. cineraria* pods that showed antifungal activity against *Lasiodiplodia theobromae* and *Aspergillus fumigates* two famous spoiling agents of mango, banana, papaya, strawberry, and orange (Solanki *et al.*, 2018).

The active ingredients of *P. glandulosa* leaves extract were purified and tested for their antibacterial, antifungal, anti-infective and anti-parasitic effects. Various

phytochemicals showed various effectiveness. A purified dihydroindolizinium salt was proven effective against *Plasmodium falciparum*, two of the extracted chemicals showed activity against *Leishmania donovani*, and juliprosine inhibited the growth of the fungi *Cryptococcus neoformans* and the bacterial species *Mycobacterium intracellulare*. It is worth noting that none if the active ingredients showed toxicity to the mammalian VERO cells (Rahman *et al.*, 2011). Another active ingredient of *P. glandulosa* was purified in an earlier study, 2,3-dihydro-1H-indolizinium chloride has shown antifungal activity against *Cryptococcus neoformans* and *Aspergillus fumigatus*, and antibacterial activity against methicillin resistant S. *aureus* (MRSA) and *Mycobacterium intracellulare* (Samoylenko *et al.*, 2009).

An experiment conducted in India in 2013 showed that the oral median lethal dose of the ethanolic extract of *P. juliflora* leaves in mice is 3807.9 mg/kg and in rats more than 5000 mg/Kg, which proves that the extract is not toxic. Experiments on rodents proved that the plant's extract has effective anti-inflammation properties (Choudhary & Nagori, 2013).

A study conducted on *A. alternata* isolated from tobacco leaves in 2009 have shown that the aqueous extract of *P. juliflora* leaves has significantly decreased the mycelium growth of the fungus. Methanolic and ethanolic extracts showed also significant antifungal activity. As when alkaloids were extracted, the alkaloid solution showed a complete inhibition of the fungi growth at concentration of 1 mg/ml while commonly used fungicides in the market are usually used at a concentration of 2 mg/ml (Raghavendra *et al.*, 2009).

Different concentrations of *P. juliflora* ethanolic leaf extracts were tested for their antimicrobial activity against different bacterial strains including five Gram-positive strains: *Staphylococcus epidermis, Staphylococcus aureus, Streptococccus sp*.

Micrococcus luteus and *Bacillus subtilis.* Five Gram-negative strains were also tested: *Salmonella typimurium, Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli*, and *Pseudomonas sp*. Results showed good inhibition activity that is comparable to that of commonly used antibiotics such as streptomycin and penicillin (Sathiya & Muthuchelian, 2008).

Basie *et al*. (2014) has tested the antifungal effect of different plants leaf extracts against *Colletotrichum musae*, which is the causative agent of postharvest banana anthracnose. *Prosopis juliflora* extract showed the best results on fungal growth inhibition followed by *Acacia albida*. An earlier study conducted in 2010, proved *in-vitro* efficacy of *P. juliflora* extract against soil pathogenic fungi including: *F. oxysporum, Rhizoctonia solani, A. alternata* and *Curvularia lunata* (Lakshmi *et al.*, 2010).

Prosopis juliflora aqueous leaf extract was also tested as an effective mouth-rinse solution. The efficacy of the extract against oral and periodontal bacterial pathogens was higher than that of available mouth-rinse solutions in the market. According to the study, *P. juliflora* extract inhibited growth of *Enterococcus faecalis, S. aureus*, *Prevotella intermedia*, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (Osuru *et al.*, 2011).

Powder of leaves, stem and flower of *P. juliflora* were tested for their effect to control root rot in soil, fungi used were *Fusarium spp., Rhizoctonia solani* and *Macrophomina phaseolina*. Results showed significant decrease in root rot incidence and significant improvement in the plants growth parameters including shoot and root weight and height, leaves size and number of nodules per plant (Ikram & Dawar, 2013).

Prosopis juliflora alkaloids extracts have shown efficacy in binding to an enzyme called acetyl cholinesterase and in inhibiting its activity, this enzyme is a main

player in the neurodegenerative disorder known as Alzheimer disease. During the progress of Alzheimer, the brain synapsis and the neuromuscular junctions are affected, the role of acetyl cholinesterase is to hydrolyze cationic neurotransmitters that terminate the nervous impulse. Among the *P. juliflora* alkaloids, juliflorine was proven to inhibit acetyl cholinesterase in a dose dependent manner and therefore, to delay the onset of the disease (Choudhary *et al.*, 2005).

In a study conducted in 2014, the ethanolic extracts of *P. juliflora* roots and leaves have shown great results in controlling the hatching of nematode eggs. Gastrointestinal infection of ruminant with nematodes affect badly their growth and is of concern to farmers, especially, in poorer area where the control by chemicals is not affordable. Having a natural and cheap plant extracts that might help in controlling nematodes is of major importance (Sylvester *et al.*, 2014). Another study conducted earlier has shown the activity of the acetone leaves extract of *P. juliflora* against the mosquito larvae (*Anopheles stephensi*), this mosquito is an important vector of malaria and its control by regular pesticides is causing air pollution, the results were very promising in terms of finding a biological mosquito controller (Senthilkumar *et al.*, 2009).

Greenhouse gases production is a major environmental concern nowadays, methane (CH_4) is one of the concerning gases that are naturally and continuously released to the atmosphere during the digestive fermentation of ruminants. A novel assay has shown that the alkaloids extracts of *P. juliflora* can significantly decrease the methane production from wheat bran, which add one more value for those plants extracts (de Jesus Pereira *et al.*, 2017).

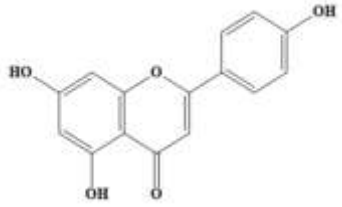
Overall, *P. juliflora* rich chemical composition makes it a strong candidate not only for the food industry but also for the cosmetic, agricultural and pharmaceutical

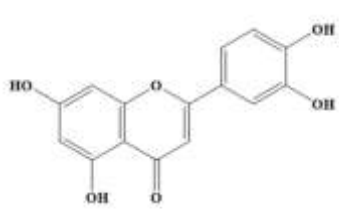
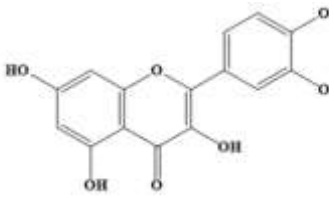
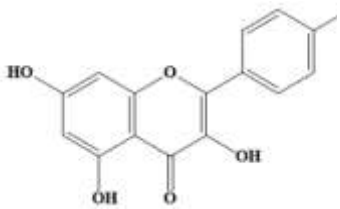
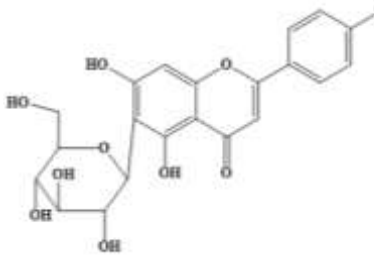
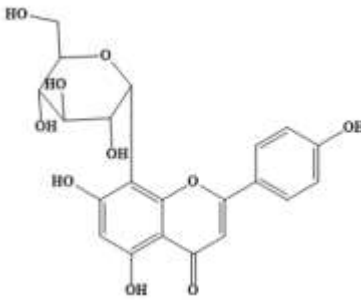
industries. Being a valuable source of phenolic compound and knowing the power of those phenolic compounds in scavenging reactive oxygen species (ROS) makes *P. juliflora* extracts capable of initiating multiple antioxidative mechanisms which might slow down skin aging process, and can act as anti-carcinogenic, anti-inflammatory, anti-allergic and antimicrobial agent (de Brito Damasceno *et al.*, 2018).

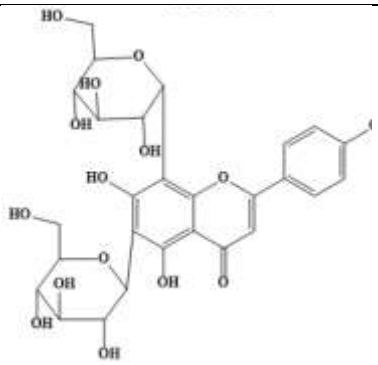
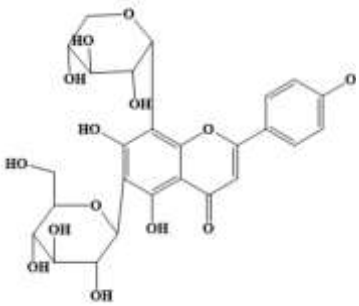
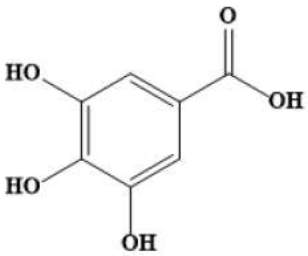
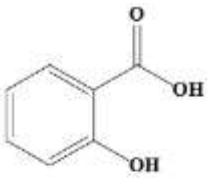
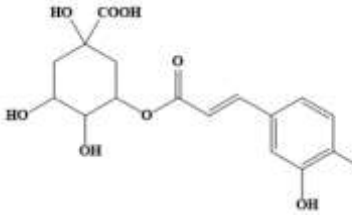
2.2.4. Prosopis juliflora chemical composition

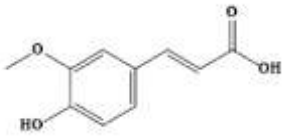
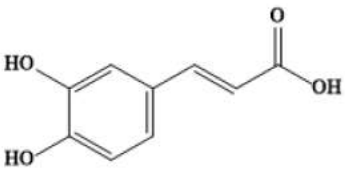
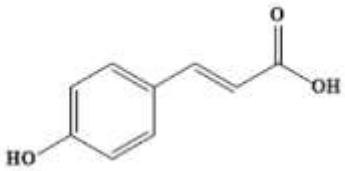
Prosopis juliflora contains many phytochemicals with pharmaceutical properties. Leaf extracts screening in previous works revealed the presence of different phenolic compounds including: tannins, acids, glycosides, flavonoids and alkaloids including piperidine alkaloids (Sathiya & Muthuchelian, 2008; Raghavendra *et al.*, 2009). The main piperidine alkaloids purified from the different parts of *P. juliflora* are named after it, this includes: julifloridine, N-methyl julifloridine, juliprosopine (juliflorine), julifloricine, juliprosine, 3′-oxo-juliprosopine, secojuliprosopinal, 3-oxo-juliprosine, juliprosinene and mesquitol (da Silva *et al.*, 2018; de Brito Damasceno *et al.*, 2018). Tables 9 and 10 summarizes the characteristics of the main phytochemicals identified in *Prosopis species* and in *P. juliflora*, respectively.

Table 9. Main characteristics of different phytochemicals identified in various *Prosopis species* (Sharifi-Rad et al., 2019).

Name	Structure	Molecular weight	Type	Characteristics
Apigenin		270.240	Flavonoid	Yellow $C_{15}H_{10}O_5$

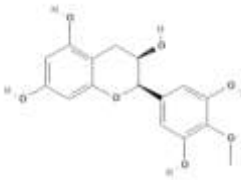
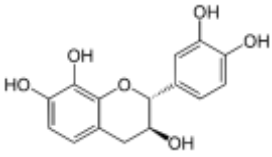
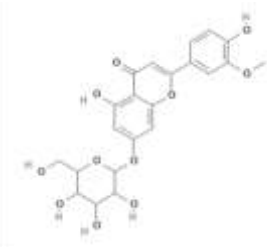
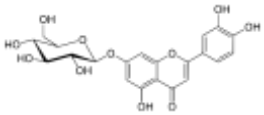
Name	Structure	Molecular weight	Type	Characteristics
Luteolin		286.24	Flavonoid	Yellow $C_{15}H_{10}O_6$
Quercetin		302.23	Flavonoid	Insoluble $C_{15}H_{10}O_7$
Kaempferol		286.23	Flavonoid	slightly soluble Yellow $C_{15}H_{10}O_6$
Isovitexin		432.40		$C_{21}H_{20}O_{10}$
Vitexin		432.38	flavonoid C-glycoside	Light yellow $C_{21}H_{20}O_{10}$

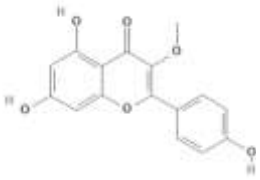
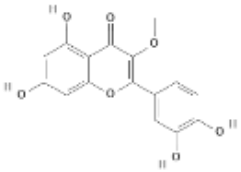
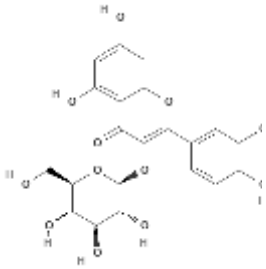
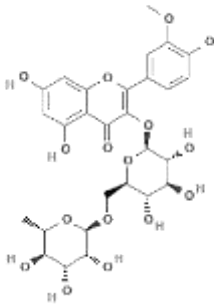
Name	Structure	Molecular weight	Type	Characteristics
Vicenin II		594.50	flavonoid C-glycoside	$C_{27}H_{30}O_{15}$
Schaftoside		564.49	flavonoid C-glycoside	$C_{26}H_{28}O_{14}$
Gallic acid		170.12	Phenolic acids	$C_7H_6O_5$ Solubility in water: 1.19 g/100 mL
Hydroxibenzoic acid		138.12	Phenolic acids	$C_7H_6O_3$ Solubility in water: 0.5 g/100 mL
Chlorogenic acid		354.31	Phenolic acids	$C_{16}H_{18}O_9$

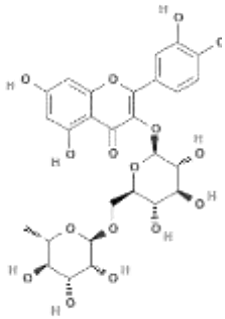
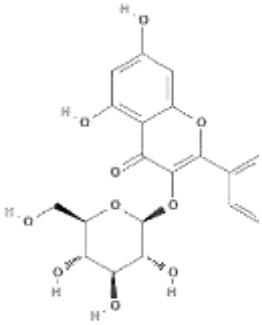
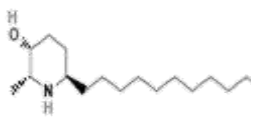
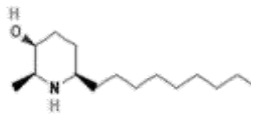
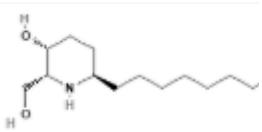
Name	Structure	Molecular weight	Type	Characteristics
Ferulic acid		194.18	Phenolic acids	C10H10O4 Solubility in water: 0.78 kg/m³
Caffeic acid		180.16	Phenolic acids	$C_9H_8O_4$
Coumaric acid		164.04	Phenolic acids	$C_9H_8O_3$

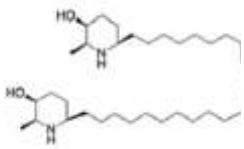
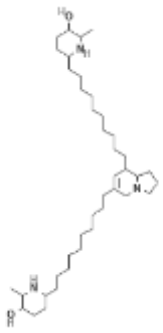
The main piperidine alkaloids purified from the different parts of *P. juliflora* are named after it, this includes: julifloridine, N-methyl julifloridine, juliprosopine (juliflorine), julifloricine, juliprosine, 3′-oxo-juliprosopine, secojuliprosopinal, 3-oxo-juliprosine, juliprosinene and mesquitol. Many of those allelochemicals are water soluble. In addition to phenolic compounds, *P. juliflora* extracts exhibited the presence of polysaccharides such as galactomannas (with different ratios of galactose and mannose) and compounds containing arabinose and glucose (da Silva *et al.*, 2018; de Brito Damasceno *et al.*, 2018).

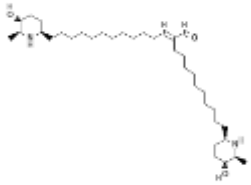
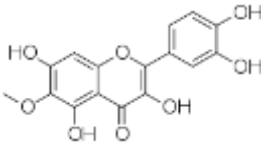
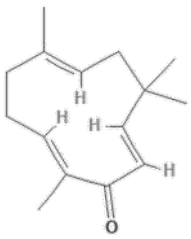
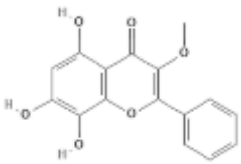
Table 10. Main characteristics of different phytochemicals identified in *P. juliflora*.

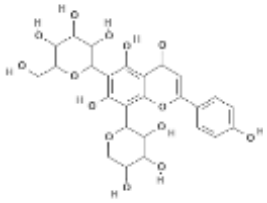
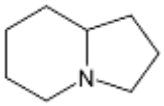
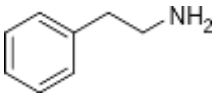
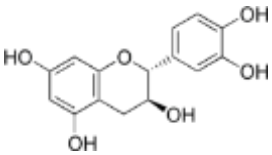
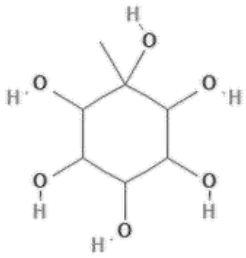
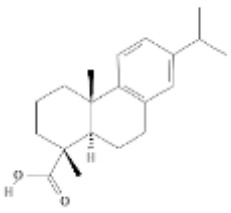
Name	Structure	Type & Molecular weight	Characteristics	References
4'-Methyl-epigallocatechin		Catechin 320.29	$C_{16}H_{16}O_7$	(Sharifi-Rad *et al.*, 2019)
Mesquitol		Flavonoid 290.26	$C_{15}H_{14}O_6$	(Sharifi-Rad *et al.*, 2019)
Apigenin-6,8-di-C-glycoside		594.50	$C_{27}H_{30}O_{15}$ same as Vicenin 2	(Sharifi-Rad *et al.*, 2019)
Chrysoeriol 7-O-glucoside		462.40	$C_{22}H_{22}O_{11}$	(Sharifi-Rad *et al.*, 2019)
Luteolin 7-O-glucoside		Glycosyloxy -flavone 448.40	$C_{21}H_{20}O_{11}$ Known as Cynaroside	(Sharifi-Rad *et al.*, 2019)

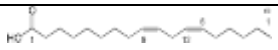
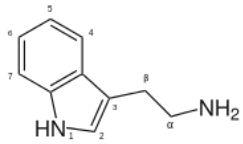
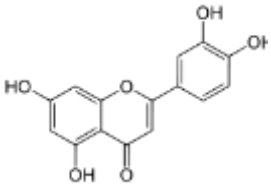
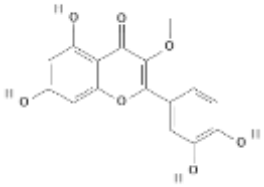
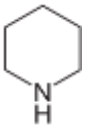
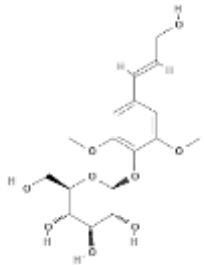
Name	Structure	Type & Molecular weight	Characteristics	References
Kaempferol 3-O-methyl ether		Apigenin 300.26	Known as Isokaempferide $C_{16}H_{12}O_6$	(Sharifi-Rad *et al.*, 2019)
Quercitin 3-O-methyl ether		Flavonoid 316.26	$C_{16}H_{12}O_7$	(Sharifi-Rad *et al.*, 2019)
Isorhamnetin-*3-O-glucoside*		478.40	$C_{22}H_{22}O_{12}$	(Sharifi-Rad *et al.*, 2019)
Isorhamnetin-*3-O-rutinoside*		624.50	Known as Narcissin $C_{28}H_{32}O_{16}$	(Sharifi-Rad *et al.*, 2019)

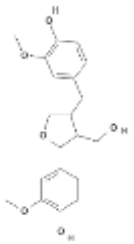
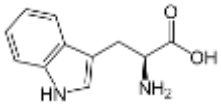
Name	Structure	Type & Molecular weight	Characteristics	References
Quercitin 3-O-rutinoside		610.50	Known as Rutin $C_{27}H_{30}O_{16}$	(Sharifi-Rad *et al.*, 2019)
Quercetin-3-glucoside		Flavonoid 463.40	Known as Isoquercetin $C_{21}H_{19}O_{12}$	(Sharifi-Rad *et al.*, 2019)
Julifloridine		Alkaloid 299.50	$C_{18}H_{37}NO_2$	(Henciya *et al.*, 2017)
N-methyl julifloridine		Alkaloid 313.75	$C_{19}H_{39}NO_2$	(Henciya *et al.*, 2017)
Prosafrinine		Alkaloid 297.50	$C_{18}H_{36}NO_2$	(Henciya *et al.*, 2017)
Prosopinine		Alkaloid 287.44	$C_{16}H_{33}NO_2$	(Henciya *et al.*, 2017)

Name	Structure	Type & Molecular weight	Characteristics	References
3″-Oxo-juliprosopine		Alkaloid 644.56	$C_{40}H_{74}N_3O_3$	(Henciya *et al.*, 2017)
3-Oxo-juliprosine		Alkaloid 625.55	$C_{40}H_{71}N_3O_2$	(Henciya *et al.*, 2017)
Juliprosinine		Alkaloid 626.55	$C_{40}H_{72}N_3O_2$	(Henciya *et al.*, 2017)
Prosoflorine		Alkaloid 627.32	$C_{40}H_{73}N_3O_2$	(Henciya *et al.*, 2017)
Juliprosine		Alkaloid 628.55	$C_{40}H_{72}N_3O_2$	(Henciya *et al.*, 2017)
Juliprosopine		Alkaloid 630.59	$C_{40}H_{76}N_3O_2$ Known as juliflorine	(Henciya *et al.*, 2017)
Julifloricine		Alkaloid 630.00	$C_{40}H_{75}N_3O_2$	(Henciya *et al.*, 2017)

Name	Structure	Type & Molecular weight	Characteristics	References
Secojuliprosopinal		Alkaloid 576.90	$C_{36}H_{68}N_2O_3$	(Henciya *et al.*, 2017)
Juliprosinene		Alkaloid 646.40	$C_{39}H_{68}N_3O_2$	(Henciya *et al.*, 2017)
Patuletin		Alkaloid 332.26	$C_{16}H_{12}O_8$	(Henciya *et al.*, 2017)
Zerumbone		Alkaloid 218.33	$C_{15}H_{22}O$	(Henciya *et al.*, 2017)
5,7,8-Trihydroxy-3-methoxyflavone		Alkaloid 300.26	$C_{16}H_{12}O_6$	(Henciya *et al.*, 2017)

Name	Structure	Type & Molecular weight	Characteristics	References
Schaftoside		Alkaloid 564.50	$C_{26}H_{28}O_{14}$	(Henciya *et al.*, 2017)
Indolizidine		Alkaloid 124.21	$C_8H_{15}N$	(Henciya *et al.*, 2017)
Phenethylamin		Alkaloid 121.18	$C_8H_{11}N$	(Henciya *et al.*, 2017)
Catechin		Flavonoid 290.26	$C_{15}H_{14}O_6$	(Henciya *et al.*, 2017)
Myo-Inositol, 4-C-methyl		194.18	Known as Laminitol $C_7H_{14}O_6$	(Henciya *et al.*, 2017)
Dehydroabietic acid		300.40	$C_{20}H_{28}O_2$	(Henciya *et al.*, 2017)

Name	Structure	Type & Molecular weight	Characteristics	References
Linoleic acid		Fatty acid 280.44	$C_{18}H_{32}O_2$	(Henciya *et al.*, 2017)
Tryptamine		Alkaloid 160.20	$C_{10}H_{12}N_2$	(Sirmah, 2018)
Luteolin		Flavonoid 286.23	$C_{15}H_{10}O_6$	(Dave & Bhandari, 2013)
Quercetin 3-O-methyl ether		Flavonoid 316.26	$C_{16}H_{12}O_7$	(Dave & Bhandari, 2013)
Piperidine		Amine 85.15	$C_5H_{11}N$	(Sirmah, 2018)
Syringin		Monosaccharide 372.40	$C_{17}H_{24}O_9$	(de Brito Damasceno *et al.*, 2018)

Name	Structure	Type & Molecular weight	Characteristics	References
Lariciresinol		Ligan 360.40	$C_{20}H_{24}O_6$	(de Brito Damasceno *et al.*, 2018)
Tryptophan		Amino acid 204.23	$C_{11}H_{12}N_2O_2$	(de Brito Damasceno *et al.*, 2018)

2.3. QATAR FRUITS AND VEGETABLES EXPENSES

Qatar population increased from 373,395 in 1986 to 2.7millions in 2020 (Planning and Statistics Authority Qatar). Table 11 shows the increase in fruits and vegetables import in Qatar according to the population change.

Qatar imports a large amount of its fruits consumption and most of its vegetables needs. Budget spent of fruits and vegetables import has shown a significant increase in the last decade due to the increase in population. The percent increase in population in Qatar between 2008 and 2020 is 92.8%, this increase together with the increase in fresh produces prices lead to a rise in both the amounts imported and the total cost. The drop in the amounts of fruits and vegetables imported to Qatar in 2017 can be explained by the blockage; however, numbers went back to a normal increase by 2018. Data shows that imported fruits have costed 225.8% more money in 2020 than in 2008, as Qatar will continue to import many of its fruits' consumption, it is vital to come up with a

coating material that extend fruits' shelf-life until consumed (Planning and Statistics Authority Qatar).

Table 11. Amounts and economical values of imported fruits and vegetable to Qatar during the last decade (Planning and Statistics Authority Qatar).

Year	Population in MM	Fruits		Vegetables	
		Weight (Tons)	Value (MM QAR)	Weight (Tons)	Value (MM QAR)
2008	1.4	102,422	344	205,400	404
2009	1.6	118,767	315	224,950	437
2010	1.7	130,833	359	260,989	508
2011	1.7	128,753	371	278,279	613
2012	1.8	134,064	453	274,849	663
2013	2.0	139,198	519	294,758	770
2014	2.2	160,237	595	334,755	881
2015	2.4	184,485	711	379,690	1,033
2016	2.6	209,528	853	415,654	998
2017	2.7	189,212	905	349,113	936
2018	2.8	241,712	1,089	403,329	1,000
2019	2.8	262,523	1,084	411,674	841
2020	2.7	260,401	1,121	402,995	871

The difference between the increase of vegetables and fruits demands are due to the development of the agricultural domain in Qatar, especially from 2017 onward, local

agriculture focuses more on the production of vegetables which lower the demands for imported vegetables. The total amount of money spent on imported fruits and vegetables in Qatar in 2020 is 1,992 MM QR (Planning and Statistics Authority Qatar). This number indicates that any losses due to spoilage cost the country millions that would have been saved by the development of a safe formulation to increase fruits and vegetables shelf-life.

In this study, *in-vivo* work focused on extending the shelf-life of chosen commodities including the two main vegetables that are locally grown which are cucumbers and tomatoes, and exotic imported fruits that are sensitive to post-harvest diseases including mango and strawberries, preserving lemons was also evaluated (Table 12).

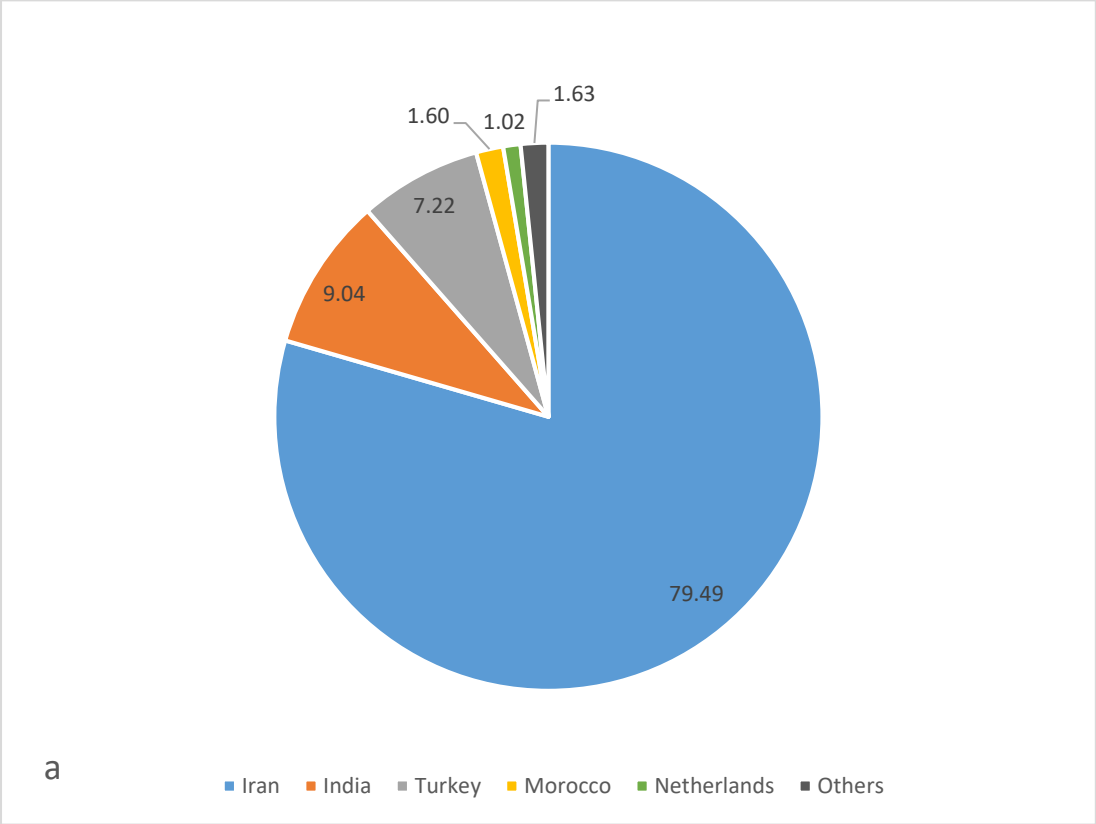
Table 12. Qatar imported cucumber, tomato, strawberry, mango and citrus fruits amounts and economical values per year (Planning and Statistics Authority Qatar).

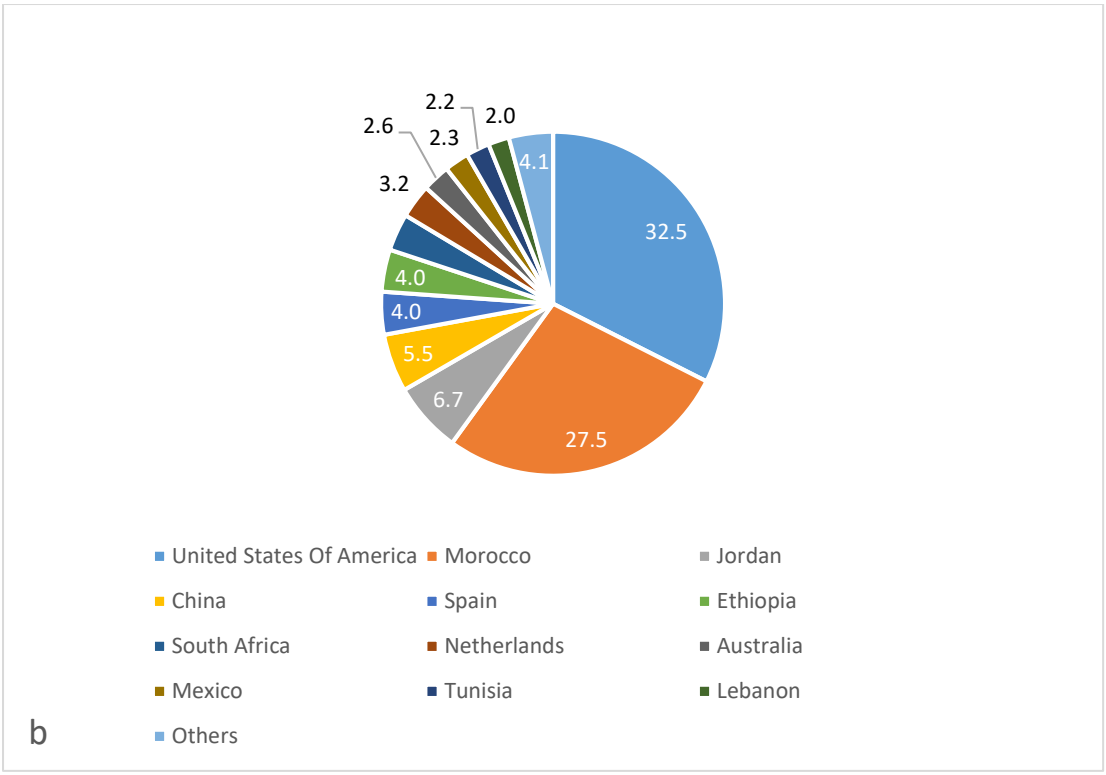
Year	Cucumber		Tomato		Strawberry		Mango		Citrus fruits	
	Weight (tons)	Value (MM QR)	Weight (tons)	Value (MM QR)	Weight (tons)	Value (MM QR)	Weight (tons)	Value (MM QR)	Weight (tons)	Value (MM QR)
2015	9.8	11.3	52.4	102.6	1.1	19.8	6.0	29.8	53.7	127.3
2016	11.5	15.8	57.3	116.2	1.5	29.1	8.5	44.2	58.1	141.6
2017	7.1	14.9	33.8	103.6	1.9	42.3	7.7	45.5	56.0	167.6
2018	14.1	19.7	48.9	107.0	1.8	37.9	7.8	51.6	63.0	181.4
2019	12.3	19.3	52.4	96.6	2.4	49.6	9.4	59.4	67.9	186.3
2020	12.6	20.5	55.8	91.5	2.5	54.1	10.8	70.8	66.1	197.3

Qatar has imported around 66.1tons of citrus fruits including lemons and oranges in 2020. The total cost of the imported amount is 197.3 MM QR, which makes 9.9% of

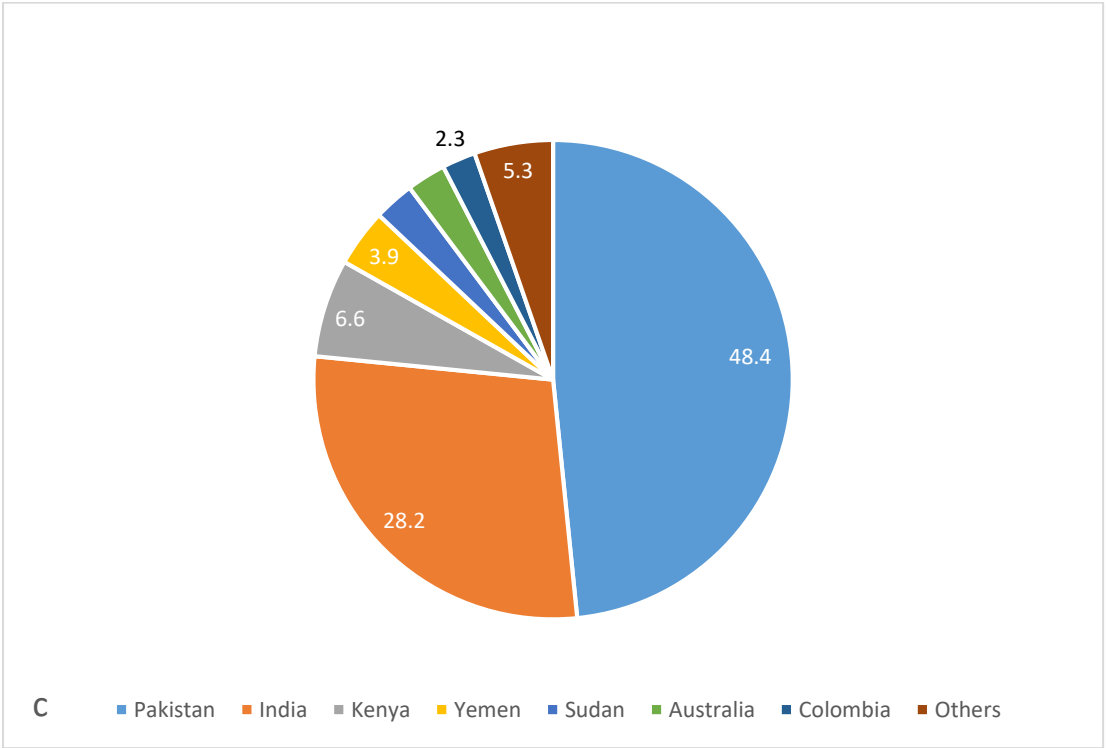
the total budget spent on fruits and vegetables, this makes citrus fruits one of the largest imported categories. Tomatoes, strawberries, and mangoes imports costs in 2020 made 4.6%, 2.7% and 3.5% of the total budget spent on fruits and vegetables respectively. The spoilage by any percentage of the imported amounts cost the country millions. Even locally grown commodities should be protected from postharvest diseases especially with the high costs of vegetables growth in Qatar where water and appropriate growing conditions for agriculture are lacking.

Qatar has imported 90.6% of its cucumbers from Iran in 2020. While other commodities were imported from different countries, figure 1 shows the main countries from which, tomatoes, strawberries, mangoes and citrus fruits were imported in 2020 (Planning and Statistics Authority Qatar).




2.2
2.0
2.6
2.3
4.1
3.2
32.5
4.0
4.0
5.5
6.7
27.5
United States Of America
Morocco
Jordan
China
Spain
Ethiopia
South Africa
Netherlands
Australia
Mexico
Tunisia
Lebanon
Others

b


2.3
5.3
3.9
6.6
48.4
28.2
Pakistan
India
Kenya
Yemen
Sudan
Australia
Colombia
Others

c

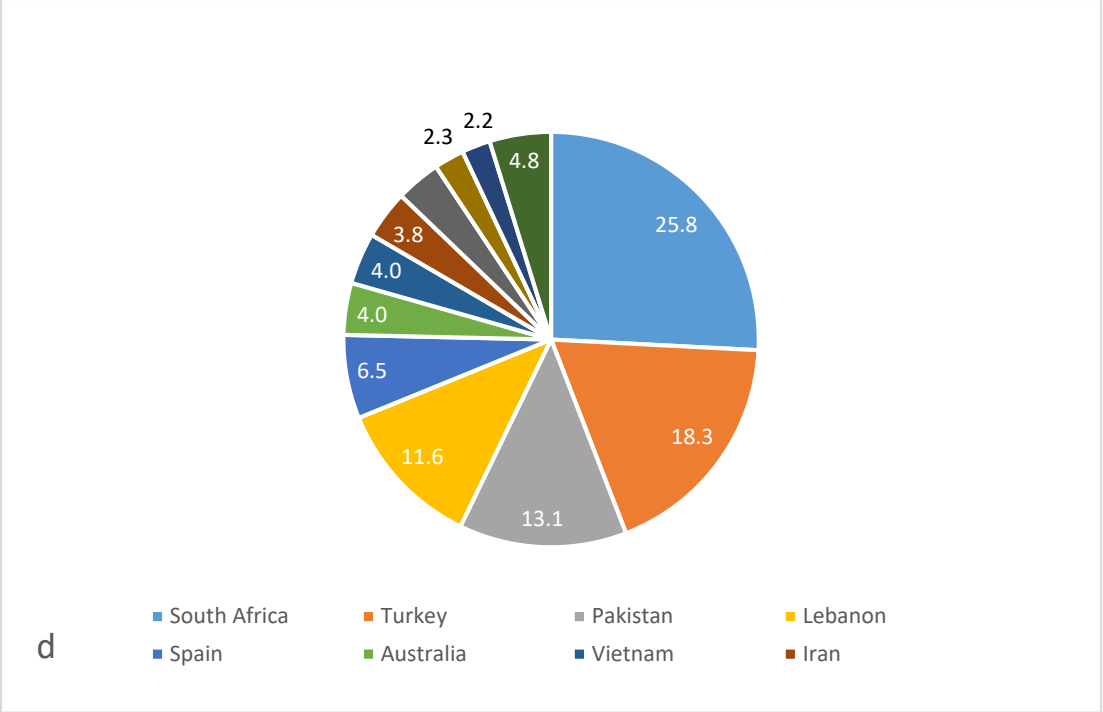


Figure 1. The main countries from which Qatar has imported tomatoes (a), strawberries (b), mangoes (c) and citrus fruits (d) in 2020 (Planning and Statistics Authority Qatar).

2.4. RIBOTYPING AS MOLECULAR IDENTIFICATION METHOD

Ribotyping is the molecular techniques used to identify plants, fungi and bacteria at the molecular level using the DNA sequences of species-specific conserved regions of the ribosomal RNA (Bouchet *et al.*, 2008). The workflow adopted in this project to identify fungal species started with PCR amplification by universal primers (ITS1/ITS4) followed by DNA sequencing of the conserved internal transcribed spacer (ITS1-5.8S-ITS2 rRNA gene cluster). Analysis of representative sequences was conducted by comparing them to NCBI database.

PCR of conserved regions followed by sequencing and BLAST analysis have been widely used in the confirmation of the species of various species. A successful classification has occurred with *Suidasia medanensis* house dust in Malaysia recently

(Ernieenor *et al.*, 2018). The same sequence of steps has been used with universal primers to monitor the specificity of a fungal strain known as *Clonostachys rosea*, this fungus is used as in the environment as a bio-controller against harmful strains of *Fusarium* (Demissie *et al.*, 2019). *Colletotrichum* is the main spoiling agent of mango around the world causing symptoms known as post-harvest anthracnose. A study using the ITS1/ITS4 primers has successfully classified the isolated fungi after BLAST analysis as *C. karstii* (Lima *et al.*, 2017). The same set of ITS primers has been used in many studies for fungi classification, *B. cinerea* isolated from different fruits samples in Turkey has been molecularly identified using the same technique (Polat *et al.*, 2018). This indicate the accuracy of the chosen methods for various types of fungi. In this research the same set of primers have been used to identify the DNA fingerprint of the *P. juliflora* tree used to collect samples from. This approach is known worldwide as the internal transcribed spacer (ITS) of the nuclear ribosomal DNA is one of the most commonly used DNA markers in plant phylogenetic and DNA barcoding analyses (Cheng *et al.*, 2015).

2.5. CHARACTERISTICS OF FRUITS EVALUATED IN *IN-VIVO* EXPERIMENTS

When the ovaries of flowering plants get mature, they form fruits. Fruits are usually consumed raw; among them, we have produces that are not usually known as fruits such as wheat, corn, beans and tomatoes. The incidence of several chronic diseases such as cardiovascular diseases, obesity, infections, neurological diseases and cancer can be lowered by following a daily diet rich with fruits and vegetables (Mailafia *et al.*, 2017). When *in-vitro* effective *P. juliflora* extract was tested *in-vivo*, parameters tested included efficacy to extend fresh produces shelf-life, efficacy in lowering disease incidence in controlled experiments and usefulness in extending storage period, and

efficacy in conserving fruits storage parameters. Chosen fruits are: tomato, cucumber, strawberry, mango and lemon.

Different spoilage microorganisms have different nutrients requirements, therefore the various compositions of fruits and vegetables allows their contamination with various microorganisms. Cucumbers and tomatoes are produced in large amounts in the region, and they are among the highly consumed vegetables in the Middle East, therefore, it is of major importance to control their spoilage and to extend their shelf-life periods. Mangoes and strawberries are among the expensive imported fruits that can be easily spoiled, which was the reason behind choosing them in our *in-vivo* analysis. Lemon were also studied because they are imported and stored for long periods of time in countries where they cannot be grown.

2.5.1. Cucumber

Cucurbits are members of Cucurbitaceae include zucchini, melon, squash and cucumber. Many microbial diseases can affect these products. Table 13 summarizes the various diseases that may affect these groups of plants, the causative agents, and the main symptoms. It is important to always store vegetables of this family at a temperature of about 10˚C, because storage at lower temperatures would cause them chilling injuries and eventually increases their chances of spoilage (Tournas, 2005; Mondal *et al.*, 2020).

The types of diseases that might affect fruits and vegetables vary according to their composition, table 14 summarizes the main nutrients in cucumber according to the United States Department of Agriculture (USDA, 2020a).

Table 13**.** The various diseases affecting the cucurbits family and the causal microorganisms (Tournas, 2005).

Disease	Spoiling agent	Symptoms
Rhizopus soft rot	*Rhizopus spp.*	Hard mycelium with dark sporangial structure
Cottony leak	*Pythium spp.*	Watery dark green lesions
Alternaria rot	*Alternaria spp.*	Spots of creamy color with gray mycelium
Anthracnose	*Colletotrichum spp.*	Watery lesions with pinkish mass of spores
Scab	*Cladosporium spp.*	Dark lesion with gummy excretion
Black rot	*Mycosphaerella spp.*	Greenish brown watery lesions that gets rigid and black

Table 14. Nutrients in 100g of raw cucumber with peel (USDA, 2018).

Nutrient	Unit	Value per 100 g
Water	g	95.23
Energy	kcal	15
Protein	g	0.65
Total lipid (fat)	g	0.11
Carbohydrate, by difference	g	3.63
Fiber, total dietary	g	0.5
Sugars, total	g	1.67
Calcium, Ca	mg	16

Nutrient	Unit	Value per 100 g
Iron, Fe	mg	0.28
Magnesium, Mg	mg	13
Phosphorus, P	mg	24
Potassium, K	mg	147
Sodium, Na	mg	2
Zinc, Zn	mg	0.2
Vitamin C, total ascorbic acid	mg	2.8
Thiamin	mg	0.027
Riboflavin	mg	0.033
Niacin	mg	0.098
Vitamin B-6	mg	0.04
Folate, DFE	µg	7
Vitamin A, RAE	µg	5
Vitamin A, IU	IU	105
Vitamin E (alpha-tocopherol)	mg	0.03
Vitamin K (phylloquinone)	µg	16.4
Fatty acids, total saturated	g	0.037
Fatty acids (monounsaturated)	g	0.005
Fatty acids, total polyunsaturated	g	0.032

2.5.2. Tomatoes

Tomatoes or *Lycopersicon esculentum* are a part of the human diet worldwide, it is the most common consumed vegetable in the Mediterranean region. Tomatoes are used in the manufacturing of tomatoes derived products such as ketchup. The fruit

acquire its red color by lycopene synthesis during ripening, tomatoes ripening is the process during which chlorophylls are degraded from the fruit and acids, sugars (glucose, fructose, sucrose), and carotenoids are formed (Guil-Guerrero & Rebolloso-Fuentes, 2009; Rivero Meza *et al.*, 2021). Table 15 summarizes the most common fungal diseases that may affect tomatoes.

Table 15**.** The various diseases affecting tomatoes and the causal microorganisms (Tournas, 2005).

Disease	Spoiling agent	Symptoms
Rhizopus soft rot	*Rhizopus spp.*	Watery texture, fermented smell, white mycelium, dark spots of spores.
Sour rot	*Geotrichum spp.*	Greenish gray lesions and sour smell
Alternaria rot	*Alternaria spp.*	Dark firm lesions
Cladosporium rot	*Cladosporium spp.*	Wounds
Gray mold rot	*Botrytis spp.*	Wound

The types of diseases that might affect fruits and vegetables vary according to their composition. Nowadays, there is a large variety of tomato types that have various compositions, the composition of the tomato depends on the cultivation method and region as well as the sampling period. In general different types of tomatoes contain: protein, lipids, glucose, fructose, sucrose, vitamin C, carotenoids (carotene, lycopene, and neurosporene), minerals (sodium, potassium, calcium, magnesium, phosphorus, sulfur, manganese, iron, copper, zinc, and selenium), nitrate, and oxalic acid (Rivero Meza *et al.*, 2021).

2.5.3. *Strawberry*

Fragaria X ananassa or strawberries are widely consumed raw berries, they are also very famous in their processed forms such as jam and juices. *Botrytis* is among the most common fungal pathogens that affect strawberries and cause a disease called grey mold (Tomas-Grau *et al.,* 2020). Fungal diseases that affect strawberries are summarized in table 16.

Table 16**.** The various diseases affecting strawberries and the causal microorganisms (Abdelfattah *et al.,* 2016).

Disease	Spoiling agent	Symptoms
Grey mold	*Botrytis cinerea*	Brown spots with grey cotton like growth
Powdery mildew	*Podosphaera aphanis*	White patches in the leaves, fruits fail to ripen.
Anthracnose	*Colletotrichum acutatum*	Watery lesions.
Blackspot	*Rhizoctonia spp.*	Hard brown spot, leaf blight, web blight, and fruit rot.
Verticillium wilt	*Verticillium dahliae*	Sudden death of plant
Blossom blight	*Cladosporium spp.*	Black rot and necrosis of flower.
Fusarium wilt	*Fusarium spp.*	Sudden death of plant
Rhizopus rot	*Rhizopus spp.*	Watery fruit covered with white mycelium and black spores.

Table 17. Nutrients in 100g of fresh, raw strawberries (USDA, 2020b).

Nutrient	Unit	value per 100 g
Water	g	90.95
Energy	kcal	32
Protein	g	0.67
Ash	g	0.4
Total lipid	g	0.3
Carbohydrate	g	7.68
Dietary fiber	g	2
Sugars	g	4.89
Sucrose	g	0.47
Glucose	g	1.99
Fructose	g	2.44
Calcium	mg	16
Iron	mg	0.41
Magnesium	mg	13
Phosphorus	mg	24
Potassium	mg	153
Sodium	mg	1
Zinc	mg	0.14
Copper	mg	0.048
Manganese	mg	0.386
Selenium	µg	0.4
Vitamin C	mg	58.8

Nutrient	Unit	value per 100 g
Thiamin	mg	0.024
Riboflavin	mg	0.022
Niacin	mg	0.386
Pantothenic acid	mg	0.125
Vitamin B6	mg	0.047
Folate	µg	24
Choline	mg	5.7
Betaine	mg	0.2
Vitamin B12	µg	0
Vitamin A, RAE	µg	1
Lutein + zeaxanthin	µg	26
Vitamin E, α-tocopherol	mg	0.29
β-tocopherol	mg	0.01
γ-tocopherol	mg	0.08
δ-tocopherol	mg	0.01
Vitamin K, phylloquinone	µg	2.2

It is important to understand the nutritional composition of each of the studied fruits to understand the diversity of infectious fungi. Strawberries are rich with antioxidants such as: vitamin C, folate and phenolic constitutes. Table 17 summarizes the nutritional composition of fresh strawberries. This fruit contains also non-nutritive phytochemicals including: flavonoids, phenolic acids, tannins, and lignins (Giampieri *et al.*, 2012).

2.5.4. Mango

Mango (*Mangifera indica*) is a tropical fruit rich with fibers, vitamin C, vitamin A, polyphenols, carotenoids, carbohydrates, and calcium. Various types of mangoes have wide range of nutritional facts, and some are more rich with carbohydrates and other nutrients than others. India is the largest producer of mangoes in the world (Jha *et al.*, 2010). Global production of mango has reached around 50 million tons in 2016 (Zahedi *et al.*, 2019). Mangoes are eaten at all ripening stages and used in a wide range of cuisine. However, mango availability in the market is negatively affected because of its susceptibility to postharvest diseases and to chill injuries. The short shelf life of mangoes increases its prices in markets that depends totally on imported fruits (Shah & Hashmi, 2020). Due to their nutritional composition, mangoes are prone to various bacterial and fungal diseases (Table 18).

Table 18. The various diseases affecting mangoes and the causal microorganisms (Đinh, 2002).

Disease	Pathogen	Time of infection
Alternaria rot	*Alternaria alternata*	Postharvest
Anthracnose	*Colletotrichum gloeosporioides*	Preharvest
Bacterial black spot	*Xanthomonas campestris*	Preharvest
Bacterial soft rot	*Erwinia* spp.	Postharvest
Black mould rot	*Aspergillus niger*	Postharvest
Blue mould	*Penicillium* spp.	Postharvest
Grey mould	*Botrytis cinerea*	Postharvest
Mucor rot	*Mucor* spp.	Postharvest

Disease	Pathogen	Time of infection
Phylosticta rot	*Guignardia mangiferae*	Preharvest
Powdery mildew	*Oidium mangiferae*	Preharvest
Scab	*Elsinoe mangiferae*	Preharvest
Soft brown rot	*Hendersoria creberrima*	Postharvest
Sooty mould	*Capnodium* spp. *and Meliola* spp.	Preharvest
Stem end rot	*Botryodiplodia theobromae; C. gloeosporioides; Dothiorella* spp.	Preharvest & postharvest
Stemphylium rot	*Stemphylium vesicarium*	Postharvest
Transit rot	*Rhizopus* spp.	Postharvest

2.5.5. Lemon

Citrus limon or lemon fruits are rich with contents of health-promoting properties, including anti-inflammatory and antioxidant phytochemicals. Both the pulp and the peel of lemons are rich in vitamin C (ascorbic acid), polyphenols (narirutin, diosmin, phlorin, etc.) and carotenoids (Zeta carotene, beta-carotene and alpha-carotene). Table 19 shows the detailed composition of lemon fruit. Shelf-life of lemons at low temperature is between 6 and 8 weeks (Naeem *et al.*, 2019). The most occurring diseases that affect citrus fruits in general and lemons in particular are green rot caused by *Penicillium digitatum* and blue mold caused by *Penicillium italicum*, In addition to sour rot caused by *Geotrichum citri-aurantii* (Martínez-Blay *et al.*, 2020). Other types of fungi can occasionally infect lemons and cause postharvest diseases.

Table 19. Nutrients in 100g of fresh, raw lemon fruits (USDA, 2016).

Nutrient	Unit	value per 100 g
Water	g	88.98
Energy	kcal	29
Protein	g	1.1
Total lipid	g	0.3
Carbohydrate	g	9.32
Dietary fiber	g	2.8
Sugars	g	2.5
Calcium	mg	26
Iron	mg	0.6
Magnesium	mg	8
Phosphorus	mg	16
Potassium	mg	138
Sodium	mg	2
Zinc	mg	0.06
Copper	mg	0.036
Selenium	µg	0.4
Vitamin C	mg	53
Thiamin	mg	0.04
Riboflavin	mg	0.02
Niacin	mg	0.1
Vitamin B6	mg	0.08
Folate	µg	11

Nutrient	Unit	value per 100 g
Choline	mg	5.1
Vitamin B12	µg	0
Vitamin A, RAE	µg	1
Carotene, beta	µg	3
Carotene, alpha	µg	1
Cryptoxanthin, beta	µg	20
Lutein + zeaxanthin	µg	11
Vitamin E	mg	0.15
Fatty acids, total saturated	g	0.039

2.6. USAGE OF CHITOSAN IN FRUITS COATING

Chitosan (poly β-(1-4)N-acetyl-d-glucosamine) is a non-toxic biodegradable polymer derived from shrimp shells and other sea crustaceans and it has known fungicidal properties. Chitosan is widely applied as coating material to prevent postharvest fruit decay (Priyadarshi & Rhim, 2020). The antifungal effect of chitosan is related to its de-acetylation level (Sharif *et al.*, 2018). Fungal strains inhibited by chitosan shows various inhibition modes of action. Previous studies described morphological changes induced by chitosan on fungal hyphae and spores. The antimicrobial activity of chitosan depends on the interactions between the positive charges of the polymer and the negative charges of the bacterial and fungal plasma membranes. Studied antifungal mechanisms showed that chitosan binds first to the target surface membrane to cover it and when the threshold concentration is reached, chitosan modifies the permeability of the fungal membrane to release cellular contents which leads to death (Troncoso-Rojas & Tiznado-Hernández, 2014).

The antifungal activity of chitosan has been tested against several microorganisms. Sharif *et al*. (2018) has summarized some of the case studies during which chitosan was successfully used as an antifungal agent (Table 20).

Table 20. Chitosan usage as antifungal agent against different pathogenic fungi in different crops (Sharif *et al.*, 2018).

Crop	Chitosan Concentration	Pathogen/Pest	Mode of chitosan Application
Banana	1.0% (*w*/*v*)	*Anthracnose*	*In-vivo*
Carrots	2 or 4% (*w*/*v*)	*Sclerotinia sclerotiorum*	*In-vitro*
Cucumber	0.2 g L^{-1}	*Botrytis cinerea*	Foliar spray
Cucumber	2% (*w*/*v*)	*Sphaerotheca fuliginea*	Petri dish treatment
Chili pepper	0.32% (*w*/*v*)	*Colletotrichum capsici*	*In-vivo*
Eggplant	20 mL	*Ralstonia solanacearum*	Cotton leaf disk elicitation method
Mango	1% (*w*/*v*)	*Colletotrichum gloeosporioides*	Post-harvest coating
Orange	2% (*w*/*v*)	*Penicillium italicum* and *Penicillium digitatum*	Post-harvest coating
Pear	25 g/L	*A. kikuchiana* and *P. piricola*	Post-harvest treatment
Papaya	1.5% (*w*/*v*)	*C. gloeosporioides*	*In-situ*
Palm	1 mg mL^{-1}	*F. oxysporum*	Soil inoculation

Crop	Chitosan Concentration	Pathogen/Pest	Mode of chitosan Application
Peach	0.5 g L^{-1}	*Monilinia fructicola*	Dipping in solution
Tomato	1 mg/mL	*Alternaria solani*	Foliar application
Tomato	0.1% (*w*/*v*)	*F. oxysporum f. sp. Lycopersici*	Foliar application

Food security in Qatar is one of the main objectives of the country's vision. Being able to extend fruits and vegetables shelf-life using the extracts of an available plant would protect the Qatari environment and lessen economical losses. The agricultural domain in Qatar is evolving, yet it is facing multiple difficulties including the climate conditions and the arid land nature. Therefore, providing the local fresh produce supply will always be costly. This project's main objective is coming up with a naturally based anti-spoilage formulation, which will boost the Qatari economy, enhance food security measures and solve a worldwide agricultural problem. The project is divided into four main objectives including the identification of an *in-vitro* active antimicrobial crude extract from *P. juliflora*, the exploration of the identified extract *in-vivo* on fruits and vegetables, studying the genetic variation of *P. juliflora* in Qatar, and fractionating the crude extract to identify biologically active fractions and their stability.

CHAPTER 3: MATERIALS AND METHODS

3.1. SCREENING THE ANTIMICROBIAL EFFECTIVENESS OF THE FRESH JUICE AND OF THE AQUEOUS AND ETHANOLIC EXTRACTS OF THE LEAVES AND PERICARPS OF *P. JULIFLORA*.

3.1.1. Food spoiling microbial isolates tested

For the *in-vitro* antimicrobial analysis, the effects of the six crude extracts prepared was tested against bacterial, fungal and yeast strains that are known to cause food contaminations. Fungal strains included *Penicillium* strains, *Fusarium, Alternaria, Botrytis*, *Cladosporium*, *Geotrichum*, *Colletotrichum*, *Aspergillus species*. Bacterial strains include: *Escherichia*, *Bacillus*, *Staphylococcus*, *Proteus*, and *Pseudomonas*. The antimicrobial activity of the crude extracts was also tested against *C. albicans*.

3.1.2. Fungal strains isolation and identification

Prosopis extracts were tested for their antifungal activity against species that were isolated and microscopically identified during a previous study (Table 21) (Saleh & Al-Thani, 2019).

Table 21**.** Fungal strains used in the study and their origins.

	Fungi	Origin	Country of origin
1	*Alternaria*	Cucumber	Qatar
2	*Aspergillus*	Cucumber	Lebanon
3	*Botrytis*	Tomato	Lebanon
4	*Cladosporium*	Tomato	Morocco
5	*Colletotrichum*	Orange	Morocco

	Fungi	Origin	Country of origin
6	*Geotrichum*	Tomato	Netherland
7	*Fusarium*	Cucumber	Qatar
8	*Penicillium 1*	Orange	Australia
9	*Penicillium 2*	Cucumber	Qatar

3.1.3. Fungal strains molecular identification

3.1.3.1.DNA extraction

Fresh fungi cultures of 4 day-old were used to collect hyphae for DNA extraction. Hyphae of each fungal strain were aseptically collected by a spatula into a sterile mortar previously cooled at -20˚C. Liquid nitrogen was added to freeze dry the hyphae upon immediate crashing into a fine powder that was transferred into a sterile eppendorf tube to extract the DNA according to the kit manufacturer instructions (DNeasy Plant Mini Kit-QIAGEN-USA).

Four hundred micro liter of buffer AP1 and 4µl of RNase A were added to each tube then tubes were vortexed and incubated for 20min at 65°C. Tubes were invert every five minutes during incubation. After incubation, 130µl of buffer P3 was added to each tube and tubes were incubated in ice for 5min. Tubes were then centrifuged for 5 min at 20,000xg. Lysates were pipetted into a QIAshredder spin columns placed in a 2ml collection tubes and centrifuged for 2min at 20,000xg. Flow-through of each column was transferred into a new tube without disturbing pellets. One point five volumes of Buffer AW1 was added to each tube and mixed by pipetting. Then 650µl of the mixture was transferred to a DNeasy Mini spin column placed in a 2ml collection tube, columns were centrifuge for 1min at ≥6000xg. Flow-through was discarded and the rest of the sample was processed the same way. Spin columns were

placed into new 2ml collection tubes and 500µl buffer AW2 was added to each column, columns were centrifuged for 1min at ≥6000xg and flow-through was discarded. Another 500µl of buffer AW2 were added and columns were centrifuged for 2min at 20,000xg. Spin columns were transferred to new 1.5ml or 2ml micro-centrifuge tubes and 100µl buffer AE was added for elution in each tube. Tubes were incubate at room temperature for 15min. Tubes were centrifuged for 1 min at ≥6000xg. Another 100µl buffer AE for was added in each tube and kept overnight before centrifuging for 1 min at ≥6000xg. Elution 1 and elution 2 results were run on 1% agarose gel to confirm DNA presence. DNA samples were labeled properly and stored at -20˚C for further use.

3.1.3.2.PCR

Sterile PCR tubes were labeled and 8.5µL of DNAse free (Thermo Fisher Scientific, US) water was added to each tube. Then 12.5µL of master mix solution and 1.5µL MgCl2 (Thermo Fisher Scientific, US) was added to each tube. ITS1 and ITS4 primers were used for the cycle (1.5µL of each). Finally, 1µL of the appropriate DNA sample was added in each tube. Tubes were centrifuged for 1minute to elute all liquid inside.

Tubes were placed in the PCR machine and the below cycle was run for 25 cycles:

Initialization: 94˚C: 5min

Denaturation: 94˚C: 1min

Annealing: 55˚C: 2min

Extension: 72˚C: 2min

Final elongation: 72˚C: 10min

Final hold: 4˚C

3.1.3.3.Gel electrophoresis

One percent agarose gel was prepared using 1X Tris/Borate/EDTA buffer (TBE) (Thermo Fisher Scientific, US), the mixture was heated by microwave between 30s and 60s (just before boiling), 4µl of Siber stain (Thermo Fisher Scientific, US) were added. The mixture was poured in the gel rack with combs. The solidified gel was transferred into the electrophoresis apparatus and covered with 1X TBE. On a Parafilm paper 1µl of orange die (Thermo Fisher Scientific, US) was mixed with 5µl of the DNA sample and the 6µl were inoculated into the gel's well. Once the gel is loaded, it was run for 30min at 100V for genomic DNA or 1h at 50V for PCR products. Gel was visualized under UV light.

3.1.3.4.PCR product purification

PCR products obtained were purified using Invitrogen Quick PCR Purification Kit (QIAGEN, Germany) as indicated by the manufacturer. Buffers were prepared first as instructed then PCR tubes were labeled and 20µl of the appropriate PCR product was added in each tube. 80µl (4X volume) of Buffer B2 was added in each tube and tubes were mixed by vortex. The mixtures were transferred into PureLink Spin columns kept in collection tubes and centrifuged at 10.000xg for 1 minute. Flow-through was discarded and columns were re-inserted in collection tubes and 650µl of wash buffer 1 (W1) was added in each tube. Tubes were centrifuged again at 10.000xg for a minute and flow-through was discarded. Collection tubes were re-inserted and tubes were centrifuged at maximum speed for 3 minutes. Columns were placed into labeled 1.7ml Elution Tubes and 30µl of elution buffer were added to the center of each column. Columns were incubated at room temperature for 1 minute and then centrifuged at maximum speed for 2 minutes, elution is the purified PCR product that

was stored at 4˚C until used. A sample of the purified PCR products were run on 1% agarose gel to check purification and to determine bands approximate size.

3.1.3.5.Sanger Sequencing

First, the PCR 96-well pate should be labeled. Each fungal strain occupy two well one for the forward primer and one for the reverse primer. According to the Sanger sequencer (3130/3130xl DNA Analyzers, Thermofisher Scientific, USA) instructions, volume in each of the 96 PCR well plate should be 5µl. Amount of DNA of the purified PCR products used depends on the size of the of the DNA fragments estimated by gel electrophoresis, noting that PCR products amounts should be determined by Nanodrop (ND2000, Thermo Scientific, USA) quantification method. Amount of primer in each well should be 10µM.

3.1.3.6.Bioinformatics

BioEdit software was used to read the sequencing results. Basic Local Alignment Search Tool (BLAST) network services of the National Centre for Biotechnology Information (NCBI) database was used to compare the obtained sequences. The fungal species were designated to the sequenced analyses based on similarity with the best aligned sequence of BLAST search.

3.1.4. Samples collection and pre-treatment

Prosopis juliflora leaves and fruits were collected from healthy mature plants from the field of Qatar University campus after the proper permission. One spot of trees was chosen from the field to maintain consistency throughout the project. Leaves were washed thoroughly with tap water followed by sterilized distilled water and left to dry in a sterilized oven at 45˚C. Fruits were washed first with tap water then sterilized with 70% ethanol to be washed then sterilized distilled water. Fruits were also left to dry in a sterilized oven at 45˚C. Dried plant parts were grinded separately and powder of

leaves and pericarps were stored in sterile jars at 4˚C until extracts preparation (Choudhary & Nagori, 2013).

3.1.5. Crude Extracts preparation

Crude extracts can be prepared using various solvents, based on a thorough literature, three extraction methods were applied (aqueous and ethanolic extracts and fresh plants juice). As two plant parts were used, six different crude extract solutions were investigated in the first part of this research.

3.1.5.1. Aqueous extract preparation method

Glass bottles were filled with 200ml distilled water and autoclaved. Twenty grams of either leave's powder or fruits' powder were aseptically weighed and added to the 200ml sterile distilled water. Bottles were incubated at 70˚C with shaking (50cycles/min) for 3 hours then temperature was lowered to 45˚C with shaking (50cycles/min) for 48 hours. Supernatants were filled in 50ml tubes and centrifuged at 4500rpm for 2 to 5 min depending on the solution turbidity. Pellets were avoided and supernatants were filtered using Whatman filter papers and poured into 150 mm glass Petri-plates and transferred to a pre-sterilized oven at 45˚C to evaporate the solvent. The dried (powdery or gummy) material was aseptically scratched from the surface of the petri-plates using a sterile scalpel. Dry aqueous leaves and fruits extracts were weighed and stored in sterile labeled test tubes. The bottles were re-filled with 100ml of sterile distilled water each and incubated at 45˚C with shaking (50 cycles/min) for an extra 24 hours. Second elution was treated the same way. Leaves and fruits aqueous extracts were then re-suspended in sterile distilled water to prepare stock solutions of 125 mg/ml. Suspension was sterilized using syringe filter 0.2µm (Sigma Aldrich-Germany) before being used (Sayago *et al.*, 2012; Sana *et al.*, 2016).

3.1.5.2.Ethanol extract preparation method (preliminary trial)

The same aqueous extraction method was repeated for ethanolic extract but 70% ethanol was used as a solvent during extraction rather than distilled water and temperature was 45°C throughout the extraction period. Ethanolic crude extract was re-suspended into 4% Dimethyl sulfoxide (DMSO) (Sigma Aldrich-Germany) solution to prepare a stock solution of 150mg/ml.

3.1.5.3.Ethanol extract preparation method (final trial)

To avoid DMSO in the antifungal trials, ethanolic crude extract was re-suspended into sterile distilled water to prepare stock solutions of 150mg/ml. (Sayago *et al.,* 2012; Sana *et al.,* 2016). Tubes were then centrifuged and the water soluble supernatant was taken and stored at 4˚C to be used in the analysis, while the non-water soluble pellet was incubated at 45˚C to dry, the pellet was then weighed and the new concentration of the solution was re-calculated. It was noticed that the mass of the pellet was around 9.5% of the initial total mass of the ethanolic extract which makes the final concentration of the stock solution around 125mg/ml. Suspension was sterilized using syringe filter 0.2µm. Figure 2 summarizes the final leaves ethanolic extraction method used.

3.1.5.4.Fresh plants' juice preparation method

Leaves and fruits were freshly collected, washed, sterilized using 70% ethanol then washed with sterile distilled water and dried under the hood. Sterile distilled water was used to soak leaves and fruits for 24 hours. Mixtures were then blended into smooth mixtures using sterilized blender. Fruit and leaves shakes concentrations were 514.3mg/ml and 312.5mg/ml respectively. Equal amounts of the shake and 20mM tris-HCl were mixed and centrifuged at 4500rpm for 25min.

Supernatant was kept at 4˚C until being directly used for media preparation. (Solanki *et al.*, 2018).

One molar Tris-HCl stock solution was prepared by mixing 12.1g of Tris-base (Sigma Aldrich-Germany) in 50ml distilled water, pH was adjusted using a digital pH-meter (Jenway, UK) by adding 1M hydrochloric acid (HCl) (Sigma Aldrich-Germany) solution drop by drop. Once pH reached 7.2 the volume was adjusted to 100ml by adding distilled water. Bottle was autoclaved and stored at 4˚C to be used in the preparation of the necessary amounts of 20mM Tris-HCl.

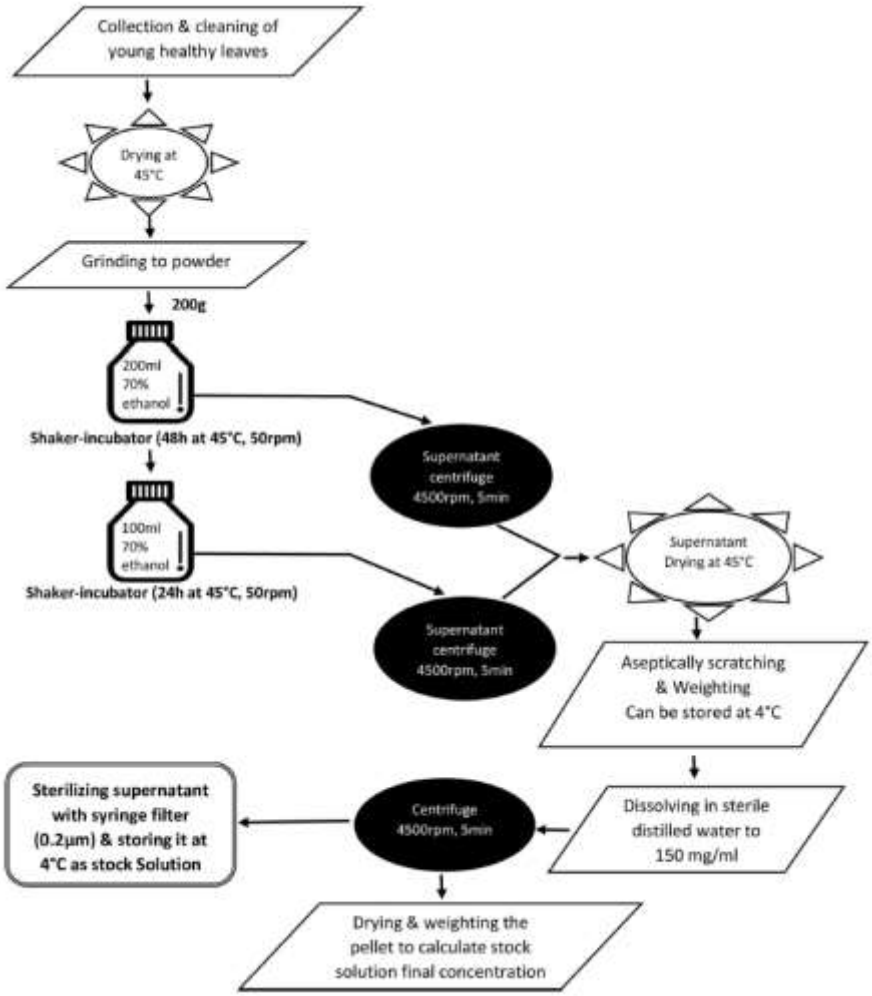


Figure 2. Flow diagram of *P. juliflora* water-soluble leaf ethanolic extract preparation method.

3.1.5.5.Determination of extraction yield

The extraction method yield of the crude extract of interest was determined using the following formula: Extraction yield $=$

$$\frac{Weight\ of\ the\ extracted\ powder\ or\ gummy\ material(g)\ \text{X}\ 100}{Weight\ of\ leaves\ powder\ used\ (g)}$$

3.1.6. *In-vitro* antimicrobial effect of crude extracts

3.1.6.1.Disk antifungal method

Potato dextrose agar (PDA) (Difco-USA) plates of 20mg/ml of extract (aqueous and ethanolic, leaves and fruits) were prepared by adding the appropriate volume of stock solutions previously prepared to 50˚C PDA media (Sana *et al.*, 2016). As for the tris-HCl fresh fruits and leaves juices, the solution was directly used to dissolve PDA powder and were autoclaved and used. Once the media solidify, a disk of 6mm of agar was aseptically removed from the center of each petri-dish. Four day-old fungi culture were used to obtain 6mm disks that were transferred aseptically to replace the agar disks. Plates were labeled with extracts name and fungi strains tested. The experiment was run in four replications. Plates with 1ml of Clotrimazole (antifungal ear drops of 10mg/ml) per plate were used as positive control. Pure PDA plates were also inoculated as negative control. PDA plates with tri-HCl buffer were used as negative control plates for the plates prepared from fresh leaves and fruits juice eluted by tri-HCL. Plates with 4% DMSO were used as negative control for plates prepared using leaves and fruits ethanolic extract using the preliminary trial extraction method. All plates were incubated upside down at 25˚C for 5 days. Percentage inhibition of mycelial growth (PIMG) was then calculated using the formula below, note that mean diameter mycelial growth was determined by measuring the diameter of the infected area in two perpendicular directions (Soylu *et al.*, 2010).

$\text{PIMG} = \frac{(dc-dt)X100}{dc}$ /dc: Average mycelium diameter in the negative control plate.

dt: Average mycelium diameter in the treated plate.

PIMG of various fungi using different crude extract solutions were recorded for comparison to determine the activity of each of the six crude extracts on each of the food spoiling fungi tested (Askarne *et al.*, 2012; Bill *et al.*, 2014).

3.1.6.2.Pour plate method for antifungal activity

For the fungal strains that cannot be tested using the disk antifungal method because of the spread of their spores, pour plate method was used. Calculation was made to prepare PDA plates of 20mg/ml of each of the extracts (Aqueous leaves and fruits and ethanolic leaves and fruits). The media was left in the oven to cool down to 50˚C before adding the extracts and the spore suspensions. Spores were collected by taking one disk of fungi (6mm) from a 4 to 5 day-old *Penicillium* plate to 5ml sterile distilled water, tubes were vortexed to suspend the spores. A serial dilution was prepared and the amount of spores was calculated using heamatocytometer (Hausser Scientific, UK). Knowing that final concentration of spores in a suspension (C) is calculated as follow:

C=Average number of spores per square X dilution factor $X10^4$/

- Average number of spores was found by counting the number of spores in five of the nine heamatocytometer compartments and the average was calculated.
- Dilution factor: is two, as 6µl of spore suspension was mixed with 6µl of cotton blue and the volume was inoculated into the heamatocytometer.

Final spore concentration per plate should be around 30 spores per plate, calculation was made to add the appropriate volume of spores. In the case of *Penicillium*, dilution 10^{-4} was used to inoculate the media. First spores were soaked with a solution of 20mg/ml of the LE extract for 1.5h and then they were inoculated into the PDA with 20mg/ml PJ-WS-LE extract of 50˚C that was poured in petri-dishes. Experiment was conducted on four replications. Plates were incubated at 25˚C for 4 days, number of thallus per plate was then calculated and the percent inhibition was calculated as follow:

$$\text{Percent spores inhibition} = \frac{(Nc-Nt)X100}{Nc}$$ Nc: Number of spores on the control plate

Nt: Number of spores in the treated plate

3.1.6.3.Antibacterial effectiveness

Disk diffusion method was used to characterize the antimicrobial effect of the crude extracts on bacterial isolates. Bacterial lawns of each isolate was prepared on Mueller-Hinton agar (HIMEDIA-India). 24h fresh solid bacterial cultures were used to prepare bacterial suspensions by taking two loopful of bacteria into 5ml of sterile distilled water and incubating the suspension at 37˚C for 30 minutes before using a sterile cotton swab to inoculate the suspension in all directions on Mueller-Hinton agar plates. 5mm sterile paper disks (Thermo Scientific™ Oxoid™ Blank Antimicrobial Susceptibility Disks, US) were inoculated with 20µl of different concentrations of the crude extract (1, 5, 10, 20, and 50mg/ml). Disks were then transferred with sterile forceps into the inoculated agar plates. Disks with sterile distilled water were used as negative control and 70% Dettol disks were used as positive control. The antibiogram of the bacterial isolates were also determined to compare crude extracts to antibiotics (Ampicillin (AMP), Amoxicillin (AMX), Bacitracin (B), Carbenicillin (CB), and Cephalothin (CR)) (Oxoid-USA). Pates were incubated at 37˚C. The experiment was

conducted in triplicates. Diameters of the various inhibition zones were recorded for comparison and percentages of inhibition were calculated (Raghavendra *et al.*, 2009).

3.1.6.4.Anti-yeast effect

The same disk diffusion method was used to test *C. albican* susceptibility to the extracts (Giri & Kindo, 2014).

3.1.7. PJ-WS-LE extract stability

Powder and liquid extract of the extract(s) that has (have) shown good antifungal activity were stored at 4°C for six month. Their antifungal activity was re-evaluated using the agar diffusion method to determine active phytochemical stability with time.

3.1.8. Determination of minimum inhibitory concentration

Based on the results of the previous experiment, the minimum inhibitory concentration (MIC) of the active crude extract was determined against each of the affected microorganisms. The MIC test was conducted in sterile 96-well plate, in each well, 100µl of nutrient broth (Difco-USA) was added for bacterial MIC determination and 100µl of potato dextrose broth (PDB) (HIMEDIA-India) was added for fungi and yeast MIC determination. Different concentrations of the crude extracts were added in each well, and wells were inoculated with the microorganisms.

Fungal spore suspensions were adjusted to the range of 10^4spores/ml using 4 days old fungal plate and sterile distilled water, the spores concentration was adjusted using a heamatocytometer. As for bacterial suspensions, two loopful of fresh bacterial culture were added to 5ml sterile distilled water and incubated at 37˚C for 30min before being used. Clear media was used as negative control and fungicidal and antibiotics were used as positive control for fungal and bacterial testing, respectively.

Resazurin (HIMEDIA-India) was added in all wells to monitor bacterial and fungal growth, Resazurin is a blue die that becomes pink and fluorescent upon cells activity. Resazurin stock solution was prepared by dissolving 0.27g of Resazurin powder in 40ml of sterile distilled water. MIC was observed and recorded (Stefanovic *et al.*, 2015).

For inoculation, 3ml of the 10^4 spores/ml suspension was mixed with 1ml Resazurin stock solution, 20µl of the mixture was added in each of the treatment wells of the 96-well plate. As in the case of bacteria, 3ml of the bacterial suspension was mixed with 1ml of Resazurin and 20µl of the mixture was also added in each of the treatment wells. The experimental design followed was as per the figure 3. Four replication of each concentration were prepared. In the no spores/bacteria control wells, spores/bacteria and stain suspension was not added, only 10µl of Resazurin stock solution was added. For the negative control, no extract was added. For the positive control 10µl of Clatrimazole (1%) was added in each well in the case of fungi and 10µl of Ampicillin (7.5mg/ml) was used in the case of bacteria.

H	**G**	**F**	**E**	**D**	**C**	**B**	**A**	
50mg/ml				3.125mg/ml				**1**
40mg/ml				2.5mg/ml				**2**
32mg/ml				2mg/ml				**3**
25mg/ml				1mg/ml				**4**
20mg/ml				0.5mg/ml				**5**
16mg/ml				0.25mg/ml				**6**
12.5mg/ml				0.125mg/ml				**7**
10mg/ml				No spores/bacteria				**8**
8mg/ml				Negative control				**9**
6.25mg/ml				Positive control				**10**
5mg/ml								**11**
4mg/ml								**12**

Figure 3. MIC determination experimental design.

Well-plates were incubated at 25˚C and results were recorded within 48h, the MIC is the last concentration that does not show a change of Resazurin color within the incubation time.

3.1.9. Crude extract effect on fungal spores' germination

Spores suspensions of various fungal strains were inoculated into tubes of different concentrations of the previously proven active extract(s). These concentrations were: 2, 4, and 8mg/ml. Negative control tubes were also prepared (no extract). The experiment was conducted in triplicates following the experimental design in table 22.

Table 22. Spores germination experimental design.

	Negative control	2mg/ml	4mg/ml	8mg/ml
PDB	1700µl	1668 µl	1636 µl	1573 µl
LE extract of 125mg/ml stock	0	31.7 µl	63.5 µl	127 µl
Spores suspension	300 µl	300 µl	300 µl	300 µl
Resazurin	50 µl	50 µl	50 µl	50 µl

Spores suspensions were adjusted to 10^4spores/ml. Test tubes were incubated at 25˚C with 150rpm shaking for 24 hours. Slides were prepared from each test tube, and conidia were stained by cotton blue (6µl of the test tube + 6µl cotton blue). The number of germinated conidia was counted out of 100 random conidia in three slides for each test tube and averages were calculated. A spore is considered germinated if the length of the germination tube is at least half of the length of the spore itself. Slides were also prepared from the negative control tubes before incubation, to find the start

number of originally germinated spores. Spores' germination percentages and percentage inhibition of the extract(s) were calculated using the below formula (Bazie *et al.*, 2014; Zhimo *et al.*, 2016).

$$\text{Percentage inhibition}(\%) = \frac{(Gc-Gt)X100}{Gc}$$ Gc: Germination rate in the control plate

Gt: Germination rate in the treated plate

3.1.10. Mode of action of fungal or bacterial inhibition

Extracts with inhibition effect were further tested to determine if the effect is fungicidal or fungistatic. Fungal mycelia that fail to grow or was inhibited by a plant extract were transferred to a clean PDA plate (without extracts) and incubated at 25˚C for 7 days. Positive growth of the sub-culture proves fungistatic activity, while negative growth demonstrates a fungicidal effect (Bill *et al.*, 2014).

Mode of inhibition of the active extract on bacteria and fungal spores was also determined. The active extract might inhibits fungal spores and bacterial growth by killing them or just by blocking their growth temporary. Spores incubated in the presence of 8mg/ml of the previously proven effective extract(s) for 48h were used to inoculate clean PDA plates, 100µl off the suspension were spread on each plate, experiment was conducted in four replications. Spores suspension from the negative control tubes were also inoculated for comparison. Plates were incubated at 25˚C for 5 days. As for bacteria, strains incubated in 8mg/ml of the previously proven effective extract(s) for 48h were inoculated into clean nutrient agar NA (HiMedia, India) plates, experiment was conducted in four replications by spreading 100µl of the bacterial on each plate. Control tubes without plant extract were also inoculated for comparison. Plates were incubated at 37˚C for 48h.

3.1.11. The effect of effective crude extract(s) on the microscopic morphology of the studied fungal species using a scanning electron microscope (SEM).

Studying the effect of the *in-vitro* effective extract on the morphology of the studied fungi was needed to evaluate if fungi that showed fungal growth in the presence of the extract are still affected or not, this will help to predict the *in-vivo* behavior of various fungi upon exposure to the extract. Two test tubes of each of the nine fungal species evaluated were prepared as in table 23.

Table 23. Pre-setting of germination tubes used in SEM observation.

	Negative control	8mg/ml
PDB	1700µl	1540 µl
LE extract of 100mg/ml stock	0	160 µl
Spores suspension	300 µl	300 µl

Tubes were incubated at 25˚C with 140rpm shaking for 24h. Tubes were then centrifuged (5000rpm) and the pellet was washed twice with PBS (Thermo Fisher Scientific, US) pH7.4. Cells were fixed by re-eluting the pellet in a solution of 2.5% glutaraldehyde + 3.6% formalin, tubes were incubated at 4˚C for 18h. Tubes were then centrifuged (5000rpm) and washed three times with PBS. Pellets were dehydrated in serial ethanol dilution (25, 50, 70, 80, 90, and 100%), the pellet was incubated in each concentration for around 30min. Samples were then smeared on silver holders in thin films and left to air dry before gold coating them using Agar Sputter Coater. The SEM observations were made using Nova NanoSEM 450 (ELECMI, Spain).

3.1.12. Crude extracts cytotoxicity

3.1.12.1. Cells Thawing

In cells culture labs, work place should always be sterilized using 70% ethanol and all material used should also be sterilized before being kept in the hood. Working hood with all needed items except media were sterilized by UV light for 5min.

To prepare media for cells thawing, culture media was prepared using 10% fetal bovine serum (FBS), 1% Ampicillin antibiotic (Thermo Fisher Scientific, US), and DMEM media (Thermo Fisher Scientific, US). To prepare full media, 50ml of FBS and 5ml of antibiotic were added to a bottle of DMEM media of 445ml. The bottle was then labeled and stored at 4˚C if not fully used.

Cells vials were removed from -80˚C to be defrosted. Defrosting was done by hands. Once cell start getting defrosted, 1ml of full media was added followed by another one in two minutes. Cells were mixed very gently and transferred into test tubes. Cells were kept in the 2ml medium for 2 minutes until they adapt then medium volume was increased gradually up to 5ml. Tubes were centrifuged at 100xg for 5 minutes and supernatant was gently discarded. One ml of medium was added to each test tube to mix the cells. Then 9ml of full medium was added to a flask to which cells were gently transferred. Cells were checked under the microscope for good confluency and flasks were incubated at 37˚C with 5% CO2 and 16% oxygen.

3.1.12.2. Changing media

Under sterile condition, medium was removed from the flask and discarded. The flask was washed twice with 10ml of PBS pH7, then PBS was discarded. Finally, 12ml of full medium was added to the cells and flask was returned to the incubator at 37˚C with 5% CO2 and 16% oxygen.

3.1.12.3. Cells Splitting

Flasks were always labeled with: passing number, cells name, date, and user's name. First, medium was removed and discarded and flasks were washed twice with 5ml PBS that was also discarded. To detach the cells, 2ml of trypsine (Thermo Fisher Scientific, US) was added to each flask and left for 2 to 3 minutes. Flasks have to be checked under the microscope to insure detachment before proceeding. Flasks can be kept in the incubator to facilitate detachment. Then 5ml of full medium was added and the total volume of 7ml was transferred gently to a labeled test tube. Test tubes were centrifuged at 200xg for 5 minutes. New flasks were labeled and filled with 10 to 12ml of full medium. After centrifuge, supernatant was discarded and 1ml of full medium was added to the test tube to re-suspend the cells. For 1 to 1 dilution, 0.5ml of the cells suspension should be transferred to each new flask. Finally, flasks were shacked gently and incubated at 37˚C with 5% CO2 and 16% oxygen.

3.1.12.4. Cells Seeding

Flasks of around 90% confluency can be used for cells seeding assay. First, medium was removed and discarded and flasks were washed with 5ml of PBS twice. Cell were detached using 2ml of Trypsine for 2 to 3 minutes. Then 5ml of full medium was added and the total volume of 7ml was transferred gently to a labeled test tube that was centrifuged at 200xg for 5 minutes. Supernatant was discarded and 1ml of full media to re-suspended cells.

Ten µl of each tube were transferred to a labeled Eppendorf tube and mixed with 10µl of Trypan blue (Thermo Fisher Scientific, US). 12µl of the mixture were transferred to the heamocytometer chamber and slide was inserted and cells were counted. The appropriate number of cells that should be seeded per well in a treatment assay should be 60.000cells/well. To obtain the appropriate amount of cells per well,

24ml of cells suspension should be prepared in test tubes to inoculate a 24-well plate. The total amount of cells per 24ml should be (60.000X24) 1.44 10^6 cells. The cells counter gives the number of viable cells per ml and calculation was conducted to find the amount of cells suspension needed to prepare the required volume. One ml of the prepared dilution was added in each well of the 24-well plate. Plates were incubated at 37˚C, 5% CO2, 16% O2. Plates were left until cells reach 50% confluency (24 to 48h). To inoculate 96-well plate, the total volume per well is 0.2ml, and calculation should be conducted as for the 24-well plate to have 12.000cells/well.

3.1.12.5. Cells Treatment

In cells treatment, 3% DMEM medium was used (3% Serum and 1% antibiotic). Medium was discarded. Fresh 3% medium with various concentrations of the effective tested crude extract(s) were added to the wells. Negative control (cells without treatment) and positive control (cells with 10% DMSO) should always be a part of the experiment. Treatments were replicated four times. Plates were incubated at 37˚C, 5% CO2, 16% O2. Plates can be incubated for 24h for acute effect analysis and for 48h to 72h for chronic effect analysis.

3.1.12.6. Coomassie stain assay

After the incubation period medium was discarded and plated were washed twice with 1X PBS (1ml per well). One ml of the fixer (70% ethanol) was added to each well and plates were incubated at room temperature with shaking for 30min. Fixer was discarded and 0.5ml of Coomassie stain (Thermo Fisher Scientific, US) was added to each well then plates were incubated at room temperature with shaking for one hour. Plates were then washed gently and left to air dry before being examined under the microscope (Zakharchenko *et al.*, 2013).

3.2. STUDYING THE *IN-VIVO* ANTIMICROBIAL AND QUALITY ENHANCING ACTIVITIES OF THE EFFECTIVE CHOSEN CRUDE EXTRACT

3.2.1. The crude extract used

For the *in-vivo* experiments, only the proven extract as the most effective *in-vitro* and which was free of organic solvents was chosen for this part of the analysis. *Prosopis juliflora* water soluble leaf ethanolic (PJ-WS-LE) extract was prepared as described in figure 2 and used to evaluate its antimicrobial and protective effectiveness on fruits and vegetables.

3.2.2. Effect of PJ-WS-LE extract on strawberries and cucumbers shelf-life at room temperature

Sixty samples of fresh good looking imported strawberries (Morocco) and locally grown (Qatari) cucumber samples were divided into two groups. A control group (30 samples) at which these samples were kept in sterile well ventilated box at room temperature (22 to 24°C), and a treated group (30 samples) where samples were first sprayed thoroughly with 8mg/ml PJ-WS-LE solution and left to dry in a sterile drainer before being moved to a sterile well ventilated box at room temperature.

Samples were monitored every 24hours to determine shelf-life and to determine fungal spoiling agents. Each sample that shows fungal growth was removed from the box and the fungal contaminant was transferred with a sterile needle to a clean PDA plate before discarding the sample. PDA plates were incubated at 25˚C for 5 days then fungal genus was microscopically determined. The number of samples of a specific shelf life (hours) was recorded to calculate average shelf life of control and experimental groups. Percentage of edible samples at 48h for strawberry samples and at 5 day for cucumber samples was also determined.

3.2.3. Effect of PJ-WS-LE extract on strawberries shelf-life at refrigerator temperature

Sixty samples of fresh good looking imported strawberries (Morocco) were divided into two groups and treated and handled as descried above in section 3.2.2. The only difference is the storage temperature, both treated and non-treated batches were stored at 4˚C in the fridge. Experiment was terminated at day 10 and the firmness of the good remaining control and experimental samples was measured.

3.2.4. Effect of PJ-WS-LE extract against *A. alternata* and *B. cineraria* induced infection in cherry tomatoes (small scale experiment)

Organic locally grown cherry tomato samples were purchased from the market. Only freshly arrived and undamaged fruits were used in the experiment. Samples were washed with disinfecting soap and water, after thorough rinsing with tap water, samples were rinsed with sterile distilled water. Clean cherry tomato samples were moisture-dried using heat sterilized towels and categorized based on their shapes (round or long) and their weight ranges. Eighty-four samples were divided into 14-treatment category of six samples each, samples in each category had similar shapes and weight ranges. The complete randomized block design used is described in table 24.

Negative control plants were untreated but sterilized plants. *Botrytis* control and *Alternaria* control groups are samples treated with 15µl of spores suspension alone on the tomato calyx. Spores suspension were prepared by flooding a mature plate of *Botrytis* or *Alternaria* with around 10ml of sterile distilled water, spore suspension concentration was adjusted between 10^4 to 10^6spores/ml using a heamatocytometer. Wounded samples were injured using a sterile needle of a syringe in two different places near the calyx of each tomato sample.

Table 24. Number of cherry tomato samples in each of the 14 categories of the randomized block design of the controlled experiment.

	Wounded Samples	Not Wounded Samples
Botrytis control	6	6
Botrytis curative effect	6	6
Botrytis preventive effect	6	6
Alternaria control	6	6
Alternaria curative effect	6	6
Alternaria preventive effect	6	6
Negative control	6	6

To test the curative effect of the extract, 15µl of the spores suspension was applied on the calyx first and left to dry for 2 hours after which the calyx was sprayed with a solution of 8mg/ml of PJ-WS-LE extract. To test the preventive effect of the extract, the samples were first sprayed with a solution of 8mg/ml of PJ-WS-LE extract and left for 2 hours to dry then 15µl of the spores suspension was applied. Plants were all kept in sterile paper boxes that were closed tightly and left at room temperature (22 to 24°C) to be monitored on a daily basis. The experiment was ended in 2 weeks.

3.2.5. Curative and preventive effects of PJ-WS-LE extract against *A. alternata* and *C. gloeosporioides* induced infection in mangoes (large scale experiment)

To simulate fruits physical damage during transportation or in the field, wounded mango plants were used in the experiment, the wounds were made by three needle pricks (2mm deep) on the inoculation site of each plant using sterile syringe.

Complete randomized block design was used, each treatment was made of triplicate of 10 fruits each. The mango (*Mangifera indica*) type known as Chaunsa imported from Pakistan was used in the controlled experiment. Fruits were purchased from the whole sale market upon arrival to the country. Only mature and undamaged fruits were chosen for the experiment. Fruits were first washed with tap water and got sprayed twice by 70% ethanol to be then washed with sterile distilled water and left to air dry.

PJ-WS-LE extract was first tested for its ability to prevent fungal contamination in wounded mango fruits (preventive effect). Therefore, wounds were sprayed with 8mg/ml PJ-WS-LE extract and left to dry. Once dry the fruits were sprayed again with the extract and left to dry. Control fruits were treated with sterile distilled water without extract. After two hours all wounds were inoculated with 20µl of conidia aqueous solution (10^6 spore/ml).

PJ-WS-LE extract was then tested for its ability to cure fungal contamination in wounded fruits. Therefore, wounds were treated first with 20µl of conidia aqueous solution (10^6 spore/ml) and left to dry. Wounds were then sprayed twice with 8mg/ml *PJ-WS-LE* extract.

All mango samples were stored in sterilized plastic trays inside an incubator at 25°C and 75% humidity, the qualitative observations have been recorded at the interval of 24h for 5 days for *C. gloeosporioides* inoculated fruits and for 10 days for *A. alternata* inoculated fruits. Three parameters were recorded at the end of the experiment: disease incidence (DI), disease severity (DS), and percentage plant extract efficacy (%EE). Mean diameter mycelial growth was determined by measuring the diameter of the infected area in two perpendicular directions (Askarne *et al.*, 2012; Sayago *et al.*, 2012; Bill *et al.*, 2014; Xiao *et al.*, 2018).

$$\mathrm{DI} = \frac{(\text{Number of rotten fruits})\mathrm{X}100}{\text{Total number of fruits}}$$

$$\text{DS} = \frac{(\text{Average lesion diameter of treated plants})\text{X}100}{\text{Average Lesion diameter of Control plants})}$$

$$\%\text{EE} = \frac{(\text{Disease incidence in Control batch} - \text{Disease incidence in treated batch})\text{X}100}{\text{Disease incidence in Control batch}}$$

At the end of the trial, remaining mango fruits were tested for their flesh quality using a penetrometer to test the flesh firmness. The stainless steel probe of the instrument was inserted in three different points towards the equator of the fruit and numbers in Newton were recorded and compared with standards fruits firmness to judge fruits quality (Bill *et al.*, 2014).

3.2.6. *In-vivo* long term preservative activity of PJ-WS-LE extract used as coating material individually and when embedded in chitosan

3.2.6.1.Sample preparation

One hundred forty fresh good-looking (healthy) samples of strawberry, cucumber, and lemon and 84 samples of mango were bought from local market and divided into four groups of 30 samples each in the case of strawberries, cucumbers and lemons and 18 samples each in the case of mangoes. Cucumbers used were Qatari grown cucumbers purchased fresh upon their arrival to the market. Mango samples used in this trial were Neelam Mango imported from India, good looking none damaged samples were bought from the whole-sale market upon arrival from the country of origin directly. Strawberry samples used in our experiments are Driscoll's brand imported from the USA, fresh good looking samples were only used for the trial. As for Lemons, fresh samples imported from Turkey were purchased from the market for the trials.

Two control and two experimental batches were stored at chosen temperatures as indicated in table 25 (Mohammadi *et al.*, 2016).

Table 25. Experimental design of the long-term preservations effectiveness of PJ-WS-LE extract embedded in 1% chitosan strawberries, mangoes and lemons and in 0.5% chitosan for cucumbers.

	Cucumber	Strawberry	Mango	Lemon
Number of samples per treatment condition	30	30	18	30
Control batch A (negative control)	None	None	None	None
Experimental batch B	Sprayed with 8mg/ml PJ-WS-LE extract	Sprayed with 8mg/ml PJ-WS-LE extract	Sprayed with 8mg/ml PJ-WS-LE extract	Sprayed with 8mg/ml PJ-WS-LE extract
Control batch C	0.5% chitosan coating	1% chitosan coating	1% chitosan coating	1% chitosan coating
Experimental batch D	0.5% chitosan + 8mg/ml PJ-WS-LE extract coating	1% chitosan + 8mg/ml PJ-WS-LE extract coating	1% chitosan + 8mg/ml PJ-WS-LE extract coating	1% chitosan + 8mg/ml PJ-WS-LE extract coating
Storage temperature	8°C	4°C	4°C	4°C
Storage interval	7 days	7 days	10 days	10 days

The experiment was laid out in a completely randomized design, every five samples of cucumbers, lemons and strawberries and every 3 samples of mangoes made

a single replicate and were stored together in one sterile box or bag, each treatment was performed in 6 replications and the entire experiment was repeated twice

3.2.6.2.Preparation of coating solutions

Chitosan coating solution was prepared at 0.5% or 1% concentration by dissolving chitosan powder (CAS 9012-76-4, Himedia, India) in distilled water with 1% glacial acetic acid (IsoLab, Germany), the solution was stirred with a magnetic stirrer overnight. The pH of the solution was adjusted to 5.6 using 0.1M NAOH (Sigma Aldrich, Germany). For PJ-WS-LE extract chitosan embedded coating solution, specific volume of the extract stock solution sterilized by filtration was added to achieve a final extract concentration of 8mg/ml PJ-WS-LE extract in 0.5% or 1% chitosan (Chien *et al.*, 2007).

3.2.6.3.Storage quality parameters monitoring

At day zero, five samples of strawberry, cucumber, and lemon and three samples of mangoes were used to evaluate the shelf-life quality parameters of fresh samples. The various measured parameters were recorded to be used in samples monitoring during the entire storage period. Five samples were withdrawn at every storage interval of 10 days for lemons and 7 days for strawberries and cucumbers for microbiological and physicochemical evaluation. Mango samples were also evaluated every 10 days using three samples per evaluation. Weight of all samples were taken at time zero after treatment and then later again at each storage intervals. Microbiological and Physiological qualities were taken between 6 and 8 times depending on the decay rate throughout the experiment. Parameters tested per samples type are shown in table 26.

Table 26. Parameters tested for each fruits sample.

	Weight loss	Surface total fungal count (CFU)	Surface total aerobic	Firmness	Sensory quality	pH	Total soluble solids	Total Antioxidant	Respiration rate
Cucumber	+	+	+	+	+	+	+	+	+
Strawberry	+	+	+	+	+	+	+	+	+
Mango	+			+	+	+	+	+	
Lemon	+				+	+	+	+	

3.2.6.4.Evaluation of sensory quality

The evaluation of the sensory quality was carried out on a 5-point scale for samples' taste, smell, color change and overall quality. The four attributes were evaluated using the withdrawn samples at every storage interval. Each sample was given a score using the following scale: 5 points for "extremely liked", 4 points for "liked", 3 points for "acceptable" 2 points for "disliked" and 1 point for "extremely disliked". Average score per batch per week was then calculated (Patel & Panigrahi, 2019).

3.2.6.5.Estimation of weight loss

All samples used in the trials were given numbers and had their weight taken at day zero using a digital balance (Sigma Aldrich, Germany). Weights of the remaining samples were measured at every storage interval. Total weight loss is the variation between primary and final weights of the sample during a certain storage

interval. Average percent weight loss per batch was calculated (Patel & Panigrahi, 2019).

3.2.6.6.Estimation of total aerobic bacterial count

Strawberry and cucumber samples withdrawn at every measurement interval were washed from the outside with 10ml of sterile distilled water (SDW). Washing water was serially diluted (10^{-1} to10^{-3}) and spread on NA plates in duplicates for total aerobic bacterial count. Plates were incubated at 37°C for 48h. Colony forming unit (CFU) per sample was calculated and average total aerobic bacterial count CFU per treatment batch was also calculated at each time interval (Moradi *et al.*, 2019).

3.2.6.7.Estimation of total fungal count

The washing water previously prepared was also spread on PDA plates for total mold and yeast count. PDA plates were incubated at 25°C for 5 days. Mold CFU and yeast CFU per sample were calculated separately. Average mold CFU and yeast CFU per treatment batch was calculated at each time interval (Moradi *et al.*, 2019).

3.2.6.8.Evaluation of respiration rate

Strawberry and cucumber samples of each treatment batch were placed in closed containers and the level of carbon dioxide produced was measured using carbon dioxide meter (OMEGA AMQ-102, UK). A reading of the CO_2 in ppm was recorded every two seconds for around 50min. Respiration rate (ppm/s) was calculated using the slope of the trend line passing by the collected data, and weekly results were compared (Mohammadi *et al.*, 2016).

3.2.6.9.Determination of samples firmness

Withdrawn samples of strawberries had their firmness measured using a penetrometer (Agriculture Solutions, USA). The probe of the penetrometer was inserted

in two different locations in the middle of each fruit, and the average reading in Newton (N) was recorded. Average samples firmness (N) in each treatment batch was calculated at every measurement interval (Emamifar *et al.*, 2019).

3.2.6.10. pH measurement

Strawberry, cucumber, mango and lemon samples withdrawn at every measurement interval were blended into juice, which was filtered and had its pH measured using a digital pH meter (Jenway, UK). Average samples pH per treatment batch was calculated on a weekly basis. A pH 7 buffer solution was used to calibrate the pH meter before measurements (Naeem *et al.*, 2019).

3.2.6.11. Total soluble solids (TSS) measurement

Juice samples previously prepared and homogenized had their TSS in brix measured. Two drops per sample's juice were focused on a refractometer (ANTAHI, New Zealand) and measurements were taken. Average samples TSS (%Brix) per treatment batch was calculated on a weekly basis. Distilled water was used to calibrate the refractometer before measurements (Moradi *et al.*, 2019).

3.2.6.12. DPPH radical scavenging assay

A 100µl of each diluted juice sample was mixed with 1ml of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (TCI, US) solution (100mg/l) and incubated under dark conditions for 45 min at 37°C. Samples were centrifuged and the supernatant change in color intensity was measured using a spectrophotometer (Jenway, UK) at 517nm against methanol (Sigma Aldrich-Germany) as a blank. A 100µl of methanol in 1ml DPPH was used as a control. Average % radical scavenging activity per treatment batch was calculated every week (Mohammadi *et al.*, 2016). Percentage radical scavenging activity of each samples was calculated using the below formula:

% radical scavenging activity = (absorbance of control- absorbance of sample)*100/ absorbance of control.

3.2.6.13. Cucumbers water content

Thirty samples of fresh good looking cucumbers were purchased from the market upon arrival from Qatari farm. Samples were weighted and each cucumber was cut into two halves and kept in a paper bag inside an oven at 65°C. When completely dry, samples final weights were taken and percentage water content was calculated.

3.2.7. Effect of PJ-WS-LE extract on chickpeas seeds germination

3.2.7.1.Soil and extract used

Agricultural soil was bought from the market and autoclaved in the lab before being used. PJ-WS-LE extract stock solution was prepared as previously described to be used in the preparation of for seeds priming solution (20mg/ml) and seeds watering solution (8mg/ml).

3.2.7.2.Seeds germination test

Thirty chickpeas seeds were distantly distributed in sterile petri-dishes on a sterile filter papers, seed were covered with another sterile filter paper and sprayed with sterile distilled water. Petri-dishes were covered and kept in the dark for 5 days. Percentage of seeds germination was calculated.

3.2.7.3.Seeds priming

Seeds were primed for 10min or 30min before being left to air dry prior to planting. Primed seeds were soaked in a sterilized solution of 20mg/ml of PF-WS-LE extract. Control non-primed seeds were also used (Rafi *et al.*, 2015).

3.2.7.4.Seeds planting

Pots of 8cm diameter were used for planting, 300g of wet soil was added to each pot. Seeds were sown as one seed per pot. Two experimental batches were

prepared, one to be watered with 20ml of sterile distilled water (SDW) per pot every 48h. The sown seeds of the second batch were watered with 20ml of a solution of 8mg/ml PJ-WS-LE extract every 48h for the first 10 days to be then watered with the solution of 8mg/ml PJ-WS-LE extract once per week and with 20ml of SDW the remaining times (Rafi *et al.*, 2015). Pots were kept in the growth chamber in a completely randomized design with five replications per treatment. Pots belonging to the six-treatment category were labeled as indicated in table 27. Growth chamber cycle was 16h light at 24°C and 8h dark at 18°C. Pots were incubated for 30 days. Germination and growth parameters including chlorophyll content, shoot length, root length, shoot and root weights (fresh and dry), and number of leaflets per leaf were recorded after 30 days of seed germination (Rafi *et al.*, 2015).

Table 27. Treatment categories of the chickpeas seeds priming experiment and pots labels.

	Seeds primed 10min	Seeds primed 30min	Seeds not primed
Plants watered with SDW	1.1, 1.2, 1.3, 1.4, 1.5, 1.6	2.1, 2.2, 2.3, 2.4, 2.5, 2.6	3.1, 3.2, 3.3, 3.4, 3.5, 3.6
Plants watered with 8m/ml PJ-WS-LE extract or SDW	4.1, 4.2, 4.3, 4.4, 4.5, 4.6	5.1, 5.2, 5.3, 5.4, 5.5, 5.6	6.1, 6.2, 6.3, 6.4, 6.5, 6.6

3.2.7.5.Growth parameters measurement

Chlorophyll levels of each plants were measured using chlorophyll meter (SPAD 502 Plus) (konica Minolta, Japan), three measurement were taken per plant

from random leaves at the top, middle and bottom of each plant, average chlorophyll content per plant was calculated. Similarly, average number of leaflets per each plant leaves was determined by counting three leaves per plant (top, middle and bottom leaves) and finding the average. Heights of the stems and roots length of each plants were measured at day 30. Weights of fresh roots and stems were also recorded, roots and stems were then separately kept in paper bags and incubated at 45°C to dry. Weights of dry stems and roots were then measured.

3.3. EVALUATING THE GENETIC VARIATION OF *P. JULIFLORA* IN THE STATE OF QATAR (DOHA MUNICIPALITY) AND TESTING THE ANTIMICROBIAL EFFECTIVENESS OF CRUDE EXTRACTS PREPARED FROM SAMPLES TAKEN FROM DIFFERENT LOCATIONS

3.3.1. Samples collection and processing

Seven samples of *P. juliflora* leaves were collected from six different locations in Doha, Qatar, during five field trips. Leaf samples were kept in sterile labeled bags until reaching the lab where few leaflets were washed and sterilized with 70% ethanol for DNA extraction. Table 28 shows the samples details. Figure 4 shows the location sites of the samples collection on the map of Qatar, Doha.

Table 28. *P. juliflora* leaves collection details.

Sample Name	Latitude	Longitude	Location	Sampling trip number
S1	25°22'24"N	51°29'48"E	Qatar University field	1
S2	25°22'27"N	51°29'43"E	Qatar University field	2
S3	25°17'50"N	51°27'05"E	Al Rayyan	2
S4	25°17'30"N	51°28'40"E	Al Amir street	3
S5	25°14'05"N	51°29'01"E	Abu Hamour	4
S6	25°20'16"N	51°28'34"E	Al Duhail	4
S7	25°20'26"N	51°29'58"E	Rawdat Al Faras	5

3.3.2. Prosopis juliflora DNA fingerprinting

Leaves of the seven samples had their DNA extracted and their ribosomal DNA amplified by PCR using (ITS1/ITS4) primers. PCR products were sequenced using Sanger sequencing (Archak, 2000). Detailed methods were described in section 3.1.3.

3.3.3. Bioinformatics

BioEdit software was used to read the sequencing results as earlier mentioned. Basic Local Alignment Search Tool (BLAST) network services of the National Centre for Biotechnology Information (NCBI) database was also used to compare the obtained sequences to database and to obtain accession numbers. The various *P. juliflora* ribosomal sequences obtained were also uploaded on MEGA-X software and the phylogenetic analysis was carried out by constructing a phylogeny tree using the neighbor-joining (NJ) algorithm (500 bootstrap replications) (Xu *et al.*, 2017). ITS

rDNA of *P. chrysogenum* (strain code PchrQU17) and *P. citrinum* (strain code PcitQU17) were used as outgroups (Saleh & Abu-Dieyeh, 2021).

3.3.4. MICs evaluation of crude extract prepared using P. juliflora leaves samples from various locations against some chosen fungi, bacteria and yeast

Eight samples of *P. juliflora* leaves were collected from the same six locations described in figure 4. PJ-WS-LE extract was prepared as described in figure 2 using each of the samples. The MIC of each of the eight extracts was determined as described in 3.1.8 with the adjustment of using nine concentrations (1:1 dilutions) ranging from 42mg/ml to 0.16mg/ml. The MICs of the extracts were measured on a chosen set of microorganisms including: *C. albicans*, *S. aureus*, *A. alternata*, and *C. gloeosporioides*. MICs were compared.

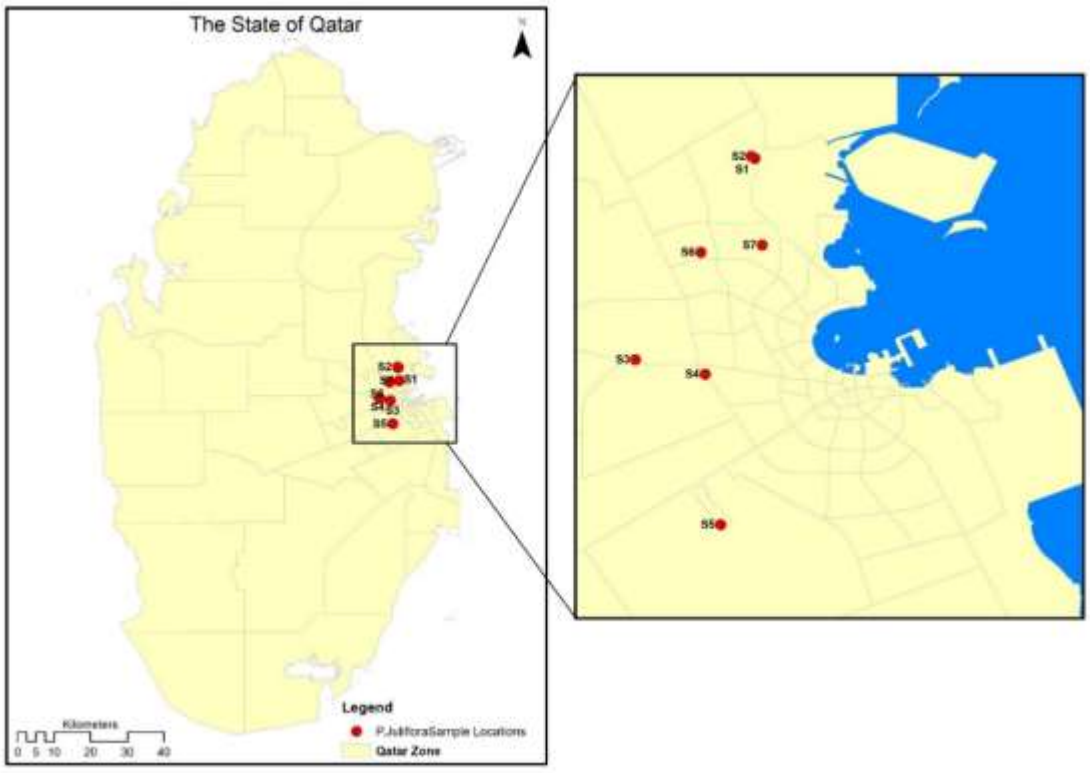


Figure 4. Location map of collection sites of *P. juliflora* leaf samples (ArcGIS software).

3.4. FRACTIONATION OF PJ-WS-LE EXTRACT AND TESTING THE ANTIMICROBIAL EFFECTIVENESS AND STABILITY OF THE VARIOUS FRACTIONS

3.4.1. Preparation of PJ-WS-LE- extract

PJ-WS-LE extract was prepared as previously described. Aseptically collected extract was dissolved in SDW and centrifuged to precipitate non-water soluble pellets. Pellets were dried and weighted and final concentration was calculated and adjusted to 36mg/ml. The solution was filtered with 0.2µm syringe filter and stored at 4°C until used. The loading solution was diluted to 13.2mg/ml and centrifuged at highest speed before being loaded to avoid any insoluble impurity.

3.4.2. Fast protein liquid chromatography (FPLC) reagents

Two eluent solutions A and B were prepared. Eluent A is 0.1% trifluoro-acetic acid (TFA) (Sigma Aldrich, Germany), 2% acetonitrile (ACN) (Sigma Aldrich, Germany) solution prepared by mixing 1ml TFA to 20ml ACN and 979ml of sterile double distilled water. Eluent B is a 70% ACN solution. System cleaning solution is 20% ethanol.

3.4.3. PJ-WS-LE solution fractionation by FPLC

The preparative protein purification chromatography system, AKTA avant chromatography system FPLC (Cytiva, USA) was used with a reversed-phase chromatography column (RPC, 3ml) (GE-Healthcare Bio-Sciences AB, Sweden). Before every run, the system was cleaned with 20% ethanol and then an empty run with eluent B was repeated until obtaining a straight line.

After loading 0.5 ml of the PJ-WS-LE loading solution, the system was run with a constant flow of 2ml/min, and fractionation was started after 12min with 10ml per fraction, 12 conical tubes (50ml) were used for fractions collections. Fractions 2 to

fraction 7 showed clear UV absorbance peaks and were collected to test their antimicrobial efficacy. Cycle 1 was repeated twice to demonstrate results reproducibility (Wiesner *et al.*, 2009).

Upon identification of biologically active fraction(s), the fractionation cycle was repeated again with a less steep concentration gradient change around the active fraction elution time with a collection size of 2ml per fraction, a deep 96-well plate was used for fractions collection in cycle 2.

3.4.4. FPLC fractions processing

Tubes containing the fractions obtained were incubated in a sterile oven at 55°c to evaporate the ACN solvent. Obtained pellets of cycle 1 were re-dissolved in 1ml SDW to be used. Obtained pellets of cycle 2 were re-dissolved in 0.5ml SDW to be used.

3.4.5. Antimicrobial effectiveness of the various fractions

The 96-well plate method was used to test the antimicrobial activity of the six fractions F2 to F7 of fractionation cycle 1 and of the twelve fractions (B8, to C7) of fractionation cycle 2. A mixture of all tested fractions (total) and PJ-WS-LE crude extract were also tested using the same experimental design. The total solution was prepared by mixing 100µl of each fraction. Four replication of each fraction were prepared. Each fraction of cycle 1 including the crude extract was serially diluted nine times in PDB medium, crude extract dilutions ranged from 13mg/ml to 0.05mg/ml, fractions were diluted from their original elution volume by dilution factors ranging from 1/2 to 1/512. The last three rows if the 96-well plate were used as control rows, row 10 contain no extract fractions, row 11 contains antimicrobial agent and the last row does not contain any spores. Representative microorganisms chosen for the analysis were: *A. alternata*, *S. aureus* and *C. albicans*.

As for fractionation cycle 2, first the sub-fractions were tested against *C. albicans*. Upon identification of the active fraction(s), active fraction(s) was (were) tested against: *A. niger*, *P. chrysogenum*, *B. cinerea*, *F. oxysporum*, *P. citrinum*, *C. gloeosporioides*, *C. cladosporioides*, *G. candidum*, *E. coli*, *P. mirabilis*, and *B. Subtilis.* Since the volumes obtained were lower, only six dilutions of each fraction were prepared, while nine dilutions of the crude extract with the three above described controls were run in parallel. The same experimental design described for testing fractions of cycle 2 was used. An ineffective fraction was used as a negative control. Each test was repeated twice.

3.4.6. Testing for active fraction thermostability

Active fraction F6 was incubated for 24h at temperatures ranging from 50°C to 80°C and then 96-well plate test was repeated against *C. albicans* using the same method described above.

CHAPTER 4: RESULTS AND DISCUSSION

4.1. SCREENING THE ANTIMICROBIAL EFFECTIVENESS OF THE FRESH JUICE AND OF THE AQUEOUS AND ETHANOLIC EXTRACTS OF THE LEAVES AND PERICARPS OF *P. JULIFLORA*.

4.1.1. Food spoiling fungal isolates molecular identification

The fungal stains used were previously isolated from fruits and vegetables and microscopically identified (Appendix 1). The DNA of all isolated fungi was extracted and PCR was used to amplify conserved genes (ITS1-5.8S-ITS2 rRNA gene cluster). Purified PCR products are shown in figure 5.

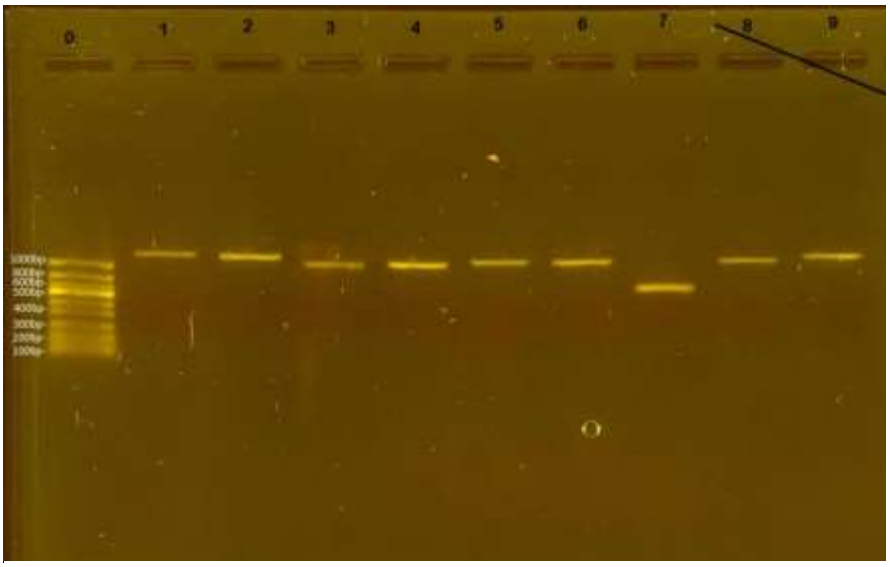


Figure 5**.** Gel electrophoresis results of purified PCR products of the nine fungal strains used in this study in 1% agarose gel. (0.50bp DNA ladder (Thermo Fisher Scientific, USA) 1.*Aspergillus*, 2.*Penicillium* 1, 3.*Botrytis*, 4.*Fusarium*, 5.*Alternaria*, 6.*Penicillium* 2, 7.*Geotrichum*, 8.*Colletotrichum*, and 9.*Cladosporium*.

PCR products were subject to sequencing followed by blasting on NCBI website, isolates were identified at the species level as indicated in table 29.

Table 29. PCR products blasting results indicating species of fungal isolates used.

Serial #	Fungal genus (Microscopic identification)	Sequence length	Fungal species (molecular identification)
1	*Aspergillus*	720bp	*Aspergillus niger*
2	*Penicillium 2*	1115bp	*Penicillium chrysogenum*
3	*Botrytis*	1006bp	*Botrytis cinerea*
4	*Fusarium*	904bp	*Fusarium oxysporum*
5	*Alternaria*	899bp	*Alternaria alternata*
6	*Penicillium 1*	1011bp	*Penicillium citrinum*
7	*Colletotrichum*	677bp	*Colletotrichum gloeosporioides*
8	*Cladosporium*	1081bp	*Cladosporium cladosporioides*
9	*Geotrichum*	357pb	*Geotrichum candidum*

4.1.2. In-vitro antimicrobial effect of crude extracts

4.1.2.1. Antifungal effect

PDA plates with 20 mg/ml of *P. juliflora* extracts (aqueous and ethanolic, leaves and fruits) were used to test the antifungal efficacy of the extracts on all fungal strains identified except *Penicillium* strains for which pour plate method was used. Within the same trial, plates made by PDA powder and *P. juliflora* leaves and fruits fresh juices were also used. Results of percentage inhibition of mycelial growth are shown in figures 6 and 7. Comparison shows that leaves extracts were more effective than fruits extracts. Fruits extracts enhanced the growth of some fungal species such as: *Cladosporium cladosporioides*, *Fusarium oxysporum*, *Colletotrichum gloeosporioides*,

and *Geotrichum candidum* (Figure 7). It is worth noting that the results presented were of 5 days reading, however, at 48 hours *B. cinerea* for example showed a much faster growth in the presence of fruits extracts (43.09mm diameter) than the control (14.72mm diameter) although both have reached maximum growth in 5 days. Therefore, fruits extracts were not subjected to any further analysis as antifungal agents.

Among the leaves extracts, ethanolic extract showed the best results with 100% inhibition of the growth of *B. cinerea* and *A. alternata* (Figure 7). Inhibition rate was 64%, 60.8% and 59.8% to *Geotrichum candidum*, *Colletotrichum gloeosporioides* and *Colletotrichum gloeosporioides* respectively. Therefore, leaves ethanolic extract was chosen for further study its efficacy as an antifungal natural product.

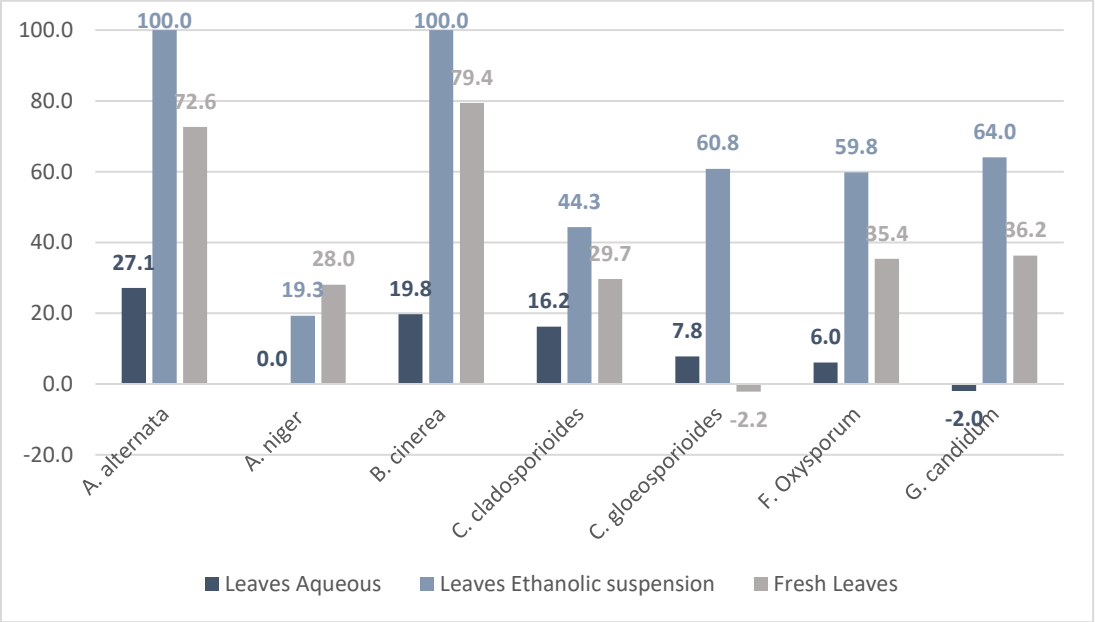


Figure 6. Percentage inhibition of mycelial growth (PIMG) of the seven tested fungal species using *P. juliflora* leaves extracts prepared in three different methods. Aqueous and ethanolic extract concentration used was 20mg/ml.

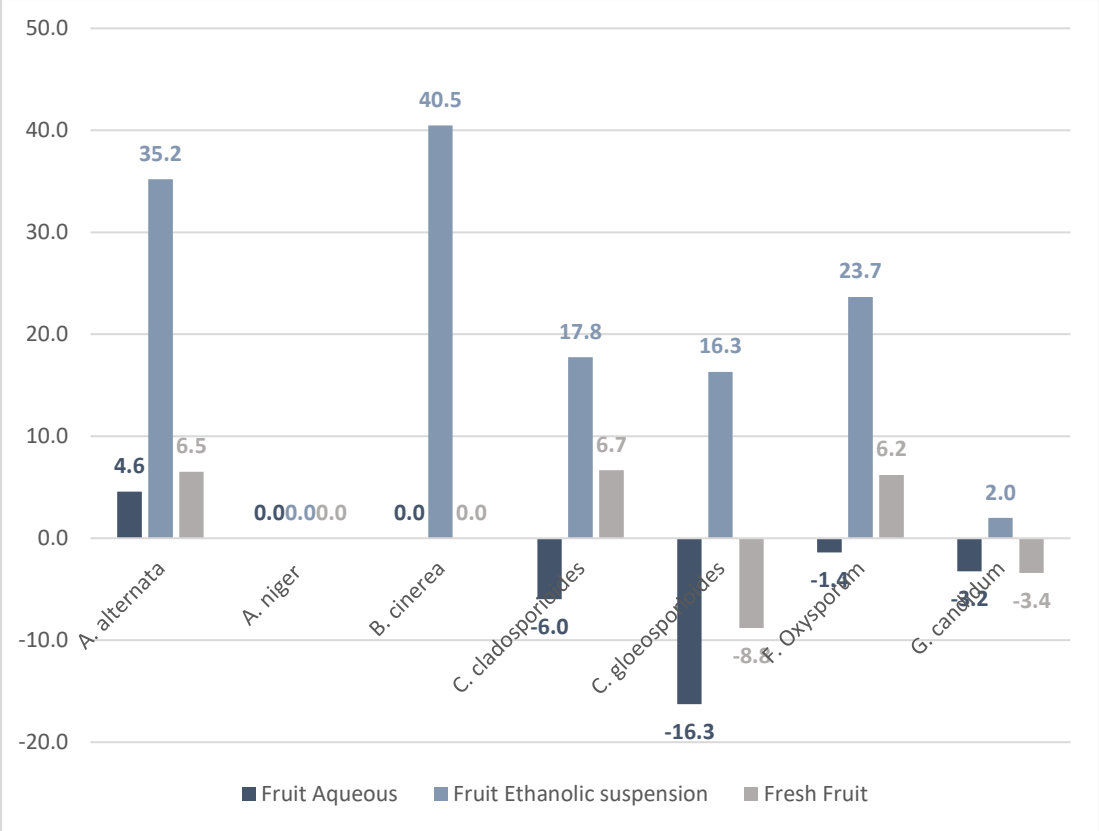


Figure 7. Percentage inhibition of mycelial growth (PIMG) using *Prosopis juliflora* fruits extracts extracted in three different forms. Aqueous and ethanolic extract concentration used is 20mg/ml.

It is worth noting that during the running of the control plates that contain the solvents only without plants extract, PDA plates with 4% DMSO concentration, which was the concentration used to dissolve the ethanolic extract, showed antifungal activity as shown in figure 8. The high inhibition rate showed that DMSO should not be used as a solvent. To avoid DMSO the leaves ethanolic extract was dissolved in water only and centrifuged to precipitate the non-water soluble particles and then exclude them from the analysis. PJ-WS-LE extract was used for a final anti-fungal assay. Results of five days old plates are shown in figure 9.

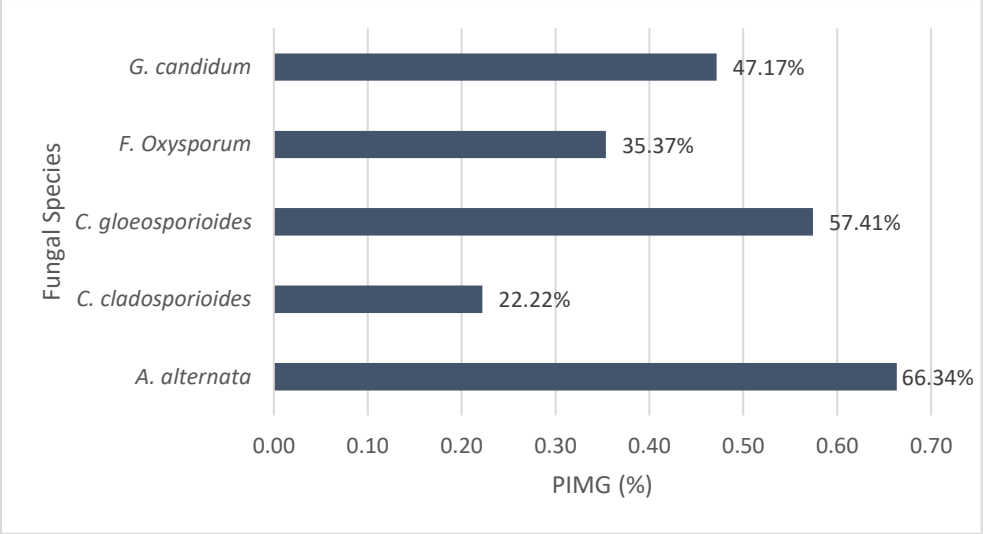


Figure 8. Percentage inhibition of mycelial growth (PIMG) of 4% DMSO.

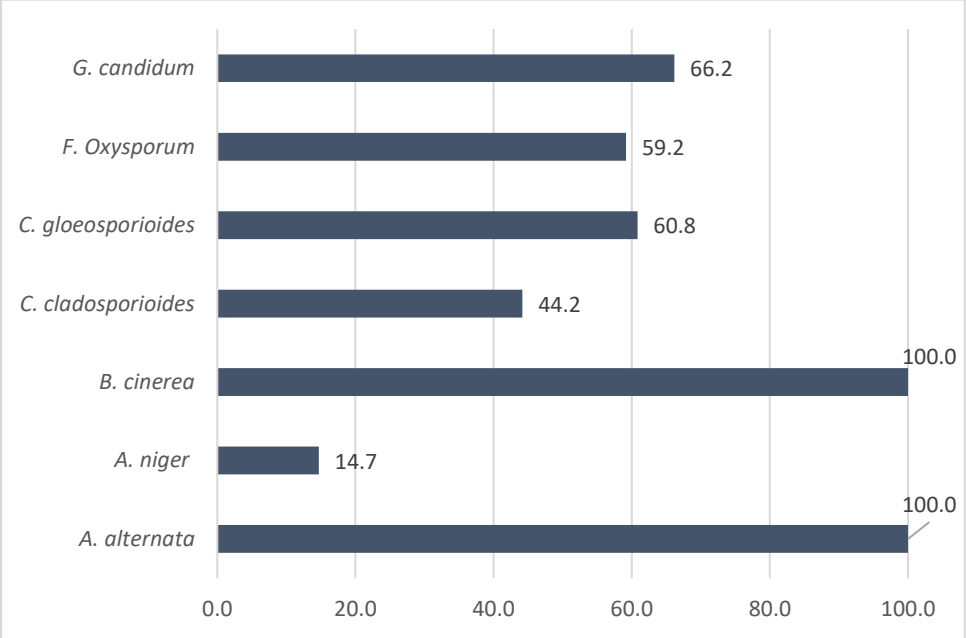


Figure 9. Percentage inhibition of mycelial growth (PIMG) of 20mg/ml of PJ-WS-LE extract.

The homogeneous water soluble leaves ethanoic extract showed results very similar to those obtained in the first leaves ethanolic extract trial which indicates that the active phytochemicals of this extract are water-soluble. Results showed 100% inhibition of the growth of *B. cinerea* and *A. alternata* and high inhibition rates to *G.*

candidum and *C. gloeosporioides*. Trial was conducted in four replications for each fungal species (Appendix 2). Mycelial diameters in plates containing leaves ethanolic extracts and mycelium diameter in control plates were shown with the standard deviation error bars in figure 10. T-test showed significant difference between the mycelium diameters of the controls and the experimental plates for all fungal strains ($p \leq 0.05$) except for *A. niger* ($p = 0.279 > 0.05$).

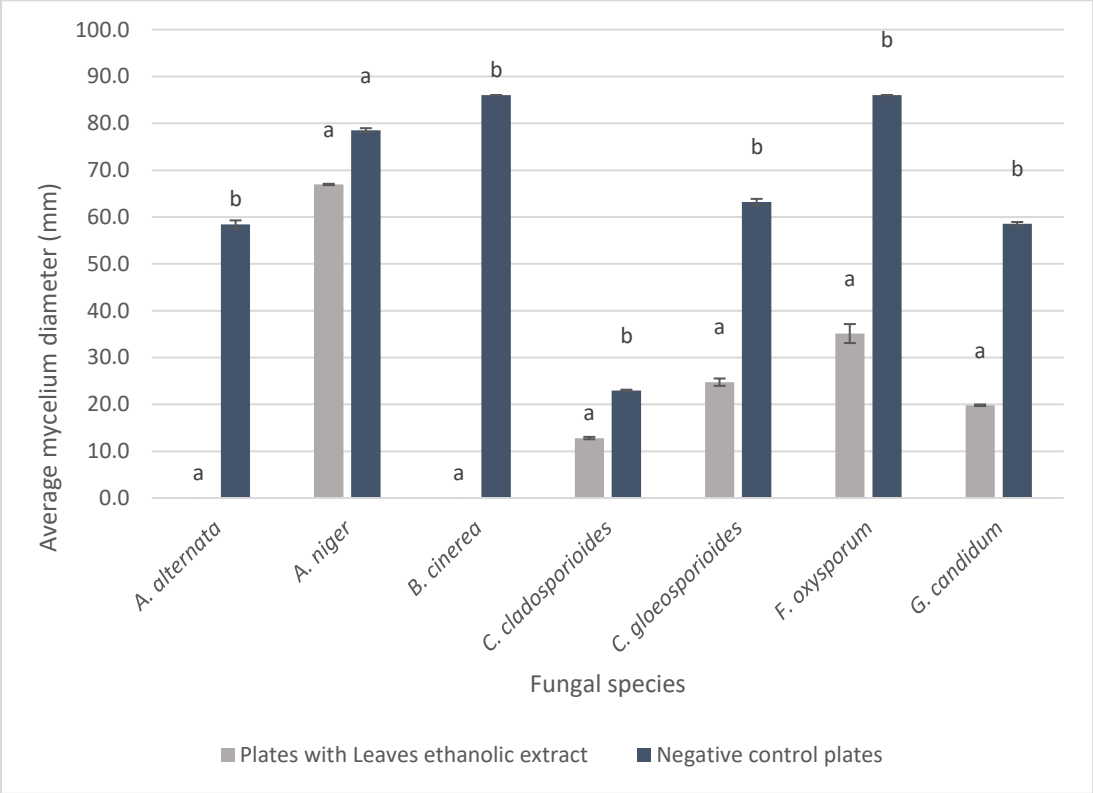


Figure 10**.** Average mycelium diameter (mm) ± SE (N =4) of seven fungal species in the

presence and absence of PJ-WS-LE extract after 5 days incubation at 25°C.

[ab] Treatment columns with different letters have their values significantly different as shown by t-test at $p = 0.05$.

For the pour plate method results, *P. citrinum* was 100% inhibited upon soaking its spores for 1.5h in 20mg/ml of the PJ-WS-LE extract following by pouring them in PDA plates of the same PJ-WS-LE extract concentration. As for the case of *P. chrysogenum* the fungal strain was not affected at all by the extract.

Different parts of *P. juliflora* were evaluated for their antimicrobial activity in various studies, yet studies had their gaps including the usage of organic solvents in extracts preparation which might be toxic to the environment and to human health, in addition, some studies lack control batches. Preliminary studies showed antifungal effect of various extracts of *P. juliflora*. Raghavendra *et al.*(2009) indicated that the aqueous leaf extract of *P. juliflora* significantly decreased mycelial growth of *A. alternata* isolated from tobacco leaves with a PIMG of 71.59% at a concentration of 24%. This inhibition figure is comparable to our results in which the PIMG of *A. alternata* was found to be 80.57% after exposure to 2mg/ml of PJ-WS-LE extract and 100% after exposure to 20mg/ml concentration. On the other hand, a study, examined the effect of the methanolic extract of *P. juliflora* leaves on soil-borne pathogenic fungus *Sclerotium rolfsii* had no significant antifungal activity against this species (Sana *et al.*, 2016). Bazie *et al*. (2014) tested the antifungal effect of leaf extracts from different plant species against *Colletotrichum musae*, the causative agent of postharvest banana anthracnose, and they found that the methanolic extract of *P. juliflora* leaves showed the best results in fungal growth inhibition followed by *Acacia albida*. A study in 2009 examined the antifungal effect of leaf extracts from multiple plant species against different *Fusarium species* found that using a fresh infusion of *P. juliflora* leaves resulted in a PIMG of 80.25% against a species of *F. oxysporum*, compared to 59.2% obtained in our study. The extraction methods including the final solvent and the concentrations used in the two studies are different (Satish *et al.*, 2009). Fresh extract

from the maceration of *P. juliflora* leaves had a low antifungal efficacy against *Alternaria solani*, with a PIMG of 27.14% at a 10% concentration (Rex *et al.*, 2019). While our extraction method showed a very high antifungal efficacy (100% inhibition) against a strain of *Alternaria* (*A. alternata*)

4.1.2.2. Disk diffusion method (antibacterial and antifungal effect)

Disk diffusion method was used to characterize the antimicrobial effect of the crude extracts on seven bacterial isolates: two strains of *E. coli*, *P. mirabilis*, *S. aureus*, *B. Subtilis*, and *P. aeruginosa* were used. An initial screening showed that fruits extracts couldn't inhibit bacterial growth but ethanolic leaves extract was more effective than aqueous leaves extract. The various concentrations of PJ-WS-LE showed good inhibition of four of the tested strains as shown in figure 11. *B. subtilis* was more inhibited by the extract than by 70% Dettol. As for *S. aureus* the diameter of the inhibition zone of 50mg/ml was almost comparable to that of 70% Dettol.

The antibiogram showed that the bacterial strains tested were multi-drug resistant, especially the strains that were the most sensitive to the leaves extract (Table 30). All disk diffusion results were conducted in triplicates (Appendix 3).

The effect of PJ-WS-LE extract against *C. albicans* was also tested using disk diffusion method. Experiment was conducted in triplicates and dose-dependent inhibition of the yeast strain was very clear with the highest diameter of inhibition (18.43mm) found with 50mg/ml extract while diameter of inhibitory zone resulted from 70% Dettol was 22.5mm.

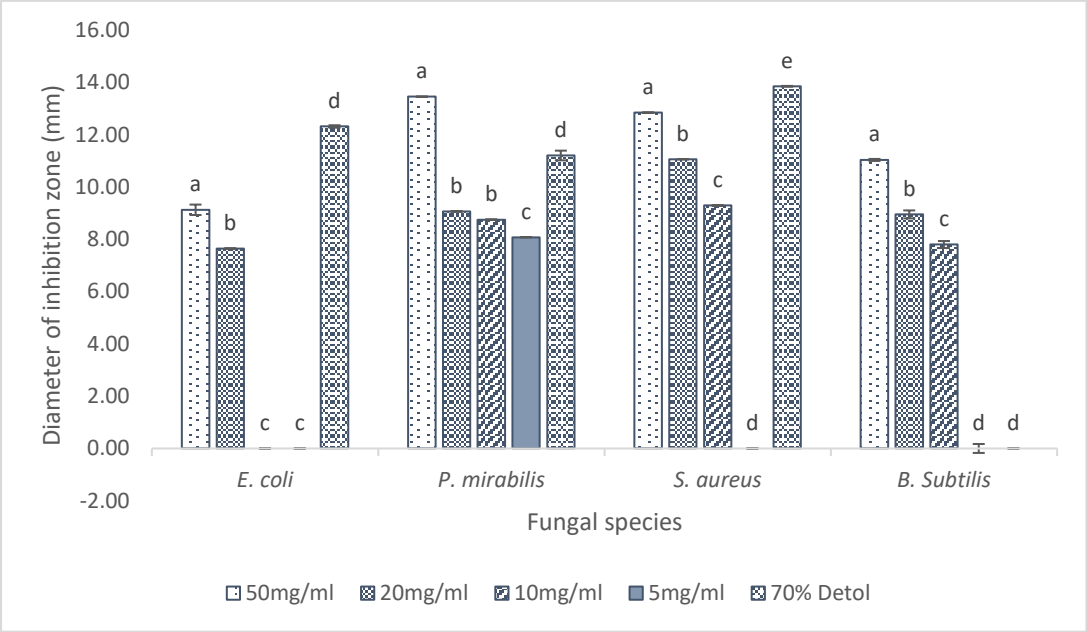


Figure 11. Diameter of the inhibition zone (with standard deviation bars, N =3) of different concentrations of PJ-WS-LE extract against four bacterial strains using disk diffusion method.

[abcde] Treatment columns with different letters have their values significantly different as shown by the one way ANOVA test at p=0.05.

Table 30. Antibiogram of seven bacterial strains to (Ampicillin (AMP), Amoxicillin (AMX), Bacitracin (B), Carbenicillin (CB), and Cephalothin (CR)).

	AMP		AMX		B		CB		CR	
E. coli 1	22.6	(S)	21.75	(I)	0	(R)	25.1	(I)	0	(R)
E. coli 2	21.7	(I)	17.28	(R)	0	(R)	17.59	(R)	24.68	(S)
P. microbilis	28.3	(S)	29.9	(S)	0	(R)	30.97	(S)	20.75	(S)
S. aureus	9.36	(R)	9.03	(R)	0	(R)	12.57	(R)	16.62	(I)
B. Subtilis	10.64	(R)	10.2	(R)	8.39	(I)	10.26	(R)	28.15	(S)
P. aeruginosa	0	(R)	0	(R)	0	(R)	21.55	(S)	0	(R)

(S) means bacteria is susceptible to the antibiotic, (R) means bacteria is resistant to the antibiotic and (I) means that the bacteria has intermediate resistance to the antibiotic.

Disk diffusion method results showed that the diameters of inhibition zones of Gram-positive bacteria (*S. aureus* and *B. subtilis*) around PJ-WS-LE extract were higher than some of those caused by commonly used antimicrobials including ampicillin, Amoxicillin, Bacitracin and Carbenicillin. Singh and Verma (2011) examined the effect of 100mg/ml of ethanolic extract of the leaves, pods and flowers of *P. juliflora* against different bacterial strains. The disk diffusion method showed that the inhibitory zone diameters of *E. coli* and *S. aureus* treated with 100mg/ml of leaf ethanolic extract were 12.81±0.45mm and 12.72±0.67mm, respectively. These numbers were close to our results showed inhibitory zone diameters of *E. coli* and *S. aureus* treated with 50mg/ml of PJ-WS-LE extract were 9.13±0.20mm and 12.85±0.01mm, respectively. Note that half the amount of extract prepared in this study was as effective as Singh and Verma's extract in the case of *S. aureus. D*ifferent concentrations of *P. juliflora* leaf ethanolic extract were tested for their antimicrobial

activity against bacterial strains including *S. epidermis*, *S. aureus*, *Streptococcus spp*. *Micrococcus luteus*, *B. subtilis*, *Salmonella typimurium*, *Klebsiella pneumonia*, *E. coli*, and *Pseudomonas spp* in two different studies. Results showed growth inhibition activity. It is noted that *S. aureus* was more inhibited by extract in the present work, while *E. coli* and *B. subtilis* showed similar values to Sathiya and Muthuchelian's study (2008), while Thakur *et al.* (2014) have used higher extract concentrations. It is important to know that the novel extraction method used in this study was different from the other two studies in which leaf extract was dissolved in ethanol or in DMSO, which may had antimicrobial effects themselves regardless of the extracted phytochemcials (Sathiya & Muthuchelian, 2008; Thakur *et al.*, 2014).

A study by Osuru *et al.* (2011) analyzed the aqueous extract of *P. juliflora* leaves as a possible mouthwash solution, results showed that extract inhibited growth of *Enterococcus faecalis*, *S. aureus*, *Prevotella intermedia*, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans.* The inhibitory zone diameter of *S. aureus* was 10.16±0.28mm, which is still lower than the inhibitory zone diameter obtained with the extraction method used in our study (11.06±0.01). Both extraction methods however, involved water as a final solvent.

A recent study examined the enhanced effect of *P. juliflora* methanolic extract combined with silver nanoparticles as an antimicrobial agent against strains of *Candida sp.* and *Staphylococcus sp.* Leaves of *P. juliflora* were dried, grinded, and soaked in methanol for 24h, and the extract was filtered and mixed with a solution of silver nitrate ($AgNO_3$). Upon color change, the precipitate was washed, dried, and used for further analysis. Results showed that 1µg/ml of this nano-powder was capable of totally inhibiting the growth of *MRSA*, *C. albicans* and C. *tropicalis* (Anwar *et al.*, 2019). In the present study, both *S. aureus* and *C. albicans* were completely inhibited

by our extract with an MIC of 500µg/ml. The combined effect of *P. juliflora* leaf aqueous extract with silver nanoparticles encapsulated in chitosan showed a higher diameter of inhibition zone (22mm) against *E. coli* compared to the current study (9.13mm), this was due to the combined antimicrobial effect of chitosan and the synthesized nanoparticles (Malini *et al.*, 2020). Zinc monoxide nanoparticles derived from *P. juliflora* leaf aqueous extract had also a successful antimicrobial effect against *E. coli* and *B. subtilis* with an inhibitory zone diameter of 23mm and 19mm, respectively, at 100µg/ml concentrations (Sheik Mydeen *et al.*, 2020). These findings have shown that plant extracts enhanced with nanoparticles or coating material lead to a higher antibacterial efficacy. However, nanoparticles enhanced studies lacked the control batches that test for the antimicrobial effect of the metals nanoparticles themselves. Unlike PJ-WS-LE extract where phytochemicals were dissolved in water, these materials require further analysis to test for their possible health risks and to ensure that they are safe for the environment if they are to be used in the field or as a postharvest controller.

4.1.3. Extraction yield

Among the tested extract, PJ-WS-LE extract was the most effective as an antimicrobial agent therefore, the extraction method yield was calculated each time new extract was prepared. Average extraction yield is around 11%.

4.1.4. PJ-WS-LE extract stability

Agar diffusion method was conducted using the seven previously tested fungal species using old powder and liquid extracts. Results showed a confirmed stability of the active antifungal compounds of the crude extract preserved at 4°C in powder form and in aqueous solution form. Figure 12 shows the PIMG of the seven fungal strains

using six month old extract compared to freshly prepared PJ-WS-LE extract. No significant difference was observed between the three experimental batches.

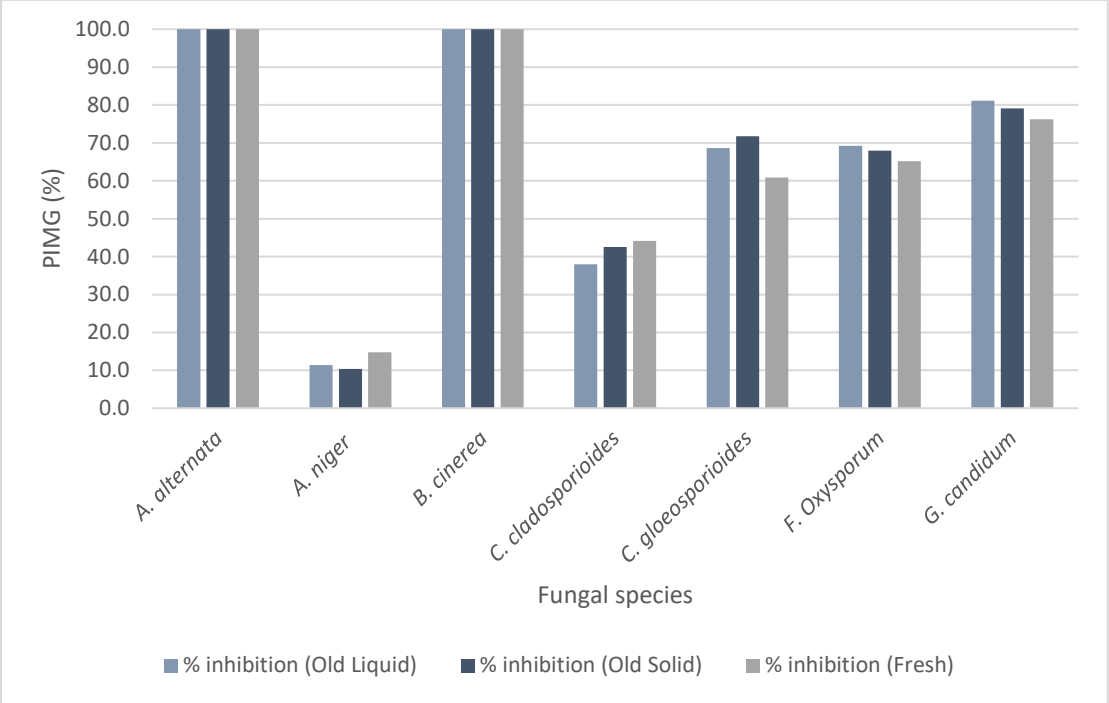


Figure 12. Percentage inhibition mycelium growth of seven fungal strains by 20mg/ml of six-month-old PJ-WS-LE extract compared to fresh extract.

4.1.5. Determination of minimum inhibitory concentration

Results of the 96-well plate experiment for fungi and bacteria are shown in appendix 4 and appendix 5. Table 31 shows the different MICs of the PJ-WS-LE extract to all tested fungi, bacteria and yeast isolates. Results were recorded after 72h of incubation. Spores that germinate at all extract concentrations had their MICs valued as >50mg/ml. Beside the three fungal species that showed total inhibition of their growth on PDA plates (*B. cinerea*, *A. alternata* and *P. citrinum*), *C. cladosporioides* and *G. candidum* had their spores germination totally inhibited in the presence of the

extract. Interestingly, *A. niger* at 48h had an MIC=2.5mg/ml, yet spores grew in all wells at 72h, which means that the extract delayed the fungal growth.

The MICs of bacterial types that showed inhibition zone in the disk diffusion method were only evaluated. Extract completely killed Gram-positive species in liquid culture even when used at very low concentrations. While Gram-negative species showed cellular activities at all extract concentrations although were partially inhibited on agar.

Table 31. Minimum inhibitory concentrations of PJ-WS-LE extract.

Fungal species	MIC (mg/ml)	Bacterial Type	MIC (mg/ml)
A. niger	>50	*B. subtilis*	0.125
P. chrysogenum	>50	*S. aureus*	0.5
B. cinerea	1	*E. coli*	>50
F. oxysporum	>50	*P. microbilis*	>50
A. alternata	1		
P. citrinum	2		
C. gloeosporioides	>50		
C. cladosporioides	4		
G. candidum	2.5		
C. albicans	0.5		

Many studies aimed to find the MIC of *P. juliflora* crude extracts, enriched extracts, and purified compounds against a scattered group of microorganisms; however, none of the studies was comprehensive enough to test for the efficacy of a

particular extract against a range of spoiling organisms. Santos *et al*. (2013) subjected the ethanolic extract of *P. juliflora* pods to acid-base treatments to obtain alkaloid-enriched extracts. The ethanolic extract in that study was suspended in acetic acid solution, and then the aqueous phase was extracted with chloroform or ethyl acetate at different pH levels. The basic chloroformic extract showed the best antimicrobial efficacy as well as a high alkaloid concentration. The MIC of our PJ-WS-LE extract on *S. aureus* and *B. subtilis* were 0.5mg/ml and 0.125mg/ml respectively, while the MICs obtained using the alkaloid enriched extract, particularly the basic chloroformic extract, were 0.05mg/ml for *S. aureus* and above 0.1mg/ml for *B. subtilis*. It is worth noting that crude extracts could be safer for human health and for the environment than concentrated phytochemicals which might exhibit toxicity. Singh & Verma (2011) found that the MIC of the alkaloid rich fraction of the ethanolic extract of *P. juliflora* leaves on strains of *S. aureus* and *B. cereus* was 0.05mg/ml, compared to our MICs of similar strains of *S. aureus* and *B. subtilis* (0.5mg/ml and 0.125mg/ml). The lower MIC results from Singh & Verma's research can be justified by the concentrated active phytochemicals in the alkaloid-rich fraction**.** Both previously discussed studies showed lower MICs compared to our study because the use of concentrated active compounds normally result in lower MICs than the usage of crude extracts. However, if crude extracts continue to demonstrate successful inhibitory activity against microorganisms, they will be of commercial interest as they have feasible extraction methods that can be conducted in developing nations at a low cost. In addition, crude extracts are likely to have lower toxicity compared to concentrated pure phytochemicals (Lima *et al.*, 2017). The investigation of another species of *Prosopis* showed that the aqueous ethanolic extract of stem bark of *Prosopis chilensis*, which are rich with tannins, has significant antimicrobial activity against *Micrococcus luteus*, *Bacillus Subtilis*, *Bacillus cereus*,

Staphylococcus aureus, *Streptococcus pneumonia*, and *Cryptococcus albidus*. The MICs of the stem bark extract against *B. subtilis* and *S. aureus* were 0.16mg/ml and 0.62mg/ml respectively, which are very close to the MICs of our PJ-WS-LE extract (0.125mg/ml and 0.5mg/ml) (Singh *et al.*, 2010). The similarity between these results suggests the presence of common active phytochemicals in the two extracts. Alkaloid extracts of *P. juliflora* showed a complete inhibition of *A. alternata* growth at a concentration of 1mg/ml while common fungicides in the market are typically used at a concentration of 2mg/ml. This number is equal to the MIC of our crude extract against *A. alternata* (Raghavendra *et al.*, 2009).

4.1.6. Crude extract effect on fungal spores' germination

Spores suspensions exposed to various concentrations of PJ-WS-LE extract for 24h with shaking at 25˚C were evaluated for germination percentage. Results are summarized in table 32. Percentages of spores germination at the start of the experiment (before incubation) were also calculated and subtracted from the final percentages of spores germinating in the presence of the leaves extract to rule out spores that were already germinated on the PDA plates, the margin of error in this calculation is what explains the minus values in the case of *A. alternata* and *G. candidum*. PJ-WS-LE extract at 8mg/ml concentration inhibited spores' germination of all fungal species that showed total inhibition in the 96-well plate experiment except for *C. cladosporioides*. There was no significant difference in *C. cladosporioides* spores germination in the presence or absence of the extract, which was not in consistence with previous experiment, it is very possible that shaking allowed the fungi to escape the extract inhibition by a certain mechanism. It is worth noting that *A. niger* showed a very low level of spores germination (4.67%) in the presence of 8mg/ml of the extract within 24h, this was in consistence with previous results that showed that *Aspergillu*s growth

is delayed when exposed to the extract and those results might change if incubation time is increased. *Colletotrichum gloeosporioides* and *P. chrysogenum* showed a dose dependent spores germination inhibition when exposed to PJ-WS-LE extract. The most resistant fungal species was *F. oxysporum* which showed only 8.9% spores germination decrease at 8mg/ml of the extract. Finally, microscopic observation showed that germinated spores even of the most resistant strains showed tress like symptoms (Appendix 6).

Table 32. Percentage of germinating spores after 24h of exposure to different concentrations of PJ-WS-LE extract.

	Control	2mg/ml	4mg/ml	8mg/ml
A. niger	44.93	17.81	9.38	4.67
P. chrysogenum	95.74	40.46	30.43	21.70
B. cinerea	79.21	0.00	0.00	0.00
F. oxysporum	99.00	99.00	99.00	89.91
A. alternata	52.96	6.94	3.87	-0.89
P. citrinus	45.15	47.46	24.16	4.58
C. gloeosporioides	59.31	26.87	19.81	14.13
C. cladosporioides	51.79	56.56	48.51	49.61
G. candidum	72.06	75.29	16.28	-0.72

4.1.7. Mode of action of fungal or bacterial inhibition

Fungal mycelia that failed to grow or was inhibited by a plant extract were transferred to a clean PDA plate (without extracts) and incubated at 25˚C for 7 days. Results are shown in figure 13. The average diameter of *A. alternata* and *B. cinerea*

disks were much lower in recovered disks than in the negative control. Some of the disks couldn't re-grow at all.

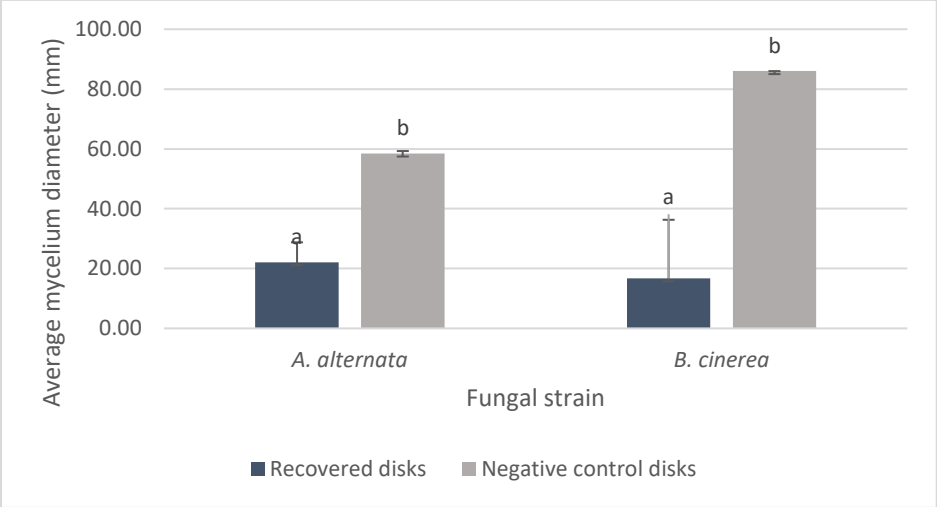


Figure 13. Average diameter ±SE of fungal plugs transferred from 20mg/ml PJ-WS-LE extract plates to clean PDA plates compared to direct fungal plugs growth on clear PDA plates.

[ab] Treatment columns with different letters have their values significantly different as shown by t-test at p=005.

When fungal spores and bacteria were incubated in the presence of PJ-WS-LE extract for 48h (8mg/ml) and then spread on clean media. Results showed that the mode of inhibition of *A. alternate*, *B. cinerea* and *C. albicans* is fungicidal, while the mode of inhibition of *G. candidum* and *C. cladosporioides* is fungistatic (Appendix 2). As for the effect of the extract on *S. aureus* and *B. subtilis* results showed bacteriostatic mode of action (Appendix 3).

4.1.8. The effect of PJ-WS-LE extract on the microscopic morphology of the studied fungal species hyphae and spores

Scanning electron microscope (SEM) was used to evaluate the effect of PJ-WS-LE extract on the hyphae and spores of nine fungal species. SEM evaluation showed damage of the external morphology of all tested fungal species. *A. niger* was not entirely inhibited by the extract as shown in previous assays. However, the typical net smooth surface of the hyphal structure seen in the non-treated control samples of *A. niger* (Figure 14a) was not maintained when it was exposed to 8mg/ml of PJ-WS-LE extract for 24h. The extract caused loss in the hyphae smoothness of this fungal species; exposed hyphae were more applanate with exfoliated flakes and small pores in some places (Figure 14b). At higher magnification (25.000X), *A. niger* spores showed surface damages and small pores (arrow) (Figure 15b) that were not seen in the control (Figure 15a). Control (non-treated) samples of *P. citrinum* and *P. chrysogenum* showed normal filamentous tubular hyphae while treated samples showed severely fractured hyphal structure with vacuolation that might indicate leakage of essential intracellular components (data not shown). Treated *Penicillium* spores were shrunken compared to control (Figure 15c) with craters of different sizes (arrows) (Figure 15d). *C. cladosporioides* is one of the fungi that its growth was inhibited by the leaf ethanolic extract *in-vitro*; this was also clearly indicated by the large holes in the treated spore membranes shown by SEM (Figure 15f), untreated *C. cladosporioides* sample showed smooth spores (Figure 15e). Treated *B. cinerea* mycelium lost their tubular shape (Figure 14c) and showed degenerative changes including applanation and formation of exfoliated flakes while their spores at higher magnification (10.000X) were totally collapsed (Figure 14d). Microscopic observation of this fungus supports the total growth inhibition observed in previous experiments.

Untreated samples of *G. candidum* showed nice tubular hyphae (Figure 14e) that were distorted and flattened when treated with the extract (Figure 14f). Tubular unique spores of *G. candidum* (Figure 15g) were collapsed when treated with the antifungal extract (Figure 15h). Spore collapse might be an indicator of loss of intracellular components as indicated earlier and therefore cell death. Growth of *F. oxysporum* was not totally inhibited by the crude extract *in-vitro*, yet SEM images showed severe damage in both mycelium and spores. Treated *F. oxysporum* with extract showed various levels of diastrophic and fractured mycelium and spores, in addition to vacuolation and pores in the case of treated spores (Figure 15j) compared to normal (Figure 15i). The treated *C. gloeosporioides* samples showed distorted mycelium with unusual surface bulges and applanation in some areas (Figure 14h) as well as damaged spores with rugged and fractured surfaces with holes (arrow) (Figure 15l). Figures 14g and 15k shows the normal shapes of *C. gloeosporioides* mycelium and spores for comparison. Finally, *A. alternata* was among the most affected tested fungi by PJ-WS-LE extract. Treated samples showed collapsed spores with large vacuolation while their mycelia lost their smoothness (Figure 14i), shriveled and distorted with large vacuolation indicates loss of intracellular components that leads to death (Figure 14j).

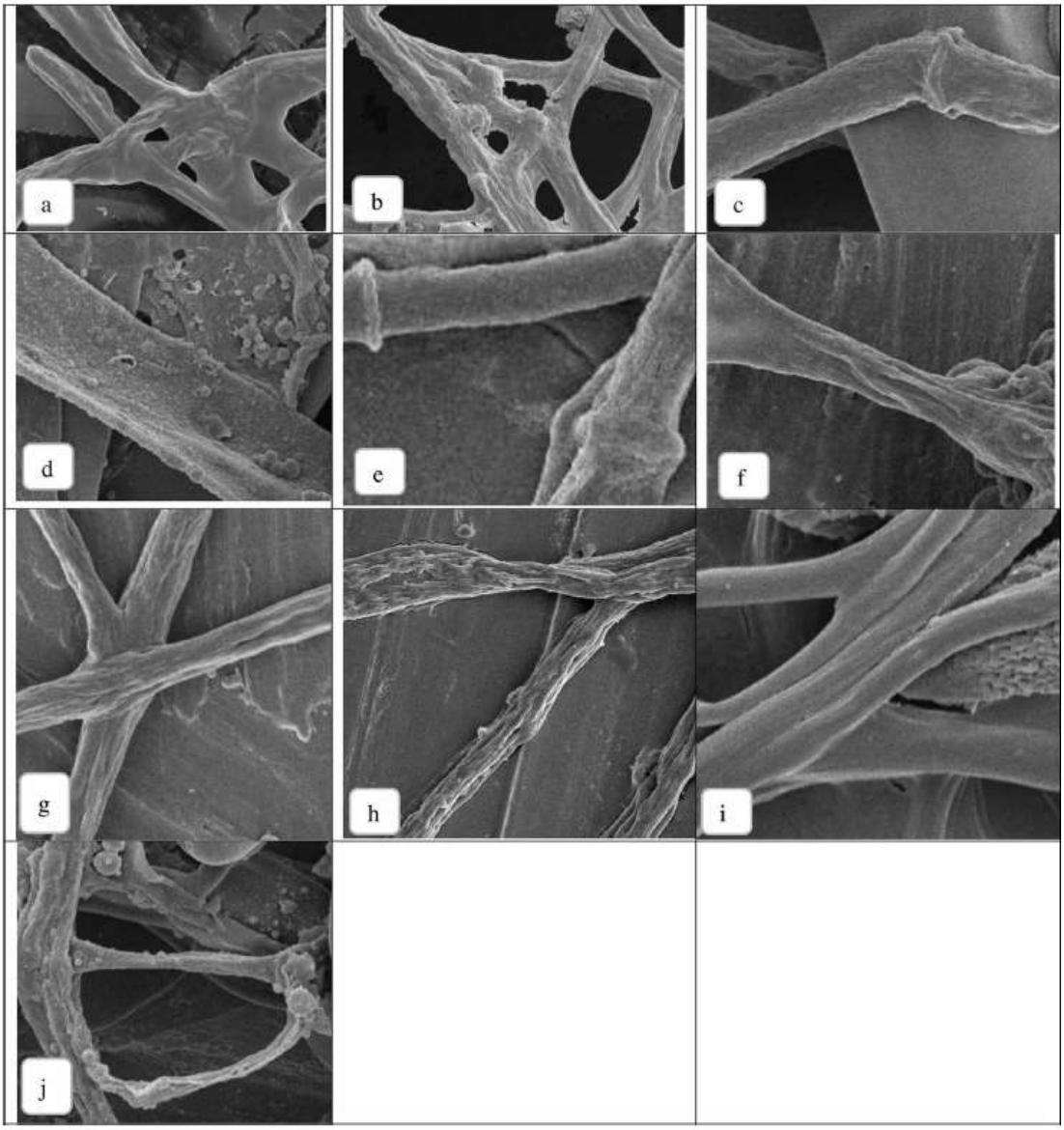


Figure 14. SEM images of the hyphae of control fungi and fungi treated with 8mg/ml PJ-WS-LE extract**.** *A. niger*: untreated (a) and treated (b) (5.000X). *B. cinerea*: untreated (c) and treated (d) (5.000X). *G. candidum*: untreated (e) and treated (f) (5.000X). *C. gloeosporioides*: untreated (g) and treated (h) (10.000X). *A. alternata*: untreated (i) and treated (j) (5.000X).

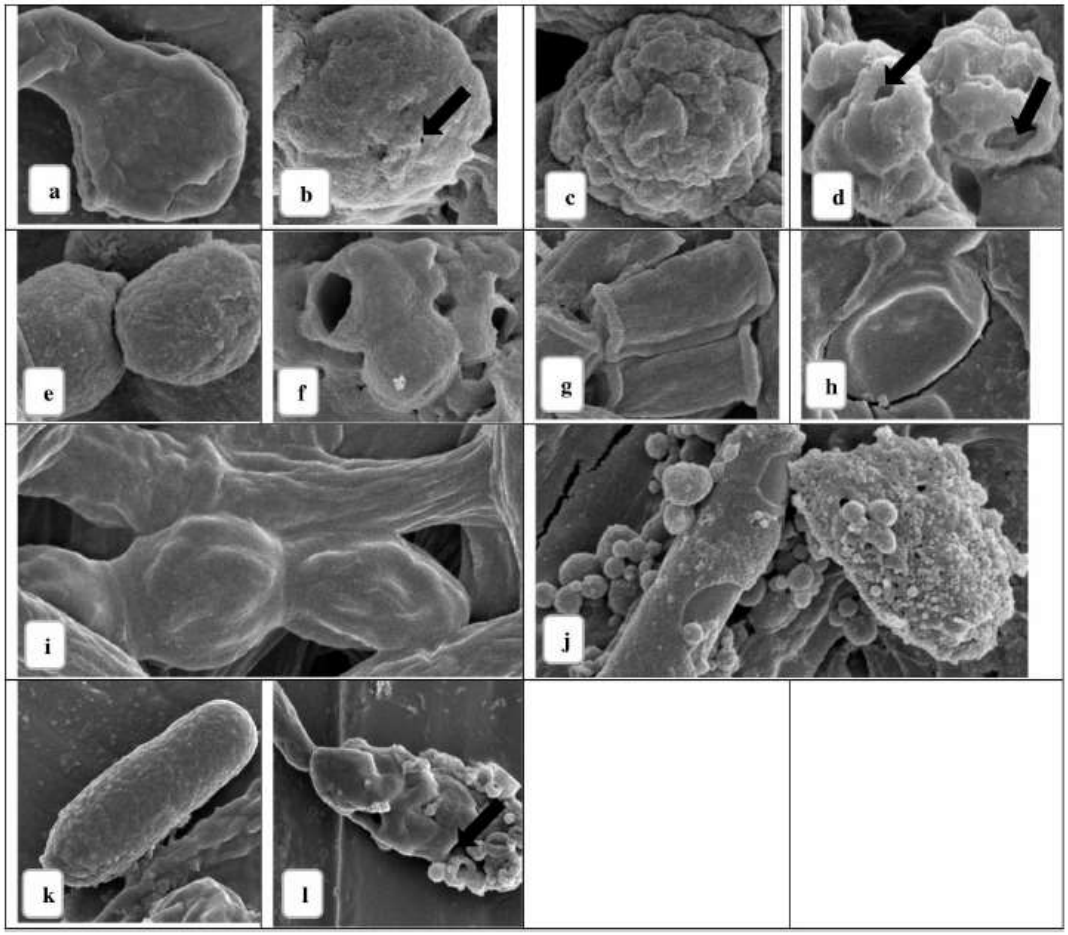


Figure 15. SEM images of the spores of control fungi and fungi treated with 8mg/ml PJ-WS-LE extract**.** *A. niger*: untreated (a) and treated (b) (25.000X). *P. citrinum*: untreated (c) and treated (d) (25.000X). *C. cladosporioides*: untreated (e) and treated (f) (20.000X). *G. candidum*: untreated (g) and treated (h) (10.000X). *F. oxysporum*: untreated (i) and treated (j). *C. gloeosporioides*: untreated (k) and treated (l) (10.000X).

Botrytis cinerea treated with PJ-WS-LE extract resulted in completely collapsed spores and degenerative changes in their mycelium that could be due to a decrease in exopolysaccharide (EPS) formation in their outer membrane. Similar results were observed when *B. cinerea* was treated with phenazine-1-carboxylic acid (PCA) produced by the *Pseudomonas aeruginosa* LV strain. Hyphae of the treated fungi lost

smoothness and formed unusual surface bulges (Simionato *et al.*, 2017). In the present work, growth of *F. oxysporum* was not affected by crude extract *in-vitro*, yet SEM images showed severe damage in mycelium and spores of both tested strains. Similarly, SEC imagery of *Fusarium sporotrichioides* treated with *Mentha piperita* essential oil showed distorted and shrunken mycelia compared with the control (Rachitha *et al.*, 2017). Another study conducted on an economically important pathogenic *Fusarium* strain known as *Fusarium verticillioides* also showed slender, shrunken, and winding hyphae that lost their linearity with some depressions on the surface (Xing *et al.*, 2014).

Various antifungal agents cause morphological changes similar to those caused by PJ-WS-LE extract. When the two pathogenic fungi *Mycrosporum gypseum* and *Trychophyton mentagrophytes* were treated with the lyophilisate of granular gland secretion from *Duttaphrynus melanostictus* frogs, the fungal cells showed cellular deformations and pores. Hyphae of *M. gypseum* showed shrinkage while those of *T. mentagrophytes* showed shrinkage and pores (Barlian *et al.*, 2011). Recently, the treatment of *Villosiclava viren*s, an emerging disease of rice panicles, with the essential oils of 18 plants showed promising results with cinnamon bark oil, cinnamon oil and *trans*-cinnamaldehyde. Scanning electron microscopic imaging of *V. viren*s treated with the vapor of one of the effective essential oils exhibited degenerative changes in the hyphal morphology including exfoliated flakes, applanation, vacuolation and shriveling. Treatment with direct contact of essential oils caused more severe exfoliated flake damage with collapse and blistering (Zheng *et al.*, 2019). The morphological changes caused by PJ-WS-LE extract are indicators if abnormal fungi growth or on stressed fungi which might lose their pathogenicity against fresh produce with time.

4.1.9. Crude extracts cytotoxicity

Coomassie stain assay did not show cellular toxicity on HaCat cells at 4mg/ml and 8mg/ml of the PJ-WS-LE extract (Figure 16).

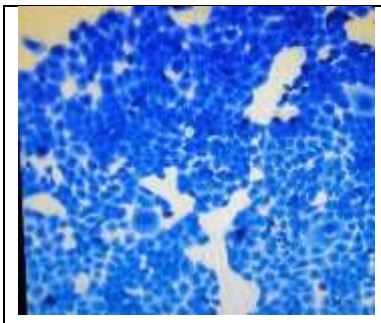
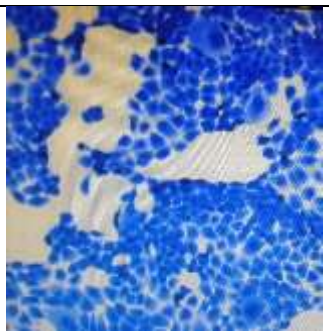
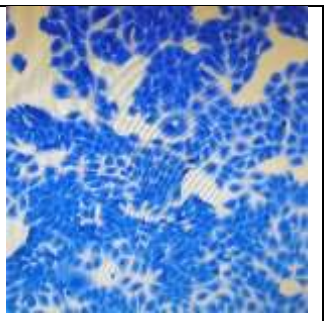
		
HaCat cells after 24h in 8mg/ml	HaCat cells after 24h in 4mg/ml	HaCat control

Figure 16. Coomassie stain assay, 24h results.

Literature showed that cattle and arid lands use of *P. juliflora* as a source of food around the world (Henciya *et al.*, 2017). In addition, the plant's leaves are used in folklore medicine in many countries beside the direct consumption of the fruits (de Brito Damasceno *et al.*, 2018). All these information support our preliminary results and imply the lack of toxic chemicals in the plant to humans and animals.

4.2. STUDYING THE IN-VIVO ANTIMICROBIAL AND QUALITY ENHANCING ACTIVITIES OF THE EFFECTIVE CHOSEN CRUDE EXTRACT

4.2.1. Effect of PJ-WS-LE extract on strawberries self-life at room temperature and at refrigerator temperature

Strawberry samples sprayed with 8mg/ml PJ-WS-LE extract showed longer shelf-life than control samples, table 33 shows the number of samples rotting every day for 5 days.

Table 33. Number of experimental and control strawberry samples lost by fungal spoilage at room temperature (22 to 24°C) every 24 hours.

Number of control samples rotting	Number of experimental samples rotting	Shelf-life (h)	Shelf-life (days)
9	3	24	1
7	6	48	2
6	7	72	3
2	5	96	4
0	2	120	5
0	1	144	6

Average shelf-life of treated strawberries at room temperature was 72h while none treated plants had an average shelf-life of 49h. T-test showed statistically significant difference between control and experimental samples shelf-life (p=0.007≤0.05). Percentage of edible samples after 48 hours was also calculated, 62.5%

of the treated samples were still consumable while only 33.3% of the non-treated samples were still edible within 48 hours (Appendix 7).

Microscopic identification of isolated fungi showed that three fungal species were behind the strawberry samples spoilage. *Botrytis* was isolated from 95.8% of the spoiled samples while *Cladosporium* and *Rhizopus* were isolated from 6.3% and 4.2% of the spoiled samples respectively.

Upon changing storage temperature, it has been proven that the active compounds in the crude extract were capable of preventing fungal growth on strawberry samples at 4°C also to extend treated samples shelf-life. Table 34 shows the number of samples rotting every day for ten days.

Table 34. Number of experimental and control strawberry samples lost by fungal spoilage at 4°C every 24 hours.

Number of Control samples rotting	Number of Experimental samples rotting	Shelf-life (h)	Shelf-life (days)
6	0	96	4
4	0	120	5
0	0	144	6
2	0	168	7
1	0	192	8
3	0	216	9
1	0	240	10

During the 10 days of the experiment, 17 control samples were rotten at different time slots to make the average shelf-life of non-treated samples 103h (4.3 days). On the other hand, all treated samples survived with a shelf-life of 240h (10 days). T-test comparing the overall shelf-life values at day 10 validate variances equality assumption and showed statistically significant difference between control and experimental samples shelf-life (p=0.00≤0.01). The percentage of edible samples at day 10 was 43.3% of the non-treated samples and 100% of the treated samples. Results showed that the crude extract was capable of totally preventing fungal growth on refrigerated strawberry samples for 10 days (Appendix 8).

Upon microscopic identification of the isolated fungi, three fungal genera were behind the control strawberry samples spoilage. *Botrytis* was isolated from 70.6% of the spoiled samples. While *Rhizopus* and *Cladosporium* were isolated from 17.6% and 5.9% of the spoiled samples respectively. Average firmness of the 13 remaining control samples was 42.6N while average firmness of the 30 remaining treated samples was 44.5N. T-test showed no significant difference in the average firmness between treated and non-treated samples ($p > 0.05$).

Figure 17 shows a comparative analysis of percentage samples loss for fungal decay per day, it is clear that low temperature has extended strawberry samples storage-life (blue curves) compared to samples kept at room temperature. Addition of the PJ-WS-LE extract has significantly protected strawberries from rotting by adding two more days to life of some of the strawberry samples at room temperature, while samples in the refrigerator were totally protected during the 10 days of the experiments.

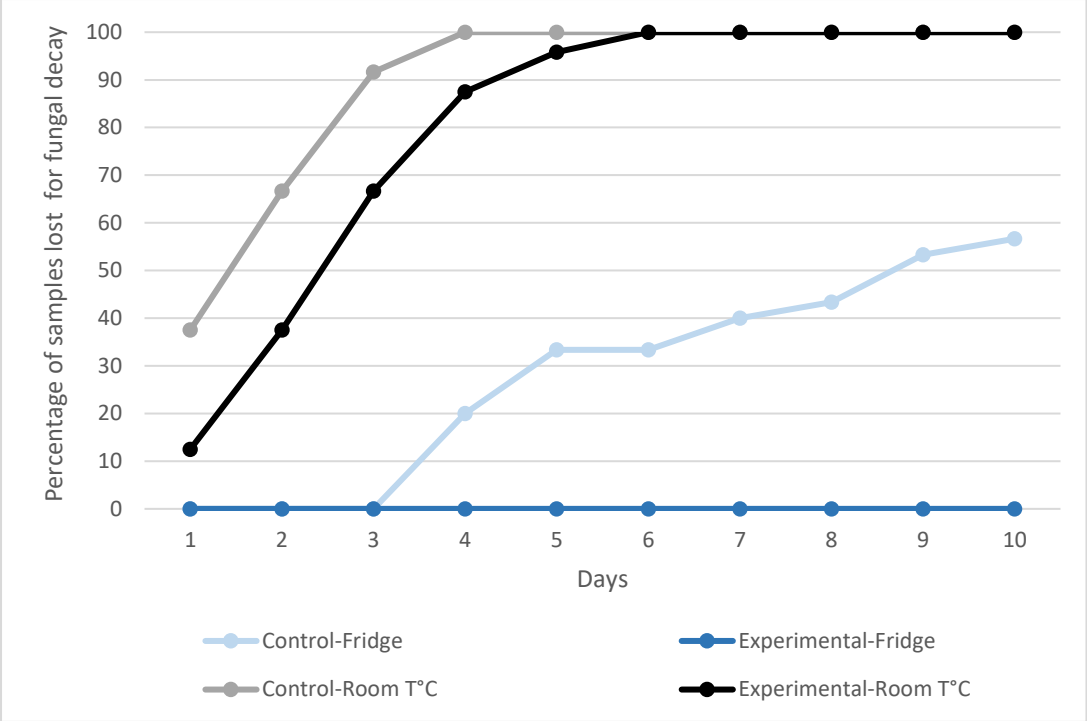


Figure 17. Cumulative percent loss of strawberry samples stored at different temperature for 10 days. Experimental samples are samples treated with 8mg/ml PJ-WS-LE extract.

In-vivo analysis results showed that strawberry samples treatment with PJ-WS-LE extract, both at room temperature and in the fridge had a slow-down effect on the rate of grey mold progression, which indicates that the active compounds in the crude extract were capable of preventing fungal growth on strawberry samples. Microscopic identification of strawberry spoiling agents showed a dominance of *Botrytis*, this indicates that 8mg/ml of the PJ-WS-LE extract had a strong protective effect on strawberry, by significantly delaying fungal growth mainly *Botrytis* at both room temperature and at 4°C. Results are in agreement with the effect of ultrasound treatment in extending strawberry shelf-life reported by Aday *et al*. (2013). A recent study showed also an increase in strawberry shelf-life of samples coated with a novel edible coatings based on bail seed gum (BSG) enriched with *Echinacea* extract (EE), samples coated

with 3% BSG and 3% EE showed better quality characteristics compared with control samples during the 20 fridge storage days of the experiment (Moradi *et al.*, 2019). The same researchers showed good efficacy of the edible coatings based on salep solution (SS) enriched with grape seed extract (GSE). At 1.5% SS and 3% GSE coating material, samples showed a microbial load within the "good for consumption" range during the 20 days of the experiment (Emamifar *et al.*, 2019). Comparison between methods should take into consideration, other than shelf-life, the feasibility of the treatment method, considering the simple extraction method of PJ-WS-LE extract and its direct spraying as aqueous solutions on fruits samples give our study an advantage among others with a similar effectiveness in shelf-life increase.

4.2.2. Effect of PJ-WS-LE extract on cucumbers shelf-life at room temperature

Cucumber samples sprayed with 8mg/ml PJ-WS-LE extract showed longer shelf-life than control samples which means that the active compounds in the crude extract were capable of preventing microbial growth on cucumber samples. Table 35 shows the number of samples rotting every day for 2 weeks.

Average shelf-life of treated cucumbers at room temperature was 176.8h hours (7.36 days) while none treated samples showed an average shelf life of 100h (4.16 days). T-test validated variances equality assumption and showed statistically significant difference between control and experimental samples shelf-life (p=0.00≤0.01). Percentage of edible samples after 5 days was also calculated, 66.7% of the treated samples were still good for consumption while only 26.7% of the non-treated samples were still edible after 5 days (Appendix 9).

Table 35. Number of experimental and control samples lost by fungal spoilage every 24 hours.

Number of control samples rotting	Number of experimental samples rotting	Shelf-life(h)
10	0	48
1	4	72
1	0	96
10	6	120
8	8	144
0	0	168
0	0	192
0	3	216
0	3	240
0	2	264
0	0	288
0	4	312

Cucumber is known by yeast and bacterial spoilage this is why many samples were removed from the experiment without showing any hyphal growth. Yet pure cultures were taken from cucumbers that showed fungal growth, microscopic identification showed only C*ladosporium* growth in 26.7% of the evaluated samples (Appendix 9). Results shows that 8mg/ml of the PJ-WS-LE extract was protecting cucumbers from natural spoiling by delaying significantly *Cladosporium* growth. In

addition, the active compounds in the treatment had efficacy against the non-identified yeast and bacteria that caused the spoilage of the rest of the samples.

Overall, untreated cucumber samples started rotting within 48 hours and all showed fungal growth after 6 days with an average shelf-life of 5 days. Extract treatment has extended the shelf-life of the samples to an average of 9 days with 76.8% average shelf-life longer. At day 5, 66.7% of the treated samples were still marketable. Results may be compared to those of a study conducted on treating cucumber samples with chitosan nanoparticles loaded with *Zataria multiflora* essential oil. Experiment was conducted at 10°C which makes shelf-life higher than those of our study yet the cost of the storage is also higher. Mohammadi *et al*. (2016) showed that uncoated cucumber samples showed signs of fungal decay (31.5% of the samples) after 9 days as the coated ones were all still good for consumption. At the end of the 21 days incubation period both treatment methods used showed extension of cucumbers shelf-life in fridge with 44.87% and 13.38% of the coated samples showing decay compared to a total loss of the control samples. Another study conducted on cucumber tested the efficacy of chitosan nanoparticles loaded with *Cinnamomum zeylanicum* essential oil against a well known fruits' rotting agent, *Phytophthora drechsleri*. In the controlled experiment, cucumbers were inoculated with *P. drechsleri*, 100% of the control samples showed fungal growth compared to 0% of the fruits coated with chitosan loaded with *C. zeylanicum* essential oil. As for fruits shelf-life at 10°C, only 21.6% of the treated samples showed decay after 21 days compared to 100% decay of the control sample (Mohammadi *et al.*, 2015). Recent study showed that starch-glucose edible coating (1.5µM starch-2.5µM D-glucose) of cucumber has slowed down the ripening process and increased shelf-life of the fruits during the long storage period at fridge temperature up to 30 days (Patel & Panigrahi, 2019). Despite the difference in storage

temperature, PJ-WS-LE extract showed the highest shelf-life increase (76.8%) compared to other studies conducted on cucumber. It is also worth noting that the extract preparation method and the coating procedure performed are simple and economically feasible, which give PJ-WS-LE extract an advantage over others.

4.2.3. Cherry tomato controlled experiment at room temperature

None of the negative control samples showed fungal growth within the two weeks timeline of the experiment, which indicates the efficacy of the pre-sterilization treatment of the samples. PJ-WS-LE extract showed efficacy in controlling growth of *A. alternata* and *B. cinerea* in artificially inoculated wounded and none-wounded samples, while most of the none-treated control samples showed fungal growth, only one treated sample showed growth of *A. alternata* and another showed growth of *B. cinerea* (Figure 18). The extract showed both curative and preventive effects on both wounded and entire samples, which indicates very promising results for the market usage. Pictures of the end results are shown in appendix 10.

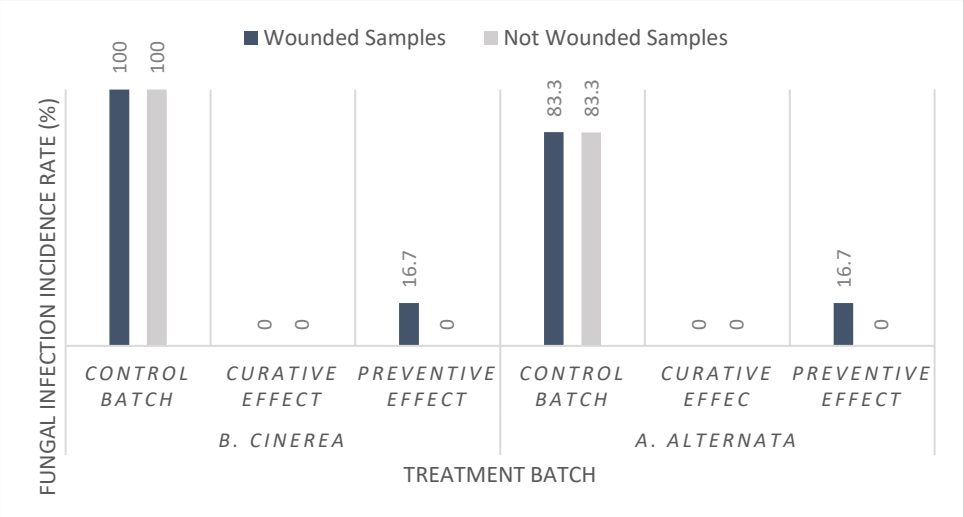


Figure 18. Percentage infection rate of wounded and none-wounded cherry tomato samples inoculated with *A. alternata* and *B. cinerea* with and without PJ-WS-LE extract treatment evaluating curative and preventive effects.

The *in-vivo* preservative effect of PJ-WS-LE extract on cherry tomatoes was comparable to the effect of low fermenting yeast (*Lachancea thermotolerans*) volatile organic compounds (VOCs) on inoculated *F. oxysporum* infection. The assay showed that 76% of the control cherry tomatoes were infected while none of the treated batches showed any infection (Zeidan *et al.*, 2018). These results are similar to those of PJ-WS-LE extract that showed 100% *Botrytis cinerea* infection and 90% *Alternaria alternata* infection in the control batches with only 4.16% of the treated batches showing infections in each of the evaluated fungal strains. However, it is more practical to apply an aqueous solution (PJ-WS-LE extract solution) as a post-harvest diseases controller than exposing fruits to VOCs.

4.2.4. Curative and preventive effects of PJ-WS-LE extract against *A. alternata* and *C. gloeosporioides* induced infection in mangoes (large scale experiment)

The efficacy of PJ-WS-LE extract in reducing *C. gloeosporioides* incidence on artificially inoculated fruits was low, preventive treatment showed a disease incidence rate reduction of 25% (extract efficacy). Although extract efficacy in controlling mango anthracnose is not total eradication, PJ-WS-LE extract exhibited significant reduction in disease severity, which was reduced by more than 50% of the control average lesion diameter of 14.7mm to preventive treatment average lesion diameter of 7.0mm by the end of the 5 days storage period (Figure 19). The effect of PJ-WS-LE extract on mango firmness was also evaluated at day 5, one-way ANOVA test showed a significant average firmness conservation (p=0.00≤0.01) in samples exposed to the extract (curative and preventive treatment) (26.74N) in comparison to the control (12.62N).

As for *A. alternata* artificially inoculated mango fruits, PJ-WS-LE extract showed high efficacy in reducing infection incidence rate from 51.7% in control samples to 10.3% in treated samples, again preventive treatment showed higher efficacy than curative treatment. PJ-WS-LE extract at 8mg/ml has an efficacy of 80% against *A. alternata* infection by the end of the 10 days storage period. Disease severity was also reduced in treated samples. Samples with preventive treatment (average lesion diameter=3.7mm) that had infection showed a disease severity of 16% compared to the untreated control samples (average lesion diameter=11.5mm) (Figure 19) (Appendix 11). The effect of PJ-WS-LE extract on mango firmness was evaluated at day 10, ANOVA test showed a significant average firmness conservation ($p \leq 0.01$) between plants exposed to the extract (curative and preventive treatment) (14.93N) and the untreated plants (7.4N).

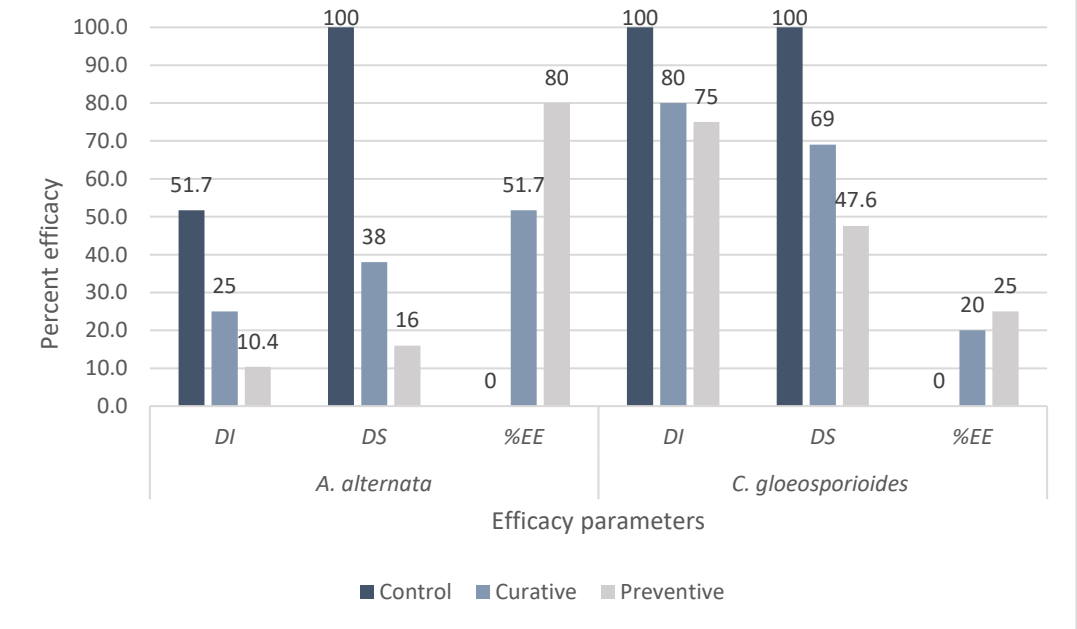


Figure 19. Curative and preventive efficacy parameters of PJ-WS-LE extract against *A. alternata* and *C. gloeosporioides* inoculated in mango samples. Disease incidence (DI), disease severity (DS), and percent plant extract efficacy (%EE).

The protective effect of PJ-WS-LE extract against anthracnose caused by *C. gloeosporioides* in artificially inoculated mango samples was 25%. Similar anthracnose incidence inhibition rate was seen in inoculated avocadoes samples treated with *Aloe vera* extract. However, when *Aloe vera* extract was mixed with 1% chitosan the disease incidence rate went down. In the present study, preventive treatment was more effective on treated *C. gloeosporioides*. Based on the results of the long term experiment involving chitosan, it is likely to have a higher extract efficacy against *C. gloeosporioides* when mixing PJ-WS-LE extract to chitosan (Bill *et al.*, 2014). As for the disease severity, PJ-WS-LE extract showed high success rate in lowering anthracnose lesion diameter by more than half with an average lesion diameter of 7mm in the preventive treatment samples. The result is somehow comparable to the final lesion diameter of treated avocado samples (diameter=8.94mm) as shown by Bill *et al*. (2014). Another study conducted on mangoes showed significant efficacy of *P. juliflora* leaves extracts in lowering anthracnose severity in inoculated samples. A comparison between the two extraction method should be considered to improve our extract efficacy, however, it is vital to examine first the kind of solvent used by other workers which might have a toxic antifungal activity on its own (Deressa & Jalata, 2015). A study conducted on Papaya samples, showed the effectiveness of different plants extracts collected from Ambo and Haramaya, Ethiopia in lowering anthracnose severity without totally inhibiting the fungal growth on plants which indicate the difficulty in controlling mango anthracnose to a 100% level. *In-vivo* experiments on mangoes showed much higher efficacy of PJ-WS-LE extract in protecting artificially inoculated samples from *A. alternata* (80%). These results were in harmony with previous *in-vitro* results showing total inhibition of *A. alternata* by PJ-WS-LE extract, while *C.*

gloeosporioides showed only decrease in mycelium growth (Saleh & Abu-Dieyeh, 2021). *A. alternata* is one of the most serious fungi that cause postharvest diseases in the world, in mangoes, it can rot on the sides and stem ends, resulting in significant postharvest losses (Li *et al.*, 2018). Controlling *A. alternata* using a naturally produced extract would add a valuable, safe and effective product to the agricultural world.

4.2.5. In-vivo long term preservative activity of PJ-WS-LE extract individually and when embedded in chitosan coating material- Case of Strawberry

4.2.5.1. Sensory evaluation

The change of sensory characteristics of strawberries stored at 4°C was evaluated on a weekly basis. Figure 20 represents the change of the score of fruits belonging to different treatment batches.

As storage period passes, the overall sensory score of all strawberry batches decline. However, samples treated with 8mg/ml of PJ-WS-LE extract (batch B) and samples coated with 8mg/ml of PJ-WS-LE extract embedded in 1% chitosan conserved better scores during the three weeks of storage. These results demonstrate more the usefulness of the plants extract tested in extending strawberries shelf life.

At the end of the three weeks of the experiment, samples coated with PJ-WS-LE extract alone and with PJ-WS-LE extract embedded in 1% chitosan conserved a liked sensory quality with sensory score averages of 4.4 and 4.3 respectively. Similar results have been recently described with BSG+EE coating and with SS+GSE coating (Emamifar *et al.*, 2019; Moradi *et al.*, 2019). This demonstrates the importance of edible coatings in maintaining good sensory quality parameters. Beside the feasibility of the extraction method, the choice of the edible coat to be adopted in preservation depends also on the availability of the natural product added to the coating material.

Known as an invasive tree in many countries (including Qatar), *P. juliflora* is widely available and its utilization is encouraged to manage its widespread (Wei *et al.*, 2018).

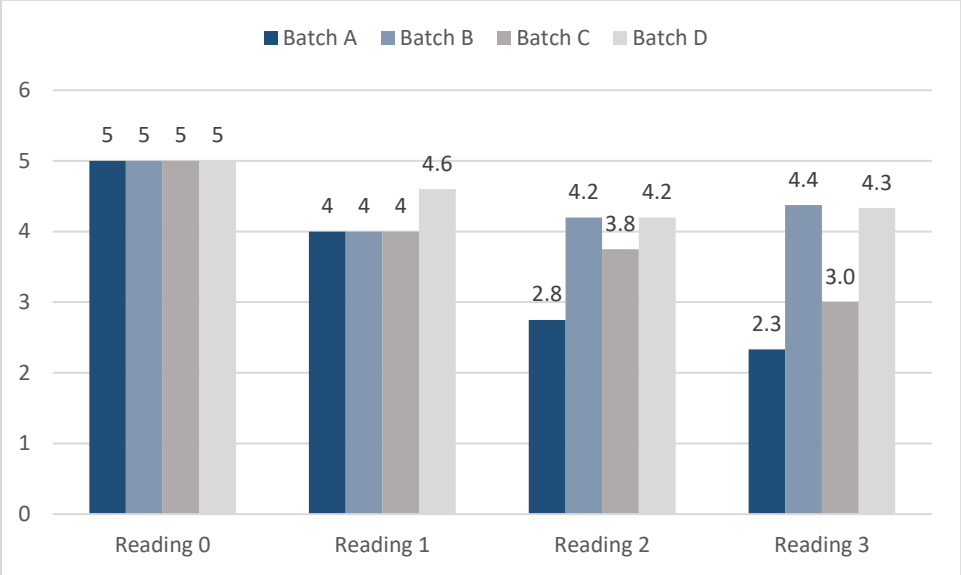


Figure 20. Average sensory scores of strawberry samples exposed to different treatments on a weekly basis (A: Negative control samples with no treatment; B: samples treated with 8mg/ml PJ-WS-LE extract; C: samples coated with 1% chitosan; D: samples coated with 1% chitosan + 8mg/ml PJ-WS-LE extract) (Score from 1 to 5 are: 5 points for "extremely liked", 4 points for "liked", 3 points for "acceptable" 2 points for "disliked" and 1 point for "extremely disliked").

4.2.5.2. Weight loss

The effect of samples coating treatment on the weight loss was monitored over the course of three weeks. Percent change in weight was calculated for each treatment batch every week (Figure 21). Weight loss increased with time, however, PJ-WS-LE extract showed efficacy in reducing weight loss independently and when embedded in chitosan.

There was a significant increase in the percentage change of weight of strawberries in all treatment categories with storage time. Strawberries coated with 1% chitosan and 8mg/ml of the PJ-WS-LE extract had the lowest change in weight with an observable increase of this change from week to another. The extract alone showed also high effectiveness in protecting fruits from weight loss. Levene's test of the data of each week supported the hypothesis of equality of variances ($p \geq 0.01$), which allows the usage of one-way ANOVA, results shows the significant effect of the type of treatment on the percentage weight change. Post-Hoc Tukey-test showed that the difference between the percentage changes in weight of the strawberry batches exposed to the four different treatments were significantly different among eachothers in week 1. In week 2, there was no significant different between the percentage change of weight of batches A and C, however, batches B and D had significantly lower weight loss. The same test was conducted for week 3, results categorized the treatment groups into two subsets (subset 1: batch A and C and subset 2: batches B and D). The two subsets have a high significant difference in their percentage weight loss averages ($p \leq 0.01$), however the batches of each subset were not significantly different from eachothers, which indicates that towards the end of the experiment the extract alone and the extract embedded in 1% chitosan (batches B and D) were as effective in protecting fruits against weight loss. Final percentage weight loss of fruits of batches B and D were 10.2% and 7.3% respectively compared to 16.8% weight loss in the control group (batch A).

The 8mg/ml PJ-WS-LE extract significantly decreased average weight loss of strawberries in both treatment groups B and D, indicating that the extract can serve as a protective layer help avoiding fruits water loss. There was a significant difference in weight loss between batches C and D. Therefore, PJ-WS-LE extract incorporated into the polysaccharide-based edible coating (chitosan) has significantly improved the water

barrier properties of chitosan by reducing fruits surface evaporation rate. Similar results were found with SS+GSE coating (Emamifar *et al.*, 2019) and with BSG+EE coating (Moradi *et al.*, 2019), which prove the importance of enriched edible coating in maintaining strawberries' weight. It is worth noting that strawberry is a sensitive fruit and that not all coating methods work for it, even those that showed good results with other fruits. For example, Parreidt *et al*. (2019) have recently developed a new alginate-based coating that protects well cantaloupe samples from weight-loss; but has an adverse effect on strawberry samples by increasing their weight loss compared to the control samples (Parreidt *et al.*, 2019). Such results highlight the importance of PJ-WS-LE extract as suitable coating material for soft fruits.

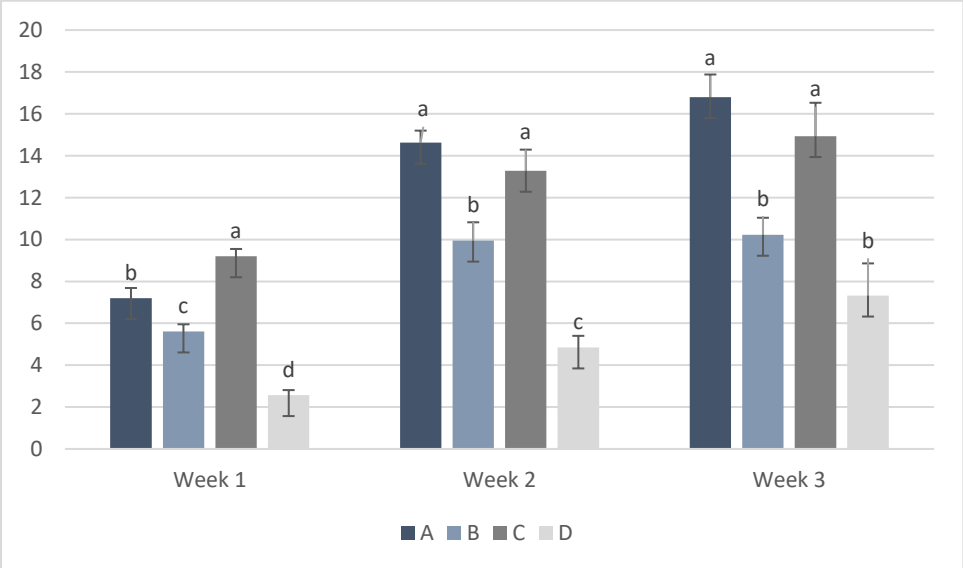


Figure 21.Average percent change in weight of the overall strawberry samples exposed to different treatments on a weekly basis ±SE. (A: Negative control samples with no treatment; B: samples treated with 8mg/ml PJ-WS-LE extract; C: samples coated with 1% chitosan; D: samples coated with 1% chitosan + 8mg/ml PJ-WS-LE extract).

[abc] Treatment columns with different letters have their values significantly different as shown by the one-way ANOVA Post-Hoc Tukey test for the data of each week, at p=0.05.

4.2.5.3. Respiration rate

The respiration rate of strawberry samples was evaluated by measuring the level of carbon dioxide produced by samples in a closed containers using a CO_2 meter, a reading of the CO_2 in ppm was taken every two seconds over 50min. Respiration rate was calculated using the slope of the trend line passing by the collected data. Figure 22 shows the results with the trend lines. Comparison between the carbon dioxide levels of the four treatment categories over the course of the three weeks is shown in figure 23. Figure 23 shows that respiration rate of strawberry samples of all treatments represented by the carbon dioxide level decreased with time during the first three weeks then slight increased in week 4 in some samples. Week 0 results indicate that chitosan treatment slow down the respiration rate although carbon dioxide level is higher in the presence of the PJ-WS-LE extract (batch D). Plants of batch B coated with the extract alone showed the highest rate of respiration throughout the three weeks.

Respiration rate is an indicator of plants normal activity. In addition, levels of oxygen and carbon dioxide in packaged fruits and vegetables affect their metabolic activities. Results showed the highest respiration rate with samples coated with 8mg/ml PJ-WS-LE extract alone. Batch D did not show similar results, this could be due to stress caused by the layer of chitosan on the respiration rate by closing plants pores (Petriccione *et al.*, 2015). Different protective treatments showed different effects on respiration rate of strawberry samples, a previous study assessed the effect of ultrasound treatment in increasing strawberry shelf-life and showed that treated samples had

significantly lower respiration rates than the control. Samples from that study were stored in tightly closed containers which might have led to gases accumulation and therefore affected respiration rate (Aday *et al.*, 2013).

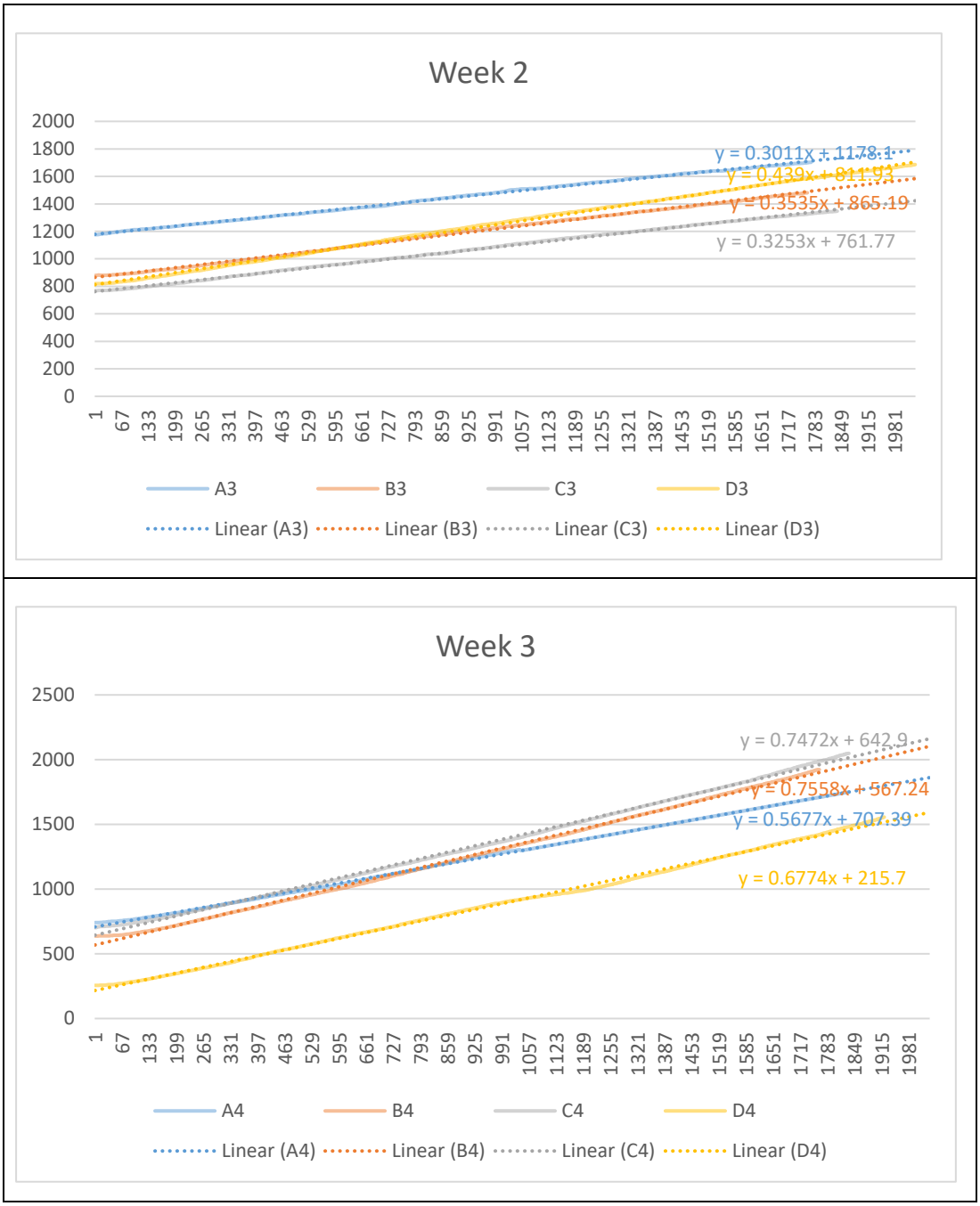


Figure 22. Levels of carbon dioxide in ppm per second of strawberry samples belonging to the four treatment categories (A: negative control; B: sprayed with 8mg/ml PJ-WS-LE extracts; C: coated with 1% chitosan; D: Coated with 8mg/ml PJ-WS-LE extracts embedded in 1% chitosan) measured during weeks 1, 2, 3 and 4 of the storage period. Dotted lines represent the trend lines and their equations.

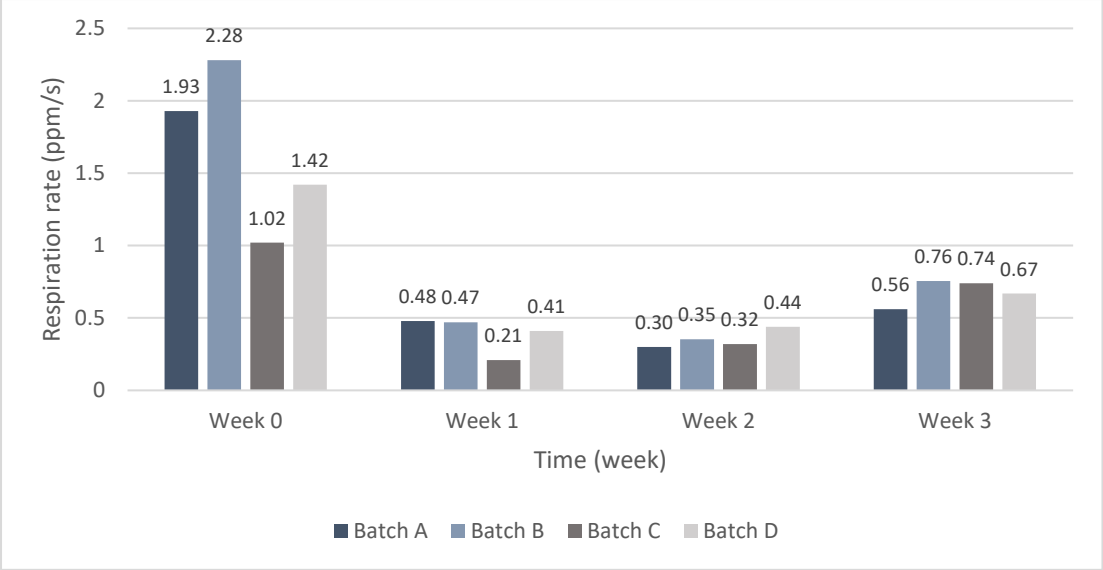


Figure 23. Average carbon dioxide levels in ppm/s of strawberry samples belonging to the four treatment categories (A: negative control; B: sprayed with 8mg/ml PJ-WS-LE extracts; C: coated with 1% chitosan; D: Coated with 8mg/ml PJ-WS-LE extracts embedded in 1% chitosan) over the four weeks of the experiment.

4.2.5.4. Total aerobic bacterial, yeast and mold count

PJ-WS-LE extract in the strawberries coating material served as an antimicrobial barrier that kills surrounding bacteria. From reading zero, samples coated for few hours showed much lower bacterial count than negative control samples and samples coated with chitosan alone figure 24.

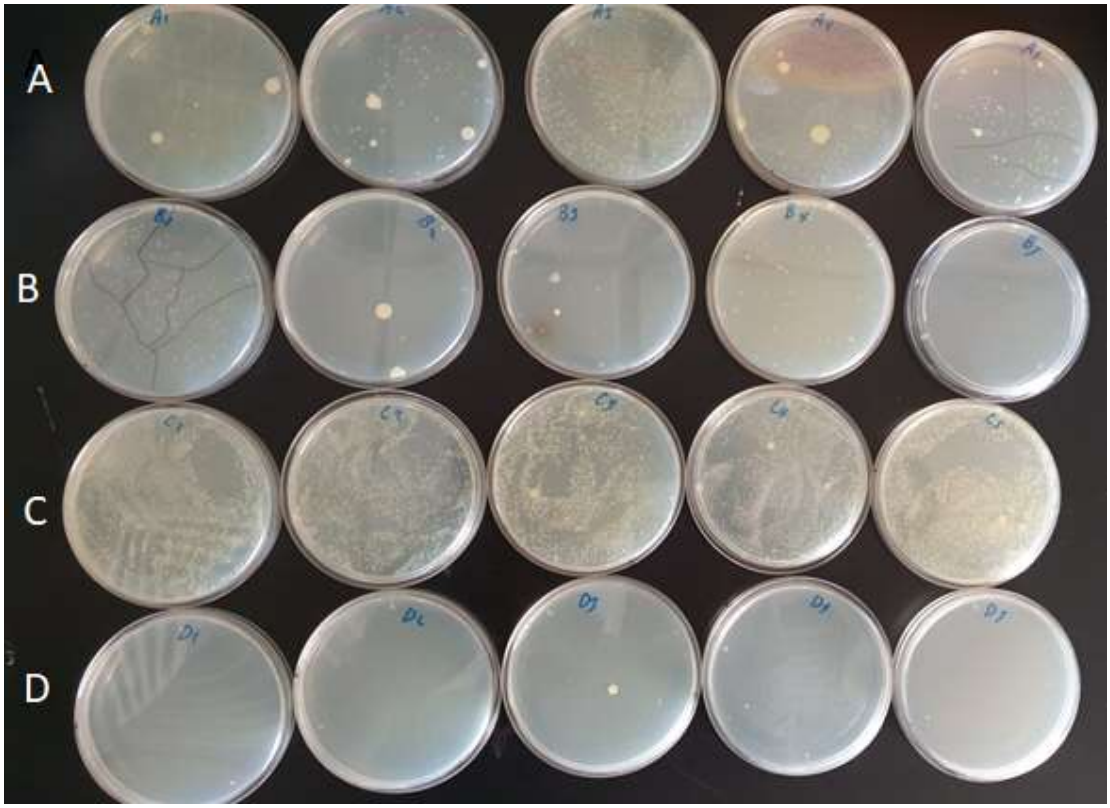


Figure 24. Nutrient agar plates showing the total aerobic bacterial growth of 100µl of the 25ml of sterile distilled water used to wash the surfaces of different strawberry samples used in taking the first reading. Plated are given the names of the treatment with the number of the replicate (1 to 5) (A: negative control; B: sprayed with 8mg/ml PJ-WS-LE extracts; C: coated with 1% chitosan; D: coated with 8mg/ml PJ-WS-LE extracts embedded in 1% chitosan).

Average aerobic count in CFU/g of each treatment batch was calculated between week 0 (day of treatment) and week 3 of storage at 4°C, results are shown in figure 25. The average CFU pattern seen in all treatments is a drastic decrease in CFU after one week of storage followed by an increase in week 2 to decrease again in week 3. Strawberry samples exposed to different treatment showed a consistent difference in their CFU throughout the weeks. Samples of batch B treated with 8mg/ml of PJ-WS-LE extract showed a very low bacteria count at the day of treatment and in week 1, the

total number remained lower than the control group (batch A) by 64.3% and 35% in weeks 2 and 3 respectively. The lowest bacterial count was shown in samples treated with 8mg/ml PJ-WS-LE extract stabilized in 1% chitosan to reach zero CFU/g average in week 3.

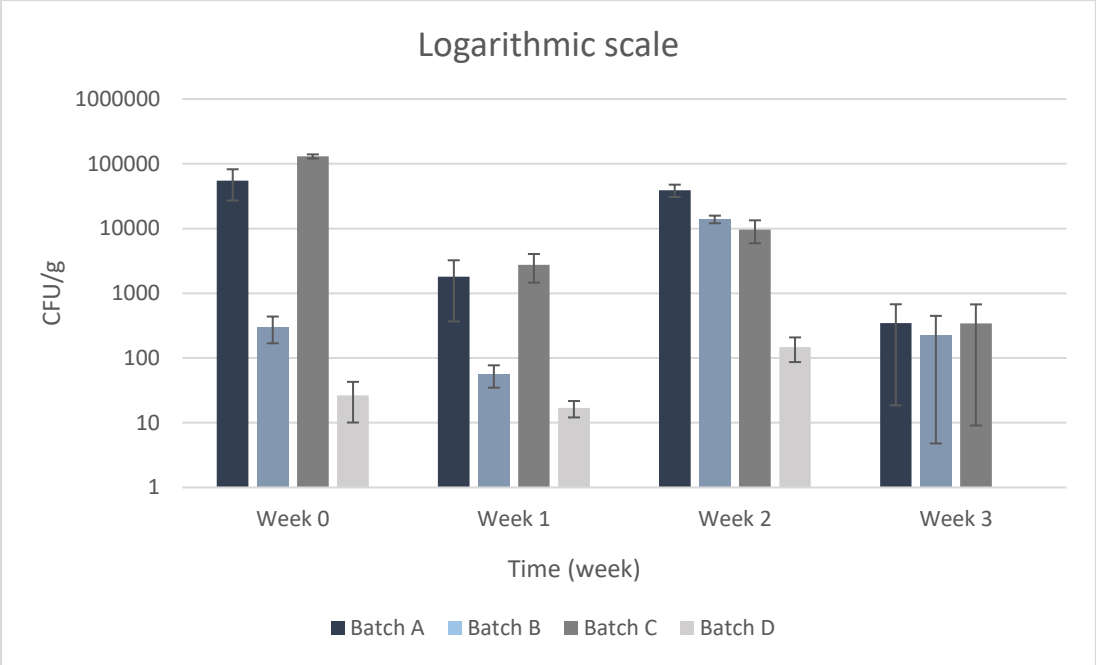


Figure 25. Average aerobic surrounding bacterial CFU in CFU/g ±SE of strawberry represented in logarithmic scale of samples exposed to different treatment during three weeks of storage (A: negative control; B: sprayed with 8mg/ml PJ-WS-LE extracts; C: coated with 1% chitosan; D: coated with 8mg/ml PJ-WS-LE extracts embedded in 1% chitosan).

PJ-WS-LE extract in the strawberries coating material served as an antimicrobial barrier that kills surrounding mold spores and yeast. From reading zero, samples coated for few hours showed much lower fungal count than negative control samples and samples coated with chitosan alone, the effect was stronger in samples of batch D coated with the extract embedded in 1% chitosan figure 26.

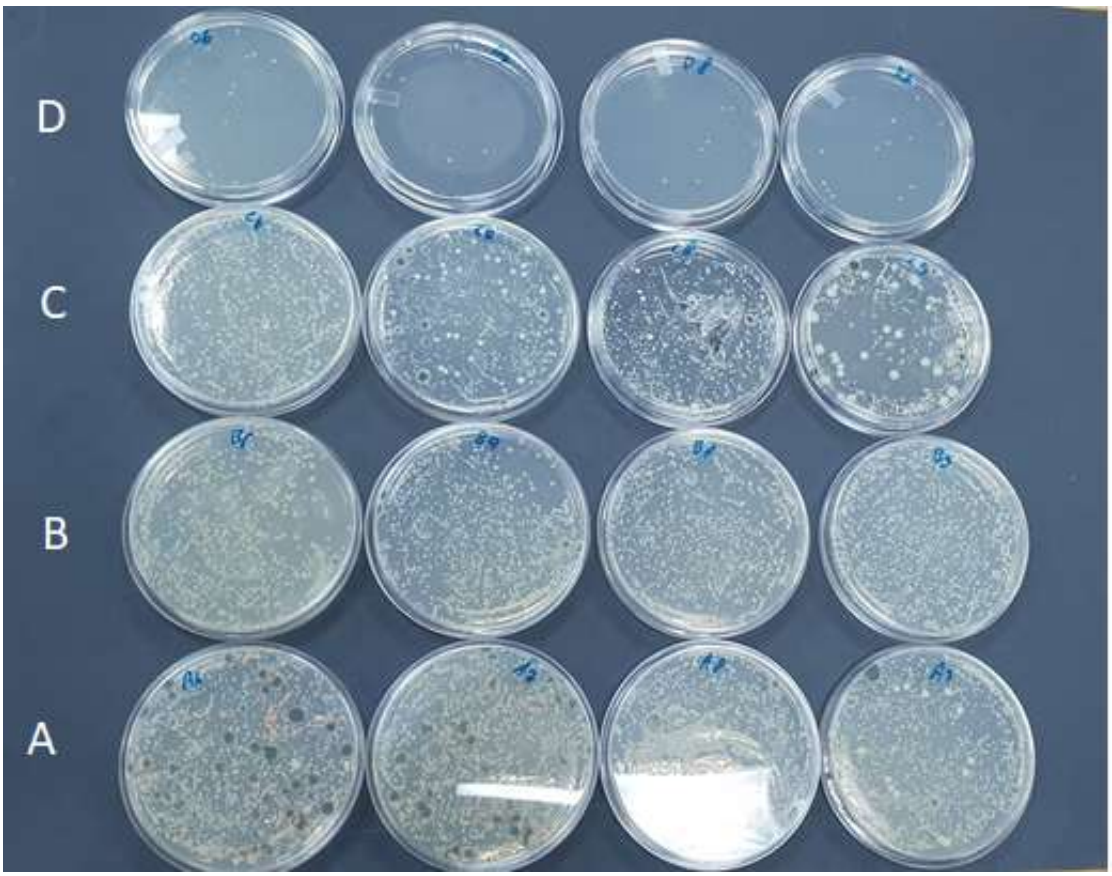


Figure 26. Potato dextrose agar plates (with ampicillin) showing the total mold and yeast growth of 100µl of the 25ml of sterile distilled water used to wash the surfaces of different strawberry samples used in taking the first reading. Plated are given the names of the treatment with replication number (1 to 5) (A: negative control; B: sprayed with 8mg/ml PJ-WS-LE extracts; C: coated with 1% chitosan; D: coated with 8mg/ml PJ-WS-LE extracts embedded in 1% chitosan).

CFU of fungi surviving on the surface of strawberry samples was calculated by washing the outside of the samples with sterile distilled water and using the wash water for serial dilution and spread plate method. Average mold and yeast CFU in CFU/g of each treatment batch was calculated between week 0 (day of

treatment) and week 3 of storage at 4°C, results are shown in figures 27 and 28. The lowest yeast and mold count was seen in batch D where it reaches zero CFU/g mold and 292 CFU/g yeast in week 3. Batch B coated with 8mg/ml of PJ-WS-LE extract alone showed lower mold and yeast count in the first three reading. However, in week 3 CFU of both mold and yeast dropped significantly in all batches to become equal in batches A, B and C, only experimental samples of batch D had zero mold count and a yeast count that was 38% lower than the control batch.

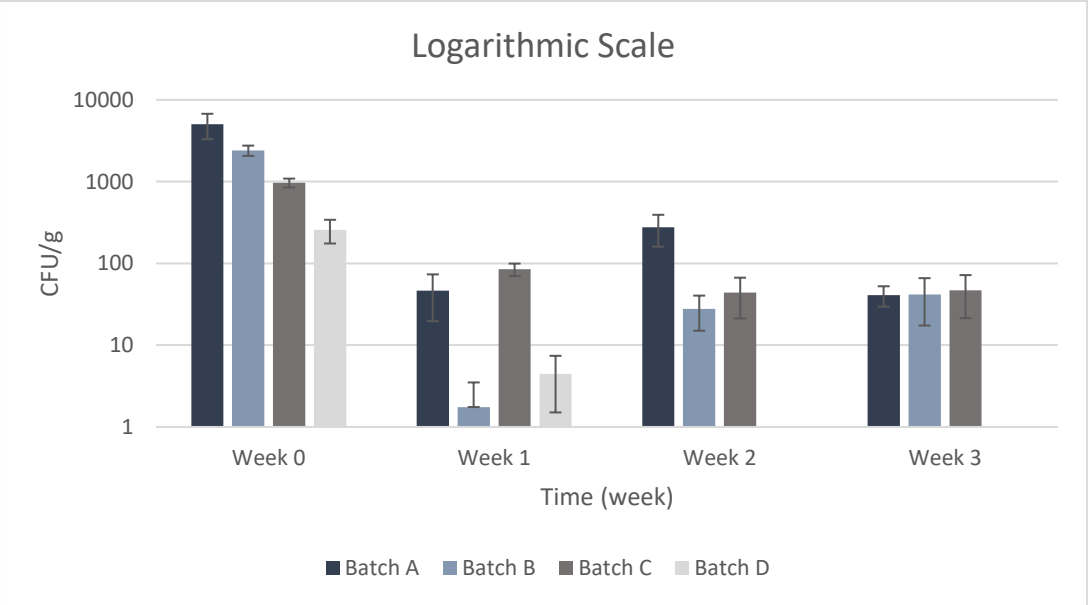


Figure 27. Average surrounding mold spores CFU in CFU/g ±SE of strawberry represented in logarithmic scale of samples exposed to different treatment during three weeks of storage (A: negative control; B: sprayed with 8mg/ml PJ-WS-LE extracts; C: coated with 1% chitosan; D: coated with 8mg/ml PJ-WS-LE extracts embedded in 1% chitosan).

One-way ANOVA test conducted to evaluate the effect of treatment type on microbial count showed significant differences at $P \leq 0.05$ with the lowest CFU

bacterial, mold and yeast counts for batch D. Mold samples showing growth upon spread plate method were subjected to microscopic identification, the number of samples showing a certain fungi genus was recorded in table 36 regardless of the number of colonies per samples. Results showed that the dominant genus in strawberry samples stored at 4°C was *Cladosporium* found in 60 samples of different treatment batches throughout the storage period followed by *Botrytis* found in 35 samples. The low mold count in experimental samples indicate the effectiveness of PJ-WS-LE extract against the dominant strains.

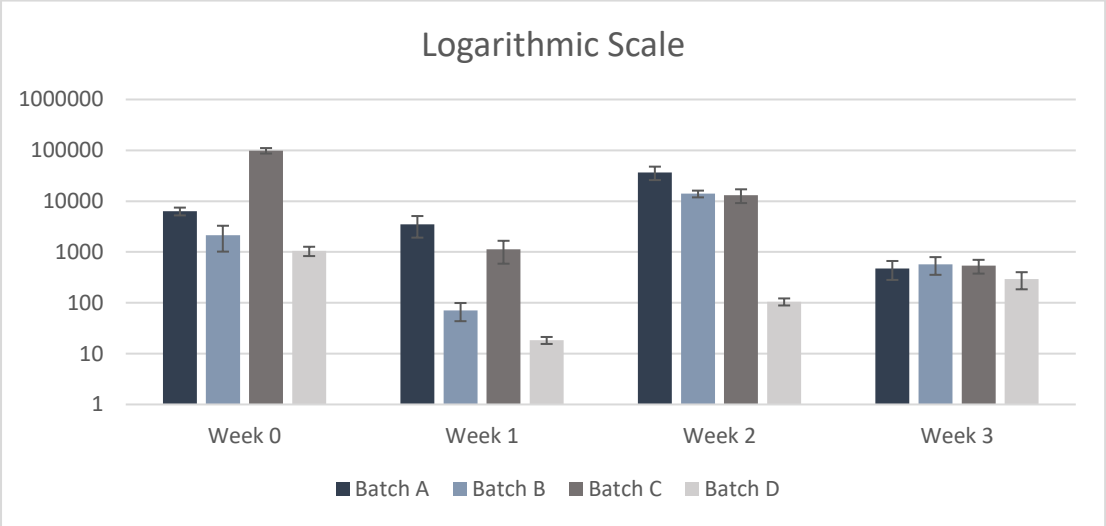


Figure 28. Average surrounding yeast CFU in CFU/g ±SE of strawberry represented in logarithmic scale of samples exposed to different treatment during three weeks of storage (A: negative control; B: sprayed with 8mg/ml PJ-WS-LE extracts; C: coated with 1% chitosan; D: Coated with 8mg/ml PJ-WS-LE extracts embedded in 1% chitosan).

Table 36. Number of strawberry samples showing specific fungal genus growth throughout their storage period.

Weeks	1				2				3				Total
Treatment Batches	A	B	C	D	A	B	C	D	A	B	C	D	
Cladosporium	2	1	3	3	9	6	6	11	6	5	6	2	60
Botrytis	2	0	3	1	3	2	0	7	6	5	6	0	35
Penicillium	1	1	1	0	0	0	0	0	0	1	1	0	5
Aspergillus	0	0	1	0	0	0	3	1	1	0	1	0	7
Rhizopus	0	0	0	0	3	0	0	0	0	0	0	0	3

Microorganisms surrounding fruits and vegetables increase in numbers during fruits ripening and play a role in fruits spoiling (Erkmenand & Bozoglu, 2016). The capability of the coating material to lower the surrounding microorganisms count could play a role in increasing plants shelf-life. PJ-WS-LE extract showed a high antibacterial activity from the first few hours after treatment. At the end of the three weeks, samples of batch B showed 64.3% lower aerobic bacterial count than the control group while samples of batch D showed zero bacterial CFU. As for fungi, batch D also showed the lowest final numbers with zero CFU/g mold count and yeast count that is 38% lower than the control batch A. The most encountered mold genus were *Cladosporium* and *Botrytis*, which proves, as shown in the *in-vitro* analysis, the extract efficacy against these two fungal types (Saleh & Abu-Dieyeh, 2021). SS+GSE and BSG+EE coating treatments of two recent studies showed that the coating materials worked as a retarding agent of microbial growth in fresh strawberries during cold

storage (4°C) by lowering microbial counts (Emamifar *et al.*, 2019; Moradi *et al.*, 2019). However, PJ-WS-LE extract showed the strongest antimicrobial efficacy with last total aerobic bacterial CFU and total mold CFU reaching zero in samples coated with chitosan enriched with 8mg/ml PJ-WS-LE extract, a level that was not possible by any of the previously described extracts. The effectiveness of different coating material on the total aerobic bacterial CFU count and total fungal CFU count in strawberry samples preserved at 4°C for 12 days were tested, the coating materials tested included *Aloe vera*, ascorbic acid, chitosan, starch, potassium sorbate and calcium chloride (Rahimi *et al.*, 2019). Comparing their results to our two-weeks results showed that the control batches had comparable total aerobic bacterial count of $46.5x10^3$CFU/g and $39.1x10^3$CFU/g in Rahimi *et al*. study and in the present study, respectively. The 12 days old results of the total aerobic bacterial count in strawberries coated with different coating material ranged between $7.5x10^3$CFU/g and $26.5x10^3$CFU/g. However, PJ-WS-LE extract embedded in chitosan showed a CFU of $14.7x10^1$CFU/g, which demonstrate the higher efficacy of the present study extract. Similar results were found with the total fungal count, the present study fungal count at week 2 reached less than 10 CFU, which was not recorded with any of their tested coating material.

4.2.5.5. Effectiveness of PJ-WS-LE extract in maintaining strawberry samples weight

A correlation between the samples weight loss pattern and the change in microbial counts throughout the study would support our hypothesis stating that PJ-WS-LE extract acts as an antimicrobial barrier around the fruits and maintain their storage quality during the experiment including the lessening of weight loss. Pearson test results showed a significant correlation between bacterial, fungal and yeast counts patterns. Most interestingly, the results showed a significant correlation between the

percentage weight change data and the total CFU counts (bacterial, mold and yeast) in the tested samples (Table 37).

Table 37. Correlation matrix between the percentage change in weight of strawberry samples and their total CFU counts of mold, yeast, and bacteria.

	Mean	SD	% Weight Change	Mold CFU	Yeast CFU	Bacteria CFU
% Weight Change	9.83	4.44	1			
Mold CFU	53.96	99.10	.469**	1		
Yeast CFU	8830.67	21193.79	.308*	.336*	1	
Bacteria CFU	6418.52	12467.89	.501**	.599**	.595**	1

** *p*-value < 0.01; * *p*-value < 0.05

The present study *in-vitro* analysis showed strong effectiveness of PJ-WS-LE extract against the most encountered fungi in this study, which were *Cladosporium spp.* and *Botrytis spp*. with MICs of 4mg/ml and 1mg/ml respectively (Saleh & Abu-Dieyeh, 2021). Saleh and Abu-Dieyeh have also demonstrated the broad-spectrum efficacy of PJ-WS-LE extract against certain species of bacteria and fungi, that explain the correlation between mold, yeast and bacterial CFU change. Being a valuable source of phenolic compound, *P. juliflora* extracts had the capacity of initiating multiple antioxidative mechanisms, which can act as anti-carcinogenic, anti-inflammatory, anti-allergic and antimicrobial agents. The antimicrobial activity of *P. juliflora* has been previously described in previous studies (de Brito Damasceno *et al.*, 2018). Previous results, together with the significant correlation proven by Pearson test imply that the

strong antimicrobial activity of the extract protects samples from fungal and bacterial rot and maintain their weights among other quality parameters.

4.2.5.6. Changes in physical and chemical properties during storage time (Firmness, pH, TSS, and antioxidant ability)

Samples were withdrawn from the trial every week to have their storage quality-parameters measured, table 38 shows the variation in the average firmness, pH, total soluble solids and total antioxidants of samples with different treatments throughout the three weeks storage at 4°C.

Negative control batch of strawberry showed a 30% loss of their firmness throughout the storage time. However, all other batches showed stability in their firmness, which showed effectiveness of the extract and chitosan in conserving fruits quality. Results of strawberry juice pH of different batches throughout the weeks showed fluctuations in numbers. However, when each batch results (all weeks results combined) were compared using one way ANOVA, the overall results didn't show significant difference in pH of strawberries of different batches (p=0.764>0.05). Total soluble solids results showed an increase of TSS level of the control (batch A) samples during the three weeks of storage. Coated samples showed a slight insignificant decrease in their TSS levels. DPPH radical scavenging activity of strawberry samples of the four treatment batches was measured on a weekly basis. The antioxidant activity increased in strawberry samples of all treatment groups with time. However, the lowest increase was found with strawberry samples of the coated batch D, which means that the PJ-WS-LE extract coat with 1% chitosan has successfully slowed down the antioxidant increase during fruits ripening.

Table 38. Average physical parameters ±SE of strawberry samples exposed to different treatments (A: negative control; B: sprayed with 8mg/ml PJ-WS-LE extracts; C: coated with 1% chitosan; D: coated with 8mg/ml PJ-WS-LE extracts embedded in 1% chitosan) for three weeks.

Treatment Batch	Week	Firmness (N)	pH	TSS (Bx)	DPPH RSA (%)
Batch A negative control	1	29.86 ± 3.5	3.61 ± 0.071	61.2 ± 3.1	14.16 ±3.5
samples	2	26.24 ± 3.1	3.77 ± 0.052	69.0 ± 3.5	18.07 ±4.5
	3	20.83 ± 2.1	3.73 ± 0.061	67.7 ± 3.2	32.90 ±2.9
Batch B coated with	1	29.53 ± 1.1	3.69 ± 0.033	61.2 ± 2.6	7.69 ±4.7
8mg/ml PJ-WS-LE	2	32.42 ± 3.2	3.66 ± 0.028	65.8 ± 4.4	17.42 ±4.0
extract	3	30.59 ± 3.8	3.74 ± 0.037	63.0 ± 1.3	33.90 ±4.2
Batch C coated with 1%	1	34.22 ± 4.4	3.65 ± 0.057	65.2 ± 5.9	11.63 ±3.2
chitosan	2	35.10 ± 6	3.65 ± 0.042	63.2 ± 4.5	16.55 ±3.9
	3	33.35 ± 2.9	3.67 ± 0.055	63.0 ± 4.6	38.46 ±2.3
Batch D coated with	1	31.6 ± 4.1	3.68 ± 0.038	65.2 ± 1.7	4.19 ±2.2
8mg/ml PJ-WS-LE	2	30.74 ± 6.2	3.74 ± 0.060	62.8 ± 1.9	18.50 ±1.2
extract in 1% chitosan	3	32.47 ± 4.4	3.68 ± 0.039	64.3 ± 3.7	26.12 ±3.9

Comparing the control (batch A) samples to coated samples showed that all three coating methods provide a protective layer that helped in maintaining firmness. Samples coated with chitosan (Batches C and D) showed 36.7% higher final firmness than that of the control group (A) samples. Firmness is usually an indicator of the physical anatomy of the fruits' cells and tissues and it reflects cell wall strength and intercellular adhesion state, a low firmness is an indicator of destruction in the stability of the cell walls (Aday *et al.*, 2013). SS+GSE and BSG+EE coating treatments showed maintenance of sample firmness with time compared to control (Emamifar *et al.*, 2019).

However, previous study showed that the higher is the ultrasound power used to treat strawberry samples, the lower is the sample's firmness with time, which reflect possible treatment-induced damage (Aday *et al.*, 2013). This highlights the importance of natural coating materials, which have minimum or no side effects of the nature of the fruits. The pH levels did not change significantly throughout the experiment, a slight increase in the pH level was noticed with time and it could be due to degradation of organic acids in the cells (Aday *et al.*, 2013). Aday *et al.* (2013) showed a slight increase in strawberry samples' pH by time with the highest increase in the control samples (Aday *et al.*, 2013).

TSS stability in coated samples could be caused by the closure of minute pores of the strawberries' skin by the coating material which decreases water loss (Patel & Panigrahi, 2019). Stability in strawberry treated samples TSS results was consistent with their average weight loss results that showed the highest weight loss with the control batch A, noting that weight loss is usually due to the loss of water content which consequently increases the total soluble solids concentration. Samples coated with SS+GSE in a recent study showed TSS increase with time, although TSS change was lower in treated samples than in the control, but showed less stability compared to samples treated with PJ-WS-LE extract (Emamifar *et al.*, 2019). Similar results were obtained with BSG+EE coating which lowered TSS increase with time but did not maintain it (Moradi *et al.*, 2019). Ultrasound, as a protective measure, showed a decrease in TSS with time, which indicates that the treatment, unlike PJ-WS-LE extract, has distorted the cell structure of the samples (Aday *et al.*, 2013).

Fruits and vegetables juices are rich with antioxidants responsible of the oxidation of free radicals by acting as oxygen scavengers. DPPH free radical scavenging activity reflects the ability of the existing antioxidants to oxidize DPPH and

therefore decolorize it (Patel & Panigrahi, 2019). As fruits mature, the antioxidants levels increase, sometimes as a mechanism of self-defense against ripening. The increased antioxidant contents is mainly due to increase in lipophilic antioxidant compounds (Naeem *et al.*, 2019). In this study, the antioxidant activity increased in strawberry samples of all treatment groups with time. However, the lowest increase was with strawberry samples of the coated batch D, which reflects the success of the treatment in slowing down fruits ripening.

4.2.6. In-vivo long term preservative activity of PJ-WS-LE extract individually and when embedded in chitosan coating material- Case of Cucumber

4.2.6.1. Sensory evaluation

The change of sensory characteristics of cucumbers stored at 8°C was evaluated on a weekly basis. Figure 29 represents the change of the score of fruits with different treatments.

As storage period passes, the overall sensory score of all cucumber batches decline. There is no significant difference between the end quality of cucumbers belonging to batch D and those of the control samples. Best end quality was seen in batch B, at week two cucumbers sprayed with the PJ-WS-LE extract alone showed an overall quality score of 4.1, further demonstrating the capability of the extract to extend cucumbers shelf-life. Apparently, the 0.5% chitosan treatment was not suitable for the cucumbers skin.

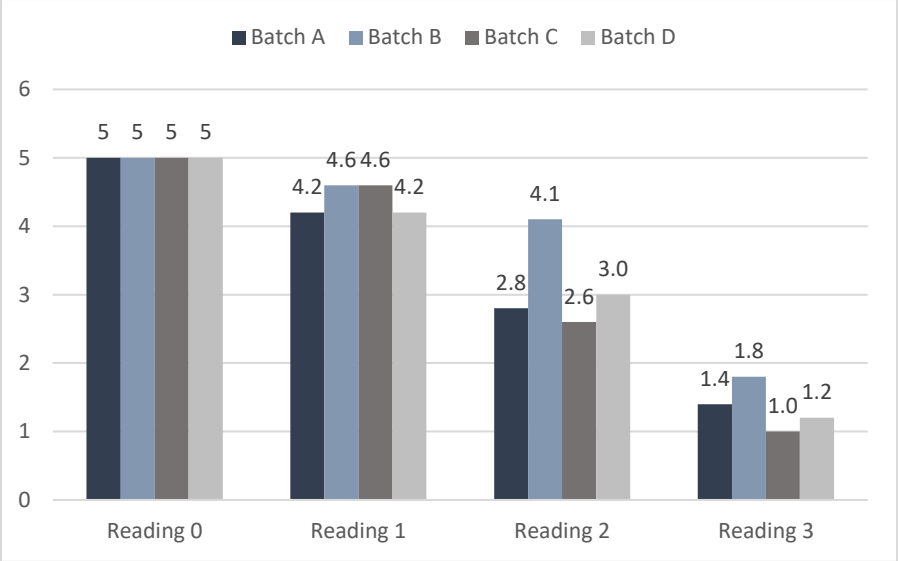


Figure 29. Sensory evaluation of cucumber samples exposed to different treatments on a weekly basis (A: Negative control samples with no treatment; B: samples treated with 8mg/ml PJ-WS-LE extract; C: samples coated with 0.5% chitosan; D: samples coated with 0.5% chitosan + 8mg/ml PJ-WS-LE extract) (Score from 1 to 5 are: 5 points for "extremely liked", 4 points for "liked", 3 points for "acceptable" 2 points for "disliked" and 1 point for "extremely disliked").

4.2.6.2. Weight loss

The effect of cucumber coating on percentage weight loss was monitored over the course of three weeks. Percentage change in weight was weekly calculated for each treatment batch (Figure 30). Cucumber samples coated with 0.5% chitosan alone or with 8mg/ml PJ-WS-LE extracts embedded in 0.5% chitosan showed unexpectedly higher weight loss than samples not coated with chitosan. Tukey test following one-way ANOVA for each week's data showed no significant difference between weight loss of batches C and D during weeks 1 and 3 (p=0.397>0.05 and p=0.064>0.05 respectively), which indicates that chitosan treatment did not suit the cucumber skin and increased weight loss. Cucumber batch with the lowest change in weight is batch

B coated with 8mg/ml PJ-WS-LE extract, in week 1 the extract showed a significant effectiveness in protecting cucumber samples against weight loss compared to the control samples (batch A) ($p \leq 0.01$). The weight loss of samples of batch B increased with time but remained the lowest among other batches.

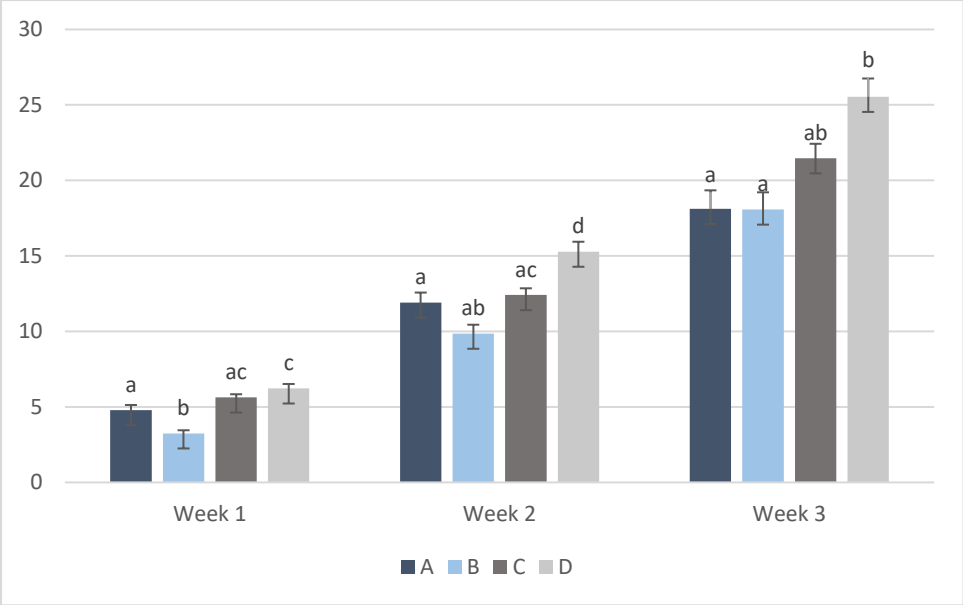


Figure 30. Average percent change in weight of the overall cucumber samples exposed to different treatments on a weekly basis ±SE. (A: Negative control samples with no treatment; B: samples treated with 8mg/ml PJ-WS-LE extract; C: samples coated with 0.5% chitosan; D: samples coated with 0.5% chitosan + 8mg/ml PJ-WS-LE extract). [abcd] Treatment columns with different letters have their values significantly different as shown by the one-way ANOVA Post-Hoc Tukey test for the data of each week at p=0.05.

An effective edible coatings should act as a barrier against water loss. Different edible coatings are formulated using different compositions including proteins, lipids, and polysaccharides (Raghav *et al.*, 2016). Every composition has its

advantages and disadvantages, lipids are hydrophobic compounds so they can limit water loss, although, their non-polymeric nature limits their ability to form films with good mechanical integrity (Yousuf *et al.*, 2021). On the other hand, polysaccharides have efficient gas barrier properties but they are highly hydrophilic which can show high water vapor permeability (Kocira *et al.*, 2021). Among polysaccharides, chitosan, obtained from alkaline deacetylation of chitin has been widely used because of its antimicrobial properties (Elieh-Ali-Komi & Hamblin, 2016). However, Qatari cucumber are grown in green houses where they are continuously watered. Water content results showed higher water content in Qatari samples. This explains why chitosan coating caused fast water loss and therefore shrinkage of the fruits which limit its usefulness as a local coating material (Gutiérrez-Pacheco *et al.*, 2020).

Samples coated with 8mg/ml PJ-WS-LE extract (batch B) showed also lower weight loss compared with other three batches during the first two weeks. In the first week, batch B weight loss was significantly lower than the control batch A. Lower weight loss in coated fruits indicates partial pores blockage which lowers in turn water loss (Patel & Panigrahi, 2019). At the end of the trial batches coated with chitosan showed the highest weight loss, which proves one more time the incompatibility of this edible coating with locally-grown cucumbers. Weight loss is usually related to multiple reactions in the fruits including browning, vitamin degradation and enzymatic activities that enhance rate of microorganisms growth including fungal decay (Mohammadi *et al.*, 2016). The coating of cucumber samples with chitosan nanoparticles loaded with *Cinnamomum zeylanicum* essential oil showed the lowest weight loss in coated samples compared to the untreated control samples (Mohammadi *et al.*, 2015). Another study conducted by Mohammadi team showed a weight loss in cucumber samples treated with chitosan nanoparticles loaded with *Zataria multiflora* essential oil of less than 10%

compared to weight loss of 12.5% in uncoated samples within 15 days (Mohammadi *et al.*, 2016). Similarly, batch B samples treated with PJ-WS-LE extract showed weight loss lower than 10% compared to 11.9% weight loss in control samples during the first 15 days.

4.2.6.3. Respiration rate

Respiration rate was calculated using the slope of the trend line passing by the collected data. Comparison between the carbon dioxide levels of the four treatment categories over the course of the three weeks is shown in figure 31.

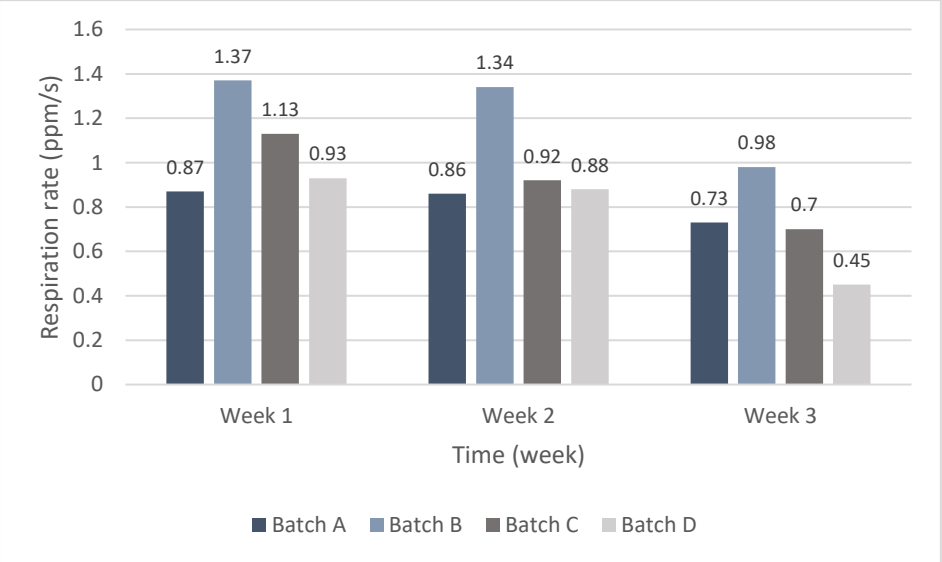


Figure 31. Respiration rate of cucumber samples belonging to the four treatment categories (A: negative control; B: sprayed with 8mg/ml PJ-WS-LE extracts; C: coated with 0.5% chitosan; D: coated with 8mg/ml PJ-WS-LE extracts embedded in 0.5% chitosan) over the three weeks of the experiment.

Figure 31 shows that the respiration rate of cucumber samples of all treatments represented by the carbon dioxide level decreases with time during the three weeks of the experiment. Week 1 results indicated that samples treated with PJ-WS-LE

(batch B) extract had the highest rate of respiration while the negative control group (batch A) was the lowest. Plants of batch B coated with the extract alone showed the highest rate of respiration throughout the three weeks which indicates the effectiveness of PJ-WS-LE extract in protecting cucumber samples and keeping their normal respiration rate. Chitosan coated samples showed the lowest rates of respiration towards the third week.

Respiration rate is an indicator of plants normal activity. The respiration rate monitoring throughout the experiment showed the best rate with batch B. It is worth noting that the PJ-WS-LE extract embedded in 0.5% chitosan had a respiration rate of 54% lower than that of batch B samples towards week 3. Similarly, cucumber samples coated with *Zataria multiflora* essential oil showed lower respiration rate (Mohammadi *et al.*, 2016).

4.2.6.4. Total aerobic bacterial, fungal and yeast count

PJ-WS-LE extract in the cucumber coating material served as an antimicrobial barrier that kills surrounding bacteria. From reading zero, samples coated for few hours showed much lower bacterial count than negative control samples and samples coated with chitosan alone, effect is stronger in samples of batch D coated with the extract embedded in 1% chitosan figure 32. Figure 32 shows that samples coated with 8mg/ml PJ-WS-LE extract alone (batch B) and extract embedded in 0.5% chitosan (batch D) have their total aerobic bacterial count dropped drastically compared to the control batches. However, the CFU of aerobic bacteria surrounding the cucumber samples of batches B and D increased in weeks 2 and 3, yet the final CFU counts were still much lower than the control batch samples (batch A) in which aerobic bacterial CFU is 5.5X more than batch B CFU and 2.8X more than batch D CFU.

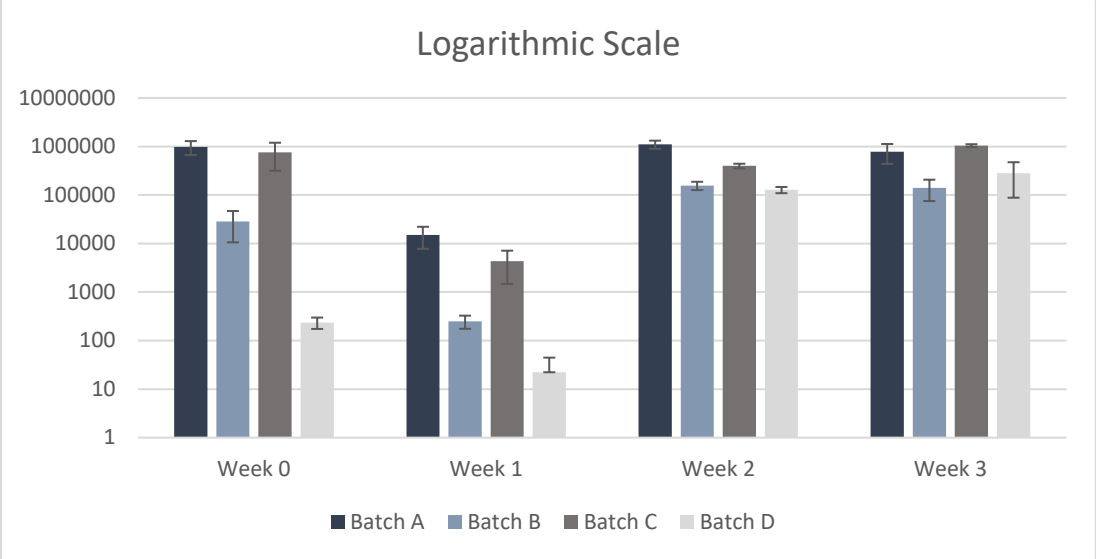


Figure 32. Average surrounding aerobic bacterial CFU in CFU/g ±SE of cucumber represented in logarithmic scale of samples exposed to different treatment during three weeks of storage (A: negative control; B: sprayed with 8mg/ml PJ-WS-LE extracts; C: coated with 0.5% chitosan; D: Coated with 8mg/ml PJ-WS-LE extracts embedded in 0.5% chitosan).

Among the factors evaluated in treated bacterial samples, was the number of strains per treatment batch. Results show decrease in the number of strains per sample in the two batches treated with PJ-WS-LE extract (batches B and D) which showed an average of only two bacterial strains per sample compared to an average of 5 strains per sample in control batches (batches A and C) (Figure 33).

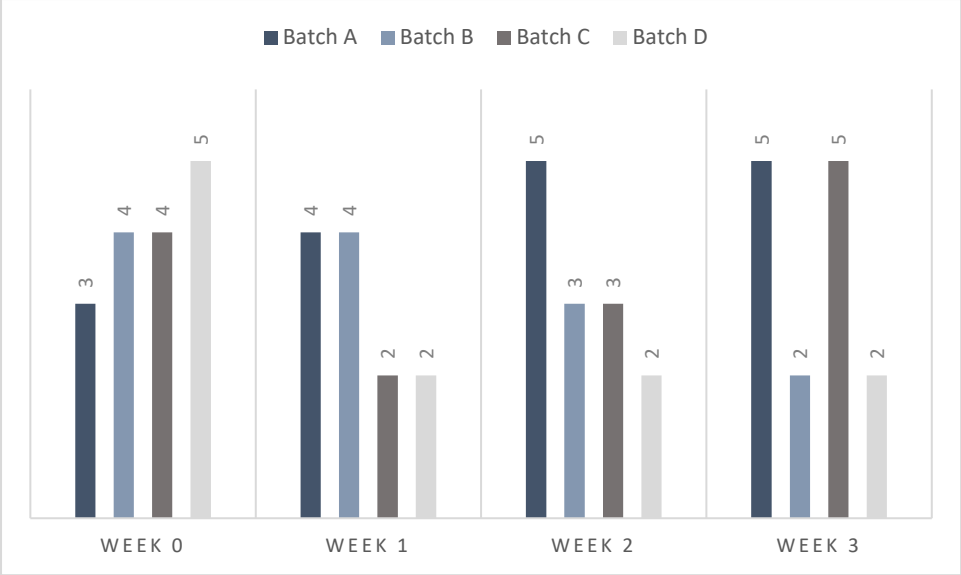


Figure 33. Average number of bacterial strains on cucumber samples exposed to different treatment during three weeks of storage (A: negative control; B: sprayed with 8mg/ml PJ-WS-LE extracts; C: coated with 0.5% chitosan; D: coated with 8mg/ml PJ-WS-LE extracts embedded in 0.5% chitosan).

Cucumber rotting involved more bacteria than mold, spread plate method of wash water used to wash the outside of cucumber samples of different batches proved this idea and showed low number of mold spores. However, samples coated with PJ-WS-LE extract showed a total count between zero and 4 CFU/g which proves the effectiveness of the extract against spores germination. For yeast count, the variation of average CFU showed a pattern similar to that of bacteria (Figure 34), with very low yeast CFU count of cucumbers of batch D during reading 0 and 1, the number increases in weeks 2 and 3 but it remained 4.4 times and 14.4 times lower than the yeast CFU of the control batch A, respectively. PJ-WS-LE extract in the cucumber coating material served as an antimicrobial barrier that kills surrounding mold spores and yeast.

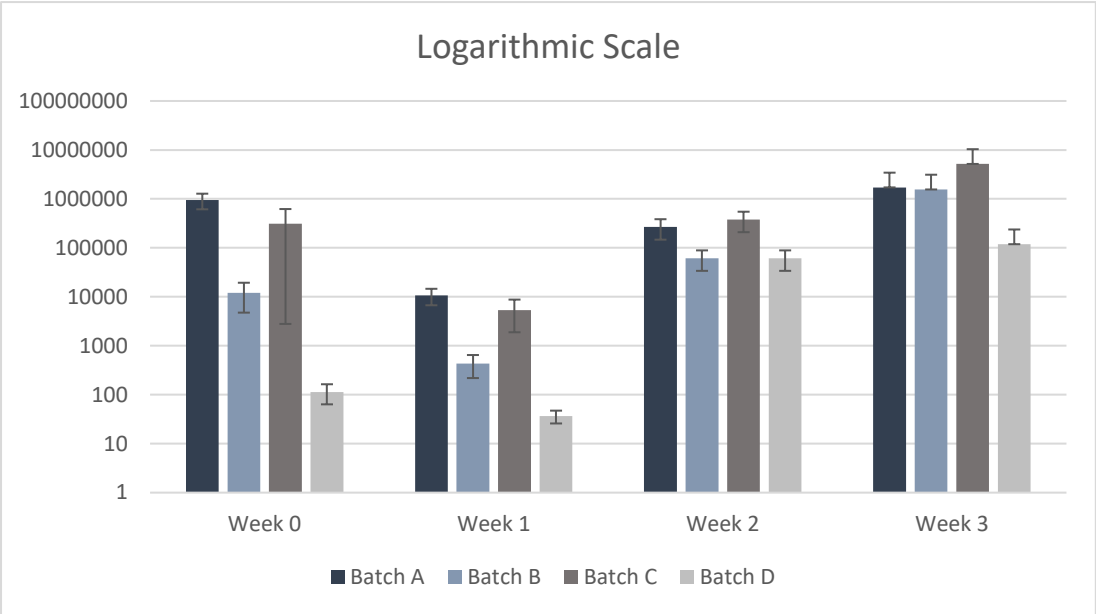


Figure 34. Average yeast CFU in CFU/g ±SE of cucumber represented in logarithmic scale of samples exposed to different treatment during three weeks of storage (A: negative control; B: sprayed with 8mg/ml PJ-WS-LE extracts; C: coated with 0.5% chitosan; D: coated with 8mg/ml PJ-WS-LE extracts embedded in 0.5% chitosan).

Mold samples showing growth upon spread plate method were subjected to microscopic identification, the number of samples showing a certain fungi genus was recorded in table 39 regardless of the number of colonies per samples. Results showed that the dominant strain in cucumber samples stored at 8°C was *Penicillium* found in 14 samples of different treatment batches throughout the storage period.

Fruits and vegetables surrounding microorganisms increase in numbers during fruits ripening and play a role in fruits spoilage. Cucumbers are usually more prone to bacterial spoilage than fungal spoilage. Total aerobic bacterial CFU monitoring between week 0 (time of treatment) and week 3 showed that PJ-WS-LE extract has effective antimicrobial qualities which lowered final CFU count by 5.5X in

batch B and 2.8X in batch D compared to the control samples (batch A). The capacity of PJ-WS-LE extract to lower the surrounding microorganisms count plays a role in increasing plants shelf-life. In addition, the average number of bacterial types per treatment batch indicated lower bacterial diversity on batches B and D samples (two types) compared to control batches A and C samples (5 types), indicating the total intolerance of some bacterial types to PJ-WS-LE extract. As for fungi, batches coated with PJ-WS-LE extract did not show fungal growth, which indicates the effectiveness of the extract against spores germination. Similar results were found with yeast count which showed a final CFU count 14.4X lower in batch D samples than the control samples. Overall results showed high antimicrobial effect of PJ-WS-LE extract. In accordance with our results, chitosan nanoparticles loaded with *Cinnamomum zeylanicum* essential oil and *Zataria multiflora* essential oil showed antimicrobial activities of the coating with lower CFU of treated samples compared to control (Mohammadi *et al.*, 2015, 2016). Noting that PJ-WS-LE extract showed the lowest bacterial and mold CFU among other treatments.

Table 39. Number of cucumber samples showing specific fungal genus growth throughout their storage period.

Weeks	1				2				3				Total
Treatment Batches	A	B	C	D	A	B	C	D	A	B	C	D	
Cladosporium	0	0	0	1	0	0	0	1	1	0	0	0	3
Penicillium	2	4	1	0	0	1	0	0	0	5	0	0	13
Aspergillus	1	0	0	0	0	0	0	0	2	0	0	0	3
Alternaria	0	0	0	0	1	1	0	0	0	0	0	0	2

4.2.6.5. Effectiveness of PJ-WS-LE extract in maintaining cucumber samples weight

A correlation between the samples weight loss pattern and the change in microbial counts throughout the study would support our hypothesis stating that PJ-WS-LE extract acts as an antimicrobial barrier around the fruits and maintain their storage quality during the experiment including the lessening of weight loss. Pearson test results showed a significant correlation between bacterial and yeast counts patterns. Interestingly, the results showed a significant correlation between the percentage weight change data and the total CFU counts of bacteria and yeast while mold CFU count is not correlated with the percentage change in weight in the tested samples (Table 40).

Table 40. Correlation matrix between percent change in weight of cucumber samples and their total CFU counts of mold, yeast, and bacteria.

Correlation Matrix

	Mean	SD	% Weight Change	Mold CFU	Yeast CFU	Bacteria CFU
% Weight Change	11.78	7.07	1			
Mold CFU	138.89	1069.19	-0.162	1		
Yeast CFU	933703.31	2089316.20	.384**	-0.059	1	
Bacteria CFU	338484.26	479181.52	.433**	-0.092	.529**	1

** *p*-value ≤0.01; * *p*-value≤0.05

Previous *in-vitro* analysis showed strong effectiveness of PJ-WS-LE extract against a variety of fungi, bacteria and even yeast with MICs ranging from 0.5mg/ml to 4mg/ml. (Saleh & Abu-Dieyeh, 2021). This explains the correlation

between yeast and bacterial CFU changes. Being a valuable source of phenolic compound gives *P. juliflora* extracts the capacity of initiating multiple antioxidative mechanisms, which can act as antimicrobial agents. The antimicrobial activity of *P. juliflora* has been previously described by many previous studies (de Brito Damasceno *et al.*, 2018). Previous results, together with the significant correlation proven by Pearson test imply that the strong antimicrobial activity of the extract protects cucumber samples from yeast and bacterial rot and maintain their weights among other quality parameters. The insignificant results in the case of mold is due to the low number of spores encountered around cucumber samples from first day which although reached zero after treatment but it didn't change widely in correlation with percentage change in weight.

4.2.6.6. Changes in physical and chemical properties during storage time (Firmness, pH, TTS, antioxidant ability)

Weekly cucumber samples monitoring included physical parameters that were measured for each treatment groups. Results of averages firmness, pH and total soluble solids of samples stored at 8°C throughout the three weeks are shown in table 41.

Negative control batch of cucumber showed 8% loss of their firmness throughout the storage period. However, all other batches showed stability in their firmness. When results of the overall storage period of each treatment batch were compared using one way ANOVA followed by Tukey test, no significant variation in the firmness averages was found (p=0.857>0.05), however, batches A and B showed average firmness of 40.2N and 40.6N respectively and batches C and D showed average firmness of 43.4N and 43.5N.

Table 41. Average physical parameters ±SE of cucumber samples exposed to different treatments (A: negative control; B: sprayed with 8mg/ml PJ-WS-LE extracts; C: coated with 1% chitosan; D: Coated with 8mg/ml PJ-WS-LE extracts embedded in 1% chitosan) throughout three weeks.

Treatment Batch	Week	Firmness (N)	pH	TSS (Bx)
Batch A negative control	1	40.8 ±7.63	6.07 ±0.056	30.8 ±0.86
samples	2	40.75 ±3.85	6.71 ±0.225	30.6 ±1.63
	3	37.38 ±7.20	6.78 ±0.073	35 ±2.14
Batch B coated with	1	38.05 ±5.36	6.38 ±0.184	31.2 ±1.77
8mg/ml PJ-WS-LE extract	2	38.72 ±3.12	6.76 ±0.075	30.8 ± 2.76
	3	38.70 ±2.29	6.83 ±0.033	32.4 ±3.01
Batch C coated with 1%	1	42.26 ±8.45	6.15 ±0.042	32.8 ±0.37
chitosan	2	41.96 ±6.04	6.89 ±0.071	30.0 ±1.58
	3	42.57 ±6.38	6.91 ±0.026	33.2 ±3.42
Batch D coated with	1	42.66 ±6.72	6.92 ±0.056	31.0 ± 1.67
8mg/ml PJ-WS-LE extract	2	39.65 ±5.25	6.87 ±0.109	35.25 ± 2.05
in 1% chitosan	3	43.29 ±5.20	6.88 ±0.098	39.2 ±2.26

The pH results of cucumber juices of different batches showed increase in numbers throughout the weeks except batch D samples that showed slight pH decrease. However, when each batch results of all weeks were combined and entered in SPSS, one way ANOVA followed by Tukey test was conducted, the overall results didn't show any significant difference in pH of cucumbers of different batches (p=0.716>0.05). Highest pHs were recoded with batch D samples (average pH=6.51).

Total soluble solids results showed an increased level in the control (batch A) samples by 12 % and of the experimental batch D samples by 21% during the three weeks of storage. Cucumber samples of batches B and C showed slight change in their TSS levels. DPPH radical scavenging activity of cucumber samples of the four treatment batches was measured on a weekly basis (data not shown). There was no significant difference in the overall DPPH RSA % among the four batches upon running one way ANOVA followed by Tukey test (p=0.774>0.05). Yet the lowest rate of antioxidant increase was found in samples of batch B**.**

Firmness is among the important sensory attributes in fruits and vegetables. Firmness is affected by pectin depolymerization leading to texture deterioration during fruits ripening (Paniagua *et al.*, 2014). There was no significant variation in the firmness averages throughout the three weeks of the experiment, however, batches A and B samples showed slightly lower firmness averages compared to batches C and D, which imply that chitosan coating played a role in hardening the cell wall of the cucumber samples. In agreement with our results, the former studies conducted on chitosan nanoparticles loaded with *Cinnamomum zeylanicum* essential oil and *Zataria multiflora* essential oil coatings showed also firmness stability in chitosan-coated samples (Mohammadi *et al.*, 2015, 2016). Furthermore, there was no significant change in pH during the experiment. Yet it was noted that samples of batches A, B and C showed pH increase with time while samples of batch D showed pH decrease, which indicates that the combination of the plant extract and the chitosan has caused different set of reactions to occur in the cucumber samples compared to other treatments. The highest increase in TSS during the experiment were found with batch D (21%) followed by batch A (12%). As increase in soluble sugars could be due to the increase in water loss and therefore weight loss, the TSS results show consistency with the average weight

loss of cucumber samples discussed earlier in which samples of batch D showed the highest average weight loss by the end of the incubation period. Lowest TSS increase was found in cucumbers coated with 8mg/ml PJ-WS-LE extract which showed the best sensory qualities during the experiment. Overall, the increase of total soluble sugars during cucumber ripening has been described, as the time passes, macromolecules produce soluble micromolecules which increases TSS (Patel & Panigrahi, 2019). It has also been reported that higher levels of sugars increase cucumber drought stress tolerance (M. Zhang *et al.*, 2007).

Total antioxidant change was not significantly changed with time, however, batch B showed the lowest DPPH radical scavenging activity increase, which reflects their slower ripening process compared to other samples. DPPH-radical scavenging activity shows usually increase as an indicator of antioxidant compounds development during postharvest storage (Mohammadi *et al.*, 2016). It is worth noting that previous studies have demonstrated that chitosan could induce increase in antioxidant activity in fruits such as tomato, orange and sweet cherry (Liu *et al.*, 2007; Meng *et al.*, 2008; Dang *et al.*, 2010).

4.2.6.7. Cucumbers water content

Results showed that samples' water content average is 96.25±0.074% of the cucumber samples net weight, which is above the literature average of 90 to 95%.

4.2.7. In-vivo long term preservative activity of PJ-WS-LE extract individually and when embedded in chitosan coating material- Case of Mango

4.2.7.1. Sensory evaluation

The change of sensory characteristics of mangoes stored at 4°C were evaluated on a weekly basis. Figure 35 represents the change of the score of fruits belonging to different treatment batches.

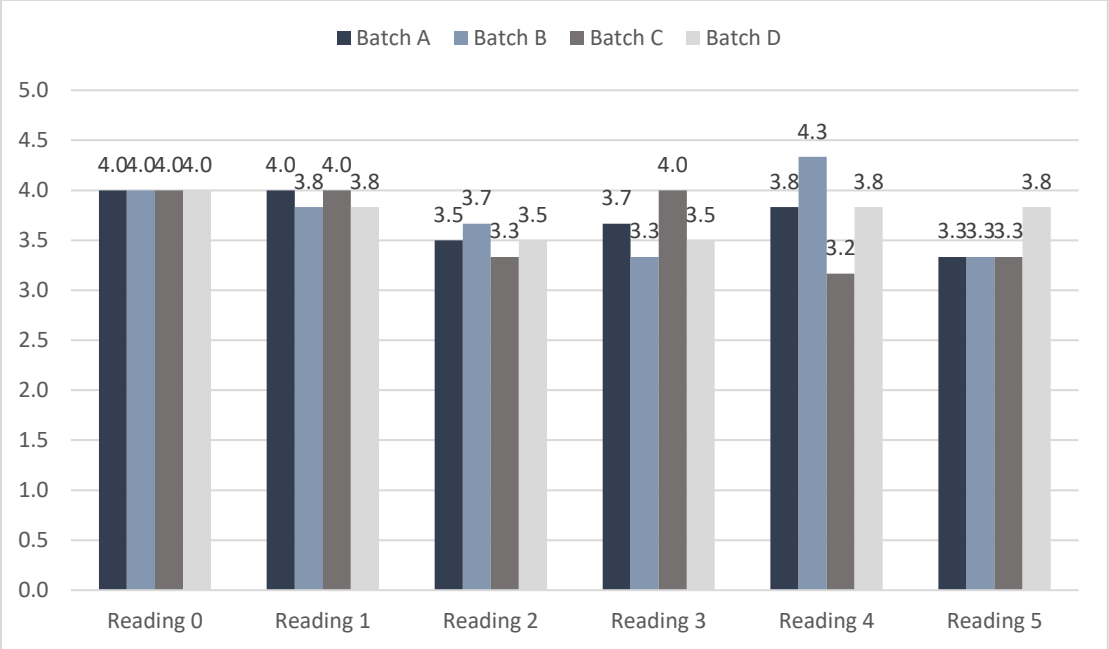


Figure 35. Sensory evaluation of mango samples exposed to different treatments on a weekly basis (A: Negative control samples with no treatment; B: samples treated with 8mg/ml PJ-WS-LE extract; C: samples coated with 1% chitosan; D: samples coated with 1% chitosan + 8mg/ml PJ-WS-LE extract) (Score from 1 to 5 are: 5 points for "extremely liked", 4 points for "liked", 3 points for "acceptable" 2 points for "disliked" and 1 point for "extremely disliked").

As storage period passes, the overall sensory score of all mango batches decline. However, samples coated with 8mg/ml of PJ-WS-LE extract embedded in 1% chitosan (batch D) conserved better scores during the five weeks of storage which highlights the coating efficacy.

4.2.7.2. Weight loss

The effect of mango samples coating treatment on the weight loss was monitored over the course of five weeks. Percentage change in weight was calculated for each treatment batch every week (Figure 36). Weight loss increased with time, however, PJ-WS-LE extract showed efficacy in reducing weight loss when embedded in chitosan.

There was a significant increase in the percentage change of weight of mango samples in all treatment categories with the passage of storage time, one-way ANOVA showed a $p \leq 0.05$. Mangoes coated with 1% chitosan and 8mg/ml of the PJ-WS-LE extract had the lowest change in weight with an observable increase of this change from a week to another. One-way ANOVA test showed a significant effect of treatment type on percentage weight change in the overall data of all weeks, post-Hoc Tukey showed that treatment D lead significantly to lower percentage of weight loss when compared to the control samples (batch A) in the overall data ($p=0.012 \leq 0.05$).

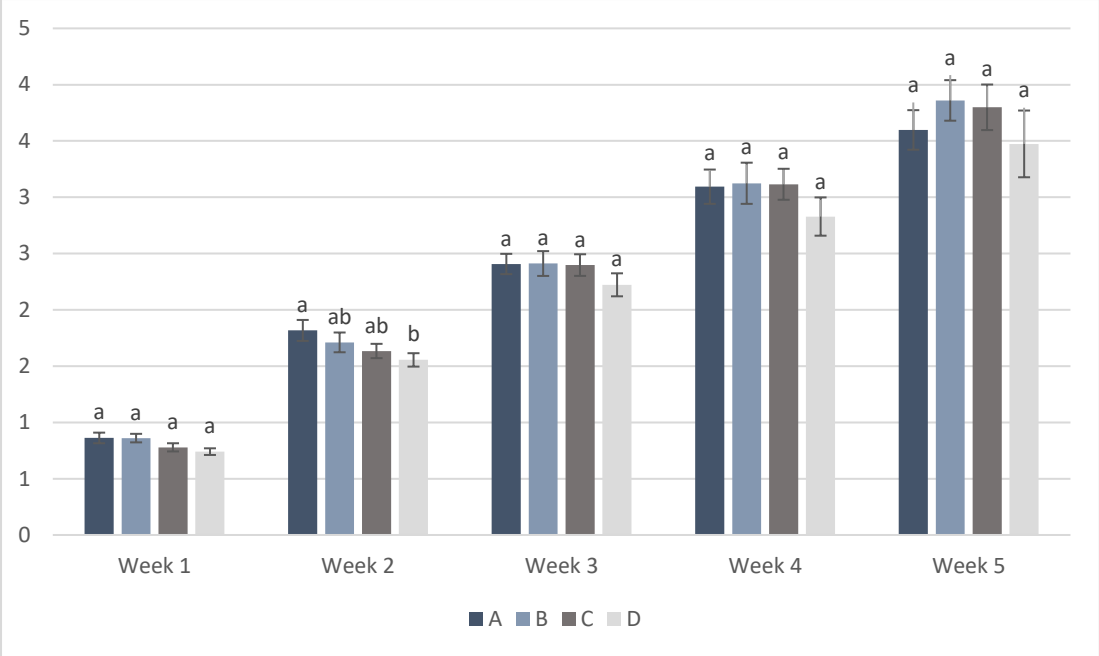


Figure 36. Average percent change in weight of the overall mango samples exposed to different treatments on a weekly basis ±SE. (A: Negative control samples with no treatment; B: samples treated with 8mg/ml PJ-WS-LE extract; C: samples coated with 0.5% chitosan; D: samples coated with 0.5% chitosan + 8mg/ml PJ-WS-LE extract). [ab] Treatment columns with different letters have their values significantly different as shown by the one-way ANOVA and Post-Hoc Tukey test for the data of each week at p=0.05.

During the postharvest storage period, water evaporation leads to fruits shriveling and weight loss (Sogvar *et al.*, 2016). Mangoes coated with 1% chitosan and 8mg/ml of the PJ-WS-LE extract had the lowest change in weight with an observable increase of this change from a week to another. At week 2, samples belonging to batch D showed significantly lower weight loss than samples of the control batch A. PJ-WS-LE extract increases water holding capacity when mixed with the edible coating of 1% chitosan. A recent study conducted on mangoes evaluated the effect of *Aloe vera* gel

embedded in 1% chitosan (CTS+AVG). Coated samples showed similarly to our results lower weight loss with time (Shah & Hashmi, 2020).

4.2.7.3. Changes in physical and chemical properties during storage time (Firmness, pH, TTS, antioxidant activity, and ascorbic acid level)

Mango samples of different treatment batches had their physical parameters monitored on a weekly basis. Results of averages firmness, pH and total soluble solids of samples stored at 4°C throughout the three weeks are shown in table 42.

Mango samples of batches A, B and C showed decrease in their firmness during the storage time while samples of batch D showed a kind of firmness stability. When results of the overall storage period of each treatment batch were compared using one way ANOVA followed by Tukey test, no significant variation in the firmness averages was found (p=0.092>005). The pH results of mango juices of different batches increased in numbers throughout the storage weeks, except of batch D, which samples decreased in their pH levels. When each batch pH results (all weeks results combined) were entered in SPSS and one way ANOVA followed by Tukey test was conducted, the overall results showed significant difference in pH of mango of different batches (p=0.001≤0.01). Tukey test showed that batch D samples have significantly lower pH than other samples (average pH=4.51).

Table 42. Average physical parameters ±SE of mango samples exposed to different treatments (A: negative control; B: sprayed with 8mg/ml PJ-WS-LE extracts; C: coated with 1% chitosan; D: coated with 8mg/ml PJ-WS-LE extracts embedded in 1% chitosan) throughout three weeks.

Treatment Batch	Week	Firmness (N)	pH	TSS (Bx)	DPPH RSA (%)
Batch A negative control samples	1	20.39 ± 7.23	4.71 ±0.053	71.3 ±6.33	36.22 ±6.9
	2	23.80 ± 3.18	4.51 ±0.051	69.0 ±0.00	37.27 ±0.5
	3	12.14 ± 1.02	4.84 ±0.111	69.3 ±6.88	62.28 ±1.5
	4	17.66 ± 5.44	4.71 ±0.063	68.3 ±3.38	64.71 ±7.1
	5	9.98 ± 0.80	4.90 ±0.090	70.7 ±3.71	-
Batch B coated with 8mg/ml PJ-WS-LE extract	1	11.41 ± 2.67	4.82 ±0.055	77.0 ±0.57	43.47 ±0.4
	2	17.58 ± 7.52	4.80 ±0.072	72.7 ±1.67	34.54 ±3.1
	3	12.58 ± 0.88	4.87 ±0.082	63.7 ±2.33	68.21 ±4.0
	4	12.3 ± 1.12	4.94 ±0.117	73.0 ±3.05	70.97 ±3.8
	5	7.26 ± 0.83	5.19 ±0.113	76.7 ±3.75	-
Batch C coated with 1% chitosan	1	18.07 ± 4.47	4.67 ±0.034	74.9 ±2.02	54.33 ±7.4
	2	7.43 ± 2.21	4.92 ±0.119	69.7 ±2.40	37.88 ±2.7
	3	10.18 ± 2.37	4.99 ±0.013	70.7 ±2.18	44.65 ±3.2
	4	13.72 ± 2.08	4.74 ±0.052	75.3 ±3.71	74.82 ±6.0
	5	14.49 ± 3.73	5.02 ±0.043	77.3 ±8.96	-
Batch D coated with 8mg/ml PJ-WS-LE extract in 1% chitosan	1	20.91 ± 10.1	4.69 ±0.020	73.3 ±3.33	58.04 ±3.7
	2	15.33 ± 2.15	4.71 ±0.022	69.7 ±0.88	73.44 ±5.1
	3	19.07 ± 1.40	4.65 ±0.119	75.7±9.92	55.29 ±4.0
	4	23.45 ± 6.37	4.26 ±0.083	81.7 ±1.66	80.95 ±3.6
	5	21.43 ± 5.22	4.31 ±0.172	86.7 ±3.33	-

Total soluble solids results showed fluctuation in the TSS levels throughout the five weeks of storage. One way ANOVA test followed by Tukey test of the overall results of each batch showed a significant difference in the average TSS levels (p=0.009), Tukey test results showed that samples of batch D have significantly higher

TSS levels when compared to others with an average TSS level of 81.7Bx compared to an average TSS level of 69.4Bx in the case of the control batch A samples. Having the highest TSS with samples of batch D is also consistent with the cucumber results.

DPPH radical scavenging activity of mango samples of the four treatment batches was measured for four weeks. The antioxidant activity increased in mango samples of all treatment groups with time. The highest increase was found with mango samples of the coated batch D followed by samples of batch C. Loss of firmness of fruits occurs during ripening by the polygalacturonases (PG) and pectin methylesterase (PME) enzymes activation, which leads to the degradation of the middle lamella between parenchyma cells, cell wall disruption, and loss of cellular turgidity (Harker *et al.*, 2010). Samples firmness was evaluated with time, all batches samples showed decrease in firmness with time except batch D samples which maintain their hardness during the five weeks of the experiment giving a value to the coating material. Similarly, mango samples coated with (CTS+AVG) were significantly firmer (Shah & Hashmi, 2020). The pH results of mango juices of different batches showed an increase in numbers throughout the storage weeks, except of batch D samples which had significantly lower pH values which indicates that different ripening chemical reactions are occurring inside the coated samples. Coating material have also led to higher soluble sugar levels in mango juice samples which might have played a role in quality maintenance. Tukey test results showed that samples of batch D have significantly higher TSS levels compared to others. Finally, mangoes coating increased DPPH radical scavenging activity. The highest increase was with mango samples of the coated batch D followed by samples of batch C. Similarly, (CTS+AVG) coated mango fruits showed higher antioxidant activity compared to control samples (Shah & Hashmi, 2020).

4.2.8. In-vivo long term preservative activity of PJ-WS-LE extract individually and when embedded in chitosan coating material- Case of Lemon

4.2.8.1. Sensory evaluation

The change of sensory characteristics of lemons stored at 4°C was evaluated on a weekly basis. The quality scores of the non-rotten samples of all treatment batches was maintained during the storage period.

4.2.8.2. Weight loss

The effect of samples coating treatment on the weight loss was monitored over the course of six weeks. Percentage change in weight was calculated for each treatment batch every week (Figure 37). Weight loss increased with time, however, PJ-WS-LE extract showed efficacy in reducing weight loss individually (Batch B) and when embedded in chitosan (Batch D).

There was a significant increase in the percent change of weight of lemon in all treatment categories with storage time. Lemons coated with 1% chitosan and 8mg/ml of the PJ-WS-LE extract had the lowest change in weight with an observable increase of this change from week 1 to week 6. The extract alone showed also high effectiveness in protecting fruits from weight loss.

The significance of the difference in percentage weight loss of different batches was evaluated on a weekly basis using one-way ANOVA followed by Tukey test. Significant differences between treatments varied from week to week (Figure 37). In general, Tukey test results divide the treatments into two subsets (subset 1: batch A and batch C; subset 2: batch B and batch D), subset 2 have significantly lower weight loss than subset 1 which proves the effectiveness of the PJ-WS-LE extract treatment. In week 6, the low number of samples allowed a significant difference, however, batch

D showed 1.02% change in its weight compared to 1.64% change in the weight of the control samples (batch A). Up to week 5, batch D coating showed significant efficacy in protecting fruits from weight loss.

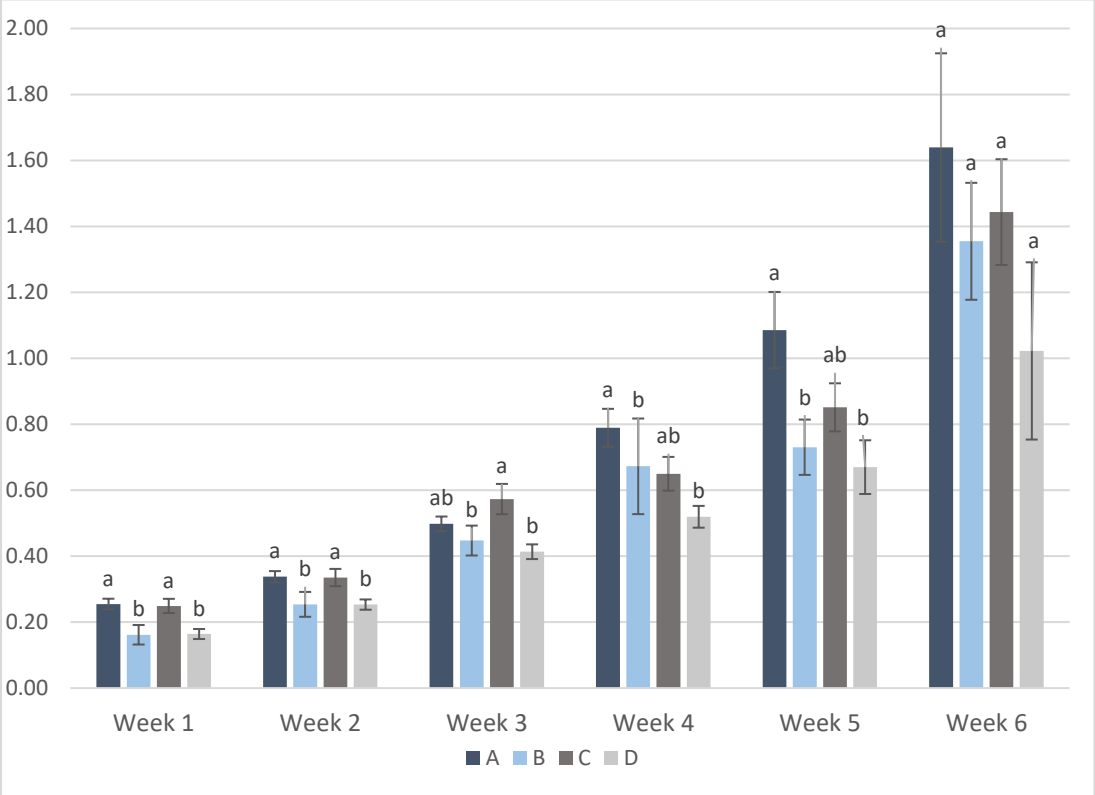


Figure 37. Average percent change in weight of the overall lemon samples exposed to different treatments on a weekly basis ±SE. (A: Negative control samples with no treatment; B: samples treated with 8mg/ml PJ-WS-LE extract; C: samples coated with 0.5% chitosan; D: samples coated with 0.5% chitosan + 8mg/ml PJ-WS-LE extract).

[ab] Treatment columns with different letters have their values significantly different as shown by by the one-way ANOVA and Post-Hoc Tukey test for the data of each week at p=0.05.

4.2.8.3. Changes in physical and chemical properties during storage time (Firmness, pH, TTS, antioxidant activity and ascorbic acid level)

pH levels of juice samples of lemons belonging to different batches showed increase in numbers throughout the storage weeks. Figure 38 shows the average pH change of lemon juice samples. The highest pH was recorded with the last average reading of samples of batch C, it is also noticed that average pH increase is higher in treated samples than the control samples (batch A).

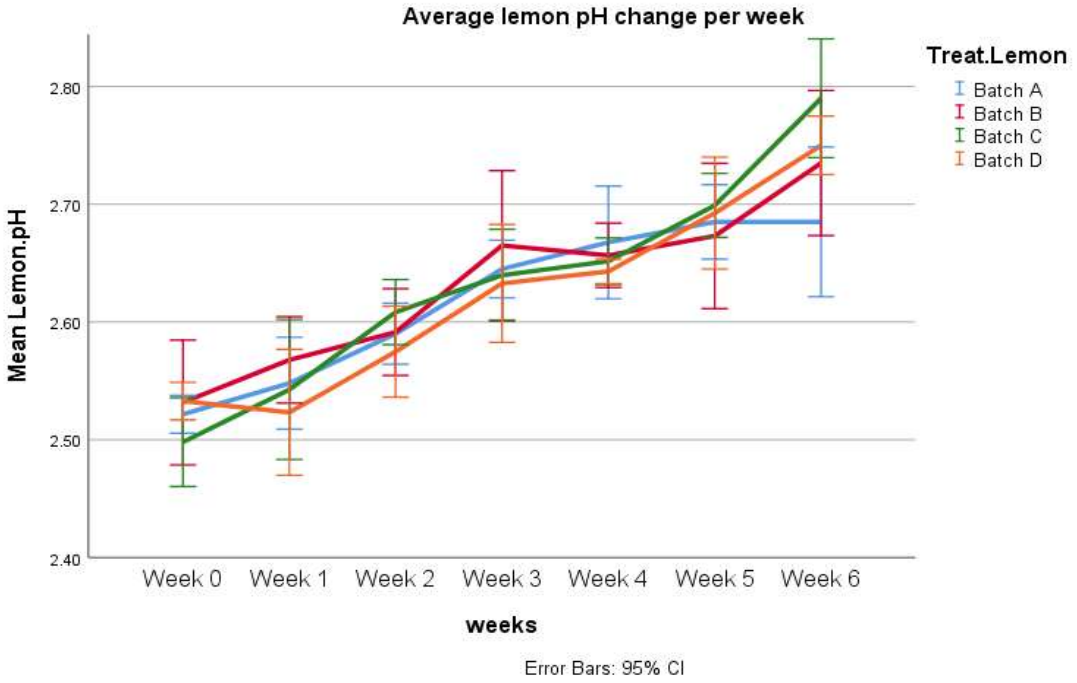


Figure 38. Average pH levels of lemon samples exposed to different treatments on a weekly basis

±SE. (A: Negative control samples with no treatment; B: samples treated with 8mg/ml PJ-WS-LE extract; C: samples coated with 0.5% chitosan; D: samples coated with 0.5% chitosan + 8mg/ml PJ-WS-LE extract).

Total soluble solids results showed fluctuation in the TSS levels throughout the six weeks of storage. One-way ANOVA test followed by Tukey test of the overall results of each batch showed a significant difference in the average TSS levels (p=0.017). TSS levels average obtained are: 78.6Bx and 78.2Bx for batches A and C respectively while batches B and D showed TSS levels of 75.5Bx and 75.1Bx. DPPH radical scavenging activity of lemon samples of the four treatment batches was measured for six weeks. There was no significant difference in the antioxidant activity of plants belonging to these four treatment batches.

The pH levels of juice samples of lemon belonging to different batches showed increase in numbers throughout the storage weeks. Average pH increase is higher in treated samples than the control samples (batch A). A recent study tested the effect of different edible coatings on lemon samples pH, showed different results with a pH reduction with time (Naeem *et al.*, 2019). In addition, Lemon samples of batches B and D showed lower TSS than samples of batches A and C. TSS results were similar to those obtained with Naeem *et al*. (2019), where coated samples exhibited lower TSS increase with time. The results are perfectly consistent with the lemons' average weight loss results that showed lower weight loss with batches B and D treated with PJ-WS-LE extract, which indicates the role of the extract in protecting lemons and keeping their quality during storage time. Finally, there was no significant difference in the antioxidant activity of plants belonging to the four treatment batches. The total antioxidant capacity was increasing with time in all batches, which is normal as antioxidant capacity usually increase with fruits ripening mainly due to the increase in lipophilic antioxidants compounds (Uckoo *et al.*, 2015). A recent study showed that edible coatings reduced the rate of antioxidants increase during storage (Naeem *et al.*, 2019).

4.2.9. Effect of PJ-WS-LE extract on chickpeas seeds germination

4.2.9.1. Effect of seeds priming and plants watering solution on plants' chlorophyll content

Thirty days old plants primed with 20mg/ml PJ-WS-LE extract for 10min and 30min showed variation in their average total chlorophyll contents. Plants watered with SDW alone showed also different chlorophyll levels than plants watered with the extract (Figure 39).

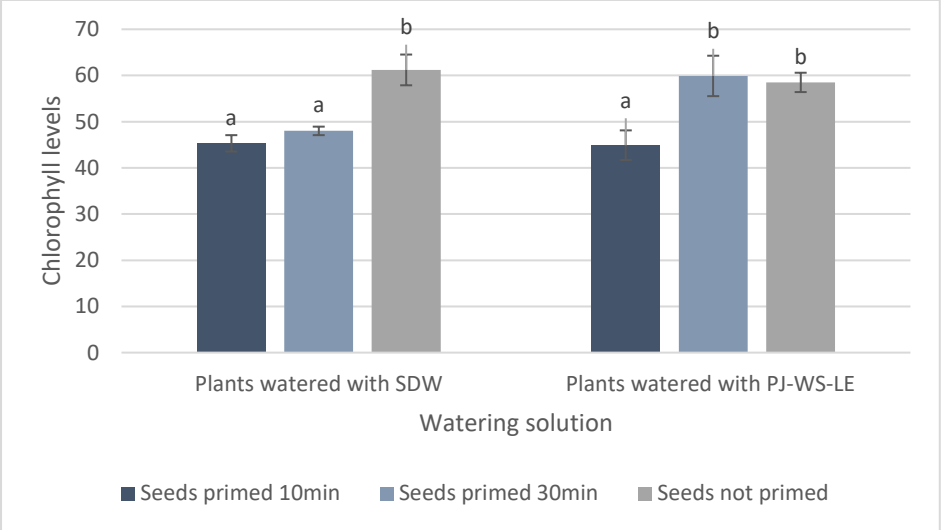


Figure 39. Average chlorophyll levels ±SE of chickpeas plants primed for different time slots and watered with different solutions.

[ab] Treatment columns with different letters have their values significantly different as shown by the one-way ANOVA Post-Hoc Tukey test for the data of each treatment at p=0.05.

Chickpeas seeds priming with PJ-WS-LE extract did not enhance plants average chlorophyll levels. One way ANOVA was performed to check significance of the priming duration of plants watered with the same solution. Dunnett Post-Hoc test

results showed that control non-primed seeds have significantly the best chlorophyll content for plants watered with SDW. Results indicates that priming chickpeas seeds with PJ-WS-LE extract did not improve plants chlorophyll content. However, when Dunnett Post-Hoc test was run on samples watered with 8mg/ml PJ-WS-LE extract solution, seeds primed with the extract for 30min before being planted showed results similar to unprimed seeds (p=0.48≥0.05), only seeds primed for 10min were significantly of a lower chlorophyll content quality than the rest of the seeds.

It is worth noting in this experiment that although priming chickpeas seed with PJ-WS-LE extract did not give them any advantage, but it also did not badly affect the seeds. Priming the seeds with the extract for 30min, helped their adaptation to the extract, which therefore helped in getting their average chlorophyll level back to normal. *Prosopis juliflora* is a plant rich with allelopathic chemicals, the insignificant effect of using PJ-WS-LE extract solution as watering solution might indicate that this extract does not contain allelopathic chemicals that might harm surrounding crops and it implies that it could be safe for usage not only post-harvesting but also in the field.

4.2.9.2. Effect of seeds priming and plants watering solution on plants' leaflets number

The number of leaflets per plants' leaf reflect healthy growth and capability of absorbing chlorophyll during growth. Average number of leaflets per plants' leaf varied among different treatment groups between 9.3 and 10.4. Although seeds priming seemed to improve the number of leaflets per leaf, yet one-way ANOVA did not show any significant differences among groups (p > 0.05).

4.2.9.3. Effect of seeds priming and plants watering solution on plants' stems height and roots length

Stems height of the plants reflects how fast it grows while the length of the roots indicates roots depth in soil, excessive roots growth compared to control might reflect stress. Average stem height showed variation in a pattern similar to the chlorophyll content variation. However, when one-way ANOVA tests were performed, differences in stems heights among categories were not significant ($p > 0.05$).

Length of roots primed for 10min in both watering categories was the longest. This indicates that priming the seeds with PJ-WS-LE extract enhanced roots growth, and that 10min was more effective than 30min priming in both cases of watering with SDW or in the case of using the extract in the field. Results make sense as root depth might reflects stress adaptation. However, when ANOVA tests were performed differences in roots heights among categories were also not significant ($p > 0.05$).

4.2.9.4. Effect of seeds priming and plants watering solution on plants' shoots and roots fresh and dry weight

Averages fresh and dry weights of plants stems belonging to different categories varied in similar patterns with the highest fresh stems. As for the dry weight the highest weight was for the plants primed for 10min and watered with SDW followed by non-primed plants watered with PJ-WS-LE extract (Figure 40). Lowest average dry stems weight was seen with plants primed for 30min, which implies one more time that this treatment could be stressful on plants, knowing that statistical analysis did not show any significance for the weights variations ($p > 0.05$).

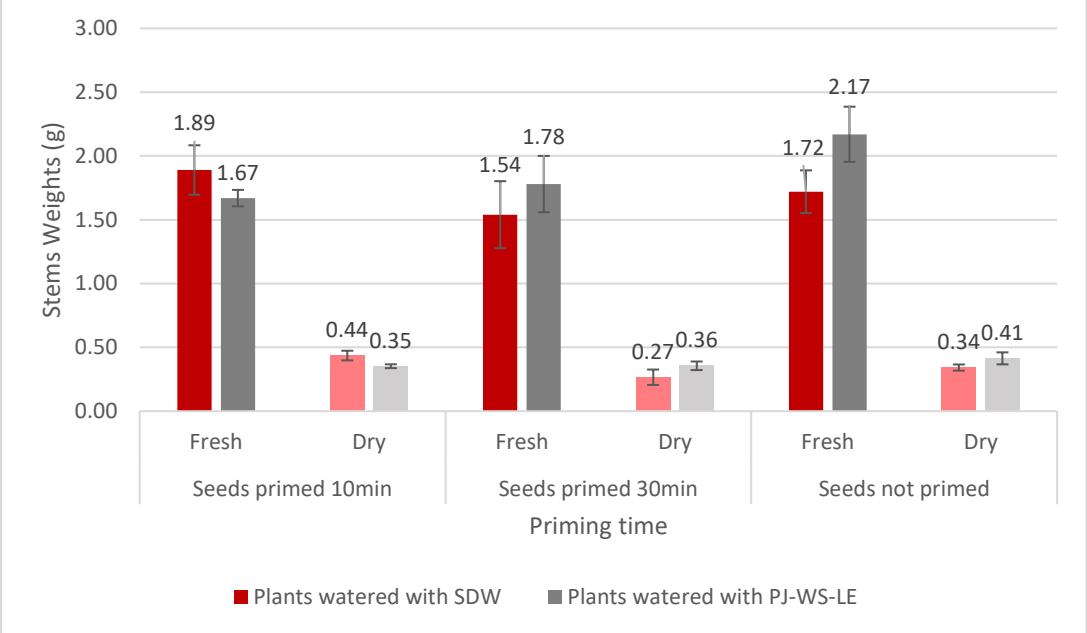


Figure 40. Average stems fresh and dry weights ±SE of chickpeas plants primed for different time slots and watered with different solutions. No significant differences among categories.

Averages fresh and dry weights of plants roots belonging to different categories varied in harmony with roots length showing the highest dry and fresh weights with plants primed for 10min (Figure 41). One-way ANOVA on plants watered with SDW showed that the average roots weight of the plants primed with PJ-WS-LE extract for 10 min is significantly higher than the other categories ($p \leq 0.05$). Overall, plants watered with SDW had significantly higher average dry roots weights than plants watered with the extract ($p = 0.019 \leq 0.05$).

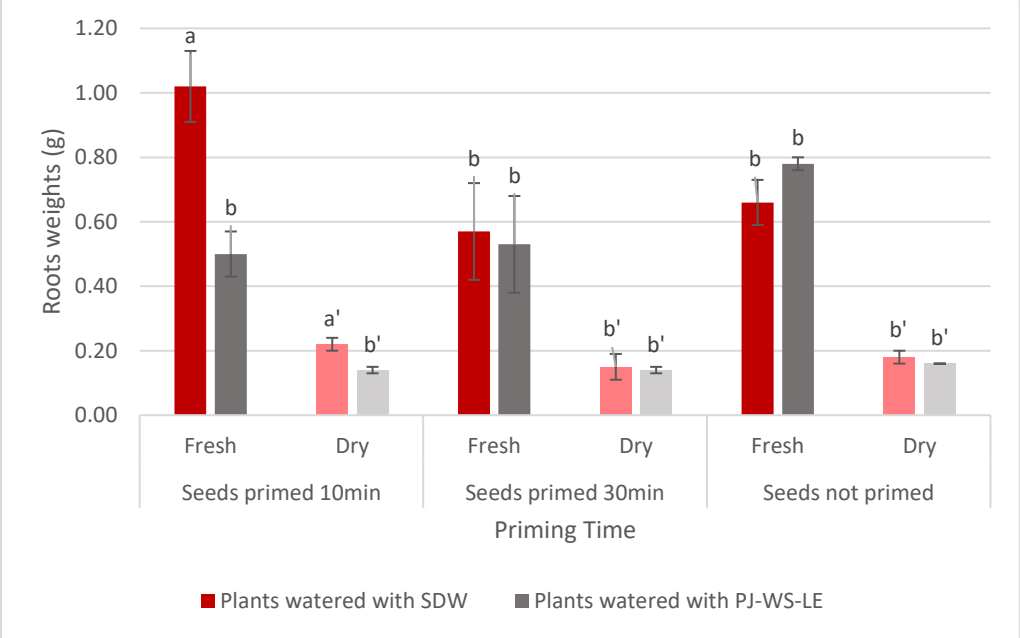


Figure 41. Average roots fresh and dry weights ±SE of chickpeas plants primed for different time slots and watered with different solutions.

aba'b' Treatment columns with different letters have their values significantly different as shown by the one-way ANOVA and Post-Hoc Tukey test at p=0.05.

Studies evaluating *P. juliflora* extracts in enhancing seeds germination and growth parameters are lacking. A previous study evaluating the possible allelopathic effect of *P. juliflora* fresh and dry leaves extracts on *Oryza sativa* (rice) seeds germination showed that the extracts in moderate amounts had a positive or neutral effect on seeds germination , however, higher amount can has a negative impact (Shaik & Mehar, 2015). These results were consistent with results of the present study on chickpeas, and they showed that *P. juliflora* extracts can be used for in field bio-pesticides purposes with no concern about negative impacts from allelopathic chemicals, which seems to be concentrated in the invasive plant roots to act as competition eradicator only when *P. juliflora* is growing.

4.3. EVALUATING THE GENETIC VARIATION OF *P. JULIFLORA* IN THE STATE OF QATAR (DOHA MUNICIPALITY) AND TESTING THE ANTIMICROBIAL EFFECTIVENESS OF CRUDE EXTRACTS PREPARED FROM SAMPLES TAKEN FROM DIFFERENT LOCATIONS

4.3.1. DNA fingerprinting results

All seven *P. juliflora* rDNA sequences were submitted to NCBI and got their accession numbers (Table 43). Upon blasting the sequences on NCBI to compare them with the database, they all showed similarity between 96% and 99% to the sequence representing *P. juliflora* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence with accession number JX139107.1. Alignments of the seven Qatari sequences with sequence number JX139107.1 are shown in appendix 12.

Table 43. PCR products blasting results.

Sample name	Sequence length (bp)	Accession number	Percentage of nucleotides identity (%) to sequence number JX139107.1
S1	932	OK184555	98.84
S2	877	OK184556	98.46
S3	532	OK184557	97.90
S4	535	OK184558	98.84
S5	533	OK184559	95.91
S6	732	OK184560	97.87

S7	723	OK184561	98.65

Sequence number JX139107.1 has originated from India. Knowing that *P. juliflora* is not a native species in Qatar, it is very likely that some of the Indian workers have brought the first seeds of the plant to the country for its resistance to similar climatic conditions and for its pharmaceutical importance among the India society (Henciya *et al.*, 2017). Neighbor joining tree showed very low variation among the *P. juliflora* isolates collected from different places in Doha (Figure 42). The sequences used to generate the tree are listed in appendix 12.

The low variation in the climate among the different places in Qatar does not allow genetic variation among the different isolates. Considering that all the trees in the country could originate from the same country (India) explains also the similarity among the sequences. A previous study on the diversity within and among the populations of *P. cineraria* and *P. juliflora* collected from different locations in Qatar was conducted on 12 samples, six of each species. Genetic variation was explored using Inter Simple Sequence Repeat (ISSR) and Random Amplified Polymorphic DNA (RAPD) markers. The analysis divided the twelve genotypes into two distinct clusters by species and showed genetic variations among samples of *P. juliflora*, however, the variation was inconsistent between the two methods used (Elmeer & Almalki, 2011). Collections of more samples and the usage of other genetic markers is needed to confirm genetic identity of *P. juliflora* in Qatar.

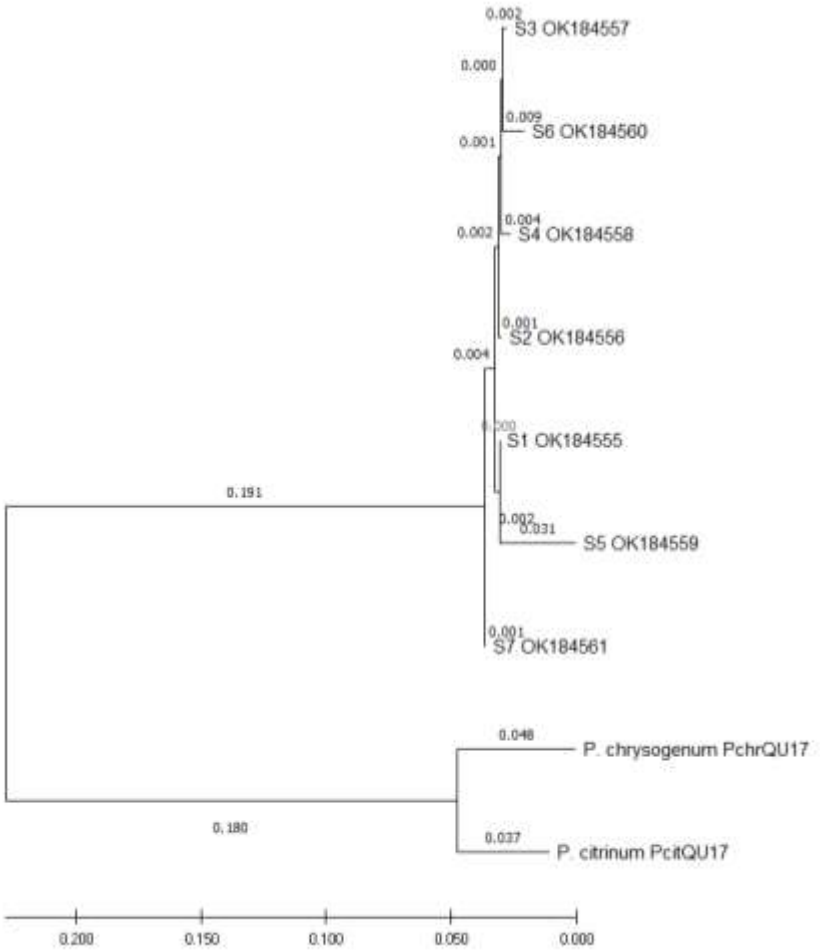


Figure 42. Phylogenetic analysis using the neighbor-joining method of *P. juliflora* based on the ITS1-5.8S-ITS2 sequences of 7 isolates collected from different locations in Doha-Qatar using *Penicillium* sequences (*P. chrysogenum* and *P. citrinum*) as an outgroup.

4.3.2. MICs evaluation of crude extract prepared using P. juliflora leaves samples from various locations against certain selected fungi, bacteria and yeast

The MICs of the extracts prepared using leaves of trees from different locations showed homogeneity in their results. The slight one raw variation shown in the case of *S. aureus* and *A. alternata* is due to the difficulty of preparing extracts of exactly the

same concentration at every run due to the difficulty in controlling how much of the extract is water-soluble. MICs of the extracts against *S. aureus* is shown to be between 0.65mg/ml and 0.32mg/ml which was consistent with our previous results (0.5mg/ml). Similarly, the MICs of the extract against *A. alternata* were within the range obtained previously, which is 1mg/ml. *C. albicans* showed MICs lower than 0.2mg/ml. Finally, *C. gloeosporioides* did not show, as previously described, total growth inhibition. However, the change in color in the 96-well plate experiment showed a dose dependent effectiveness in lowering fungal growth. All 96-well plate results are shown in appendix 13.

The results of MICs are consistent with the low genetic variation shown in the phylogeny analysis of *P. juliflora* in Qatar. The homogeneity in climate and in soil type around the collection areas and the age of the tree that is relatively new to the country, support the obtained results and imply that the types of active phytochemicals are similar in all trees. Therefore, if to be utilized, all *P. juliflora* trees in Qatar have the same antimicrobial effectiveness.

4.4. FRACTIONATION OF PJ-WS-LE EXTRACT AND TESTING THE ANTIMICROBIAL EFFECTIVENESS AND STABILITY OF THE VARIOUS FRACTIONS

4.4.1. FPLC fractionation results (run 1)

The separation chromatogram using run 1 parameters shows nice separation peaks. The groups of peaks were collected at 10min time interval and the various fractions are shown in red in figure 43. Fractions 2 to fraction 7 (1A2, 1A3, 1B1, 1B2, 1B3, and 2A1) were collected in a separate tube, dried, re-eluted in sterile distilled water and tested for their antimicrobial effectiveness using the 96-well plate method.

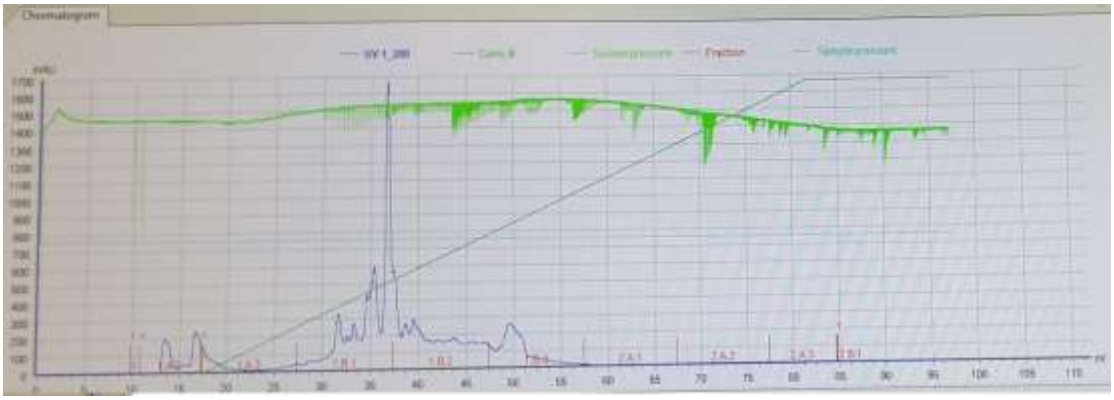


Figure 43. FPLC fractionation chromatogram of PJ-WS-LE extract run1.

4.4.2. Antimicrobial effectiveness of FPLC run 1 fractions

The six chosen fractions showed the same results against chosen bacteria (*S. aureus*), yeast (*C. albicans*) and fungi (*A. alternata*) with the strongest efficacy shown with fraction 6 (1B3). Fraction 6 showed efficacy close to the crude extract which imply that the active antimicrobial phytochemical(s) is/are in this fraction (Appendix 14).

4.4.3. FPLC fractionation results (run 2)

To further fractionate fraction 6 a less steep gradient was chosen in run 2. However, the chromatogram showed some sharper peaks for some of the inactive fractions but not of fraction 6, which got only wider as it can be seen in fraction B8 to C7 in figure 44.

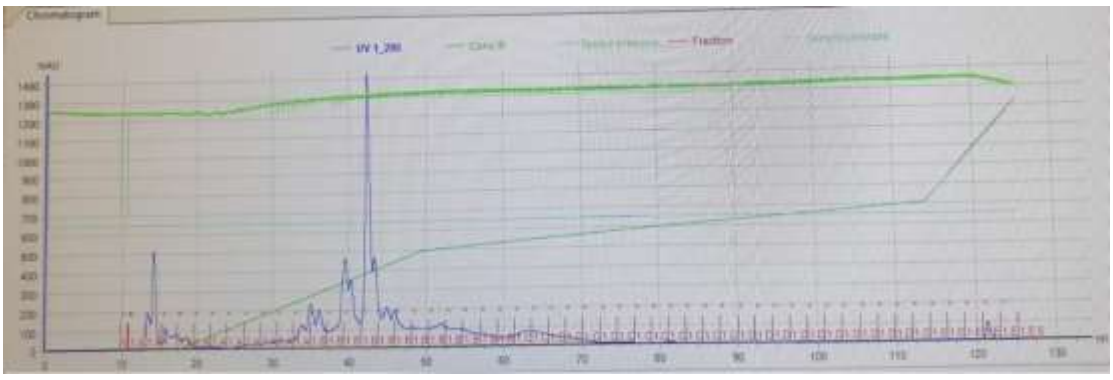


Figure 44. FPLC fractionation chromatogram of PJ-WS-LE extract run2.

4.4.4. Antimicrobial effectiveness of FPLC run 2 fractions

Fractions B8, B9, B10, B11, B12, C1, C2, C3, C4, C5, C6 and C7 were tested for their antimicrobial effectiveness against *C. albicans* using the 96-well plate method. As we approach from the previously indicated fraction 6 in run 1 antimicrobial efficacy started appearing (B9). Antimicrobial effectiveness got stronger as we approach the middle of the wide peak obtained in run 2, to reach the highest effectiveness in fractions C1, C2 and C3 (Appendix 14). Results indicated that fraction 6 is either made of one category of phytochemicals. If fraction 6 is made of a variety of phytochemicals that function in synergy, those phytochemicals would have chemical structures that allow them to elute together during fractionation.

4.4.5. Thermostability FPLC fraction 6 of the first run

Fraction 6 and the crude extract were exposed to high temperatures ranging from 50°C to 80°C. Fractions were then re-tested for their effectiveness against *C. albicans* and they all showed regular effectiveness. Results of the 96-well plate method of fraction 6 exposed to 70°C and 80°C against *C. albicans* are shown in appendix 14.

Active phytochemicals have previously been demonstrated as stable with time up to 6 months. This experiment also showed the thermostability of the active antimicrobial agents which is very important for antimicrobial agents applied in the food industry.

Previous studies showing *P. juliflora* antimicrobial activity were preliminary, and either described the effect of crude extracts on specific microorganisms or focused on alkaloids enriched extracts (Sharifi-Rad *et al.*, 2019). Water-soluble leaves ethanolic extract has not been described in literature and our fractionation was the first of its kind on leaves ethanolic extracts.

CHAPTER 5: CONCLUSIONS AND PERSPECTIVES

The outcomes of the present study indicate that PJ-WS-LE extract is a wide spectrum bio-controller that can be further investigated for different applications including: antibacterial drug against multidrug resistant bacteria, anti-yeast solutions and anti-fungal agricultural solution for in field applications and as storage coating material. *In-vitro* analysis showed solid results on the efficacy of PJ-WS-LE extract against a wide range of pathogenic microorganisms. The novel extraction method and the selection for a water-soluble end-product makes future applications easier. Coming from an invasive tree that is widely available give the extract value as an inexpensive solution for the countries that are most in need for fresh produce spoilage control. Time stability was evaluated and the extract maintained its antimicrobial effectiveness up to the six months of the trial, these results are very important for future commercialization of the extract. Scanning electron microscopy results showed that fungi that were not totally killed by the extracts had their hyphae and spores affected which implies a slower growth and lower ability to induce diseases. The *in-vitro* results were supported by the *in-vivo* results. Artificially inoculated tomato and mango samples were completely protected from the disease caused by fungi that were totally inhibited by the extract in the laboratory assays, this include *B. cineraria* and *A. alternata*. While the *C. gloeosporioides* fungal strain that was not totally inhibited *in-vitro* had their disease severity decreased in mangoes.

Strawberries coated with 8mg/ml PJ-WS-LE extract individually and strawberries coated with 8mg/ml PJ-WS-LE extract embedded in 1% chitosan maintained liked sensory characteristics during the three weeks storage period at 4°C. Both treatment batches have shown also significantly lower: weight loss percentage, total aerobic bacterial count, total yeast count and total mold count. The two batches treated with

8mg/ml PJ-WS-LE maintained somehow their firmness and their total soluble solids levels during the storage period. Strawberry samples coated with 8mg/ml PJ-WS-LE extract embedded in 1% chitosan showed the lowest increase in DPPH radical scavenging activity. PJ-WS-LE extract has beneficial effects in reducing the ripening-related symptoms and increases the storage shelf-life of strawberries at both room temperature and at 4°C, noting that shelf-life of strawberry samples stored at 4°C increased from 4.3 days average control shelf-life to 10 days with samples sprayed with the extract. The antimicrobial effectiveness of PJ-WS-LE extract against the main strawberries spoiling agents opens also doors for the application of this natural product on strawberries in field. Future studies should include field experiments.

PJ-WS-LE extract embedded in edible coating has also been demonstrated as an effective coating material to maintain mangoes quality at low temperature for up to five weeks of storage. On the other hand, spraying cucumber samples with 8mg/ml of PJ-WS-LE extract extended cucumbers shelf-life at 22°C by 77% and maintained samples acceptable quality for three weeks of storage at 8°C. Cucumber samples sprayed with the extract and stored at 8°C showed 32% less weight loss and 57% higher respiration rate than the un-treated control samples within the first week of storage. PJ-WS-LE extract in cucumber coating material served as an antimicrobial barrier that kills surrounding aerobic bacteria, mold spores and yeast, this was proven by the lower total CFU of treated samples throughout the experiment period. Future studies on PJ-WS-LE extract will involve more fruits and vegetable, and more pathogenic spoiling agents for better understanding of all possible applications of this natural bio-controller in agriculture.

PJ-WS-LE extract did show values as priming material of chickpeas seed, yet the experiment showed that there are no allelopathic phytochemicals in the extract that might slowed down chickpeas germination and growth.

Phylogeny tree showed low genetic variation of *P. juliflora* trees in the state of Qatar. Extracts prepared from leaves collected from various locations showed homogeneity in antimicrobial activity, these results are important for Qatar and indicate that all the trees have the same efficacy and they can be used to produce the biological control agent.

Fractionation work has led to a single biologically active fraction, which brings the work one step closer to the identification of the active phytochemicals. Thermostability of the active fractions was demonstrated until 80°C, which give an additive value of the extract in the food control domain.

In future work, PJ-WS-LE extract cytotoxicity should be evaluated against different cell lines including normal prostate (PNT2) cell line, fibroblast BHNF-1 cells and human neuroblastoma SH-SY5Y cells for neurotoxicity, MTT assay can be used. Animal toxicity should also be evaluated to rule out any possible toxicity. For a better understanding of the role of this natural bio-controller, antimicrobial effectiveness can be evaluated against any microbe of interest. PJ-WS-LE extract effectiveness in maintaining fruits and vegetables storage parameters can also be investigated on a wider range of crops.

Future work should include chemical identification of the active phytochemicals and further fractionation if needed. The final structure of the active phytochemical can be determined using Nuclear Magnetic Resonance (NMR). The re-evaluation of active phytochemicals individual toxicity will add also value to the end-product and would make future commercialization more approachable.

List of abbreviations

DPPH	2,2-diphenyl-1-picrylhydrazyl
ACN	Acetonitrile
ArcGIS	Aeronautical reconnaissance coverage geographical information system
AFs	Aflatoxins
CTS+AVG	Aloe vera gel embedded in 1% chitosan
A. alternata	*Alternaria alternata*
AMX	Amoxicillin
AMP	Ampicillin
ANOVA	Analysis of Variance
A. aculeatus	*Aspergillus aculeatus*
A. alliaceus	*Aspergillus alliaceus*
A. flavus	*Aspergillus flavus*
A. niger	Aspergillus niger
A. parasiticus	*Aspergillus parasiticus*
A. sclerotiorum	*Aspergillus sclerotiorum*
A. terreus	*Aspergillus terreus*
B. Subtilis	*Bacillus Subtilis*
B	Bacitracin
BSG	Bail seed gum
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BC	Before Christ
BCE	Before the Common Era

B. cinerea	*Botrytis cinerea*
C. albican	*Candida albican*
CB	Carbenicillin
CO2	Carbon dioxide
CR	Cephalothin
CTN	Citrinin
C. cladosporioides	*Cladosporium cladosporioides*
C. gloeosporioides	*Colletotrichum gloeosporioides*
C. karstii	*Colletotrichum karstii*
CFU	Colony forming unit
CFU/g	Colony forming unit per gram
C	Concentration
°C	Degree Celsius
DNA	Deoxyribonucleic acid
dc	Diameter control
dt	Diameter treated
DMSO	Dimethyl sulfoxide
DI	Disease incidence
DS	Disease severity
DMEM	Dulbecco's Modified Eagle Medium
E	East
EE	Echinacea extract
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
EU	European Union

EE	Extract efficacy
FOSC	*F. oxysporum* species complex
FPLC	Fast protein liquid chromatography
FBS	Fetal bovine serum
FAO	Food and Agriculture Organization
F. oxysporum	*Fusarium oxysporum*
G. candidum	*Geotrichum candidum*
Gc	Germination rate in the control plate
Gt	Germination rate in the treated plate
GAP	Good agricultural practices
g	Gram
g/L	Gram per liter
GSE	Grape seed extract
HACCP	Hazard analysis critical control point
HIV	Human immunodeficiency virus
HCl	Hydrochloric acid
ICFRE	Indian council of Forestry Research and Education
ISSR	Inter Simple Sequence Repeat
I	Intermediate resistant
ITS	Internal transcribed spacer
IU	International unit
Kcal	Kilocalorie
LD50	Lethal dose 50
MgCl2	Magnesium chloride
MRLs	Maximum residue limits

CH_4	Methane
MRSA	Methicillin-resistant *Staphylococcus aureus*
µM	Micro molar
µg	Microgram
µg/kg	Microgram per kilogram
µl	Microliter
µm	Micrometer
mg	Milligram
mg/mL	Milligram per milliliter
ml	Milliliter
mm	Millimeter
mM	Millimolar
MM	Millions
MIC	Minimum inhibitory concentration
min	Minutes
M	Molar
ng/Kg,	Nano gram per kilogram
NCBI	National Center for Biotechnology Information
NJ	Neighbor-joining
N	Newton
N	North
Nt	Number of spores in the treated plate
Nc	Number of spores on the control plate
NA	Nutrient agar
OTA	Ochratoxin

OCPs	Organochlorine pesticides
O2	Oxygen
PME	Pectin methylesterase
P. chrysogenum	*Penicillium chrysogenum*
P. citrinum	*Penicillium citrinum*
P. italicum	*Penicillium italicum*
L^{-1}	Per liter
mL^{-1}	Per milligram
%	Percent
PIMG	Percentage inhibition of mycelial growth
PCA	Phenazine-1-carboxylic acid
PBS	Phosphate-buffered saline
PG	Polygalacturonases
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
P. chilensis	*Prosopis chilensis*
P. cineraria	*Prosopis cineraria*
P. farcta	*Prosopis farcta*
P. glandulosa	*Prosopis glandulosa*
P. juliflora	*Prosopis juliflora*
PJ-WS-LE	*Prosopis juliflora* water soluble leaf ethanolic
P. pallida	*Prosopis pallida*
P. velutina	*Prosopis velutina*
P. mirabilis	*Proteus mirabilis*

P. aeruginosa	*Pseudomonas aeruginosa*
QAR	Qatari rial
RSA	Radical scavenging activity
RAPD	Random Amplified Polymorphic DNA
ROS	Reactive oxygen species
R	Resistant
RPC	Reverse Phased Chromatography
rpm	Revolutions per minute
RNA	Ribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
SS	Salep solution
SEM	Scanning electron microscope
S	Second
AgNO3	Silver nitrate
NAOH	Sodium hydroxide
spp.	Species
sp.	*Species*
SD	Standard deviation
SE	Standard error
S. aureus	*Staphylococcus aureus*
SPSS	Statistical Package for the Social Sciences
SDW	Sterile distilled water
S	Susceptible
xg	Times gravity
TSS	Total soluble solids

TBE	Tris/Borate/EDTA buffer
UV	Ultra violet
USA	United states of America
VOCs	Volatile organic compounds
V	Voltage
w/*v*	Weight per Volume

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ACCOMPLISHMENTS

Conferences

USA- 4th Edition of Nutrition &Food Science- USA- Nov 2021

- Novel Prosopis juliflora leaf ethanolic extract as natural antimicrobial agent against food spoiling microorganisms.

Qatar University- International Sustainability Workshop- Qatar- Oct 2021

- Novel Prosopis juliflora water-soluble leaf ethanolic extract as postharvest diseases bio-controlled: *in-vitro* results and direct applications on fruits.

Qatar's 8th International Agricultural Exhibition - Qatar-March 2021

- Application of Plant Extracts to Extend Shelf-Life Storage of Strawberries.

Publications-Research articles

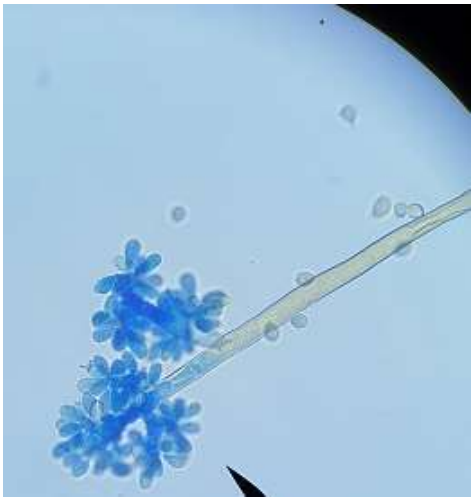
1. Saleh, I., and Abu-Dieyeh, M. (2020). Halophytes as Important Sources of Antioxidants and Anti-Cholinesterase Compounds. In M.-N. Grigore (Ed.), *Handbook of Halophytes: From Molecules to Ecosystems towards Biosaline Agriculture* (pp. 1-22)
2. Saleh, I., and Abu-Dieyeh, M. (2021). Novel Prosopis juliflora leaf ethanolic extract as natural antimicrobial agent against food spoiling microorganisms. *Scientific Reports*. 11(1).
3. Saleh, I., and Abu-Dieyeh, M. (2022). Novel Prosopis juliflora leaf ethanolic extract coating for extending postharvest shelf-life of strawberries. *Food Control*. 133.
4. Saleh, I., and Abu-Dieyeh, M. (2022). Evaluation of novel *Prosopis juliflora* water soluble leaf ethanolic extract as preservation coating material of cucumber. *Journal of Food Processing and Preservation*. In press.

Poster

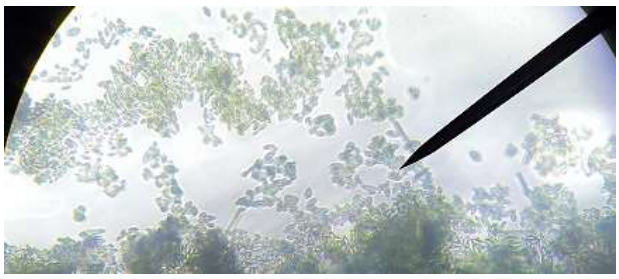
Saleh I., & Abu-Dieyeh. Novel *Prosopis Juliflora* Leaf Ethanolic extract as natural Antifungal agent against Botrytis Cinerea: Application on Strawberries' shelf-life extension. Qatar University Annual Research Forum & Exhibition. Oct 2021. doi: 10.29117/quarfe.2021.0044.

Appendices

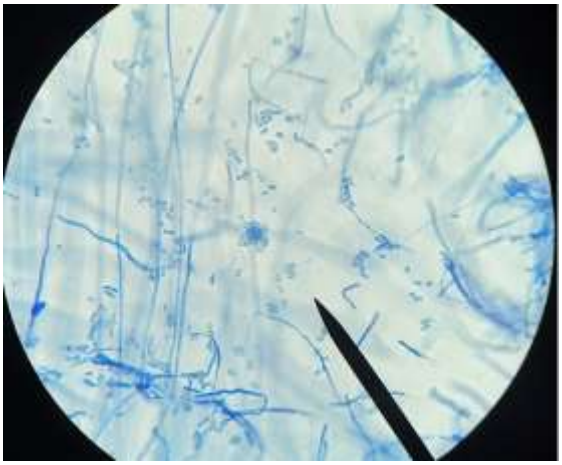
Appendix 1. Microscopic identification of the nine fungal strains isolated from different fruits and vegetable collected from the Qatari market and used in this study.



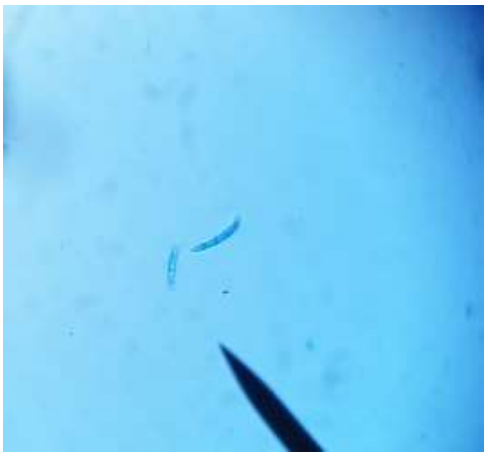
1. *Botrytis sp.*



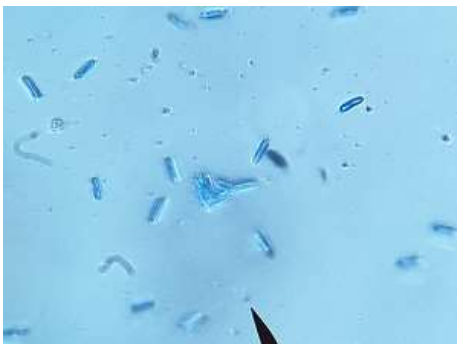
2. Cladosporium sp.



3. *Geotrichum sp.*

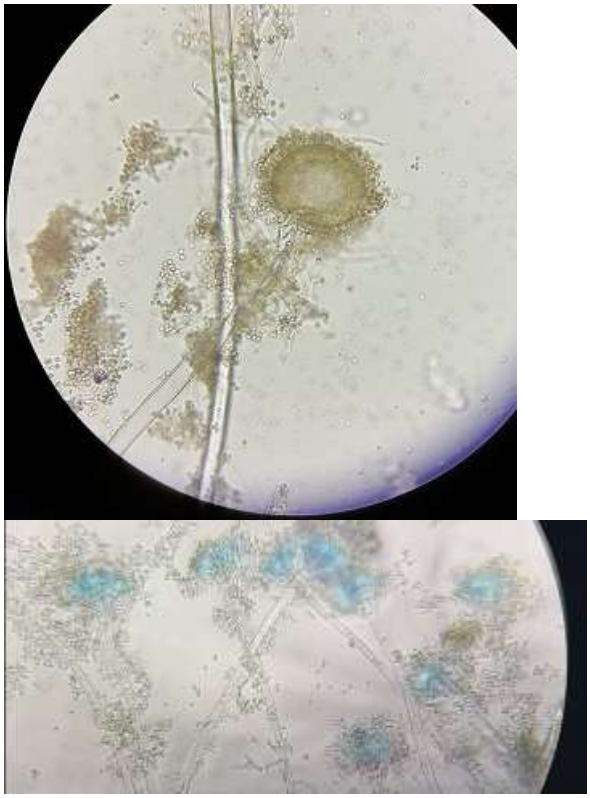


4. Fusarium sp.

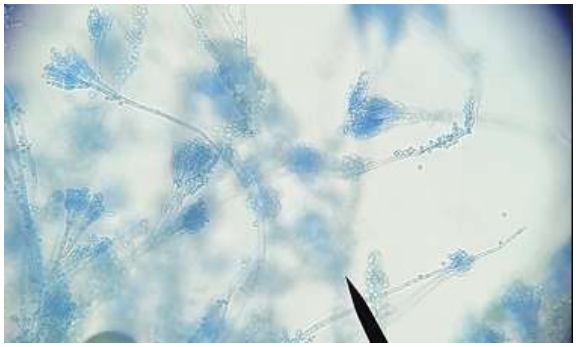


5. *Colletotrichum sp.*

6. Alternaria sp.



7. Aspergillus sp.

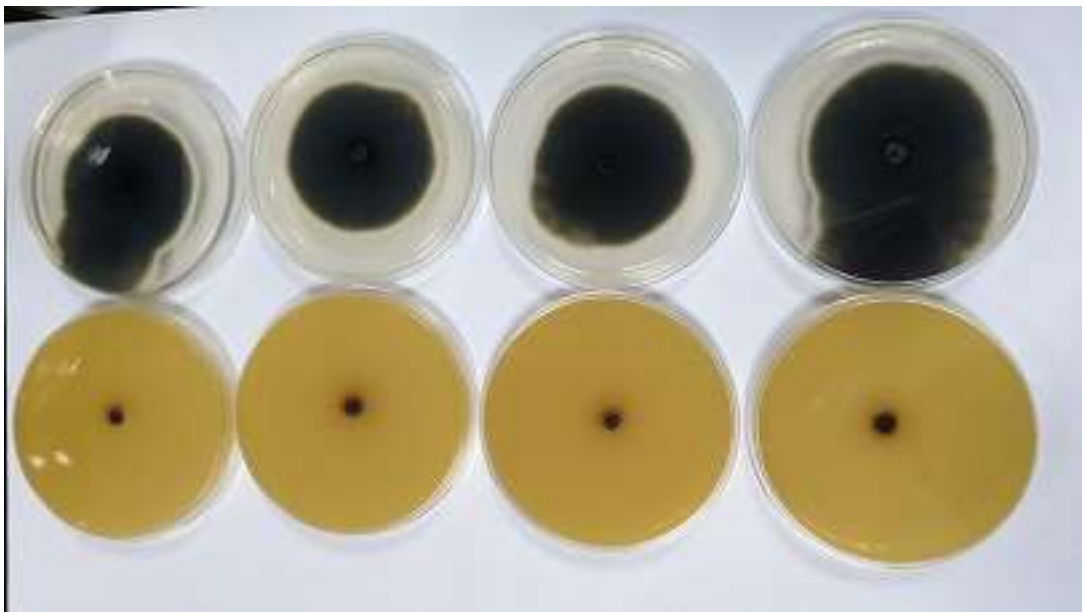


8. Penicillium sp. 1

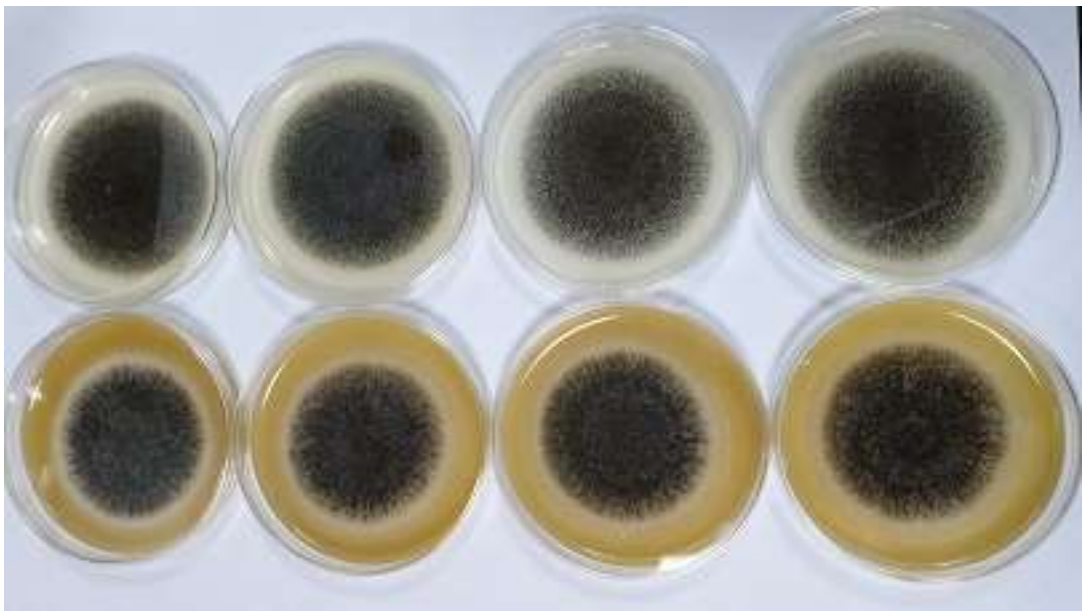
9. Penicillium sp. 2

Appendix 2. Disk antifungal assays results.

A. Four replicates results: experimental plates with 20mg/ml PJ-WS-LE extract (lower plates) vs. control PDA plates (upper plates)- 5 days old plates.

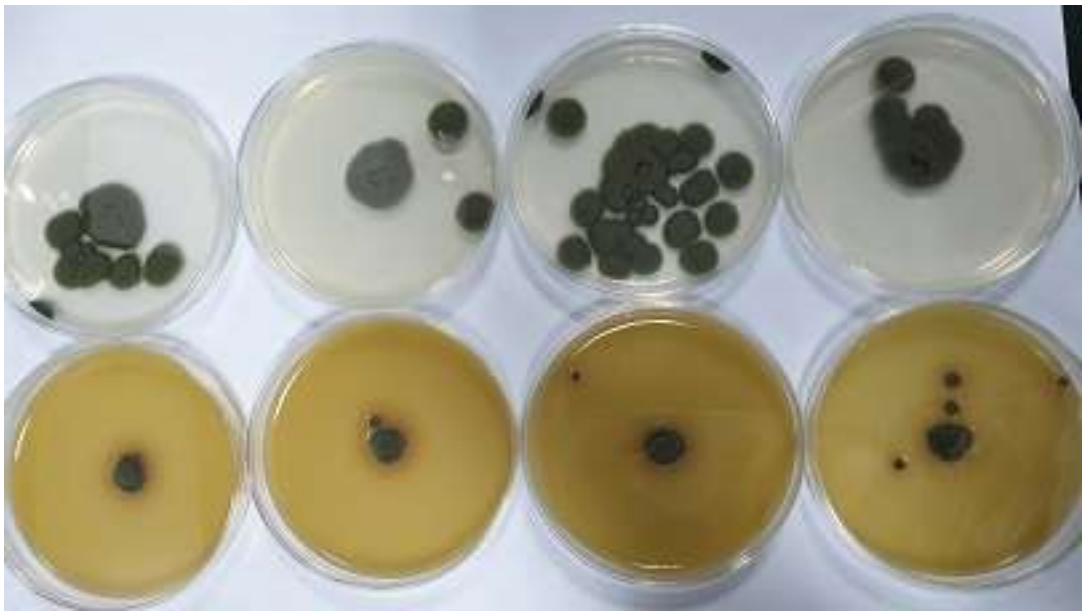


A. alternate

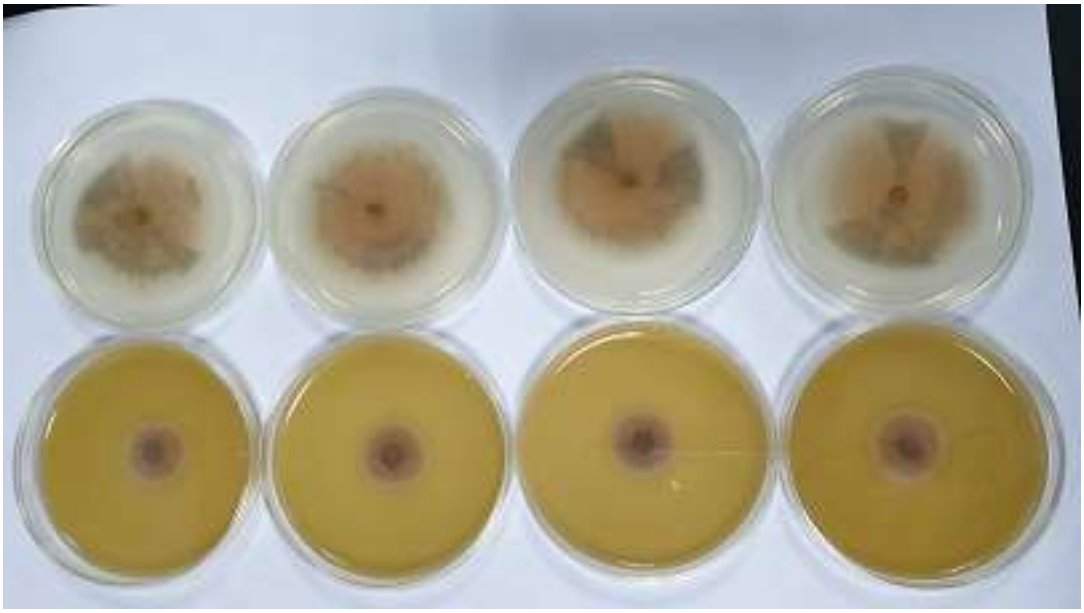


A. niger

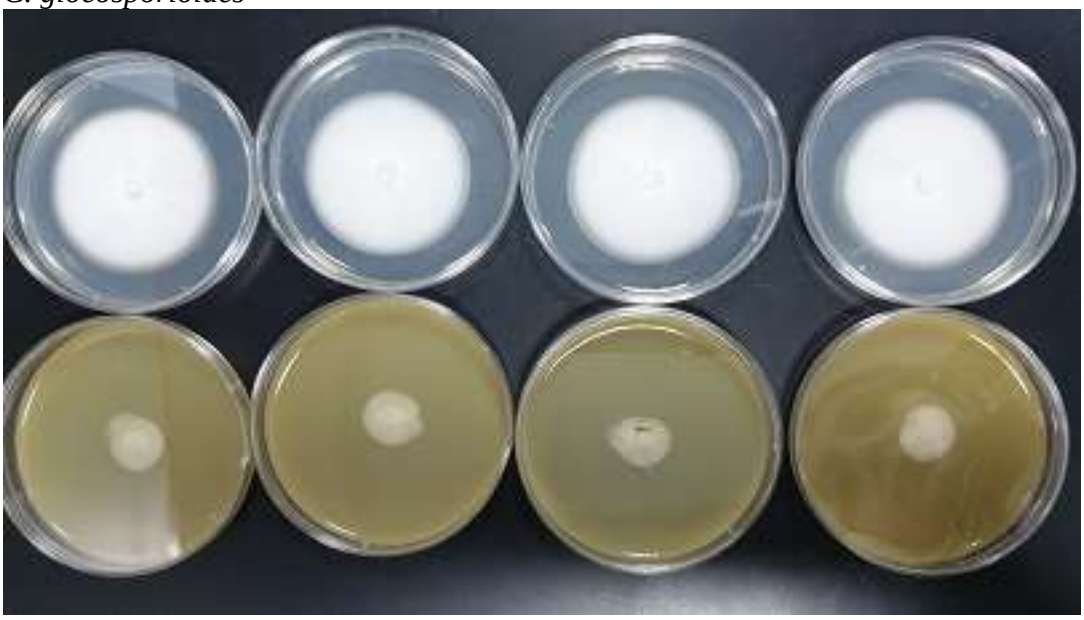
B. cinerea



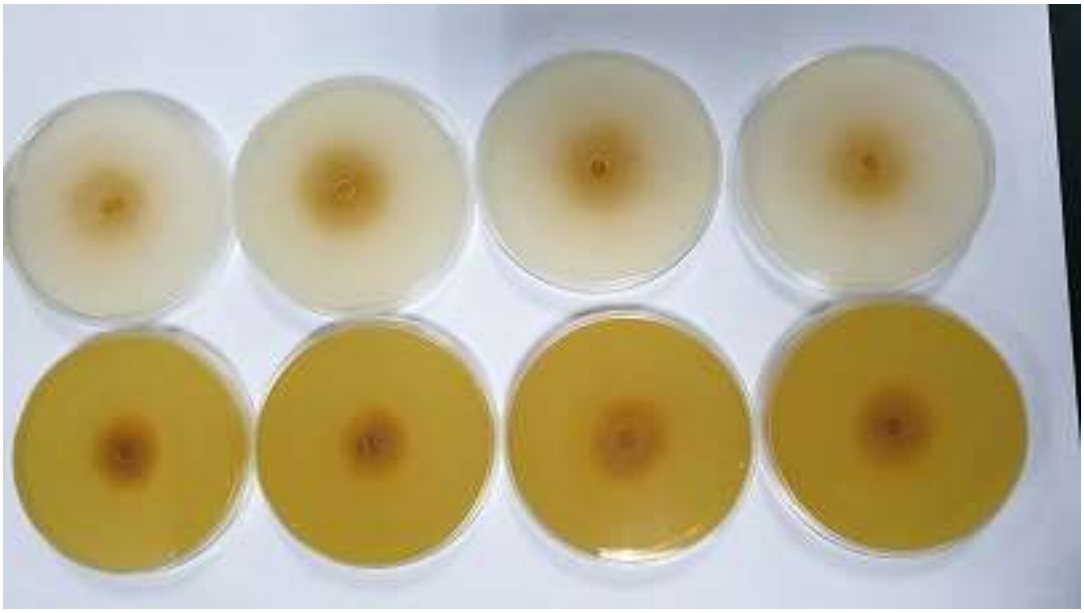
C. cladosporioides



C. gloeosporioides

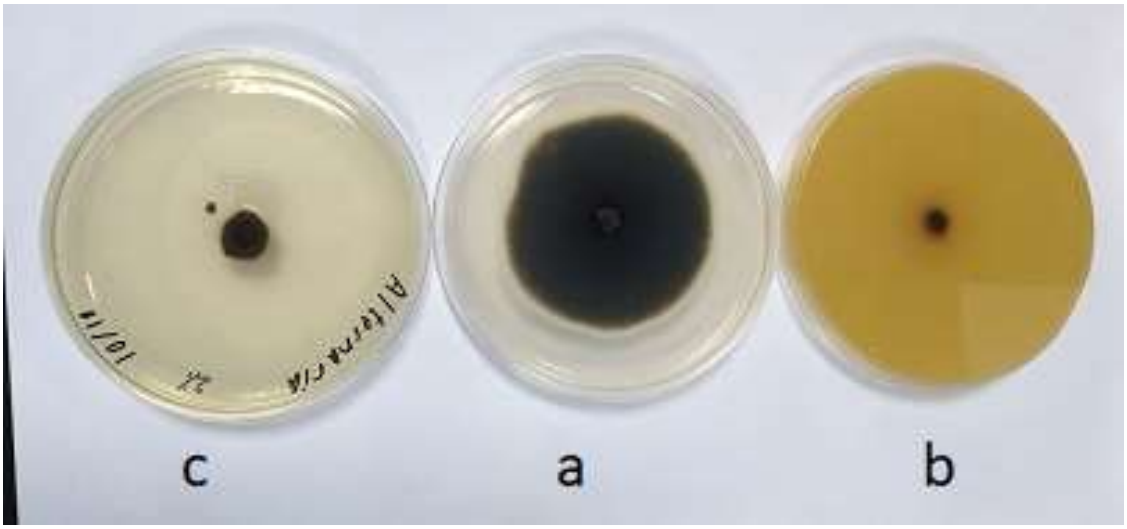


G. candidum

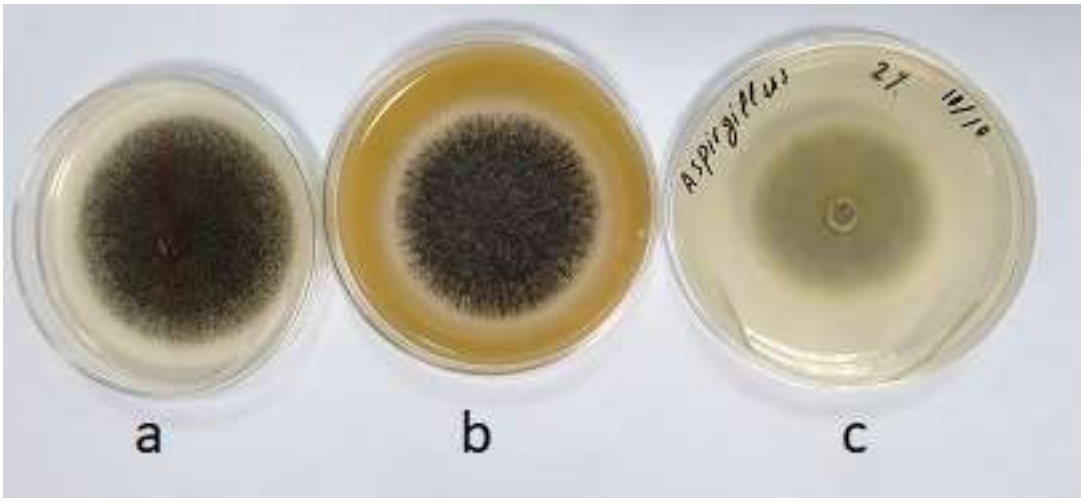


F. oxysporum

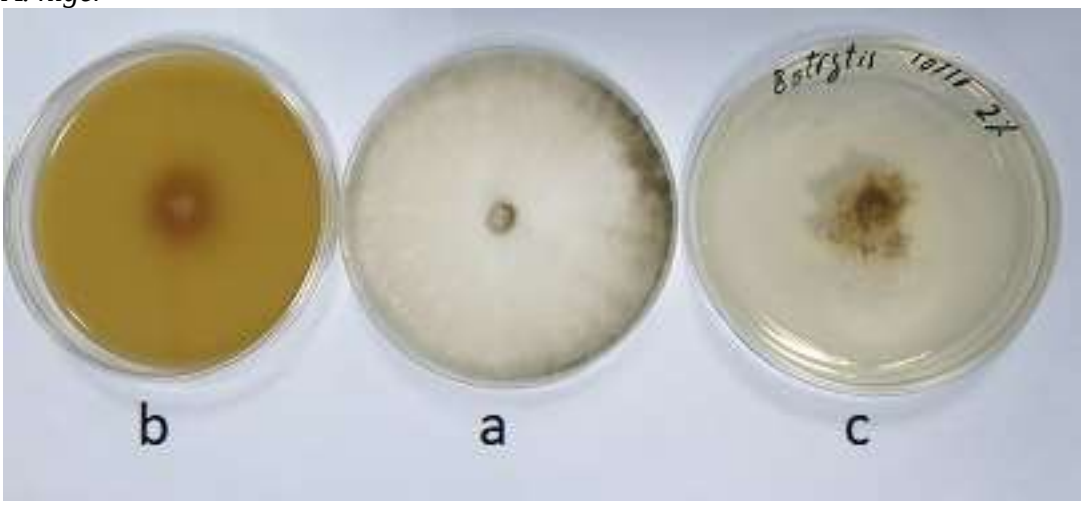
B. Comparative results of the antifungal disk method: control clean PDA plates (a) experimental plates 1 with 20mg/ml PJ-WS-LE extract (b), and experimental plates 2 with 2mg/ml PJ-WS-LE extract, labeled plates (c)- 5 days old plates



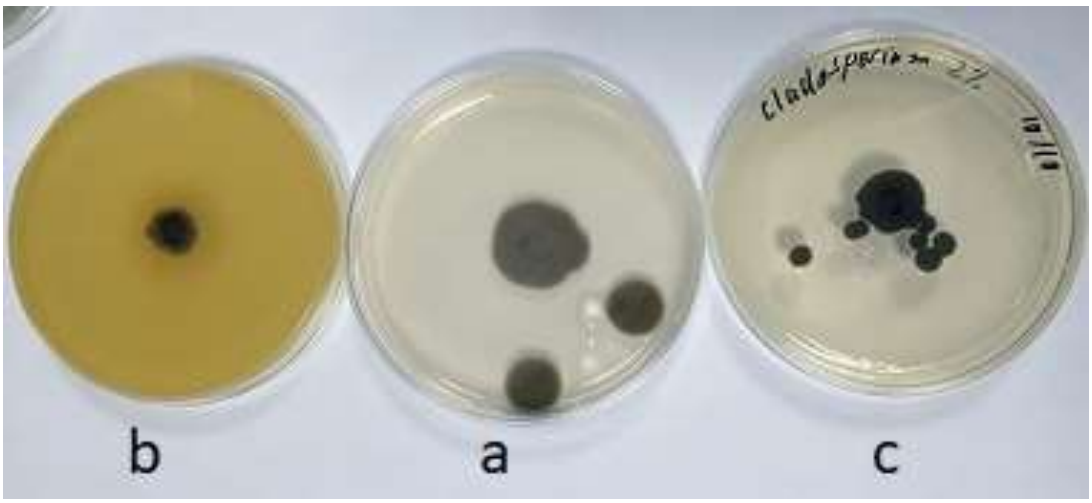
A. alternata



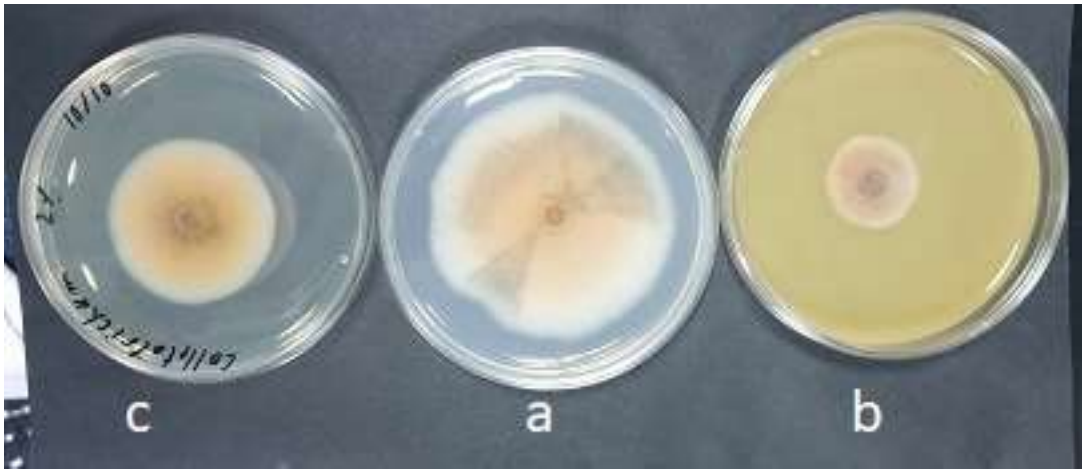
A. niger



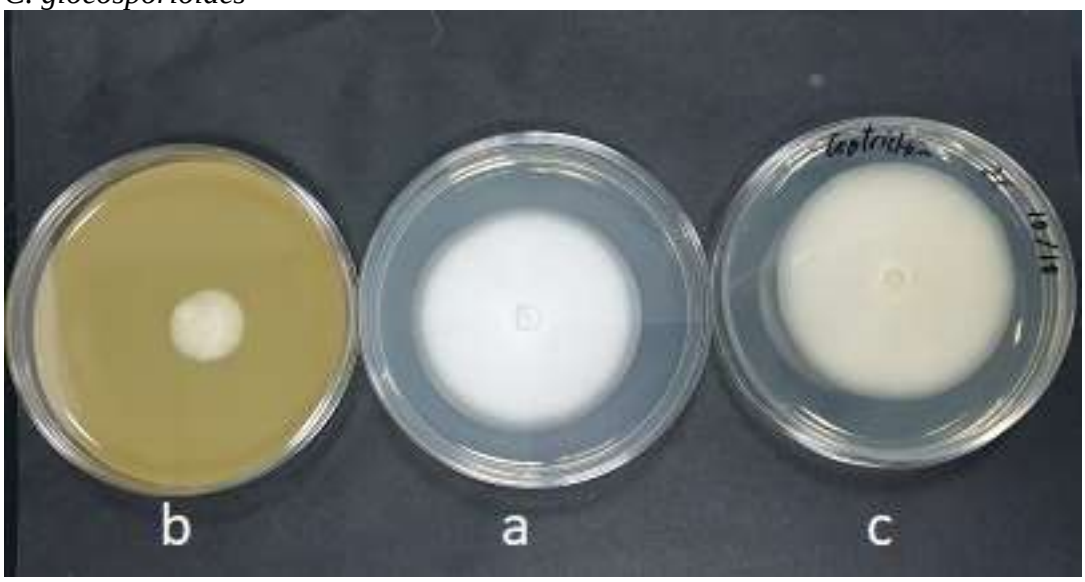
B. cinerea



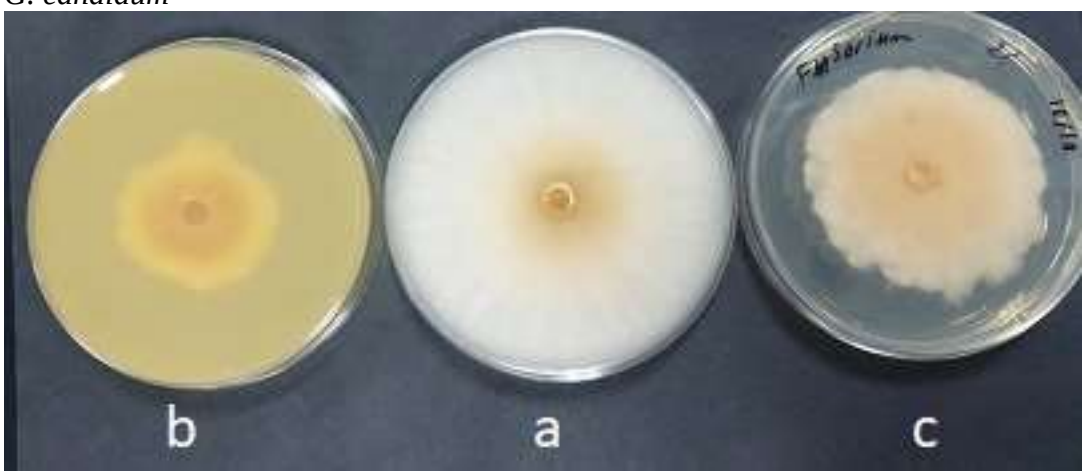
C. cladosporioides



C. gloeosporioides



G. candidum



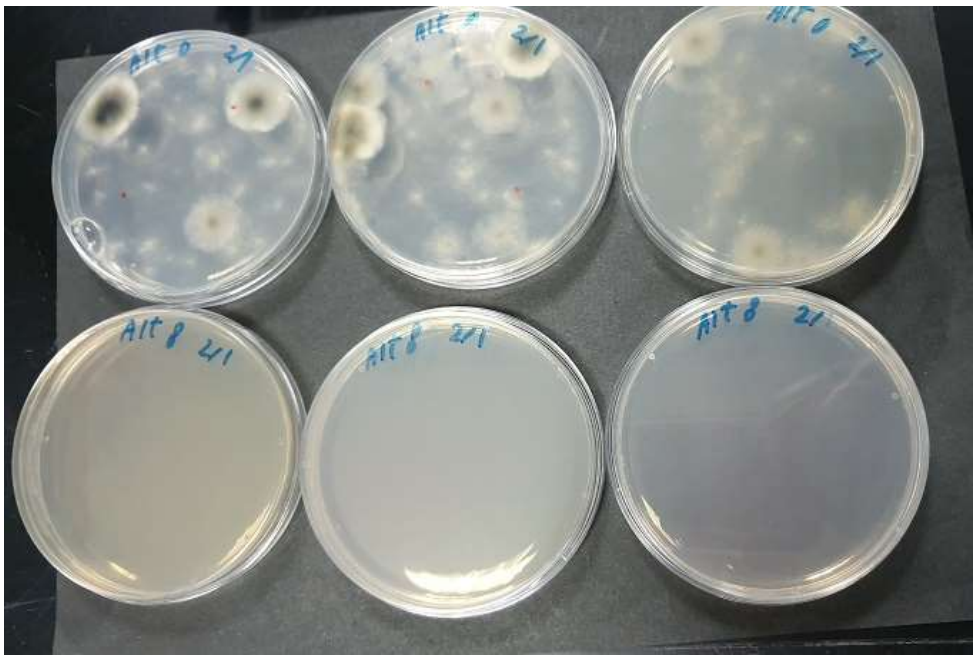
F. oxysporum

C. Pour plate method: treatment plate with 20mg/ml PJ-WS-LE extract(left-side plate) vs. control PDA plate (right-side)-72h old plates

P. citrinum

P. chrysogenum

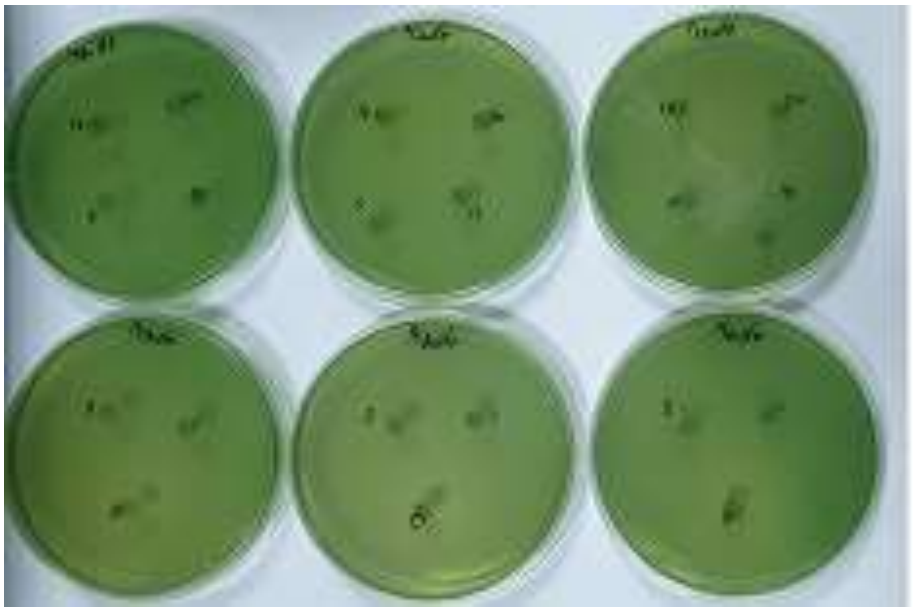
D. Results of growth of spreading fungal spores exposed to 8mg/ml PJ-WS-LE extract for 48h (down-side plates) vs. normal growth of spores exposed to sterile distilled water (up-side plates)- 72h old plates



A. alternate

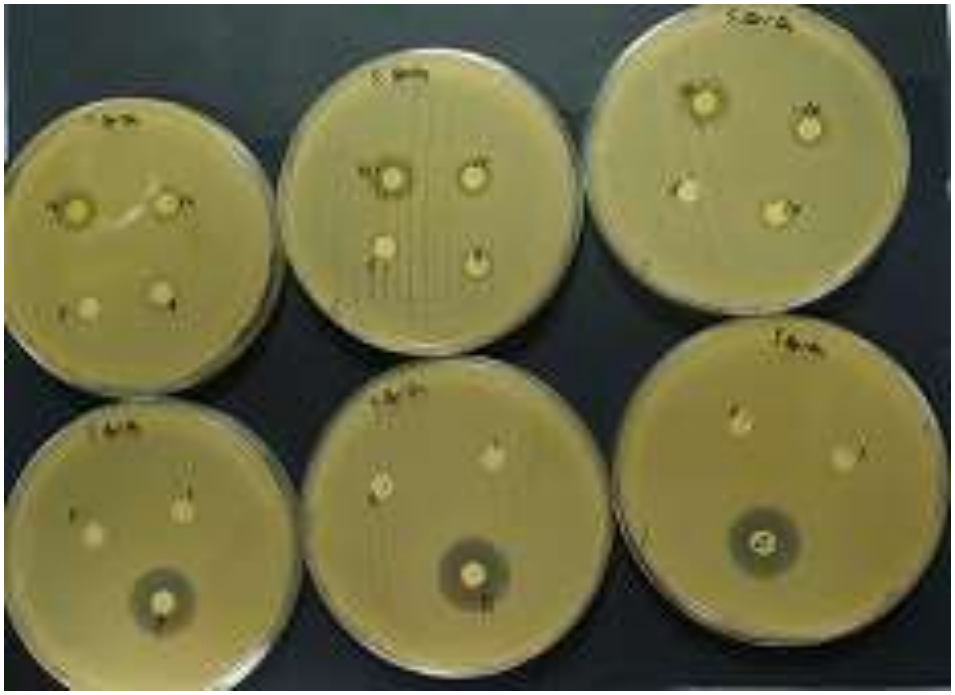
Appendix 3. Disk diffusion method results (all triplicate trials).

A. PJ-WS-LE extract was tested at different concentrations (50, 20, 20, 5, and 1mg/ml). Zero stands for 0mg/ml which is the negative control (sterile distilled water) and D stands for 70% Dettol which is a positive control. Every three plates are replication of the same experiment. Upper plate disks labeling are: 50mg/ml (upper disk to the left), 20mg/ml (upper disk to the right), 10mg/ml (lower disk to the right), 5mg/ml (lower disk to the left). Lower plates disks labeling are: 1mg/ml (upper disk to the right), 0mg/ml (upper disk to the left), Dettol (lower disk).

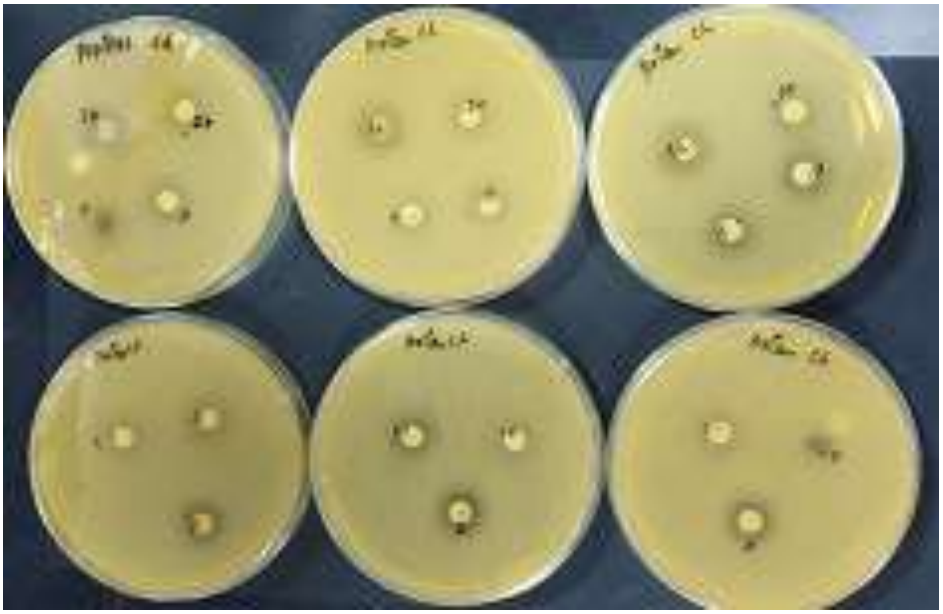


P. aeruginosa

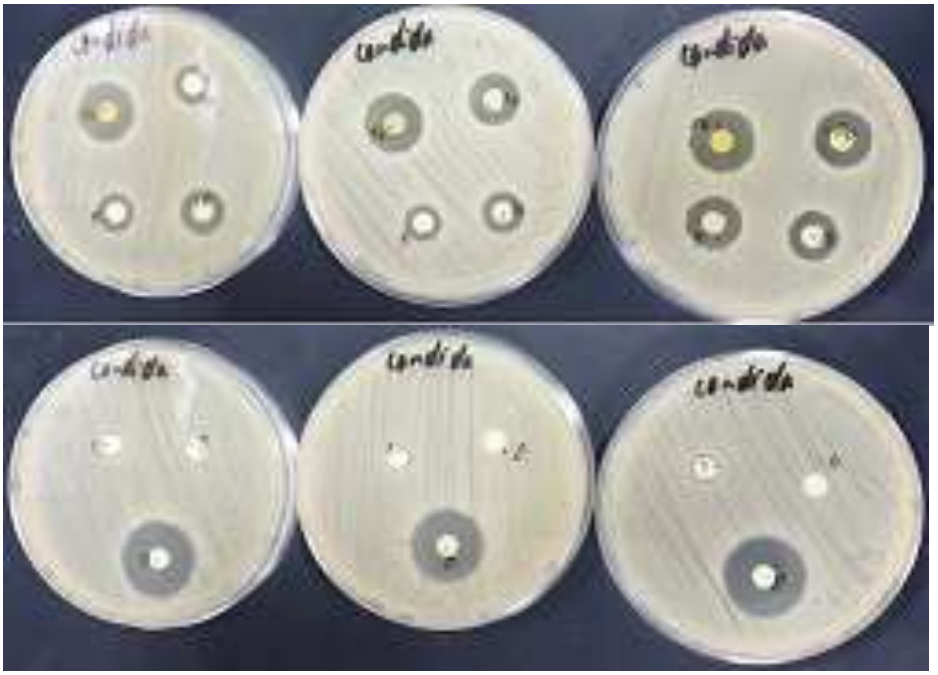
B. subtilis



S. aureus

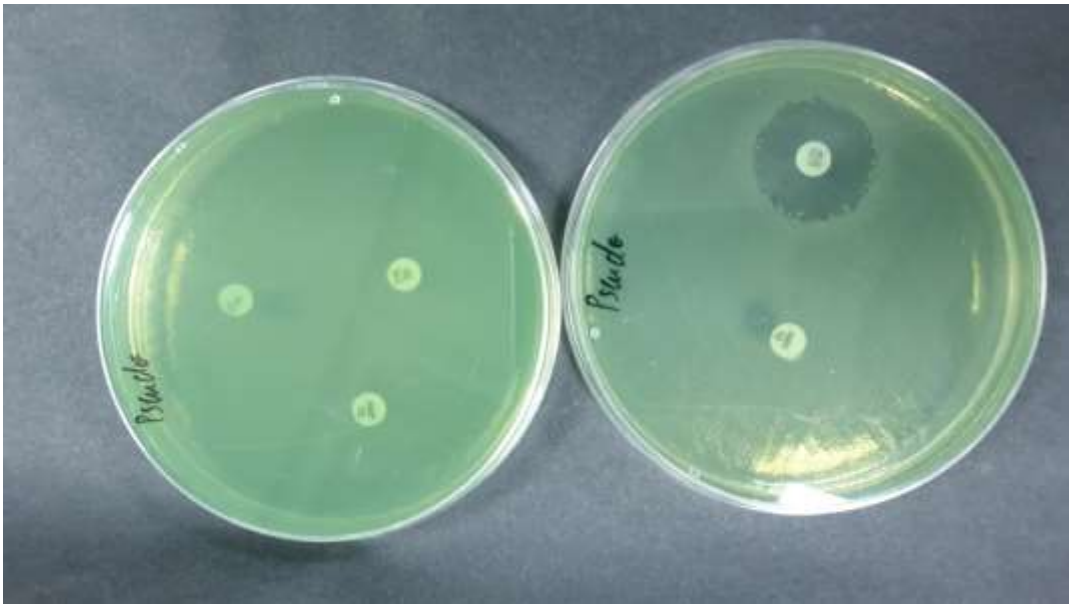


P. mirabilis

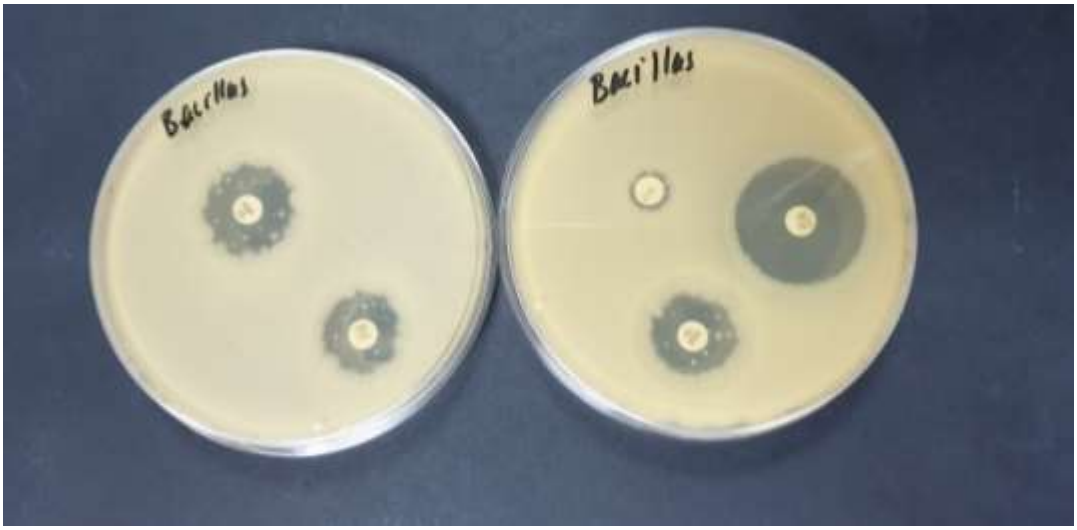


C. albicans

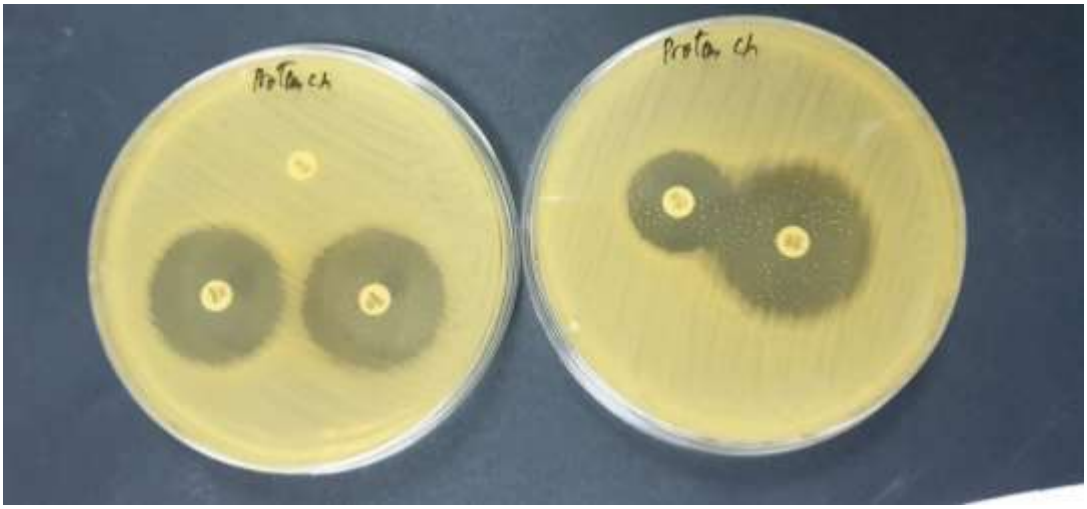
B. Antibiogram of the tested bacterial strains. (Ampicillin (AMP), Amoxicillin (AMX), Bacitracin (B), Carbenicillin (CB), and Cephalothin (CR)).



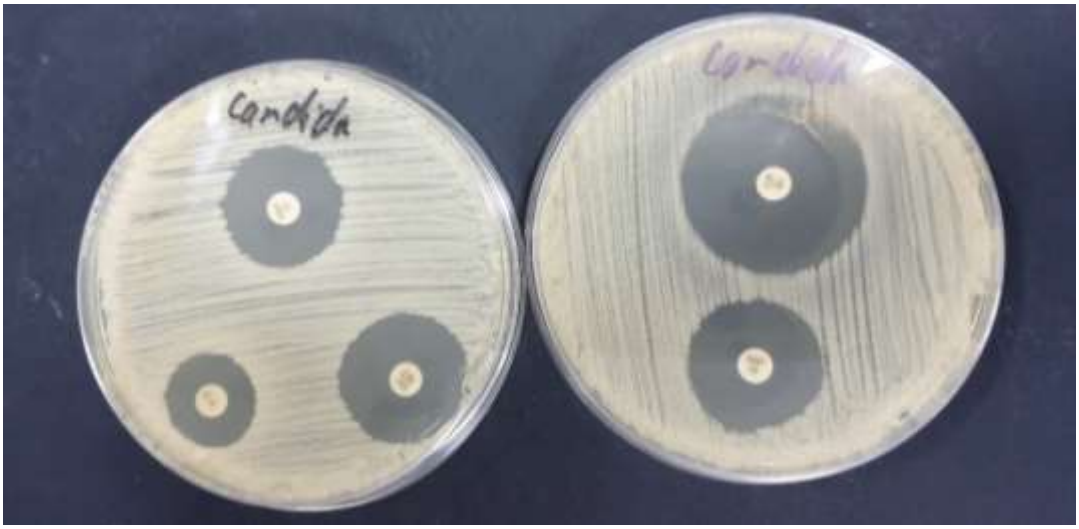
P. aeruginosa



B. subtilis



P. mirabilis



C. albicans

C. Microbial growth after 24h exposure to 8mg/ml PJ-WS-LE extract (left-side plate) vs. control microorganisms exposed to sterile distilled water (Right-side plate)- 24h old plates

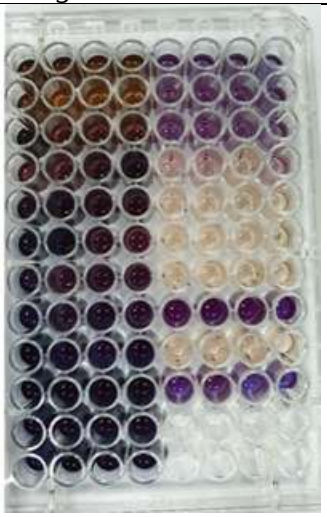
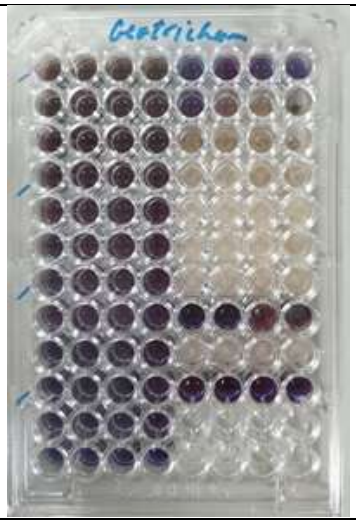
C. albicans

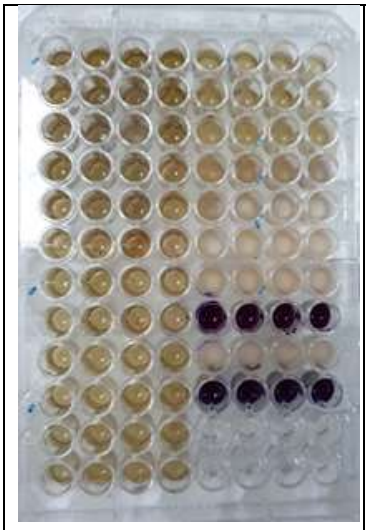
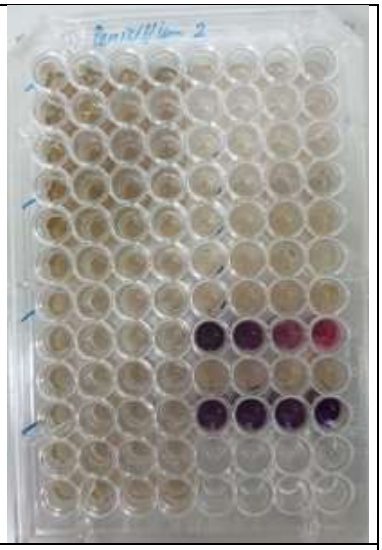
B. subtilis

S. aureus

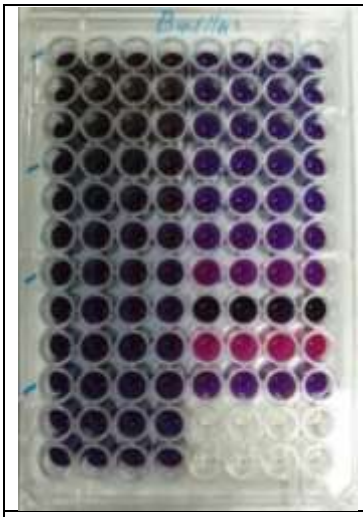
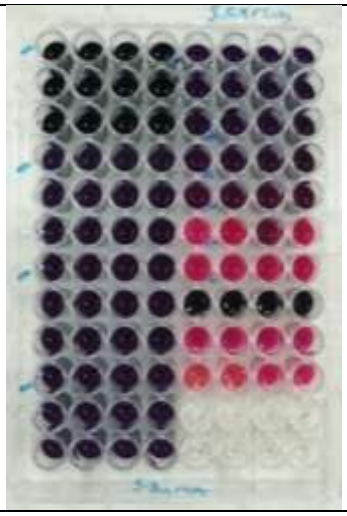
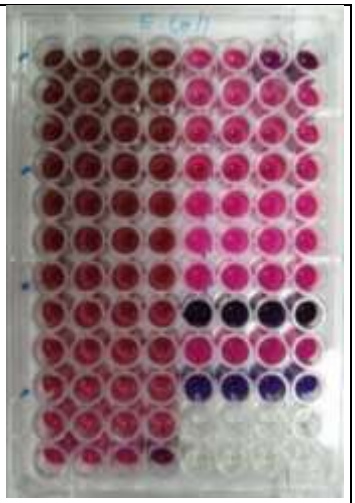
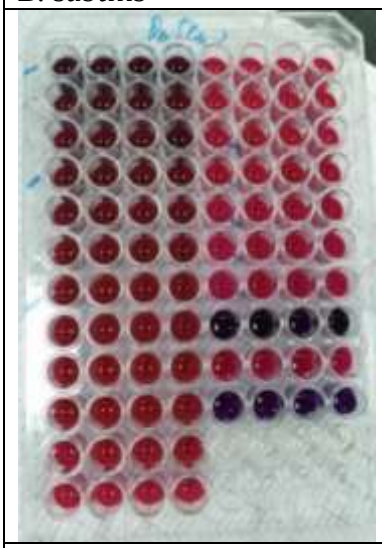
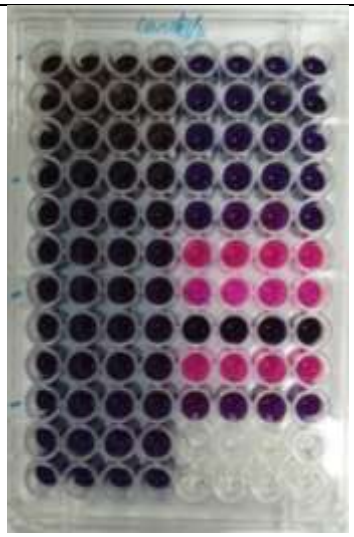
Appendix 4. 96-well plates MIC determination results-Fungi. Every 4 wells are one replicate, each plate was used for 22 different treatment, 12 on the left side of the plate

and 10 on the right side of the plate. The first 19 treatments contains 1:2 serial dilutions of PJ-WS-LE extracts starting from 50mg/ml. The last three treatments are: negative control with no microorganisms, negative control with no extract, and a positive control with chemical antimicrobial agent. Resazurin was used as an indicator of cellular activity (color will be lost). The minimum inhibitory concentration is the last concentration at which Resazurin color doesn't change.

		
A. alternata	*A. niger*	*B. cinerea*
		
C. gloeosporioides	*C. cladosporioides*	*G. candidum*

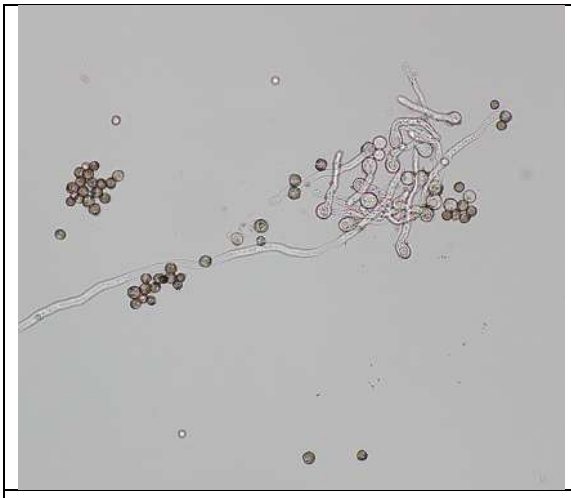
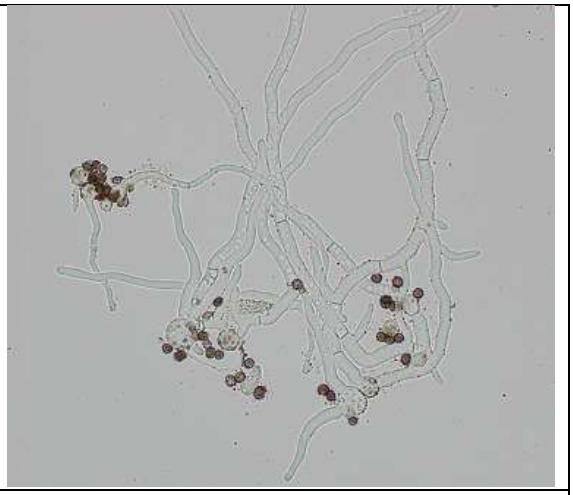
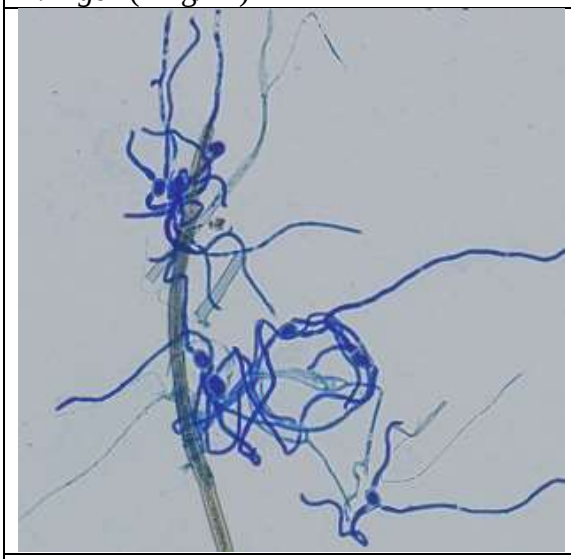
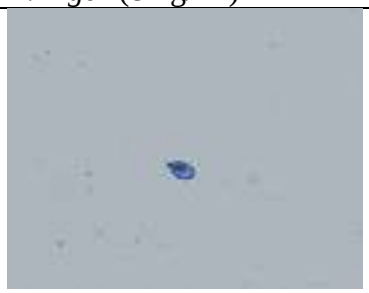
		
F. oxysporum	*P. citrinum*	*P. chrysogenum*

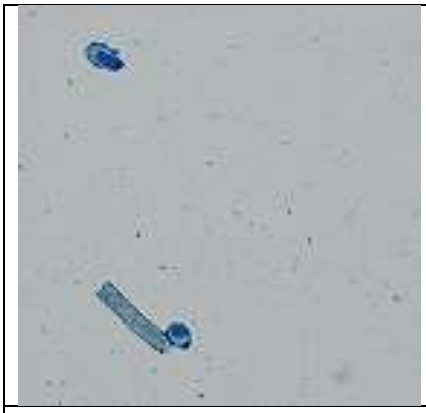
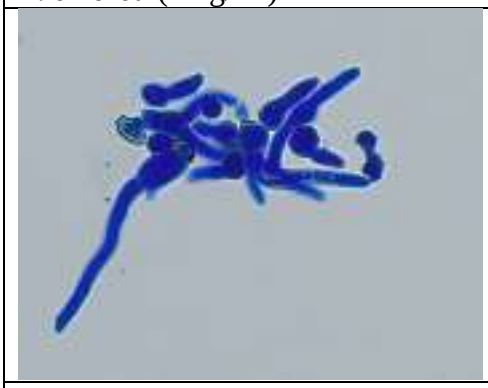
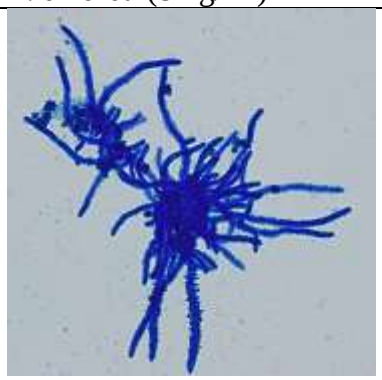
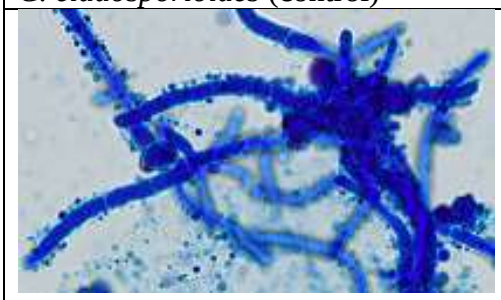
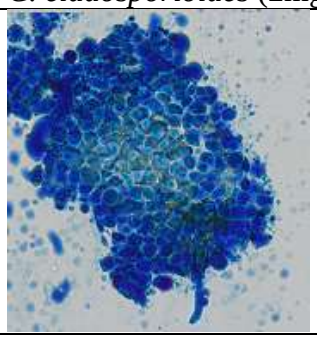
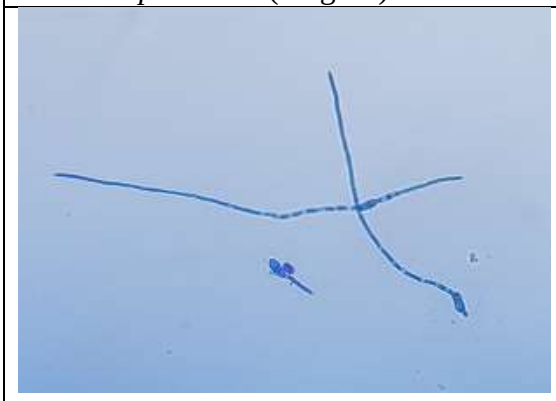
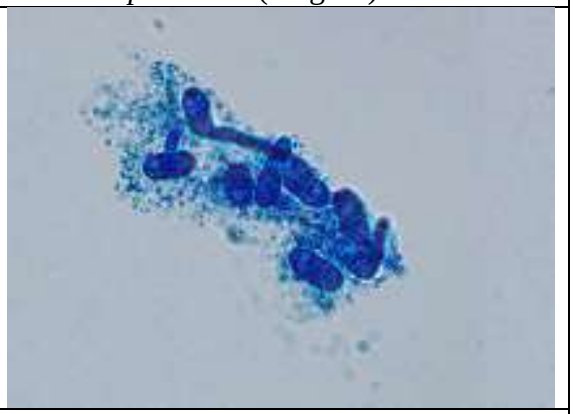
Appendix 5. 96-well plates MIC determination-bacteria and yeast. Every 4 wells are one replicate, each plate was used for 22 different treatment, 12 on the left side of the plate and 10 on the right side of the plate. The first 19 treatments contains 1:2 serial dilutions of PJ-WS-LE extracts starting from 50mg/ml. The last three treatments are: negative control with no microorganisms, negative control with no extract, and a positive control with chemical antimicrobial agent. Resazurin was used as an indicator of cellular activity (color will be lost). The minimum inhibitory concentration is the last concentration at which Resazurin color doesn't change.

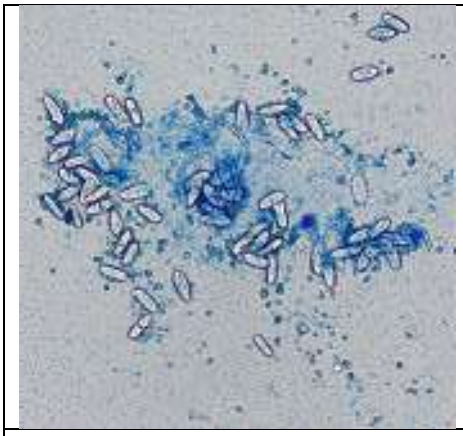
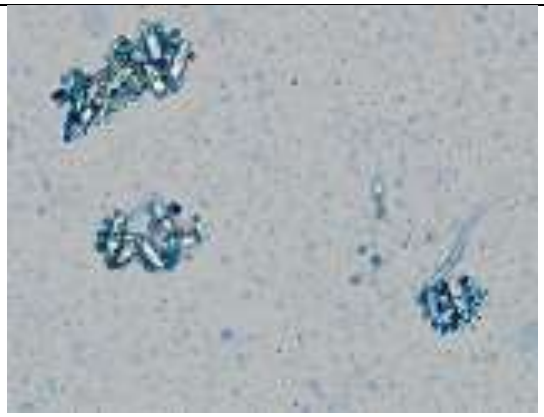
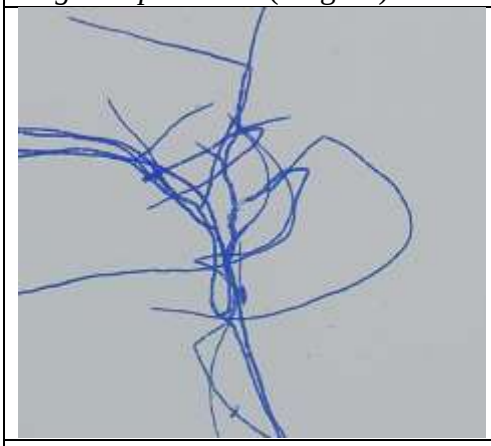
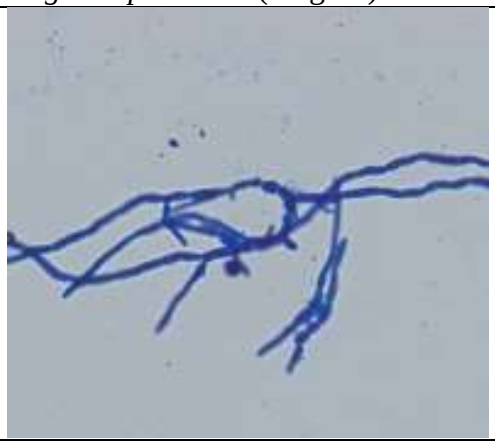
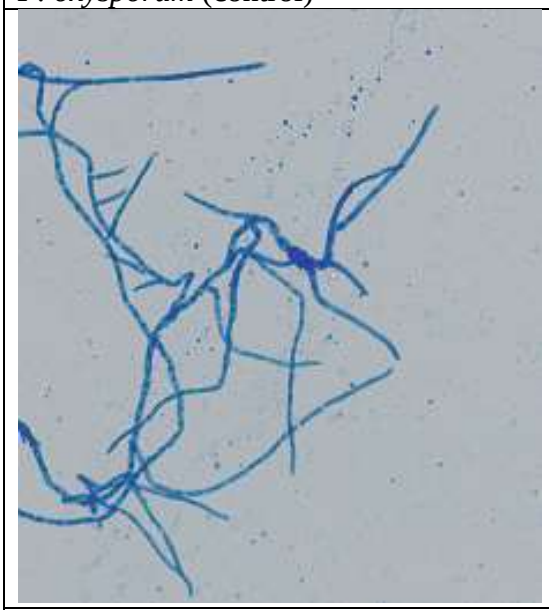
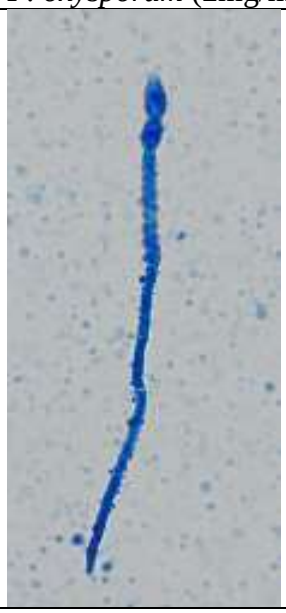
		
B. subtilis	*S. aureus*	*E. coli*
		
P. mirabilis	*C. albicans*	

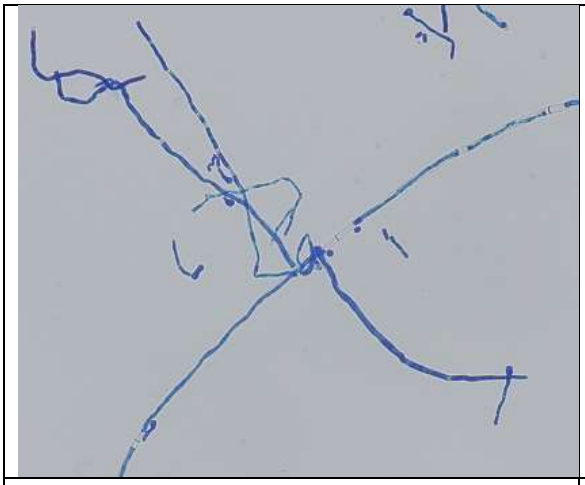
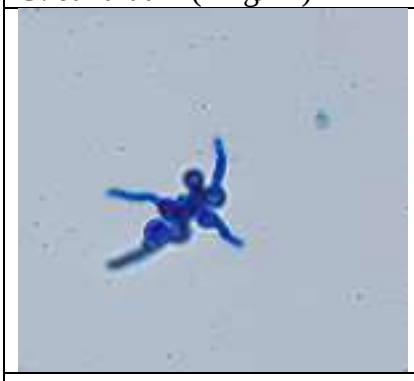
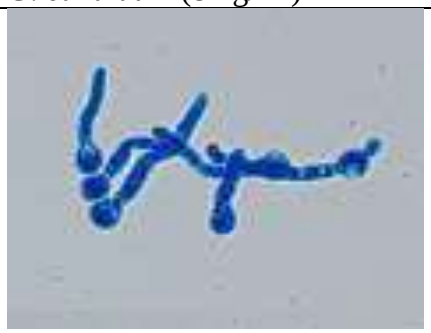
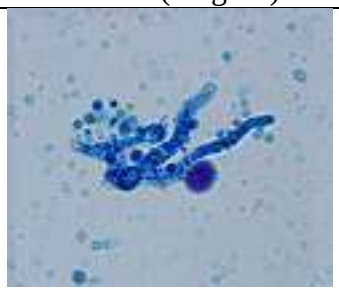
Appendix 6. Spores germination at different PJ-WS-LE extract concentrations (2, 4, and 8mg/ml). Upon 24h incubation with shacking in PDB at 25°C.

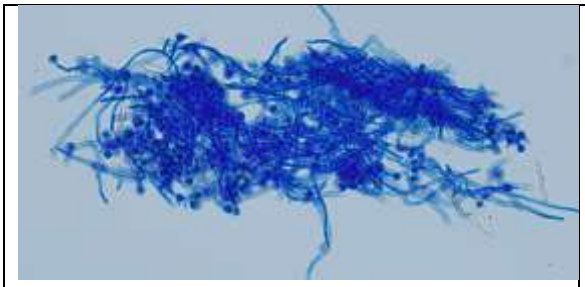
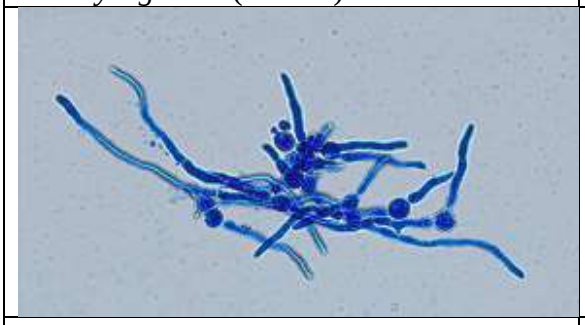
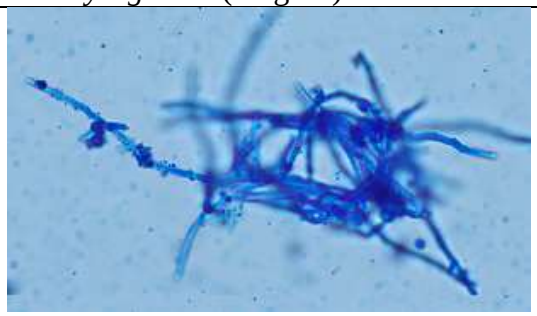
	
A. alternata (Start)	*A. alternata* (control)
	
A. alternata (2mg/ml)	*A. alternata* (4mg/ml)
	
A. alternata (8mg/ml)	

	
A. niger (control)	*A. niger* (2mg/ml)
	
A. niger (4mg/ml)	*A. niger* (8mg/ml)
	
B. cinerea (control)	*B. cinerea* (2mg/ml)

	
B. cinerea (4mg/ml)	*B. cinerea* (8mg/ml)
	
C. cladosporioides (control)	*C. cladosporioides* (2mg/ml)
	
C. cladosporioides (4mg/ml)	*C. cladosporioides* (8mg/ml)
	
C. gloeosporioides (control)	*C. gloeosporioides* (2mg/ml)

	
C. gloeosporioides (4mg/ml)	*C. gloeosporioides* (8mg/ml)
	
F. oxysporum (control)	*F. oxysporum* (2mg/ml)
	
F. oxysporum (4mg/ml)	*F. oxysporum* (8mg/ml)

	
G. candidum (control)	*G. candidum* (2mg/ml)
	
G. candidum (4mg/ml)	*G. candidum* (8mg/ml)
	
P. citrinum (control)	*P. citrinum* (2mg/ml)
	
P. citrinum (4mg/ml)	*P. citrinum* (8mg/ml)

	
P. chrysogenum (control)	*P. chrysogenum* (2mg/ml)
	
P. chrysogenum (4mg/ml)	*P. chrysogenum* (8mg/ml)

Appendix 7. *In-vivo* experiment, Strawberry shelf-life/room temperature.

Non-treated control samples after 48 hours (remaining 15 of the 30 samples at start)

Experimental samples treated with 8mg/ml PJ-WS-LE extract after 48 hours

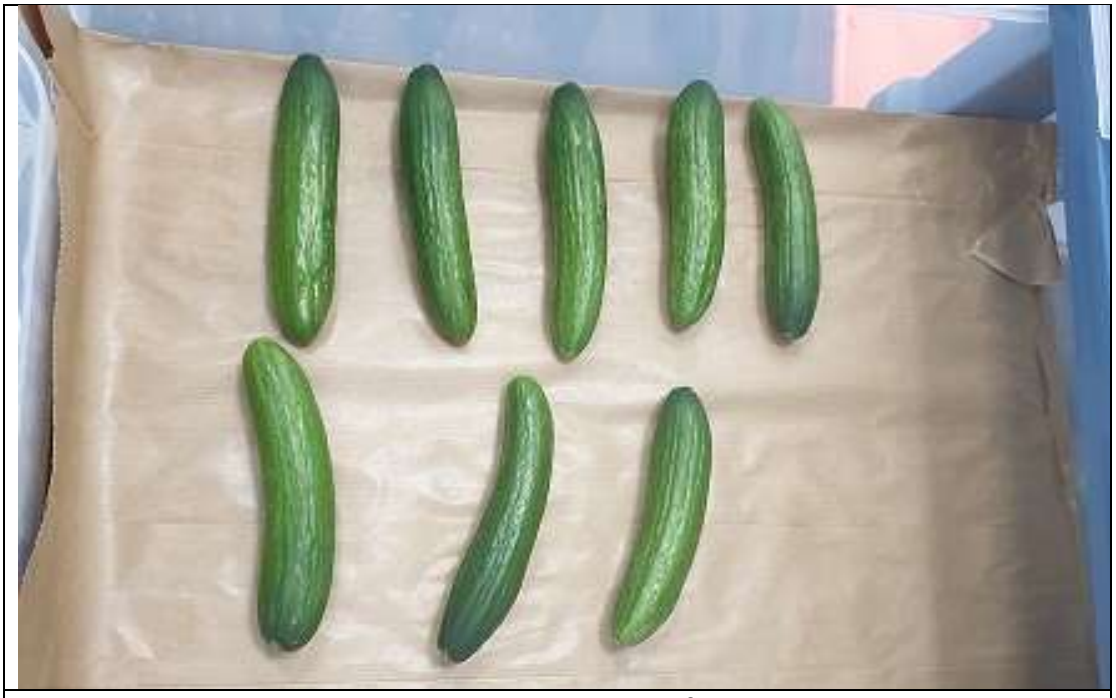
(remaining 21 of the 30 samples at start)

Appendix 8. *In-vivo* experiment, Strawberry shelf-life/ fridge temperature.

Non-treated control strawberry samples at day 10 (14 out of 30)

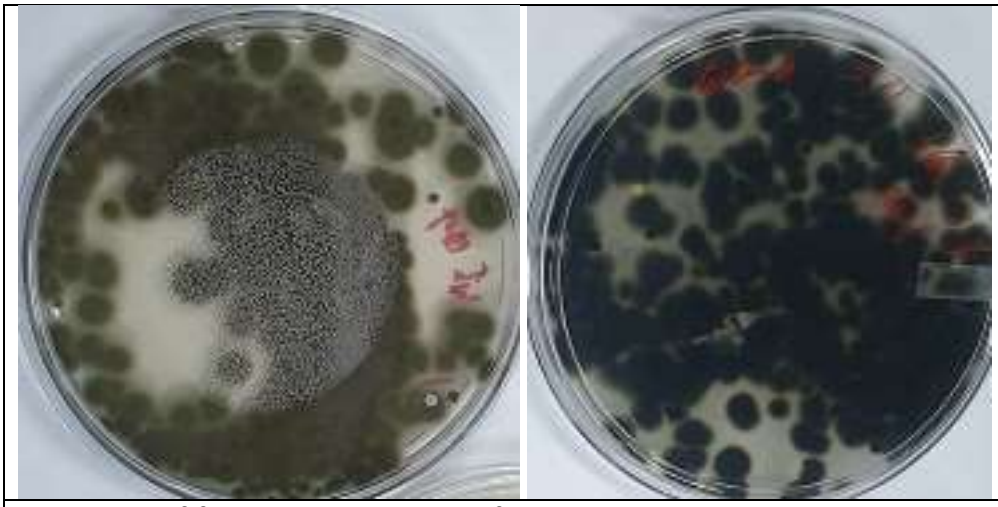
Experimental samples treated with 8mg/ml PJ-WS-LE extract at day 10 (30 out of 30)

Appendix 9. *In-vivo* experiment, cucumbers shelf-life/room temperature.

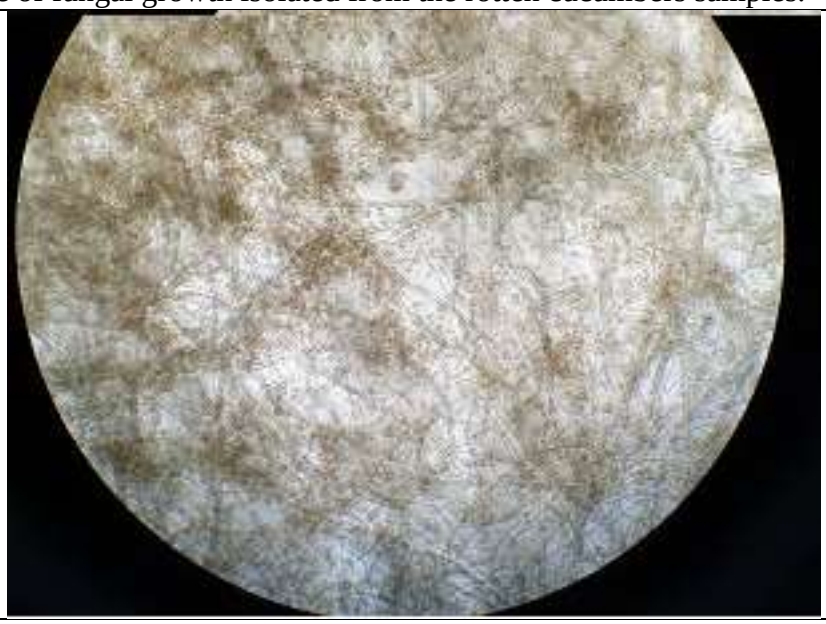


Edible non-treated control samples at day 5 (8 out of 30).

Edible experimental samples treated with 8mg/ml PJ-WS-LE at day 5 (20 out of 30).



PDA plate of fungal growth isolated from the rotten cucumbers samples.



Cladosporium sp. microscopic slide of fungal growth isolated from the rotten cucumbers samples.

Appendix 10. *In-vivo* controlled experiment, cherry tomatoes-room temperature-2 weeks results.

Negative control- Wounded

Negative control- Not Wounded

A. alternata control- Wounded

A. alternata control- Not Wounded

B. cineraria control- Wounded

B. cineraria control- Not Wounded

A. alternata preventive effect-Wounded

A. alternata preventive effect- Not Wounded

A. alternata curative effect-Wounded

A. alternata preventive effect- Not Wounded

B. cineraria preventive effect-Wounded

B. cineraria preventive effect- Not Wounded

B. cineraria curative effect-Wounded

B. cineraria s curative effect- Not Wounded

Appendix 11. *In-vivo* controlled experiment, preventive and curative effect of 8mg/ml of PJ-WS-LE extract on mangoes artificially inoculated with *A. alternata* at room temperature (representative samples).

Negative control samples.

Positive control samples.

PJ-WS-LE extract curative effect.

PJ-WS-LE extract preventive effect.

Appendix 12.

A. PCR product Sequences of the seven *P. juliflora* samples used for phylogeny tree and the *Penicillium* samples used for out-grouping.

>S1
TGTTCTTCAAGTCTCAAACCAGGGGTCCCGCCGACTGGGGTCGCGTAGTGT
CACGGGCTTTTCTCCCCGTCGAGGGAGCCCTCATCGTGAGGCCCCTTCTCT
GCGCGACGGCGCGCGCACGTCCGGTCTCTCGAGCGCATCTCATTCACTCAT
CCACCGTGGATCGTGGCCGCCGTCACTTCGGGCCCTCTTTTCGGCCGGCCG
CGAGTCGAGGCTCTCGGGAGGCCATCATCCGCCGTGCCCGCGATGGGGGC
GCTGGCGACTCTGCGTGACACCCAGGCAGACGTGCCCTCGGCCTAGTGGC
TTCGGGCGCAACTTGCGTTCAAAGACTCGATGGTTCACGGGATTCTGCAAT
TCACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAG
ATATCCGTTGCCGAGAGTCGTTTTATAGATGCAGGTGGCACGATACGCAA
CGCTGGCCGGCACCGGTGCCCGGGCGACCAGCGGCGCGCTCTGCTTGGTT
GCGGTTCCTTGACGCCTGTGGCGCCGGGGTTTTGGTTGTCGGGGGCCGGG
GGCCTGCACCGACGCACAGGGACCCGCCCCCCGGGGGTCGCGCGGGGCA
AAAGGACTTCGGGCGCCGTGGACGCCTTCGGCCTTTCTGCCCCCGGCCCG
AGACTTTGTGTCAACCCGGGTTCACGGGGGCGCGCTTTTCGCTTTGGCCTT
CCACAATGGAATCCTTACCCCAAGGTTCATTCTTCGTGACGCAACCGAAA
GAAGCACCCCGCAAGCGGGTGGACAAAACTCTGTGGGGGGGGGGGAAAG
AGAGGAGGAGCGCACGGCGCCCGCAAAATTTTGCGCCCACAACGAGGGG
GGGGGGGTGTTTTGTTTGGCCCAGGCCCGCTCCCCCACACAAAACAACAG
CCTCGCTGCGGTGGAGGGAATAGAAAAC
>S2
CTATATCTAATGTGGAAGCGCAGGGAACCGGCCGAACTGGGGGCGCGTAC
GTGTCACGGGCTTTTCTCCCCGTCGAGGGAGCCCTCATCGCTGTAGGCCCC
TTCTCTGCGCGACGGCGCGCGCACGTCCGGTCTCTCGAGCGCATCTCATTC
ACTCATCCACCGTGGATCGTGGCCGCCGTCACTTCGGGCCCTCTTTTCGGC
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AGGACTTCGGGCGCCGTGACGCCTTCGGCCTTCTGCCCCCGCCCGAGACTT
GTGTCAACCGGTTCACGGGTCGCGCTTTCGCTTTGGCATCGACATGATCCT
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CAGTCTTCGGGGGGGAGAGACGAAAGAGCCTCGGCCGCAGATCTCTCCGC
CGAACCCAGGAGGACTGTGTGTCATAGGCCGCCCCACACAAACTCGCCGC
TGGTGGTAGAACCTACCAGA
>S3
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CACGTCCGGTCTCTCGAGCGCATCTCATTCACTCATCCACCGTGGATCGTG

GCCGCCGTCACTTCGGGCCCTCTTTTCGGCCGGCCGCGAGTCGAGGCTCTC
GGGAGGCCATCATCCGCCGTGCCCGCGATGGGGGCGCTGGCGACTCTGCG
TGACACCCAGGCAGACGTGCCCTCGGCCTAGTGGCTTCGGGCGCAACTTG
CGTTCAAAGACTCGATGGTTCACGGGATTCTGCAATTCACACCAAGTATC
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GAGTCGTTTTATAGATGCAGGTGGCACGATACGCAACGCTGGCCGGCACC
GGTGCCCGGGCGACCAGCGGCGCGCTCTGCTTGGGTGCGGTTCCTTGACG
CCTGTGGGCGCCGGGGTTTTGGTTGTCG
>S4
GGTCCCCCGCCCTGGACTGGGGGGTCGCGTACGTGTCACGGGCTTTTCTCC
CGTCGAGGGAGCCCTCATCGTGCAGGCCCCTTCTCTGCGCGACGGCGCGC
GCACGTCCGGTCTCTCGAGCGCATCTCATTCACTCATCCACCGTGGATCGT
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CGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGATATCCGTTGCCG
AGAGTCGTTTTATAGATGCAGGTGGCACGATACGCAACGCTGGCCGGCAC
CGGTGCCCGGGCGACCAGCGGCGCGCTCTGCTTGGTTGCGGTTCCTTGAC
GCCTGCGGCGCCGGGGTTTTGGTTGTCGGGG
>S5
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CTCTCCCCGTCGAGGGAGCCCTCATCTTGAGGCCCCTTCTCTGCGCGACGG
CGCGCGCACGTCCGGTCTCTCGAGCGCATCTCATTCACTCATCCACCGTGG
ATCGTGGCCGCCGCCACTTCCGGCCCTCTTTTCAGACTGCCGCCAGTCGAG
GCTCTCGGGAGGCCATCATCCTGCGGACCCGCGATGGGGGCGCTGGCGAC
TCTGCCGGAACCCAGGCAGACGTGCCCTCGGCCTAGTGGCTTCGGGCGCA
ACTTGCGTTCAAAGACTCGATGGTTCACGGGATTCTGCAATTCACACCAA
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GCACCGGTGCCCGGGCGACCAGCGGCACGCTCTGCTTGGTTGCGGTTCCTT
GACGCCTGTGGCGCCGGGGTTTTGGTTG
>S6
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GTCGAGGGAGCCCTCATCGTGTTGACCCTTCTCTGCGCGACGGCGCGCGC
ACGTCCGGTCTCTCGAGCGCATCTCATTCACTCATCCACCGTGGATCGTGG
CCGCCGTCACTTCGGGCCCTCTTTTCGGCCGGCCGCGAGTCCAGGCTCTCG
GGAGGCCATCATCCGCCGTGCCCGCGATGGGGGCGCTGGCGACTCTGCGT
GACACCCAGGCAGACGTGCCCTCGGCCTAGTGGCTTCGGGCGCAACTTGC
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CATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGATATCCGTTGCCGAG
AGTCGTTTTATAGATGCAGGTGGCACGATACGCAACGCTGGCCGGCACCG
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TGTGGCGCCGGGGTTTTGGTTGTCGGGGCCGGGGCCTGCATTGACACATA
GGACCTGCCCCCCGGGCGTCCCCGGGGGGAAAAAAGTACTGGGGGGTGC
GGGGACCTCTTCCTTTCTTTCTGCCTTCCCACCCAAGACTGAGGATCAAGG
GGGATACAGGGGTCGCTTGTTTTTTTTTTTAGGCATCGACAATGATTCCCT
CCCGGATGTTTCTCCTTAAAGAAAGG
>S7

AAGACCGGCGACCAACACAACGGAACCGCGACCTGGGGTGCGTAGTGTC
ACGGGCTTTTCTCCCCGTCGAGGGAGCCCTCATCGTGAGGCCCCTTCTCTG
CGCGACGGCGCGCGCACGTCCGGTCTCTCGAGCGCATCTCATTCACTCATC
CACCGTGGATCGTGGCCGCCGTCACTTCGGGCCCTCTTTTCGGCCGGCCGC
GAGTCGAGGCTCTCGGGAGGCCATCATCCGCCGTGCCCGCGATGGGGGCG
CTGGCGACTCTGCGTGACACCCAGGCAGACGTGCCCTCGGCCTAGTGGCT
TCGGGCGCAACTTGCGTTCAAAGACTCGATGGTTCACGGGATTCTGCAATT
CACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAG
ATATCCGTTGCCGAGAGTCGTTTTATAGATGCAGGTGGCACGATACGCAA
CGCTGGCCGGCACCGGTGCCCGGGCGACCAGCGGCGCGCTCTGCTTGGTT
GCGGTTCCTTGACGCCTGTGGCGCCGGGGTTTTGGTTGTCGGGGCCGGGG
CCTGCATCGACGCACAGGACCCGCCCCCGGCGTGGCGCGGGAAAAGGACT
TCGGGCGCCGTGACCCCTTCGGCCTTCTGCCCCCCGCCCGAGACTTGAGTC
AAGCGCATCAGGGGTCGCGCTTTCGCTTTGGCATCGACGATGATCCTTCCG
CAGGTTCACCTAAGGAAG
> *P. chrysogenum*
TGCAATCTGGGAGGGATCTACTGATCGAGTCACCTGGATAAAAATTTGGG
TTGATCGGCAAGCGCCGGCCGGGCCTACAGAGCGGGTGACAAAGCCCCAT
ACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCC
CGGGATCGGAGGACGGGGCCCAACACACAAGCCGTGCTTGAGGGCAGCA
ATGACGCTCGGACAGGCATGCCCCCCCGGAATACCAGGGGGCGCAATGTGC
GTTCAAAGACTCGATGATTCACTGAATTTGCAATTCACATTACGTATCGCA
TTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAG
TTTTAAATAATTTATATTTTCACTCAGACTACAATCTTCAGACAGAGTTCG
AGGGTGTCTTCGGCGGGCGCGGGCCCGGGGGCGTAAGCCCCCCGGCGGCC
AGTTAAGGCGGGCCCGCCGAAGCAACAAGGTAAAATAAACACGGGTGGG
AGGTTGGACCCAGAGGGCCCTCACTCGGTAATGATCCTTCCGCAGGTTCA
CCTACGGAAGGAGGGCCCTCTGGGTCCAACCTCCCACCCGGTGTTTATTTT
ACCTTGTTGCTTCGGCGGGCCCGCCTTAACTGGCCGCCGGGGGGCTTACGC
CCCCGGGCCGCCCGCCGAAAAACCCTCCAACTCTGTTGGAAGAATGTAGT
CGAGGTGAAAAATAAATTATTTAAAATTTTAACACGGATCTCTTGGTTCCG
GCATCGATGAAAAACCACGAAATGGGATACGTAATGGGAATTGCAATTCG
TGAATCATCCAGTCTTTAACGCACATTGGGCCCCTGGTATTCCGGGGGGAT
GCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGTTGTGTGGTTGGGGGC
CCGGTCCTCCGATTCCGGGGGAAGGGGCCCAAAGGGAGCGGCGGCACCG
GTTCCGGTCCTCGAGCGTAAGGGGGCTTTGGTCCCCGCTCTGGTAGCCCGG
CGGGCCTTGCCGAACAACCCAAAATTTTTTATCCAGGTTGGACCCTCGGAT
CAGGTGAGGGATACCCCGCTGAACTTTAAGCATATTATATAGACGGGAGG
GAAAACTC
>*P. citrinum*

GGGGGCTTGTATCGTCCCTGATCGAGGTCACCTGAGATAATTAAAGGTTG
GGGGTCGGCTGGCGCCGGCCGGGCCTACTAGAGCGGGTGACGAAGCCCC
ATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCC
CCCCGGCGGGGGGGACGGGGCCCAACACACAAGCCGGGCTTGAGGGCAG
CAATGACGCTCGGACAGGCATGCCCTCCGGAATACCAGAGGGCGCAATGT
GCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTAGTTATC
GCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGA
AAGTTTTAACTAATTTCGTTATAGGTCTCAGACTGCAACTTCAGACAGCGT
TCAGGGGGGCCGTCGGCGGGCGCGGGGCCCGCCGAGGCAACATAGGTTC

GGGCAACACGGGTGGGAGGTTGGGCCCCGAGGGGCCCGCACTCGGTAAT
GATCCTTCCGGGGGGTTGGCCTACGGAAGGCCTCGGGGGCCAACCTCCCA
GGGGGGGGGGGGGGGGTATGTTGCCTCGGGGGGGGGGGGGGGGGGGACG
CCCCTGAAAGCTGTCTAAAGTTCCGTTGAGACCTATTACGAAATTGTTAAA
ACTTTCAACAACGGATCTCTTGGTTCGGCTCGATAAAAACGCACGCAATG
CGATACTAATGTGAATTGCAGAATTCGTGAATCATCGAGTCTTGAACGCC
ATTGCGCCCTCTGTATTCCGGAGGCATGGCTGTCGAGCCGTCATTGCTGCC
TCCAGCCCGGCTTGGGTGTTGGGGGCCCCCGTCCCCTCGCCGGGGGGGAC
TGGACCCAAAAGCTAACGGCGGCACTGCGTTCGGTTCTTCATGCGTAATG
GGGGCTTCCTCTACCGCTCCTATAAGGGCCGGGCGGGGCCGACGCACCTC
AACCTTTAATTTACTCTCACGGTTAGCCTTCCGGATTCAGGGGGAAGGGTA
TACCCCCCTG

B. Alignments of the seven *P. juliflora* samples with *P. juliflora* sample number

JX139107.1 per order of identity percentage

S1

Score	Expect	Identities	Gaps	Strand
924 bits(500)	0.0	513/519(99%)	1/519(0%)	Plus/Minus

```
Query  188  CCCCGACAACCAAAACCCCGGCGCCGCAGGCGTCAAGGAACCGCAACCAAGCAGAGCGCG  247
            ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  550  CCCCGACAACCAAAACCCCGGCGCCACAGGCGTCAAGGAACCGCAACCAAGCAGAGCGCG  491

Query  248  CCGCTGGTCGCCCGGGCACCGGTGCCGGCCAGCGTTGCGTATCGTGCCACCTGCATCTAT  307
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  490  CCGCTGGTCGCCCGGGCACCGGTGCCGGCCAGCGTTGCGTATCGTGCCACCTGCATCTAT  431

Query  308  AAAACGACTTTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATG  367
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  430  AAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATG  371

Query  368  CGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCG  427
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  370  CGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCG  311

Query  428  CCCGAAGCCACTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAGAGTCGCCAGCGCCC  487
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  310  CCCGAAGCCACTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAGAGTCGCCAGCGCCC  251

Query  488  CCATCGCGGGCACGGCGGATGATGGCCTCCCGAGAGCCTTGACTTGCGGCCGGCCGAAAA  547
            |||||||||||||||||||||||||||||||||| |||| ||||||||||||||||||||
Sbjct  250  CCATCGCGGGCACGGCGGATGATGGCCTCCCGAGAGCCTCGACTCGCGGCCGGCCGAAAA  191

Query  548  GAGGGCCCGAAGTGACGGCGGCCACGATCCACGGTGGATGAGTGAATGAGATGCGCTCGA  607
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190  GAGGGCCCGAAGTGACGGCGGCCACGATCCACGGTGGATGAGTGAATGAGATGCGCTCGA  131

Query  608  GAGACCGGACGTGCGCGCGCCGTCGCGCAGAGAAGGGGCCTCACGATGAGGGCTCCCTCG  667
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130  GAGACCGGACGTGCGCGCGCCGTCGCGCAGAGAAGGGGCCTCACGATGAGGGCTCCCTCG  71

Query  668  ACGGGGAGAAAAGCCCATGACACGTACGCGACCCCAGTC  706
            |||||||||||||||| |||||| ||||||||||||||
Sbjct  70   ACGGGGAGAAAAGCCCGTGACAC-TACGCGACCCCAGTC  33
```

S4

Score	Expect	Identities	Gaps	Strand
918 bits(497)	0.0	510/516(99%)	2/516(0%)	Plus/Minus

```
Query  188  CCCCGACAACCAAAACCCCGGCGCCGCAGGCGTCAAGGAACCGCAACCAAGCAGAGCGCG  247
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  535  CCCCGACAACCAAAACCCCGGCGCCGCAGGCGTCAAGGAACCGCAACCAAGCAGAGCGCG  476

Query  248  CCGCTGGTCGCCCGGGCACCGGTGCCGGCCAGCGTTGCGTATCGTGCCACCTGCATCTAT  307
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  475  CCGCTGGTCGCCCGGGCACCGGTGCCGGCCAGCGTTGCGTATCGTGCCACCTGCATCTAT  416

Query  308  AAAACGACTTTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATG  367
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  415  AAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATG  356

Query  368  CGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCG  427
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  355  CGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCG  296

Query  428  CCCGAAGCCACTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAGAGTCGCCAGCGCCC  487
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  295  CCCGAAGCCACTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAGAGTCGCCAGCGCCC  236

Query  488  CCATCGCGGGCACGGCGGATGATGGCCTCCCGAGAGCCTTGACTTGCGGCCGGCCGAAAA  547
            |||||||||||||||||||||||||||||||||||| |||| ||||||||||||||||
Sbjct  235  CCATCGCGGGCACGGCGGATGATGGCCTCCCGAGAGCCTCGACTCGCGGCCGGCCGAAAA  176

Query  548  GAGGGCCCGAAGTGACGGCGGCCACGATCCACGGTGGATGAGTGAATGAGATGCGCTCGA  607
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  175  GAGGGCCCGAAGTGACGGCGGCCACGATCCACGGTGGATGAGTGAATGAGATGCGCTCGA  116

Query  608  GAGACCGGACGTGCGCGCGCCGTCGCGCAGAGAAGGGGCCT-CACGATGAGGGCTCCCTC  666
            |||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||
Sbjct  115  GAGACCGGACGTGCGCGCGCCGTCGCGCAGAGAAGGGGCCTGCACGATGAGGGCTCCCTC  56

Query  667  GACGGGGAGAAAAGCCCATGACACGTACGCGACCCC  702
            |||||| |||||||||| ||||||||||||||||||
Sbjct  55   GACGGG-AGAAAAGCCCGTGACACGTACGCGACCCC  21
```

S7

Score	Expect	Identities	Gaps	Strand
917 bits(496)	0.0	511/518(99%)	2/518(0%)	Plus/Minus

```
Query  187  GCCCCGACAACCAAAACCCCGGCGCCGCAGGCGTCAAGGAACCGCAACCAAGCAGAGCGC  246
            ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  548  GCCCCGACAACCAAAACCCCGGCGCCACAGGCGTCAAGGAACCGCAACCAAGCAGAGCGC  489

Query  247  GCCGCTGGTCGCCCGGGCACCGGTGCCGGCCAGCGTTGCGTATCGTGCCACCTGCATCTA  306
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  488  GCCGCTGGTCGCCCGGGCACCGGTGCCGGCCAGCGTTGCGTATCGTGCCACCTGCATCTA  429

Query  307  TAAAACGACTTTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAAT  366
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  428  TAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAAT  369

Query  367  GCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGC  426
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  368  GCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGC  309

Query  427  GCCCGAAGCCACTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAGAGTCGCCAGCGCC  486
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  308  GCCCGAAGCCACTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAGAGTCGCCAGCGCC  249

Query  487  CCCATCGCGGGCACGGCGGATGATGGCCTCCCGAGAGCCTTGACTTGCGGCCGGCCGAAA  546
            |||||||||||||||||||||||||||||||||||| |||| ||||||||||||||||||
Sbjct  248  CCCATCGCGGGCACGGCGGATGATGGCCTCCCGAGAGCCTCGACTCGCGGCCGGCCGAAA  189

Query  547  AGAGGGCCCGAAGTGACGGCGGCCACGATCCACGGTGGATGAGTGAATGAGATGCGCTCG  606
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  188  AGAGGGCCCGAAGTGACGGCGGCCACGATCCACGGTGGATGAGTGAATGAGATGCGCTCG  129

Query  607  AGAGACCGGACGTGCGCGCGCCGTCGCGCAGAGAAGGGGCCTCACGATGAGGGCTCCCTC  666
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  128  AGAGACCGGACGTGCGCGCGCCGTCGCGCAGAGAAGGGGCCTCACGATGAGGGCTCCCTC  69

Query  667  GACGGGGAGAAAAGCCCATGACACGTACGCGACCCCAG  704
            ||||||||||||||| |||||| ||||| |||||||
Sbjct  68   GACGGGGAGAAAAGCCCGTGACAC-TACGC-ACCCCAG  33
```

S2

Score	Expect	Identities	Gaps	Strand
917 bits(496)	0.0	513/521(98%)	2/521(0%)	Plus/Minus

```
Query  187  GCCCCGACAACCAAAACCCCGGCGCCGCAGGCGTCAAGGAACCGCAACCAAGCAGAGCGC  246
            ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  556  GCCCCGACAACCAAAACCCCGGCGCCACAGGCGTCAAGGAACCGCAACCAAGCAGAGCGC  497

Query  247  GCCGCTGGTCGCCCGGGCACCGGTGCCGGCCAGCGTTGCGTATCGTGCCACCTGCATCTA  306
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  496  GCCGCTGGTCGCCCGGGCACCGGTGCCGGCCAGCGTTGCGTATCGTGCCACCTGCATCTA  437

Query  307  TAAAACGACTTTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAAT  366
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  436  TAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAAT  377

Query  367  GCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGC  426
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  376  GCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGC  317

Query  427  GCCCGAAGCCACTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAGAGTCGCCAGCGCC  486
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  316  GCCCGAAGCCACTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAGAGTCGCCAGCGCC  257

Query  487  CCCATCGCGGGCACGGCGGATGATGGCCTCCCGAGAGCCTTGACTTGCGGCCGGCCGAAA  546
            |||||||||||||||||||||||||||||||||||| |||| ||||||||||||||||||
Sbjct  256  CCCATCGCGGGCACGGCGGATGATGGCCTCCCGAGAGCCTCGACTCGCGGCCGGCCGAAA  197

Query  547  AGAGGGCCCGAAGTGACGGCGGCCACGATCCACGGTGGATGAGTGAATGAGATGCGCTCG  606
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  196  AGAGGGCCCGAAGTGACGGCGGCCACGATCCACGGTGGATGAGTGAATGAGATGCGCTCG  137

Query  607  AGAGACCGGACGTGCGCGCGCCGTCGCGCAGAGAAGGGGCCT-CA-CGATGAGGGCTCCC  664
            |||||||||||||||||||||||||||||||||||||||||| || ||||||||||||||
Sbjct  136  AGAGACCGGACGTGCGCGCGCCGTCGCGCAGAGAAGGGGCCTACAGCGATGAGGGCTCCC  77

Query  665  TCGACGGGGAGAAAAGCCCATGACACGTACGCGACCCCAGT  705
            |||||||||||||||||| |||||||||||| |||||||
Sbjct  76   TCGACGGGGAGAAAAGCCCGTGACACGTACGCGCCCCCAGT  36
```

S3

Score	Expect	Identities	Gaps	Strand
905 bits(490)	0.0	514/525(98%)	4/525(0%)	Plus/Minus

```
Query  191  CGACAACCAAAACCCCGGCGCC-GCAGGCGTCAAGGAACCGCAACCAAGCAGAGCGCGCC  249
            ||||||||||||||||||||||  ||||||||||||||||||| ||||||||||||||||
Sbjct  532  CGACAACCAAAACCCCGGCGCCCACAGGCGTCAAGGAACCGCACCCAAGCAGAGCGCGCC  473

Query  250  GCTGGTCGCCCGGGCACCGGTGCCGGCCAGCGTTGCGTATCGTGCCACCTGCATCTATAA  309
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  472  GCTGGTCGCCCGGGCACCGGTGCCGGCCAGCGTTGCGTATCGTGCCACCTGCATCTATAA  413

Query  310  AACGACTTTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCG  369
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  412  AACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCG  353

Query  370  ATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCC  429
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  352  ATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCC  293

Query  430  CGAAGCCACTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAGAGTCGCCAGCGCCCCC  489
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  292  CGAAGCCACTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAGAGTCGCCAGCGCCCCC  233

Query  490  ATCGCGGGCACGGCGGATGATGGCCTCCCGAGAGCCTTGACTTGCGGCCGGCCGAAAAGA  549
            |||||||||||||||||||||||||||||||||| |||| ||||||||||||||||||
Sbjct  232  ATCGCGGGCACGGCGGATGATGGCCTCCCGAGAGCCTCGACTCGCGGCCGGCCGAAAAGA  173

Query  550  GGGCCCGAAGTGACGGCGGCCACGATCCACGGTGGATGAGTGAATGAGATGCGCTCGAGA  609
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  172  GGGCCCGAAGTGACGGCGGCCACGATCCACGGTGGATGAGTGAATGAGATGCGCTCGAGA  113

Query  610  GACCGGACGTGCGCGCGCCGTCGCGCAGAGAAGGGGCCT-CACGATGAGGGCTCCCTCGA  668
            |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  112  GACCGGACGTGCGCGCGCCGTCGCGCAGAGAAGGGGCCTACACGATGAGGGCTCCCTCGA  53

Query  669  CGGGGAGAAAAGCCCATGACACGTACGCGACCCCA-GT-CAGTGC  711
            ||||||||||||||| |||||||||||||||||| || ||| ||
Sbjct  52   CGGGGAGAAAAGCCCGTGACACGTACGCGACCCCAAGTACAGGGC  8
```

S6

Score	Expect	Identities	Gaps	Strand
893 bits(483)	0.0	506/517(98%)	2/517(0%)	Plus/Minus

```
Query  187  GCCCCGACAACCAAAACCCCGGCGCCGCAGGCGTCAAGGAACCGCAACCAAGCAGAGCGC  246
            ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  534  GCCCCGACAACCAAAACCCCGGCGCCACAGGCGTCAAGGAACCGCAACCAAGCAGAGCGC  475

Query  247  GCCGCTGGTCGCCCGGGCACCGGTGCCGGCCAGCGTTGCGTATCGTGCCACCTGCATCTA  306
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  474  GCCGCTGGTCGCCCGGGCACCGGTGCCGGCCAGCGTTGCGTATCGTGCCACCTGCATCTA  415

Query  307  TAAAACGACTTTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAAT  366
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  414  TAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAAT  355

Query  367  GCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGC  426
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  354  GCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGC  295

Query  427  GCCCGAAGCCACTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAGAGTCGCCAGCGCC  486
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  294  GCCCGAAGCCACTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAGAGTCGCCAGCGCC  235

Query  487  CCCATCGCGGGCACGGCGGATGATGGCCTCCCGAGAGCCTTGACTTGCGGCCGGCCGAAA  546
            |||||||||||||||||||||||||||||||||||||||||| |||| ||||||||||||||
Sbjct  234  CCCATCGCGGGCACGGCGGATGATGGCCTCCCGAGAGCCTGGACTCGCGGCCGGCCGAAA  175

Query  547  AGAGGGCCCGAAGTGACGGCGGCCACGATCCACGGTGGATGAGTGAATGAGATGCGCTCG  606
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  174  AGAGGGCCCGAAGTGACGGCGGCCACGATCCACGGTGGATGAGTGAATGAGATGCGCTCG  115

Query  607  AGAGACCGGACGTGCGCGCGCCGTCGCGCAGAGAAGGGGCCTCACGATGAGGGCTCCCTC  666
            ||||||||||||||||||||||||||||||||||| |  ||||||||||||||||||||
Sbjct  114  AGAGACCGGACGTGCGCGCGCCGTCGCGCAGAGAAGGGTCAACACGATGAGGGCTCCCTC  55

Query  667  GACGGGGAGAAAAGCCCATGA-CACGTACGCGACCCC  702
            |||||| |||||||||| ||| |||| ||||||||||
Sbjct  54   GACGGG-AGAAAAGCCCGTGAACACGCACGCGACCCC  19
```

S5

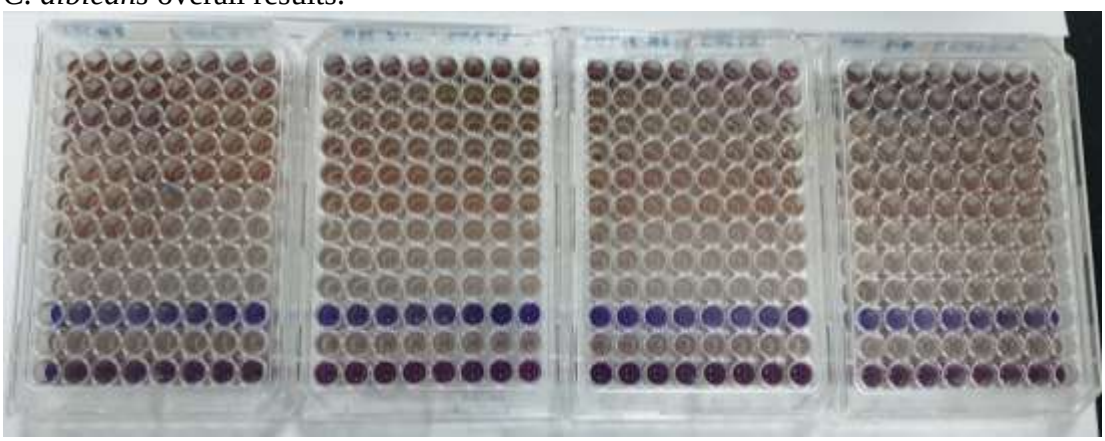
Score	Expect	Identities	Gaps	Strand
832 bits(450)	0.0	493/514(96%)	2/514(0%)	Plus/Minus

```
Query  194  CAACCAAAACCCCGGCGCCGCAGGCGTCAAGGAACCGCAACCAAGCAGAGCGCGCCGCTG  253
            |||||||||||||||||| ||||||||||||||||||||||||||||||| |||||||
Sbjct  533  CAACCAAAACCCCGGCGCCACAGGCGTCAAGGAACCGCAACCAAGCAGAGCGTGCCGCTG  474

Query  254  GTCGCCCGGGCACCGGTGCCGGCCAGCGTTGCGTATCGTGCCACCTGCATCTATAAAACG  313
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  473  GTCGCCCGGGCACCGGTGCCGGCCAGCGTTGCGTATCGTGCCACCTGCATCTATAAAACG  414

Query  314  ACTTTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATAC  373
            ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  413  ACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATAC  354

Query  374  TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAA  433
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  353  TTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAA  294

Query  434  GCCACTAGGCCGAGGGCACGTCTGCCTGGGTGTCACGCAGAGTCGCCAGCGCCCCCATCG  493
            |||||||||||||||||||||||||||||| ||  |||||||||||||||||||||||||
Sbjct  293  GCCACTAGGCCGAGGGCACGTCTGCCTGGGT-TCCGGCAGAGTCGCCAGCGCCCCCATCG  235

Query  494  CGGGCACGGCGGATGATGGCCTCCCGAGAGCCTTGACTTGCGGCCGGCCGAAAAGAGGGC  553
            ||||  ||  ||||||||||||||||||||| |||| ||||| | | ||||||||||||
Sbjct  234  CGGGTCCGCAGGATGATGGCCTCCCGAGAGCCTCGACTGGCGGCAGTCTGAAAAGAGGGC  175

Query  554  CCGAAGTGACGGCGGCCACGATCCACGGTGGATGAGTGAATGAGATGCGCTCGAGAGACC  613
            | |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  174  CGGAAGTGGCGGCGGCCACGATCCACGGTGGATGAGTGAATGAGATGCGCTCGAGAGACC  115

Query  614  GGACGTGCGCGCGCCGTCGCGCAGAGAAGGGGCCTCACGATGAGGGCTCCCTCGACGGGG  673
            |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  114  GGACGTGCGCGCGCCGTCGCGCAGAGAAGGGGCCTCAAGATGAGGGCTCCCTCGACGGGG  55

Query  674  AGAAAAGCCCATGACACGTACGCGACCCC-AGTC  706
            ||| |||||| ||||||||||||||||| ||||
Sbjct  54   AGAGAAGCCCGTGACACGTACGCGACCCCCAGTC  21
```

Appendix 13. Results of 96-well plate experiment to show MICs of PJ-WS-LE extracts prepared from the leaves of eight different trees collected from different locations in Doha against *S. aureus*, *C. albicans*, *C. gloeosporioides* and *A. alternata*. Wells 1 to 9 are 1:1 dilutions of PJ-WS-LE extracts starting from 42mg/ml to 0.16mg/ml, wells 10 are control with no bacteria, wells 11 are negative control with no extract, and wells 12 are positive control with Kanamycin.

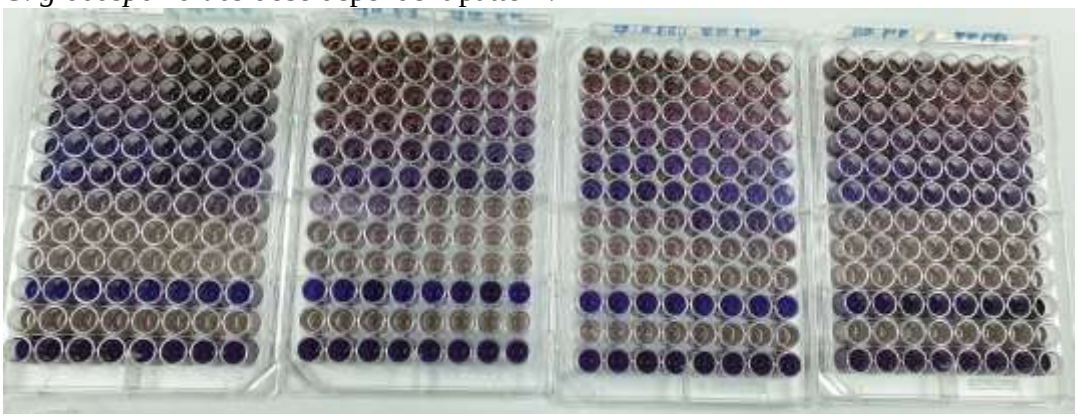
S. aureus overall results.

C. albicans overall results.



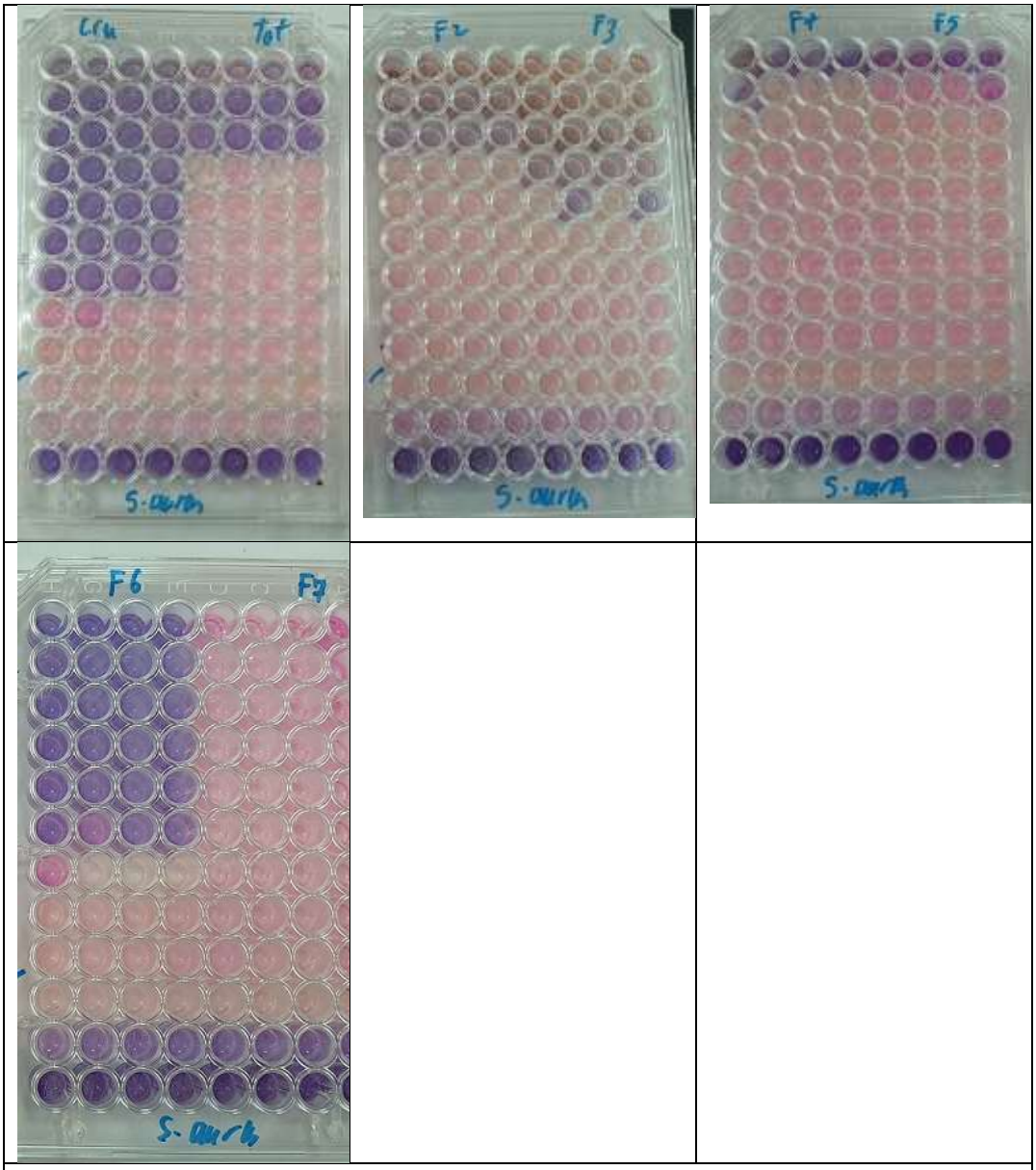
C. gloeosporioides overall results.

C. gloeosporioides dose dependent pattern.

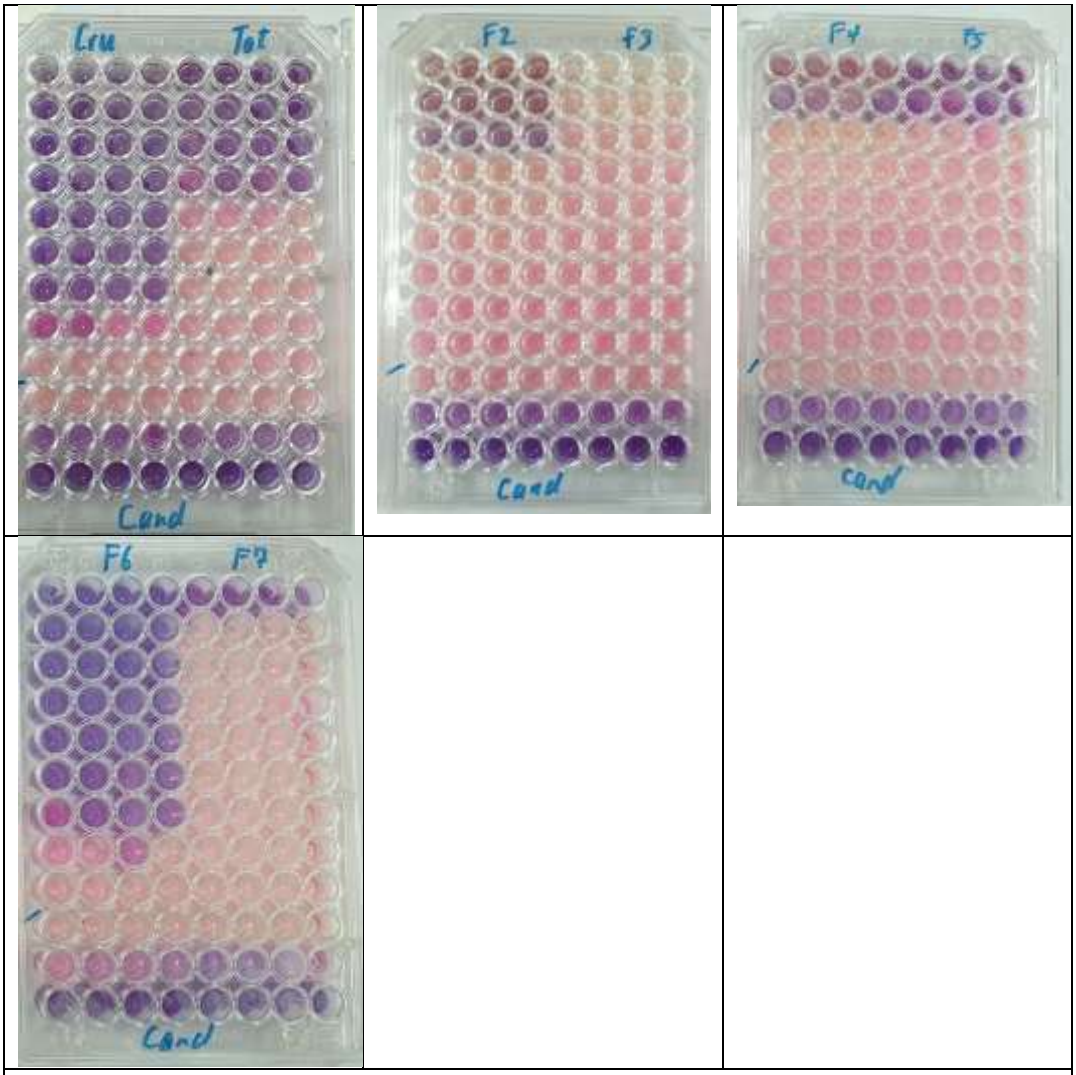


A. alternata overall results.

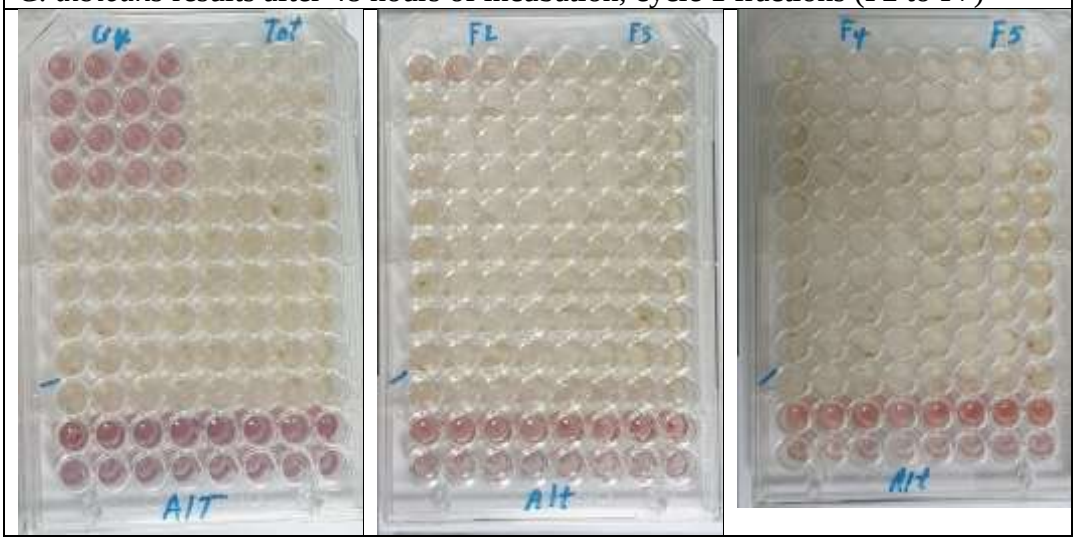
Appendix 14. 96-well plates results to determine the effectiveness of each of the FPLC fractions against representative microorganisms of bacteria, yeast and fungi.

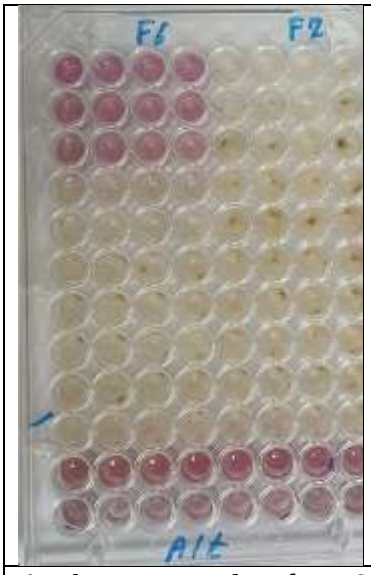


S. aureus results after 48 hours of incubation, cycle 1 fractions (F2 to F7)

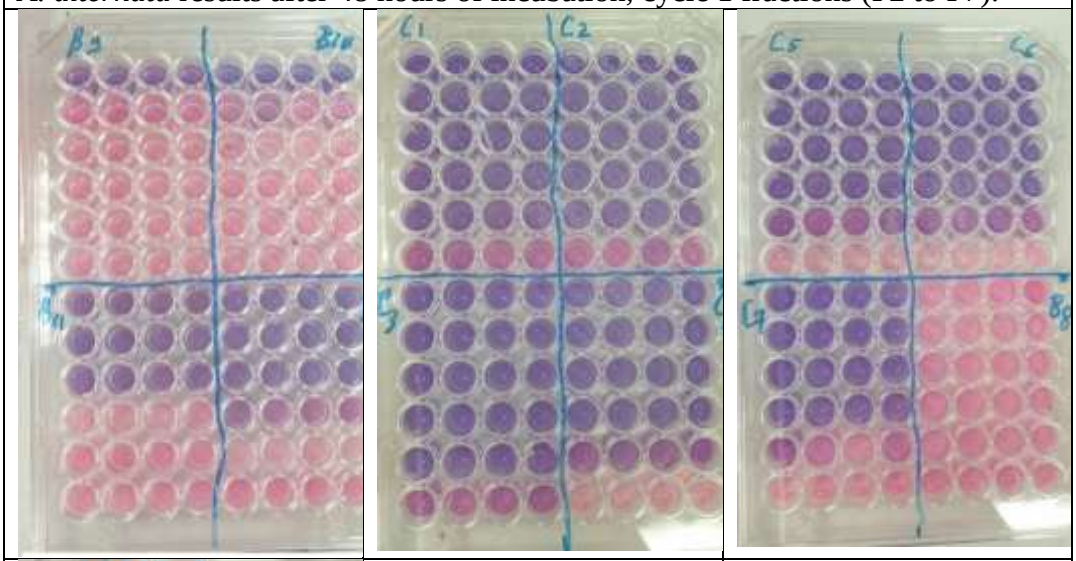


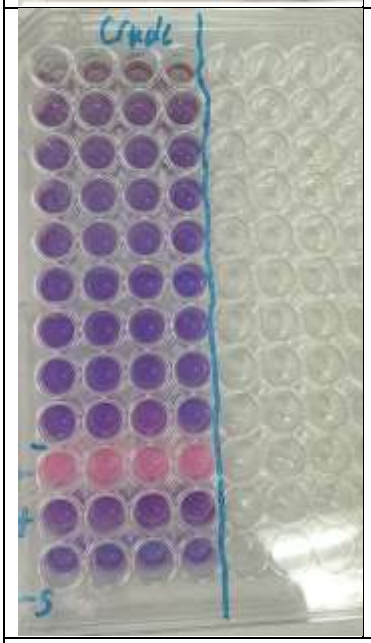
C. albicans results after 48 hours of incubation, cycle 1 fractions (F2 to F7)



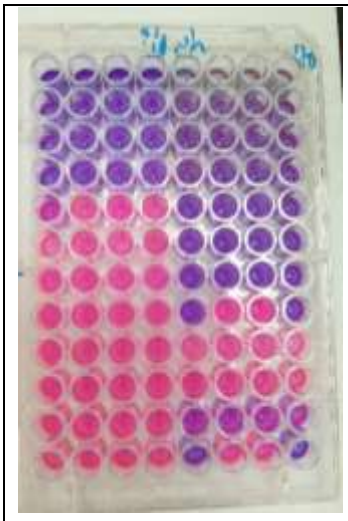


A. alternata results after 48 hours of incubation, cycle 1 fractions (F2 to F7).

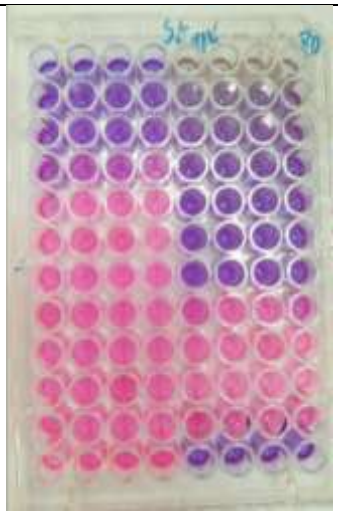




C. albicans results after 48 hours of incubation, cycle 2 fractions (B8 to C6).



a



b

Thermostability results, *S. aureus* results after 48 hours of incubation, F6 was pre-incubated at 70°C (a) and 80°C (b) for 24h before usage.