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Phytochemical profiling, antimicrobial, antibiofilm, insecticidal, and anti-leishmanial properties of aqueous extract from *Juglans regia* L. root bark: *In vitro* and *in silico* approaches

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ABSTRACT

The *Juglans regia* root aqueous extract (JRAE) phytochemical variability and the investigation of their antimicrobial, antibiofilm, insecticidal, and anti-leishmanial properties were studied. As a result, the JRAE chemical profile revealed the presence of biomolecules using UV, HPLC, and FT-IR analysis. The investigations showed marked antibacterial activities, highlighted inhibited biofilm formation, induced lethality *Leishmania amazonensis* promastigotes (50%), and possess an insecticidal activity at 100 µg/ml. A molecular docking study was performed against *S. aureus* gyrase, *S. aureus* tyrosyl-tRNA synthetase, human peroxiredoxin 5, cyclooxygenase-2, and trypanothione reductase and confirmed that the activities are mainly due to coumarin, epicatechin, juglone, epicatechin, and epicatechin presence, respectively. The stability of these complexes is essentially related to the strong hydrogen bonds formed between the most active compounds and the binding site amino acids. The identified compounds were found to pass the absorption, distribution, metabolism, and excretion property as well as five Lipinski's rules with good drug-likeness and pharmacokinetic behavior.

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Introduction

The recourse to higher plants and their preparations to cure infectious diseases is an age-old practice. Recently, different traditional medicinal plant extracts have been scientifically evaluated.^[1,2] It is widely recognized that plant therapeutic properties are due to their derivative

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bio-active compounds. Indeed, several studies have demonstrated that phenolic compounds are endowed with human health benefits.^[3,4] Currently, the new plant application search is of great interest, especially when it comes to the creation of natural plant protection projects for sustainable agriculture. Indeed, “Green Pesticides” concept refers to all types of natural tools, focused on beneficial pest control that can reduce pest population and increase food production. They are harmless, eco-friendly, and more compatible with environmental components than synthetic pesticides.^[5] Walnut tree (*Juglans regia*) is the most widespread tree nut in the world of great economic interest. The plant is a well-known plant species used in folk medicine.^[2] In Tunisia, *J. regia* is known as the “Swak” tree and its bark is frequently used for teeth cleaning and for good oral hygiene maintenance.^[6] It has been recognized that various *J. regia* plant parts are medicinally useful for a wide array of diseases including diarrhea, sinusitis, stomach ache, arthritis, asthma, eczema, scrofula, skin disorders, diabetes mellitus, anorexia, and thyroid dysfunction.^[7] Antifungal, antibacterial, anthelmintic, antiparasitic, antioxidant, antidepressant, anti-inflammatory, anticancer, and antihypoxic activities of different *J. regia* extracts were reported. Biological properties of walnut tree are related to its chemical composition^[2]; however, the use of herbal medicines must be evidenced by toxicological data.^[7]

To the best of our knowledge, there is no available study regarding the *J. regia* root bark potency. In this context, the aim of the present study was to characterize and to identify the presence of bioactive molecules in *J. regia* root bark using UV, HPLC, and FT-IR analysis. The *in vitro* antimicrobial, anti-biofilm, and antileishmania properties as well as *in vivo* insecticidal activity were also investigated. A computational study has also been done to corroborate the *in vitro* results.

Material and methods

Standards and reagents

Folin-Ciocalteu reagent, 3,5-dinitrosalicylic acid (DNS) reagent, ethanol, methanol, and hexane were purchased from Sigma-Aldrich, PubChem, and Fluka. All other used chemicals were of analytical grade and purchased locally.

Microorganisms

In this study, 11 reference bacterial strains including *Bacillus cereus* (ATCC 14,579), *Escherichia coli* (ATCC 35,218), *Enterococcus faecalis* (ATCC 29,212), *Salmonella enterica serovar Typhimurium* (ATCC 1408), *Staphylococcus aureus* (ATCC25923), *Staphylococcus epidermidis* CIP (106510), *Listeria monocytogenes* (ATCC 19,115), *Micrococcus luteus* NCIMB (8166), *Pseudomonas aeruginosa* (ATCC 27,853), *Vibrio alginolyticus* (ATCC 33787), and *Vibrio parahaemolyticus* (ATCC 1780) were used.

Plant material and extraction procedure

J. regia fresh root bark was collected from different trees in the region of Fernana (North of Tunisia) (**Latitude:** 36 38' 59.99"N, **Longitude:** 8 41' 59.99" E) in March 2015. The species were identified and deposited at the herbarium of the Faculty of Sciences of Gafsa. Samples were dried at room temperature and separately ground into powder and then stored in a smoked bottle at 4°C. The plant extract was prepared by infusing 20 g of the powdered healthy and dried material in 200 mL distilled water for 48 h, and the obtained solution was filtered (Whatman no.1) and then lyophilized.^[8] The extraction yield (X_0) was calculated as the percentage of mass of extract (m_{Extract}) relative to total mass of raw material (RM), on wet basis (m_{RM}), according to Equation:

$$X_0(\%) = (m_{\text{Extract}}/m_{\text{RM}}) * 100$$

Phytochemical screening

Qualitative analysis of secondary metabolites of the plant extract

The qualitative analysis of powdered extract was carried out with reference to phytochemical testing for the presence of various compounds by standard methods like alkaloids, glycosides, flavonoids, anthocyanins, tannins, phenolic compounds, coumarins, saponins, anthraquinone, quinones, proteins, reducing compounds, inulins, starch, resins, organic acids, carboxylic acid, oxalates, terpenoids, steroids, and sterols.^[9–11]

Phenolic compounds analysis

The total phenol content was estimated using the method described by Kassim et al. (2011).^[12] Briefly, 200 μl of aqueous extract was mixed with 1 ml Folin-Ciocalteu reagent. Then, after 5 min in the dark, 0.8 ml of 7% Na_2CO_3 was added. The absorbance was read at 760 nm after 30 min in darkness.

The total flavonoid content was estimated using the method of Kassim et al. (2011).^[12] To 500 μl of aqueous, 1500 μl of distilled water was added. At $t = 0$, 75 μl of 7% NaNO_2 was added. After 5 min 75 μl of 10% AlCl_3 was added and at 6 min 500 μl of NaOH (1 N) and 250 μl of distilled water were added. After incubation for 15 min at room temperature ($23 \pm 2^\circ\text{C}$), the absorbance was measured at 510 nm. Total phenolic and flavonoid compounds were calculated using a gallic acid and quercetin standard curve (concentration range: 50–200 $\mu\text{g}/\text{ml}$) and results were shown to be mg gallic acid or quercetin equivalents/g dry matter.

The flavonoid levels were determined using the method of Almaraz-Abarca et al. (2007).^[13] Rutin calibration curve (100–750 $\mu\text{g}/\text{ml}$) was used, and results were shown to be mg rutin equivalent/100 g of dry matter. The anthocyanin quantification was performed using the pH differential method^[14] based on the anthocyanin color changes depending on pH. Each sample absorbance was measured at 520 nm and 700 nm. The anthocyanin concentration (mg/l) was calculated according to the following formula and expressed as Cyanidin-3-glucoside equivalents:

$$A^* MW^* DF^* 1000 / \epsilon^* d$$

With: **A** is the absorbance = $(A \lambda_{520} - A \lambda_{700}) \text{pH} = 1 - (A \lambda_{520} - A \lambda_{700}) \text{pH} = 4.5$

Where **MW** is molecular weight of cyanidin 3-glucoside = 449.2 g/mol, **DF** is the dilution factor (DF = 10), **E** is the extinction coefficient of cyanidin 3-glucoside = 26 900 $\text{L mol}^{-1} \text{cm}^{-1}$, **d** = 1 cm: length of the cell.

Biochemical composition: Total carbohydrates were obtained using the DNS method.^[15] Total protein and nitrogen content was determined using the Kjeldahl method.^[16]

UV-Vis absorption spectrum: The ultraviolet-visible absorption spectrum of *J. regia* root bark aqueous extract was recorded by a UV-Vis spectro-photometer (JENWAY/7315, United Kingdom) with the wavelength range of 200 to 800 nm at 25 $^\circ\text{C}$.

Fourier-transform infrared spectroscopy (FT-IR) analysis: Sample was recorded by FTIR spectroscopy (VERTEX 70 FTIR). The sample was analyzed with an ATR A225 diamante using the middle infrared range (400–4000 cm^{-1}). The obtained data were recorded with OPUS 7.2 software.

HPLC analysis: The quantitative and qualitative analyses of the plant extract were performed by a reverse-phase HPLC on a liquid chromatography (Shimadzu, Kyoto, Japan) equipped with a vacuum degasser (DGU-14A), a quaternary pump LC-10AT, a UV-Vis detector ((DAD) SPD-M20A), a manual injector (Rheodyne), and an LC-solution (CBM-20A) software for acquisition and processing of chromatogram. The separation was performed on a C_{18} column (Shimadzu CLC-ODS (M) Kyoto, Japan: 4.6 mm \times 250 in length; 5 microns internal diameter) preceded by a guard column (Shimadzu G-ODSs (4), Kyoto, Japan: 4.6 mm \times 12.5 mm). The injection volume was 20 μL . Organic acids are eluted with a gradient of phosphoric acid (0.1%) and methanol. During this analysis, the gradient increases linearly from 0 to 5% methanol for 10 min. After the next 10 min, the gradient will

increase to 30% methanol, then up to 50% after the other 10 min. This state will last only 5 min and will end with the restoration of the initial situation following the decrease in the gradient from 50% to 10% of methanol. The last 5 min was used to recondition the column. The flow rate was 1.0 ml/min. The compound identification was done by comparing their retention times and the UV–Vis spectra to standard solutions (gallic acid, juglone, catechin, vanillic acid, epicatechin, and coumarin) used in the same conditions.

Antimicrobial activity assessment

MIC and MBC determination: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were carried.^[10] Briefly, 0–512 µg/mL of *J. regia* plant extract diluted in Mueller-Hinton medium were added to a final total volume of 100 µL per well in 96-well microtiter plates. Each tested strain (100 µL), at a final concentration of 10⁶ CFU/mL, was added to each well and incubated at 37 °C. The medium and chlorhexidine (CHX, 0.1% (w/v)) were used as the non-treated and positive controls, respectively. MIC was defined as the lowest concentration of the extract that completely inhibited growth in comparison with the non-treated control. MBC was defined as the lowest concentration that did not allow visible growth when 10 µL of the well contents were plated on agar and grown at 37 °C. All experiments were repeated thrice in duplicate.

Inhibition of the intestinal flora: The intestinal bacterial flora isolation from the fecal matter contained in the Wistar male rats large intestine, treated with 200 mg/kg of the extract, was performed. Three separate culture media named Hektoen,^[17] EMB,^[18] and Chapman^[19] were used.

Antibiofilm assay

The *J. regia* aqueous extract (100 µg/mL) effect on biofilm formation of each representative pathogenic bacterium was tested in ELISA plate (96-well).^[20] Briefly, two-fold plant extract serial dilutions were prepared with a final concentration of 100 µg/mL. A cell suspension of the tested strains was prepared as described in the MIC assay, and 100 µL (10⁶ CFU/mL) were inoculated in each well of a 96-well plate. A 0.1% CHX, phosphate buffered saline (PBS) and extract free medium (Mueller-Hinton medium supplemented with 2% glucose (w/v)) were used as the positive, non-treated and blank controls, respectively. After incubation at 37 °C for 48 h, supernatants were discarded and washed 3 times with PBS and were dried at room temperature for 45 min. The cell adhesion rate was indirectly evaluated using the crystal violet staining.^[20]

Antileishmania activity assay

The plant extract, dissolved in RPMI medium (20, 40, 60, 80, and 100 mg/mL), was distributed into the tubes containing each 400,000 parasites. The procedure was performed in triplicate for each tested concentration. The used strain was obtained from a patient's single lesion of cutaneous leishmaniasis and was identified as *Leishmania amazonensis* following its speciation using 12 isoenzymes.^[21] In the three control tubes, 1 mL of the complete RPMI medium was added. The *Leishmania* number was determined in each tube immediately using a hemocytometer. A parasite recount was performed in each tube after 48 h of incubation at 24–26°C in dark. Results were displayed as the concentration that inhibited parasite growth by 50% (IC₅₀) compared with control tubes.^[22]

Insecticidal activity

Bioassay. The larvicidal activity of *J. regia* aqueous extract against neonatal *S. littoralis* larvae was assessed by dose-mortality bioassays.^[23] Mortality was recorded after 6 days, and the LC₅₀ was calculated by Probit analysis using programs written in the R. language.^[24]

The histopathological effect of *J. Regia* extract in the midgut of *S. littoralis*: The histopathological effect of *J. regia* aqueous extract on *S. littoralis* midgut larvae was determined.^[25] The images were observed and photographed using a light microscope (Olympus Optical Co. LTD) operating at Olympus DP70 camera.

Docking studies

The organic extract contains some of the known bioactive compounds such as gallic acid (GA), juglone, catechin, vanillic acid (VA), coumarin, and epicatechin. The extract displays some potent antibacterial, antioxidant, anti-inflammatory, and anti-leishmania activities. To identify the most bioactive compounds for each biological activity, molecular docking studies have been performed. The bioactive compounds were docked into the active sites of *S. aureus* gyrase (PDB code 2 × CT) and *S. aureus* tyrosyl-tRNA synthetase (PDB code 1JIJ) for antibacterial activity,^[26,27] human peroxiredoxin 5 (PDB code 1HD2) for antioxidant activity,^[28] cyclooxygenase-2 (PDB code 1C × 2) for anti-inflammatory activities,^[29] and trypanothione reductase (TR) from *Leishmania infantum* (PDB code 2JK6) for anti-leishmania.^[30] Molecular docking calculations were performed using the Autodock package.^[31] The re-docking of the original ligands into the binding sites of *S. aureus* gyrase (PDB code 2 × CT) and *S. aureus* tyrosyl-tRNA synthetase (PDB code 1JIJ), human peroxiredoxin 5 (PDB code 1HD2), cyclooxygenase-2 (PDB code 1C × 2) for anti-inflammatory activities, trypanothione reductase (TR) (PDB code 2JK6) is well reproduced with RMSD values less than 2.00Å. The stepwise molecular docking study is reported in our previous study.^[32]

ADME and molecular target predictions

Pharmacokinetics and druglikeness are crucial steps in drug design.^[33] The investigation of the identified bioactive molecules was performed through the freely available online Swiss ADME web tool.^[32,34] Toxicity predictions were assessed using the pkCSM server (<http://biosig.unimelb.edu.au/pkcsml/prediction>). Molecular target predictions are important for finding phenotypic side effects or potential cross reactivity caused by the action of small biomolecules and were obtained by using the web tool (<http://www.swisstargetprediction.ch/>) and entering the smile formats of the desired drugs to obtain the targets. The prediction concerns the putative targets of the given molecule by utilizing 2D and 3D similarity index with known ligands.

Statistics

Statistical analysis was performed using GraphPad Prism Software (version GraphPad Prism 7.0, Northampton, MA, USA) using one-way analysis of variance (ANOVA) followed by the Tukey's test. The differences were considered significant at $p < .05$.

Results and discussion

Extraction yield

The use of different polarity solvents is highly recommended to extract various phyto-phenolic compounds with high degree of accuracy. In addition, due to their diverse chemical properties influencing the solvent solubility, no particular solvent is suggested for the optimum recuperation of the bioantioxidants. In fact, the extract obtained from water exhibited an antioxidant better capacity with absence of toxicity.^[35] Results showed that the obtained yield of *Juglans regia* was 11.3%, which is higher than other plants from several families such as *Zygophyllum album* roots (6.10%).^[36] The fluctuations between values could be attributed to genetic variability of these plants, which affect the synthesis of bioactive metabolites.^[8]

Phytochemical qualitative analysis

Nature has been a huge source of medicinal agent for thousands of years, and an impressive number of modern drugs have been isolated from these sources.^[37] Plants have the ability to produce a large variety of secondary metabolites. In the present study, qualitative analysis of *J. regia* root bark aqueous extract shows the high content presence of flavonoids, tannins,

Table 1. Qualitative phytochemical tests of the JRRAE.

Phytochemical tests		Results	
Secondary metabolites	Reagents	<i>J. regia</i> extract	Coloration
Alkaloids	Mayer	+	White precipitate
	Wagner	+	Brown precipitate
Flavonoids	Mg ²⁺	+++	Pink or red or yellow
Phenol	FeCl ₃	+++	Dark green color
Triterpenes	Liebermann–Burchard test	+	Purplish red
Sterols	Salkowski test	-	Red
Steroids	Chloroform/concentrated sulfuric acid	-	The upper layer: red Sulfuric acid layer: yellow with green fluorescence
Coumarins	UV at 366 nm	+	Intense fluorescence
Resins	CuSO ₄	+	Green color precipitate
Tannins	FeCl ₃	+++	Blackish blue
Quinones	Alc. KOH	+	Color ranging from red to blue
Carboxylic acid	NaHCO ₃	-	Effervescence
Oxylates	Acetic acid	++	Brown ring at interface
Reducing compounds	Fehling's solution	+++	Red brick precipitate
Inulins	Fehling's solution	+++	Black precipitate
Proteins	Ninhydrin	+++	Blue color
Free-antraquinones	Borntrager's reaction	++	Red
Saponins	Froth test	+	Height > 1 cm of froth
Organic acids	Bromothymol Blue	+	Yellow canary
Starch	Iodine (I ₂)	+	Blue
Anthocyanins	HCl + NH ₄ OH	+	Pink-red/purplish blue
Condensed tannins	HCl	-	Red

(+): presence, (++): abundance, (-): absence

anthraquinones, oxalates, phenols, proteins, inulins, and reducing sugar with lower levels of saponins, terpenoids, alkaloids, coumarins, resins, quinones, organic acids, starch, and anthocyanins (Table 1). These molecules are reported to have various physiological effects and medicinal properties. Notably, saponins are often used as intracellular histochemical dyes and as mild detergents.^[38] Terpenoids and flavonoids are used to treat inflammation, allergy, oxidative stress, and cancers. Further, the presence of glycosides enhances cardiac output and lowers heart diseases.^[11]

Phenolic compounds contents in *J. regia* extract

Phenolic compounds are the main predominant bioactive molecule in plants. In the current study, a preliminary screening was carried out (Table 1) then dosages, using a spectrophotometer, were done for more detailed analyses (Table 2). The total phenolic compound content of the *J. regia* root bark aqueous extract was estimated to 668.38 ± 42.21 mg gallic acid equivalent (GAE) per 100 g of dry weight (DW), which is richer in polyphenols than the previously published content of an ethyl acetate extract 3483.3 mg GAE/100 g DW.^[6] The differences are attributed to many factors that could modify the root composition, including the climate conditions, origin, ripeness of the samples, and^[38] as well as extraction methods.^[6]

The total flavonoid, flavonol, and anthocyanin contents were 267.75 ± 12.31 mg QE/100 g DW, 68 ± 3.23 mg of RE/100 g DW, and 29 ± 1.45 mg/L, respectively. The obtained results confirmed that *J. regia* is an important potential source of these bioantioxidants with values even greater than those reported for many medicinal species. Chromatographic analysis (Figure 1b) revealed the presence of several identified compounds, among them gallic acid, vanillic acid were the pre-dominant components. Flavonoids such as catechin and epicatechin have also been detected. UV-VIS spectra of JRRAE. UV-VIS spectra of JRRAE, HPLC chromatogram of JRRAE: (1) gallic acid, (2) juglone, (3) catechin, (4) vanillic acid, (5) epicatechin, (6) coumarin. IR spectrum of JRRAE. IR spectrum of JRRAE.

Table 2. Physicochemical characteristics of JRRAE.

Characteristics	Amounts
Yield (%)	11.3
Total phenols (mg of GAE/100 g DW)	668.38 ± 42.21
Flavonoids (mg QE/100 g DW)	267.75 ± 12.31 mg/l
Flavonols (mg RE/100 g DW)	68 ± 3.23
Anthocyanins (mg/L)	29 ± 1.45
Proteins (mg/mL)	31.39 ± 1.173
Reducing sugars (g/L)	17.986 ± 1.318

The obtained results are in agreement with the qualitative screening results as well as with literature data^[4,7] which reported the clear occurrence of phenolic compounds in *J. regia* peculiarly phenolic acids and flavonoids. In the literature, there were no reports of individual phenolic compounds in *J. regia* water extract, but previous studies on other species suggested that phenolic compound composition can fluctuate between locations and solvent extract.^[39] It was worth noting that many peaks remained unidentified and their identification can offer numerous information about this species. The presence of all these beneficial and promising compounds in *J. regia* water extract would be of great importance to the pharmaceutical and food industries.

Sugar and protein content

To the best of our knowledge, this was the first reports about the phytochemical content (proteins and sugars) of *J. regia* root bark. Results in Table 1 shows that the samples were rich with protein (ca. 31.39 ± 1.173 mg/mL) and contained important level of reducing sugars (17.986 ± 13.18 mg/mL).

The protein levels and carbohydrate content found here were lower than those reported from other species roots. In fact, the maturation stage, temperature, sun exposure, and climatic conditions played a major role in the plant protein content.^[40] Indeed, proteins, as an essential component of the diet of humans and other mammals, from unconventional plants should be explored and used as a raw material source for different population sectors.^[41] Furthermore, sugar content offer to *J. regia* an industrial importance. Recently, research has increased to investigate new sources of vegetable sugar from underexploited plants.^[2]

Spectral analysis of the aqueous extract of *J. regia* root bark

Ultraviolet absorption (UV) spectrum: As shown in Figure 1a, aqueous extract of *J. regia* root bark showed an absorption at 265 nm in the UV absorption spectra, suggesting the possible presence of a multitude of compounds such as phenolic compounds, mainly flavonoids (flavonols and their glycosyl derivatives), and at 500 nm that show the presence of anthocyanin, offering the natural red color.^[42] In accordance with data reported by Nour et al. (2014), a large absorbance peak at 500 nm was observed, which is a characteristic of naphthoquinones, namely juglone (5-hydroxy-1,4-naphthoquinone) which is unique to walnut.^[4] This latter shows a significant absorption in both the visible and UV domains with $\lambda_{\max} = 420 \text{ nm}$ ^[43] and $\lambda_{\max} = 255 \text{ nm}$,^[44] respectively. Also, a small peak observed in 280 nm and 375 nm is attributed to a trace of catechin and quercetin, respectively. This result was confirmed as previously reported.^[44]

Fourier infrared spectroscopy analysis (FT-IR)

The FTIR spectrum elucidates the major functional groups of polymers and determines their structural information. The FT-IR spectrum of the *J. regia* root bark aqueous extract is shown in Figure 1b and reveals a specific peak between 500 and 1500 cm^{-1} for phenolic and aromatic compounds. Peak at 1449 cm^{-1} indicates the presence of C = C bond of aromatic compound. The observed band at about 3300 cm^{-1} corresponds to O-H, as well as the H-bonded alcohols and phenols.^[12] Absorption of

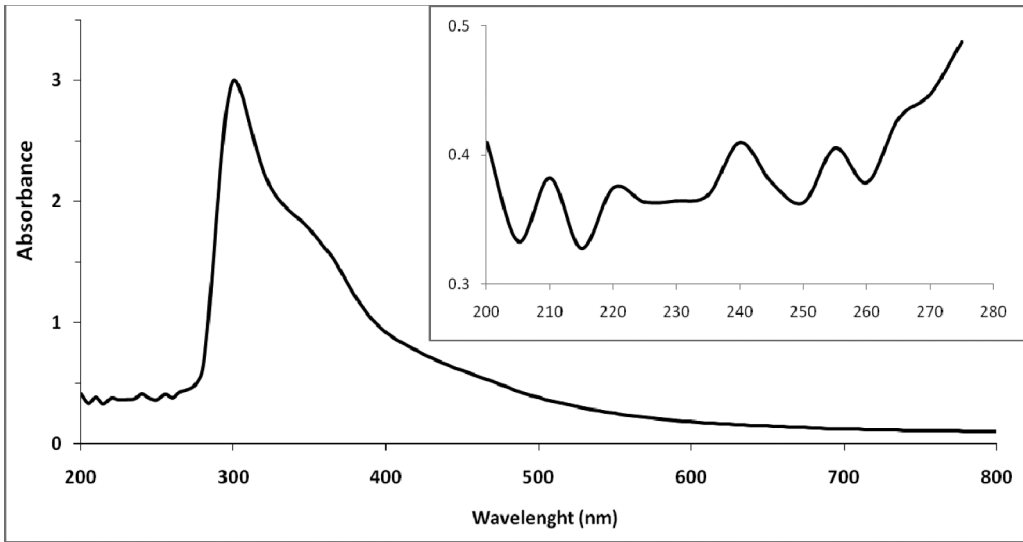


Figure 1a. UV-VIS spectra of JRRAE.

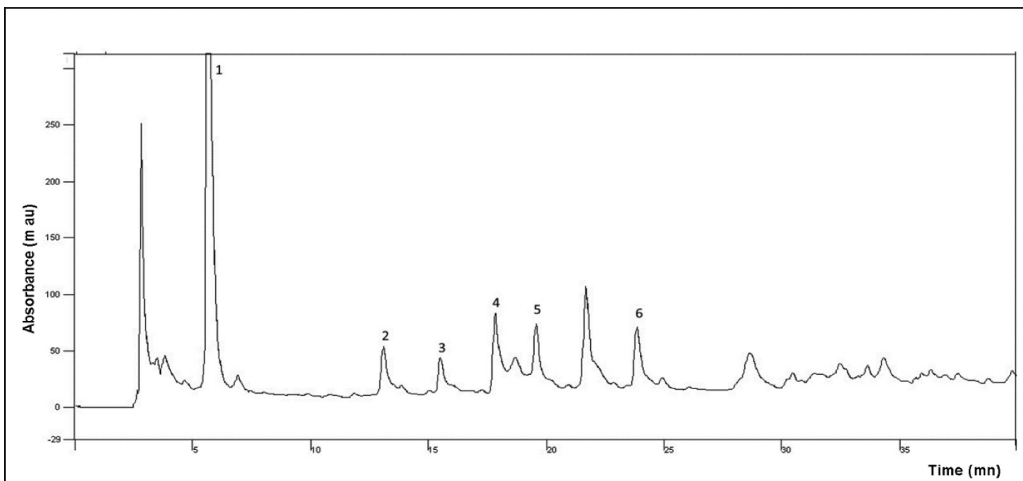


Figure 1b. UV-VIS spectra of JRRAE, HPLC chromatogram of JRRAE: (1) gallic acid, (2) juglone, (3) catechin, (4) vanillic acid, (5) epicatechin, (6) coumarin. IR spectrum of JRRAE.

C-H band of CH_2 and CH_3 groups and O-H band of carboxylic acids were observed near 2943 cm^{-1} . The absorption peaks at 1016 , 1115 , and 1410 cm^{-1} testify the C-O stretching of carboxylic acids, alcohols, and esters. Additionally, a peak at 1652 cm^{-1} revealed a characteristic band for the carbonyl group $\text{C}=\text{O}$.^[45] Another one at 2833 cm^{-1} has been observed due to the conjugation of $\text{C}=\text{C}$ and $\text{C}=\text{O}$ of carboxylic acid. These results are in agreement with the UV-Vis spectrum as well as with the HPLC spectrum.

HPLC analysis: The HPLC spectrum of the aqueous extract of *J. regia* root bark is shown in Figure 1b. Phytochemicals were separated and detected at 280 nm for catechin (+), epicatechin (-), tyrosol, and hydroxybenzoic acids,^[46] at 320 nm for *p*-coumaric, caffeic, caftaric, and ferulic acids, at 306 nm for

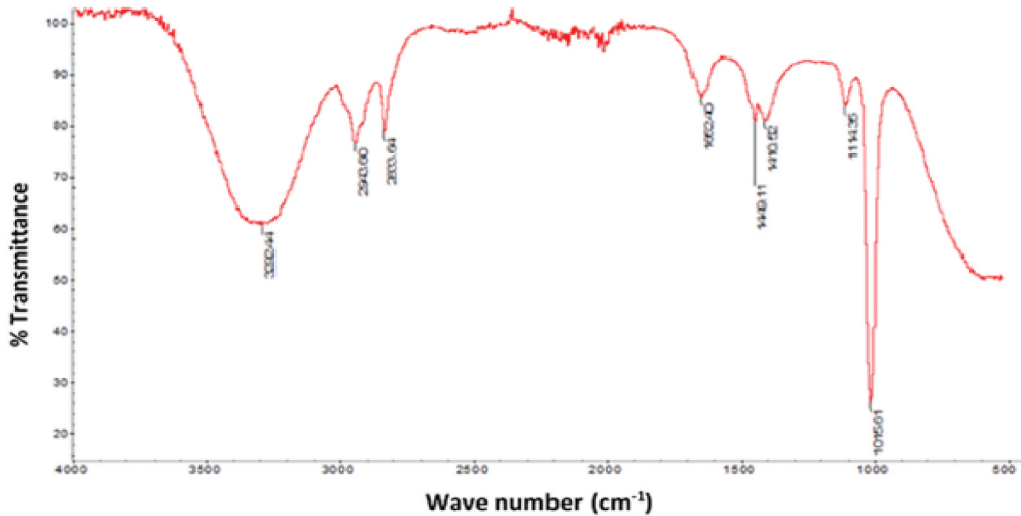


Figure 1c. IR spectrum of JRRAE.

trans-resveratrol, at 360 nm for myricetin, quercetin, and kaempferol, and at 420 nm for juglone [43]. Indeed, the chromatogram shows (Figure 1b) the presence of phenolic compounds and their derivatives, namely gallic acid and vanillic acid. Flavonoids such as catechin and epicatechin have also been detected. The areas of the obtained peaks reveal a difference between the phytochemical amounts. This clearly demonstrates that phenols amounts depend on organ age, maturation state, and environment.^[44] Interestingly, juglone and coumarin were detected in the aqueous extract of *J. regia* root bark. The obtained results are in agreement with previous UV-visible spectrum and qualitative screening results as well as with literature data^[4,7] which reported the occurrence of phenolic compounds in *J. regia* peculiarly phenolic acids and flavonoids. Nevertheless, other compounds have been detected, but we could not identify them for lack of standard solutions.

Antimicrobial activity assessment

Evaluation of MIC and MBC using the microdilution method

This study was conducted on 11 reference strains, and results are shown in Table 3. According to our findings, there is a series of fluctuations in MIC and MBC values varying with bacteria species. *Staphylococcus aureus* is the most sensitive strain toward the aqueous extract of *J. regia* root bark

Table 3. Antibacterial activity and effect of JRRAE on pathogenic bacterial adhesion evaluated by the crystal violet test.

	Extract ($\mu\text{g/mL}$)		Extract (100 $\mu\text{g/mL}$) Inhibition biofilm (%)
	MIC	MBC	
<i>Bacillus cereus</i> ATCC 14,579	32	64	25
<i>Escherichia coli</i> ATCC 35,218	256	512	60
<i>Enterococcus faecalis</i> ATCC 29,212	128	256	37
<i>Salmonella enterica</i> serovar typhimurium ATCC 1408	256	512	40
<i>Staphylococcus aureus</i> ATCC 25,923	8	16	23
<i>Staphylococcus epidermidis</i> CIP 106510	64	128	55
<i>Listeria monocytogenes</i> ATCC 19,115	16	32	30
<i>Micrococcus luteus</i> NCIMB 8166	8	16	75
<i>Pseudomonas aeruginosa</i> ATCC 27,853	128	>128	80
<i>Vibrio alginolyticus</i> ATCC 33,787	256	512	90
<i>Vibrio parahaemolyticus</i> ATCC 1780	512	>512	87

Table 4. Bacterial count in the small intestine and feces in control and treated rats for 10 days by JRRAE at 200 mg/kg.

	Bacteria in the small intestine of rats/cm ²		Bacteria/gram of feces in the large intestine	
	Control	Treated	Control	Treated
Hectoen medium	99 × 10 ³ ± 0.816	0	95.6 × 10 ³ ± 0.816	0
EMB medium	98 × 10 ⁶ ± 3.949	9,994 × 10 ⁶ ± 8.256	9.1 × 10 ⁶ ± 0.983	9,994 × 10 ⁶ ± 5.683
Chapman medium	0	0	993 ± 3.911	0

with MIC and MBC of 8 µg/mL and 16 µg/mL, respectively. In contrast, *the Vibrio* species are the most resistant with MICs and MBCs ranging from 256 to 512 µg/mL, respectively. This fluctuation can probably be linked to the unconformity of the cell wall and membrane structure and composition. Indeed, Gram-negative bacteria have an outer membrane that serves as a barrier for many molecules. This antimicrobial action of the bark extract may be more pronounced on Gram-positive than Gram-negative bacteria was also confirmed.^[47] Moreover, the presence of an efflux pump system has been established and can generate the resistance to natural compounds.^[1] Broadly, our results are in agreement with previous studies showing the potent antimicrobial effect of *J. regia* is mainly attributed to the presence of numerous flavonoids, which act essentially by enzymatic inhibition of DNA gyrase,^[11,48] juglone,^[7,43,49] alkaloids,^[50] and saponins^[11] as bioactive antibacterial agents. Additionally, *J. regia* water extract showed an antibacterial activity similar to that of chlorhexidine.

Inhibition of the intestinal flora

Results of bacterial enumerations in intestinal flora are presented in Table 4. As expected, the extract showed an inhibitory effect against bacteria of the intestinal flora, proving once again the antibacterial activity of *J. regia* root bark water extract. This is in agreement with our previous MIC and MBC described results. Additionally, we noticed that inhibition of some bacteria by the extract has favored the development of others. *Enterococci* enumerated on Hektoen selective medium in the small intestine of control rats were 99 10³ ± 0.816 bacteria/cm², whereas they were totally absent in the treated rats. Moreover, the number of *Enterobacteriaceae* on EMB medium increased in the small intestine of control rats from 98 10⁶ ± 3.949 bacteria/cm² to 9,994.10⁶ ± 8.256 bacteria/cm² in treated rats. Similar observations were made for fecal matter in the large intestine. However, *Staphylococci* were absent in the small intestine of treated rats as well as controls. The aqueous extract causes the total disappearance of *Staphylococci* in the large intestine fecal matter of treated rats. The sensibility of *Staphylococci* was demonstrated again by Alkhawajah (1997).^[47] Another time, the antibacterial activity may be attributed to the presence of numerous molecules such as flavonoids, juglone,^[7,43] alkaloids,^[50] and saponins^[11] as bioactive antibacterial agents.

Antibiofilm activity assessment

Meanwhile, medicinal herbs have proven their valuable biological properties. Indeed, plant-derived compounds could influence biofilm formation by inhibiting peptidoglycan synthesis, destroying microbial membrane structures, and modulating quorum sensing.^[1] Antibiofilm activity of *J. regia* extract was carried out on 11 referenced strains, and the results are shown in Table 3. According to our findings, 100 µg/mL of extract induced a biofilm formation inhibition as well as an elimination of the preformed biofilm. Inhibition is evaluated by bacterial biomass decrease compared to control after crystal violet staining. The aqueous extract showed varying biofilm inhibition percentages ranging from 23% for *Staphylococcus aureus* ATCC 25,923 to 90% for *Vibrio alginolyticus* ATCC 33,787. Furthermore, the biofilm proportion varies considerably according to the environmental conditions^[1] and especially according to the physicochemical properties of the involved microorganisms. Indeed, suspension medium characteristics, such as pH, concentration, and temperature, are also considered as important factors that can influence the physicochemical properties of the substrate and bacterial surfaces^[1] and consequently microbial adhesion and biofilm formation. We can conclude from the

given study that *J. regia* extract effectively penetrates into biofilms and causes lyses of test microorganisms. Previous reports confirmed the influence of *J. regia* on biofilm formation inhibition. In fact, *J. regia* extracts inhibit bacterial adhesion only by inhibition of glucan synthesis.^[48] Despite numerous reports that focused on the antimicrobial properties of plant extracts and essential oils, few studies have reported the antibiofilm activities of these compounds. Isolation and identification of effective compounds might be crucial to include them as alternatives in the control of biofilms, peculiarly in the medical field as well as in the food industries.^[20]

Antileishmania activity assay

The present study provides data on the effectiveness of the aqueous extract of *J. regia* root bark against *Leishmania amazonensis*. Results reveal that at 100 mg/mL, the extract has a potential effect on parasite viability and induced lethality to 50% of promastigotes. However, few studies were conducted on walnut leaf antileishmanial activity. To the best of our knowledge, this is the first report on the antileishmanial activities of *J. regia* root bark aqueous extract. Serakta et al. (2013) reported a significant reduction of the promastigote from *Leishmania major* in the presence of *J. regia* hydroalcoholic bark extract.^[51] Moreover, the ointment-based *J. regia* leaf extract induced a significant post-treatment decrease in the lesion size and parasite count of *Leishmania major* in infected animals, compared to control groups.^[52] These data are in agreement with our findings, which pave the way for further research in leishmaniasis curing.

It is important to mention that several factors such as drug toxicity, method of administration, and resistance of *Leishmania* spp to conventional remedies limit the leishmaniasis treatment.^[52] In fact, *J. regia* is frequently used for the management of parasitic diseases in central Italy and Morocco.^[51] Hence, this work proved that the aqueous extract of *J. regia* showed a promising anti-leishmanial activity; however, further investigations are needed to determine the mode of action of the bioactive compounds, which are behind the leishmanicidal activity.

Insecticidal activity

The bioassay of *J. regia* root bark aqueous extract was performed against the first instar larvae of *S. littoralis*. The obtained results showed that the plant extract exhibited insecticidal activity against *S. littoralis* larvae with an LC_{50} of 130.99 $\mu\text{g}/\text{cm}^2$. Larvae exposure to water, used as negative control, did not cause any mortality. In order to visualize the effect of the extract ingested by the larvae, histological sections were made in the midgut of the treated *S. littoralis* larvae. As shown in [Figure 2b](#), the *J. regia* extract caused enlargement of epithelial cells, cytoplasmic vacuolization, appearance of vesicles at the apical part of the cells toward the midgut lumen and disruption of the basement membrane. In contrast, the midgut section of the control larvae showed usual structural organization with a well-developed brush border, a clear cytoplasm, and a normal adhesive basement membrane ([Figure 2a](#)). This result is a practical point of view, since *S. littoralis* is one of the most serious agricultural pests that can attack numerous economically important crops and cause considerable damage on vegetables throughout the year in Africa, Asia, and Europe.^[53] To our knowledge, only acaricidal activity of *J. regia* leaves extracts was reported.^[2] Consequently, we report for the first time the insecticidal activity of *J. regia* root bark aqueous extract showing its potential for use as a biocontrol agent against lepidopteran pests. Delaviz reported that the juglone from leaves, extracted from *Juglans* sp., is associated with the insecticidal activity.^[49]

Molecular docking

As mentioned in materials and methods, the organic extracts display potent antibacterial, antioxidant, anti-inflammatory, and anti-leishmania activities. The organic extract contains some bioactive compounds. In attempt to identify the most active compound for each biological activity, molecular

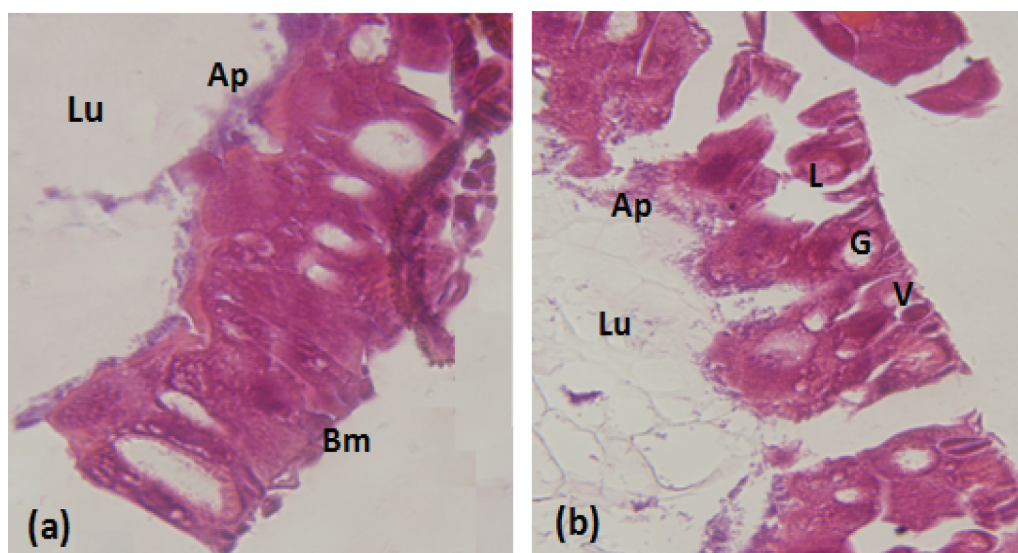


Figure 2. Histopathological effect of the extract on the intestine of *S. littoralis* larvae (a): Normal appearance. (b): Histopathological aspect of the intestine of *S. littoralis* treated larvae. **V**: vesicles formation **L**: Lysis of epithelial cells. **G**: Large vacuolation of epithelial cells. **Lu**: lumen. **Ap**: apical membrane. **Bm**: basal membrane. (Magnification $\times 40$).

docking study has been performed to determine the binding affinity of the bioactive compounds gallic acid (GA), juglone, catechin, vanillic acid (VA), coumarin, and epicatechin into the active sites of *S. aureus* gyrase (PDB code 2 \times CT) and *S. aureus* tyrosyl-tRNA synthetase (PDB code 1JII), human peroxiredoxin 5 (PDB code 1HD2), cyclooxygenase-2 (PDB code 1C \times 2) for anti-inflammatory activities, trypanothione reductase (TR) (PDB code 2JK6). **Table 5** gathers the calculated binding energies of stable complexes, ligand–receptor, number of conventional intermolecular hydrogen bonding established between the docked compounds and the active site residues of target enzymes.

All the bioactive compounds formed stable complexes into the binding sites of *S. aureus* Gyrase (pdb 2 \times CT), *S. aureus* tyrosyl-tRNA synthetase (pdb 1JII), human peroxiredoxin 5 (pdb 1HD2), cyclooxygenase-2 (pdb 1C \times 2), and trypanothione reductase (pdb 2JK6). The negative bending energies indicate that the inhibition is spontaneous and thermodynamically favorable. From the binding energies, one may easily deduce that the potency of the enzymatic inhibition of the bioactive compounds depends also on the target enzyme. For instance, the most stable ligand-receptor for *S. aureus* Gyrase (pdb 2 \times CT), *S. aureus* tyrosyl-tRNA synthetase (pdb 1JII), human peroxiredoxin 5 (pdb 1HD2), cyclooxygenase-2 (pdb 1C \times 2), and trypanothione reductase (pdb 2JK6) are obtained coumarin, epicatechin, juglone, epicatechin, and epicatechin with binding energies of -3.32 , -7.74 , -5.23 , -7.97 , and -8.43 kcal mol $^{-1}$, respectively (**Table 5** and **Figure 3**). Thus, one may conclude that the activity of the extract against *S. aureus* Gyrase (pdb 2 \times CT), *S. aureus* tyrosyl-tRNA synthetase (pdb 1JII), human peroxiredoxin 5 (pdb 1HD2), cyclooxygenase-2 (pdb 1C \times 2), and trypanothione reductase (pdb 2JK6) may be due to the presence of coumarin, epicatechin, juglone, epicatechin, and epicatechin, respectively. The stability of these complexes is mainly related to the strong hydrogen bonds formed between the most active compounds and the binding site amino acids of *S. aureus* Gyrase (pdb 2 \times CT), *S. aureus* tyrosyl-tRNA synthetase (pdb 1JII), human peroxiredoxin 5 (pdb 1HD2), cyclooxygenase-2 (pdb 1C \times 2), and trypanothione reductase (pdb 2JK6) (**Figure 3**).

Pharmacokinetic and druglikeness properties

In order to explore the drug likeliness of the most promising compounds and reduce the risk of drug attrition in late stage, the ADME property profiling of the identified compounds was investigated using SwissADME software. As outlined in **Table 6**, all compounds satisfy the Lipinski's rule of five,

Table 5. Docking binding energies, conventional hydrogen bonding, and the number of closest residues to the docked compounds into the active sites of *S. aureus* Gyrase (pdb 2 × CT), *S. aureus* tyrosyl-tRNA synthetase (pdb 1JJ), human peroxiredoxin 5 (pdb 1HD2), cyclooxygenase-2 (pdb 1C × 2), and trypanothione reductase (pdb 2JK6).

Name of compound	Binding energy (kcal/mol)	H-bonds (HBs)	Number of closest residues to the docked ligand in the active site
2 × CT			
Gallic acid	1.22	4	2
Juglone	-2.93	1	2
Catechin	-2.29	2	2
Vanillic acid	-2.15	2	2
Coumarin	-3.32	2	2
Epicatechin	-2.23	3	3
1JJ			
Gallic acid	-3.74	8	6
Juglone	-5.65	2	3
Catechin	-7.58	6	7
Vanillic acid	-3.55	4	5
Coumarin	-5.57	0	3
Epicatechin	-7.74	6	7
1HD2			
Gallic acid	-4.34	5	3
Juglone	-5.23	4	4
Catechin	-4.66	5	7
Vanillic acid	-4.23	3	4
Coumarin	-4.59	3	5
Epicatechin	-4.66	4	7
1C×2			
Gallic acid	-4.60	3	5
Juglone	-6.20	2	6
Catechin	-7.84	5	8
Vanillic acid	-4.80	3	7
Coumarin	-6.08	1	6
Epicatechin	-7.97	6	6
2JK6			
Gallic acid	-5.38	5	6
Juglone	-6.39	3	6
Catechin	-8.14	5	8
Vanillic acid	-5.47	6	8
Coumarin	-5.87	3	6
Epicatechin	-8.43	8	9

with TPSA values in the range 30.21–110.38 Å² manifesting good oral absorption with good bioavailability score. The consensus Log Po/w values were found to be less than 1.82 indicating their good lipophilic character. Their pharmacokinetics show high GI absorption for all compounds with BBB permeation only for juglone and vanillic acid, P-gp substrate for coumarin, and CYP50 inhibitor only for coumarin (CYP1A2) and gallic Acid (CYP3A4).

The BOILED-Egg graph for evaluation of passive gastrointestinal absorption and brain penetration and the bioavailability radar (Figure 4) to establish the drug likeness of compounds of have been also summarized. Results of the BOILED-Egg graph (Figure 4b) clearly revealed that only coumarin appears with blue color in the white region meaning its high probability of passive absorption by the gastrointestinal tract and was exclusively a substrate for P-glycoprotein (PGP+), however those of gallic acid, juglone, catechin, and vanillic acid appeared in red color and therefore were P-glycoprotein (PGP-) with juglone and vanillic acid in the yolk region suggesting high probability to permeate through BBB to access CNS meanwhile catechin in the white region with high probability to be passively absorbed by the gastrointestinal tract. The druglikeness behavior was assessed by the bioavailability radar (Figure 4a) for oral bioavailability prediction. The results of the druglikeness behavior revealed that all compounds are outside the pink area of the polygon only by one parameter suggesting their good oral bioavailability.

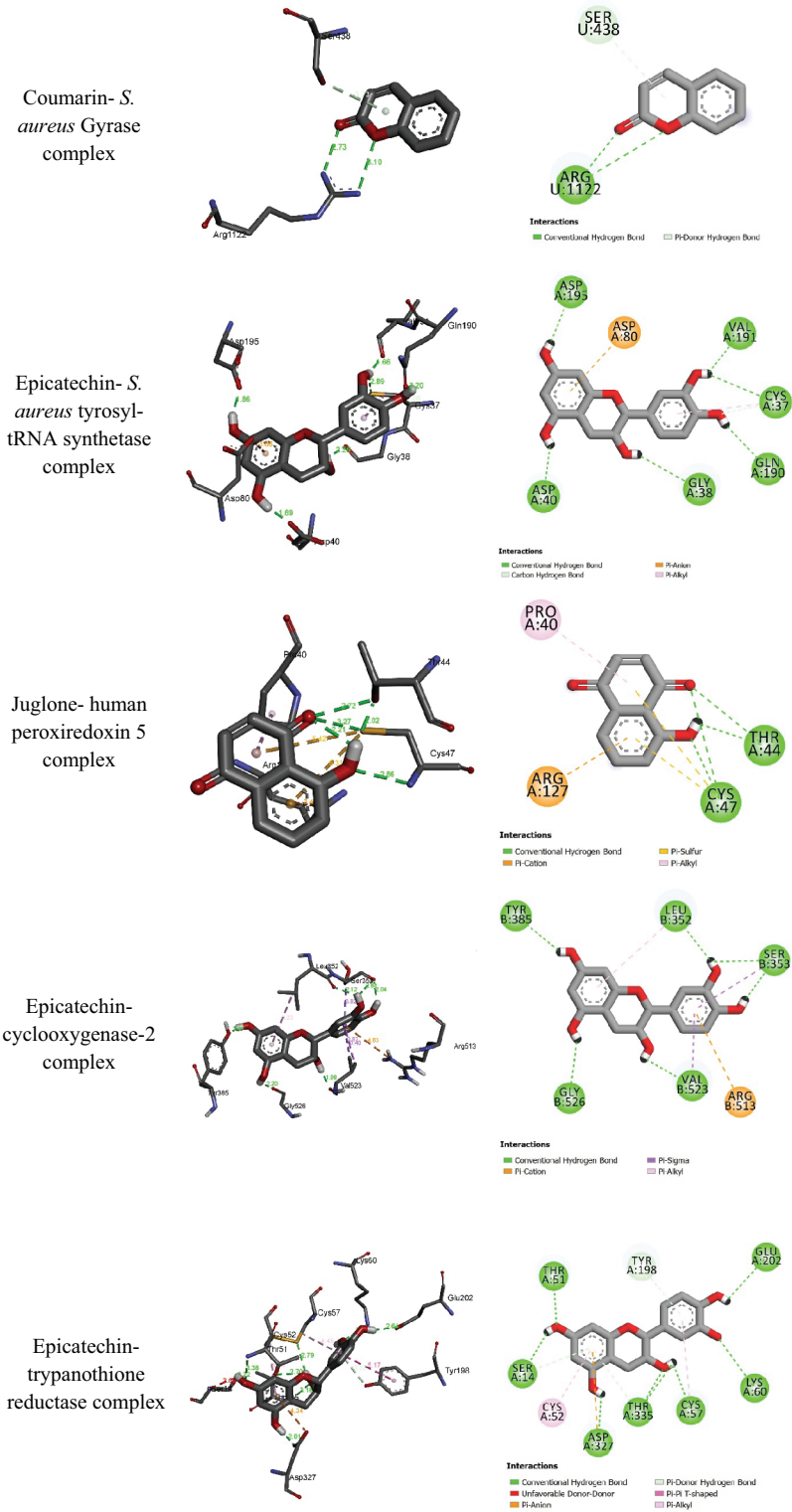


Figure 3. 3D (right) and 2D (left) binding interactions of the stable Coumarin- *S. aureus* Gyrase, Epicatechin- *S. aureus* tyrosyl-tRNA synthetase, Juglone- human peroxiredoxin 5, Epicatechin- cyclooxygenase-2, and Epicatechin-trypanothione reductase complexes.

Table 6. ADME properties of the identified compounds.

	Gallic acid	Juglone	Catechin	Vanillic acid	Coumarin
Physicochemical properties/lipophilicity/druglikeness					
Molecular weight	170.12	174.15	168.15	146.14	290.27
Num. heavy atoms	12	13	12	11	21
Num. arom. heavy atoms	6	6	6	10	12
Fraction Csp3	0.00	0.00	0.12	0.00	0.20
Num. rotatable bonds	1	0	2	0	1
Num. H-bond acceptors	5	3	4	2	6
Num. H-bond donors	4	1	2	0	5
Molar refractivity	39.47	46.27	41.92	42.48	74.33
TPSA	97.99	54.37	66.76	30.21	110.38
Consensus Log $P_{o/w}$	0.21	1.36	1.08	1.82	0.85
Lipinski's rule	Yes	Yes	Yes	Yes	Yes
Bioavailability score	0.56	0.55	0.85	0.55	0.55
Pharmacokinetics					
GI absorption	High	High	High	High	High
BBB permeant	No	Yes	No	Yes	No
P-gp substrate	No	No	No	No	Yes
CYP1A2 inhibitor	No	No	No	Yes	No
CYP2C19 inhibitor	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	No	No
CYP2D6 inhibitor	No	No	No	No	No
CYP3A4 inhibitor	Yes	No	No	No	No
Log Kp (cm/s)	-6.84	-6.00	-6.31	-6.20	-7.82

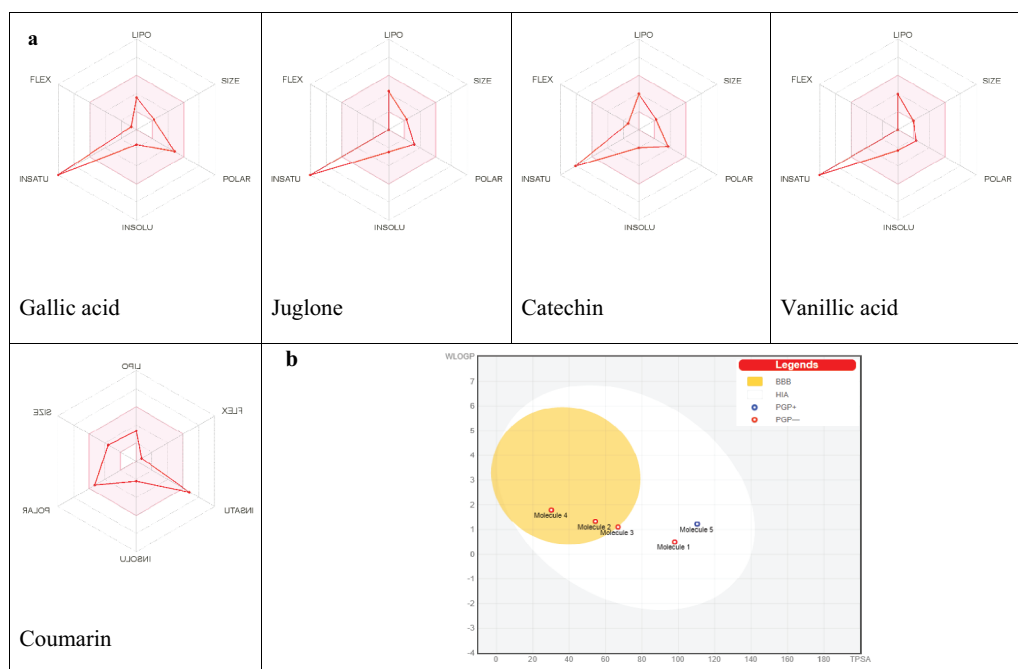


Figure 4. (A) Bioavailability radar of identified compounds based on physicochemical indices ideal for oral bioavailability. LIPO, Lipophilicity: $-0.7 < XLOGP3 < 5$; SIZE, Molecular size: $150 \text{ g/mol} < \text{mol. wt.} < 500 \text{ g/mol}$; POLAR, Polarity: $20 \text{ \AA}^2 < \text{TPSA} < 130 \text{ \AA}^2$; INSOLU, Insolubility: $0 < \text{Log S (ESOL)} < 6$; INSATU, Insaturation: $0.25 < \text{Fraction Csp3} < 1$; FLEX, Flexibility: $0 < \text{Number of rotatable bonds} < 9$. The colored zone is the suitable physicochemical space for oral bioavailability. (B) Boiled-egg (B) model of compounds.

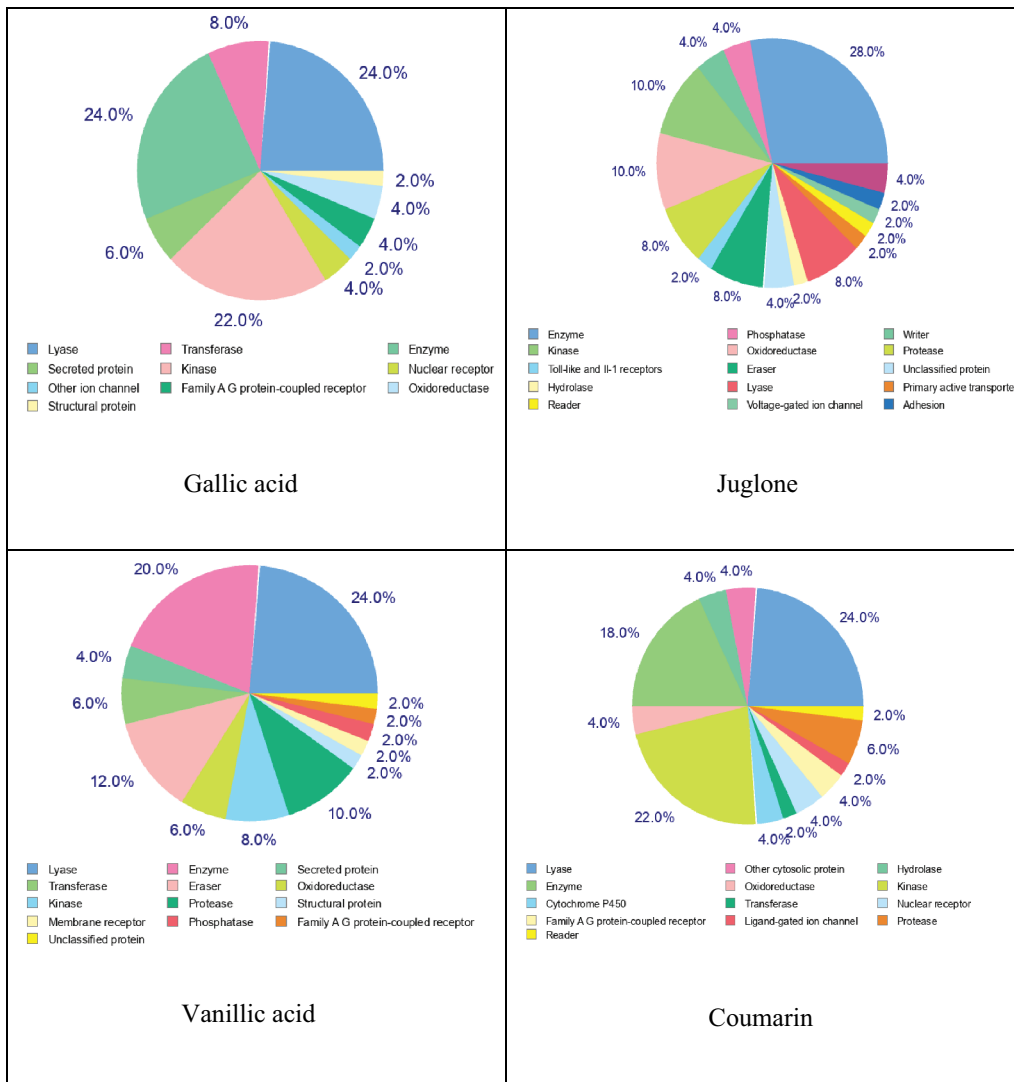


Figure 5. Top-50% of target prediction for identified compounds.

In order to estimate their targets and to rationalize their possible side-effects, the pie-chart (Figure 5) of the identified compounds has been predicted. The results of the target prediction analysis showed that the pie chart for gallic acid predicts a high level of 24% of lyase and enzyme and 22% of kinase. The pie chart for juglone predicts 28% of enzyme and 10% of kinase and oxidoreductase. Vanillic acid has attraction toward the specific binding site of lyase (28%) followed by enzyme (20%), eraser (12%), and protease (10%); however, coumarin may be binds preferentially to lyase (24%), kinase (22%), and enzyme (18%).

Conclusion

To the best of our knowledge, the results of the current work were the initial data on the characterization and biological properties of *J. regia* aqueous extract. Phytochemical characterization of *J. regia* aqueous extract by spectral analyses (UV, HPLC, and FTIR) was done, and several active phytochemicals have been found, such as flavonoids, hydrolysable tannins, reducing compounds, saponins,

terpenoids, and starch. The *J. regia* aqueous extract exhibited a strong antibacterial inhibition activity toward several pathogen strains, a marked inhibition of biofilm formation and induction of lethality of *Leishmania amazonensis* promastigotes, for the first time. In addition, *J. regia* showed the potential to possess insecticidal activity mentioned for the first time. These actions are most likely mediated by their phytochemical constituents. Based on preliminary an *in silico* analysis, our promising findings supported very well the *in vitro* data and could become a basis for further studies at *in vitro* and *in vivo* levels in order to use these compounds as potential remedies to cure various diseases. Therefore, the outcomes obtained afford a new bioactive base for the development of *J. regia* as accessible therapeutic approach against oxidative stress-related diseases.





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Disclosure statement

No potential conflict of interest was reported by the authors.

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