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RESEARCH ARTICLE

Quantitative phytochemical, antioxidant and antimicrobial properties of the seeds of *Lawsonia inermis* L.

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Abstract

Lawsonia inermis L., commonly known as Henna, is a plant that has been extensively utilized throughout the ages for its medicinal and cosmetic properties. This plant contains a variety of bioactive compounds and has attracted significant interest due to its potential antioxidant and antimicrobial activities. The aim of our study is to evaluate the antioxidant capacity and antibacterial activity of *Lawsonia inermis* seeds. *L. inermis* seed powder was extracted using hexane, dichloromethane, ethanol and water. The 4 fractions were quantified for polyphenols, flavonoids and tannins contents. The antioxidant capacity was assessed using 4 methods: 2,2'-Diphenylpicrylhydrazyl hydrate (DPPH) , 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) (ABTS), Ferric Reducing Antioxidant Power (FRAP), and Phosphomolybdate assay (PMA). Antimicrobial activity was determined by agar diffusion against *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. The liquid dilution method was used to determine the minimum inhibitory concentration, minimum bactericidal concentration and minimum fungicidal concentration. The highest content of polyphenols and tannins was obtained in the ethanolic fraction, with values of 594.01±6.07 mg GAE/g dw and 242.4±1.6 mg CE/g dw respectively. The ethanolic fraction exhibited significant scavenging of free radicals (DPPH) with an IC_{50} of 3.06±0.01 µg/mL, lower than the IC_{50} of the standard quercetin (5.30±0.02 µg/mL). Moreover, the ethanolic fraction showed antimicrobial activity against *S. aureus*, *B. cereus* and *E. faecalis* strains, with zone of inhibition values of 15.5±0.7, 14.0±0.0 and 14.5±0.7 mm respectively. The minimum inhibitory concentration of the ethanolic fraction was found to be 1.563 mg/mL against both *S. aureus* and *B. cereus* strains, while the minimum bactericidal concentration was 3.125 mg/mL against *B. cereus* and 6.25 mg/mL against *E. faecalis* strains. *L. inermis* seeds exhibit significant antioxidant capacity and demonstrate antimicrobial activity. These findings indicate the potential of *L. inermis* seeds as a valuable source of bioactive compounds with antioxidant and antimicrobial properties.

Keywords

Lawsonia inermis seeds; Polyphenols; Antioxidant capacity; Antimicrobial activity

Introduction

Microbial infections are a pressing global health concern, encompassing a broad spectrum of diseases caused by various microorganisms, including bacteria, viruses, fungi and parasites. These infections can manifest in diverse ways, from mild discomfort to severe illness, with bacterial infections being particularly prevalent. In recent decades, extensive research has been dedicated to understanding and combating microbial diseases, driven by the emergence of drug-resistant strains, recognized by the World Health Organization as a formidable threat to global health (1). Notable culprits among microbial pathogens include *Escherichia coli*, *Enterococcus faecalis* and *Bacillus cereus* (2, 3).

Simultaneously, oxidative stress, primarily mediated by free radicals or Reactive Oxygen Species (ROS), has been implicated in a variety of significant public health issues. These highly reactive chemical species can induce cellular damage and contribute to the development of ailments such as cancer, vascular diseases, and inflammatory conditions (4-6). Cells deploy a spectrum of defensive mechanisms to counteract the detrimental effects of free radicals, with antioxidants playing a pivotal role in safeguarding cellular integrity.

In the field of natural products research, there is an increasing interest in the quest for novel bioactive compounds with diverse pharmacological properties (7). Among these botanical wonders, *Lawsonia inermis*, stands out as a promising candidate, given its rich reservoirs of bioactive phytochemicals. Entrenched in traditional medicine, this plant has been highly esteemed for centuries due to its extensive therapeutic potential.

In recent years, there has been a growing interest in medicinal and aromatic plants and their extracts (7). *Lawsonia inermis* L., commonly known as Henna, is the most well-known plant of the Lythraceae family. It is an elegant shrub that grows from 2 to 6 m in height, with whitish bark. The dried leaves are slightly crumpled, 2-4 cm long, hairless, intact, and have pinnate veins. The leaves possess a mildly fragrant but not very distinctive flavor and a slight astringency, while the flowers emit a fragrant smell of white or pale pink color, reminiscent of roses, arranged in large panicles. The fruit is small, capsular, globular, reddish and contains several angular seeds in each compartment (8).

Morocco boasts a rich history of traditional medicine, marked by its diverse geography and abundant flora, with approximately 4200 species, of which 600 are recognized in traditional medicine (9). Henna stands out as a prominent medicinal plant in traditional Moroccan medicine. Various parts of the plant find application in traditional medicine systems for treating a range of ailments. The leaves are employed in addressing conditions such as diarrhea, ulcers, kidney stones, dysentery, leprosy, scabies and boils. The flowers are used for ailments like cephalalgia, burning sensations, cardiopathy, anemia, insomnia and fever. The seeds are known for their effectiveness in treating fever, diarrhea, dysentery and amnesia. Bark decoctions are employed for enlarged spleen and stubborn skin diseases, while the root is considered specific for leprosy (10). The diverse applications of *L. inermis* in traditional medicine have spurred studies investigating the therapeutic properties of different parts of the plant.

Recent pharmacological investigations into henna and its constituents have substantiated their efficacy as antibacterial, vibriocidal and antifungal agents against various strains (11, 12). Moreover, it has demonstrated potent anti-inflammatory, analgesic and antipyretic properties (12). Henna has also exhibited promise in the treatment of human breast cancer (13). Studies indicate that the extract from the leaves expedites the healing process of burns (14). While numerus studies have explored the leaves, flowers and barks of the *L. inermis* plant, there has been limited focus on the seeds. Therefore, our research is dedicated to examining this particular part of the plant.

This article aims to illuminate the significance of *Lawsonia inermis* in contemporary biomedical research, with a specific focus on its antioxidant and antimicrobial capabilities. With a history of traditional applications spanning cosmetics to traditional medicine, this plant holds considerable potential as a source of bioactive compounds. Our study endeavors to delve into these capabilities, offering tangible evidence of its health benefits.

Materials and Methods

Plant material

The plant material comprises seeds from the *L. inermis* plant (Fig. 1), harvested in 2019 from the Draa-Tafilalet region, Zagora province, Tinzouline municipality, Morocco. The authenticity of the plant material was confirmed by the botanist at the Herbarium of the Department of Botany at the Scientific Institute of Rabat, Morocco (Voucher Specimen: RAB114594), and it was deposited in the Institute's Herbarium. The dried seeds were ground using a mortar, and the resulting ground material was stored in green vials at room temperature, in a dry place and protected from moisture and light until its usage.

Fig. 1. Seeds of *L. inermis*.

Reagents and material

The solvents used, including n-hexane, dichloromethane, ethanol, methanol and dimethyl sulfoxide, were sourced from Sigma Aldrich. The spectrophotometer employed is a UV/VIS spectrophotometer (Model: UV-1800APC).

Five bacterial isolates, namely *Staphylococcus aureus* ATCC 43300, *Bacillus cereus* ATCC 14579, *Enterococcus faecalis* ATCC 19433 (Gram+), *Escherichia coli* ATCC 11775, *Pseudomonas aeruginosa* ATCC 90027 (Gram-) and along with the fungus *Candida albicans*, were obtained from the Moroccan Coordinated Collections of Microorganisms (CCMM) at the National Center for Scientific and Technical Research (CNRST) in Morocco (www.ccmm.ma).

Preparation of fractions

L. inermis seed powder (100 g) underwent extraction with 400 mL of n-hexane solvent using a Soxhlet apparatus for 8 h. The remaining residue, referred to as pomace, was then recovered and dried in an oven at 30 °C for one day. Subsequently, the pomace underwent an 8 h extraction using dichloromethane. The same extraction process was repeated using ethanol.

Utilizing the maceration method, the ethanol pomace underwent an additional extraction with 400 mL of water at room temperature for 48 h. The resulting extracts were divided into four fractions: hexane (F_1 He), chloromethane (F_2 DM), ethanolic (F_3 ETOH) and aqueous (F_4 Aq). These fractions were then evaporated using a rotary evaporator and stored in stored in opaque glass bottles until ready for use.

Phytochemical screening

The phytochemical test was conducted using staining and precipitation reactions. This test aimed to explore the main chemical groups, encompassing flavonoids, tannins, alkaloids, terpenes, saponins and coumarins (15).

Determination of flavonoids

Flavonoids were identified through the "Cyanidin" reaction. Briefly, 2 mL of each fraction was mixed with a few drops of HCl (2N) and a small amount of magnesium. The presence of flavonoids manifested as an orange to red coloring.

Determination of tannins

The presence of tannins was determined by mixing 3 mL of each fractions with a few drops of a 10% (m/v) $FeCl_3$ aqueous solution. A positive result was indicated by the development of a blue-black or blue-green color.

Detection of alkaloids

Alkaloids were identified through precipitation reactions using Bouchardat, Mayer, and Dragendorff's reagents. For each fractions, 3 mL was added to 1 mL of each reagent (Mayer, Dragendorff, Bouchardat), and the solution was allowed to stand for 10 min. A positive result was indicated by the formation of an orange precipitate with Dragendorff's reagent, a yellowish-white precipitate with Mayer's reagent, and a brown precipitate with Bouchardat's reagent.

Detection of terpenoids

Terpenoids were identified by introducing 0.3 mL of chloroform into 3 mL of each fractions, followed by the addition of 1.2 mL of concentrated H_2SO_4. The presence of a brownish-red or purple ring at the point of contact typically indicates the presence of terpenoids.

Detection of saponins

The detection of saponins was performed through the foaming test. Briefly, 10 mg of the extract was dissolved in 10 mL of the solvent used for extraction. The resulting solution was placed in a test tube, agitated for 15 seconds, and allowed to stand for 15 min. The presence of saponins was indicated by a persistent foam height greater than 1 cm.

Determination of coumarins

The test consisted of adding 0.5 mL of NH_4OH (25%) to 2 mL of each fraction and observing it under a 365 nm UV lamp. Intense fluorescence indicates the presence of coumarins

Determination of total polyphenols content

The protocol used was based on the standard procedure (16), with some modifications. Briefly, 200 µL of each fraction was added to glass hemolysis tubes, along with a mixture of 1 mL of Folin-Ciocalteu reagent (diluted 10-fold) and 800 µL of 7.5% sodium carbonate solution. The tubes were shaken and incubated for 30 min. Subsequently, the absorbance was measured at 765 nm using a spectrophotometer. Simultaneously, a calibration curve was prepared using gallic acid at various concentrations under the same experimental conditions. The results of the total polyphenols content (TPC) were expressed as mg equivalents of gallic acid/g of extract (mg GAE/g dw).

Determination of total flavonoids content

The protocol used was based on standard procedure (17), with some modifications. In a glass hemolysis tube, 500 µL of each fraction was added to 500 µL of 10% $AlCl_3$. The absorbance was immediately measured at 420 nm against the control after 1 h of incubation at room temperature. Quercetin was used to construct the standard curve and the total flavonoids content (TFC) was expressed as mg equivalents of quercetin per gram of extract (mg QE/g dw).

Determination of total condensed tannins content

The condensed tannin content (TTC) was determined using the vanillin method (18). For each fraction, 50 µL was added to 1500 µL of a 4% vanillin/methanol solution and mixed vigorously. Subsequently, 750 µL of concentrated hydrochloric acid (HCl) was added. The resulting mixture was allowed to react at room temperature for 20 min and the absorbance was measured at 500 nm against the blank. Calibration curves were developed using different concentrations prepared from catechin stock solutions. Results were expressed as milligram catechin equivalent mg of extract (mg CE/g dw).

In vitro antioxidant capacity

2,2′-Diphenyl- picrylhydrazyl hydrate method

The free radical scavenging capacity of the fractions was evaluated following the procedure (19). For each concentration or methanol (negative control), 50 µL was added to 2 mL of a DPPH solution (0.0023% methanol). After homogenization, the mixture was incubated in the dark at room temperature for 20 min and the absorbance was measured at 517 nm using a spectrophotometer. The % of DPPH free radical inhibition (PI%) was calculated using the following formula:

$$I\ \% = ((Ac-As)/Ac)*100$$

Where, I %: percentage of DPPH inhibition

Ac: Absorbance of negative control

As: Absorbance of sample

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid method)

The antioxidant capacity of the fractions against the radical ions of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was evaluated using the standard method (20). The ABTS solution was prepared by mixing 10 mL of a 7 mM methanolic solution of ABTS with 10 mL of a 70 mM methanolic solution of potassium persulfate. The reaction mixture was incubated in the dark for 16 h at room temperature. The resulting ABTS solution was then diluted with methanol to an absorbance range of 0.700-0.734 nm. Concentrations of the fractions and the positive control (ascorbic acid), ranging from 1 to 80 µg/mL, were prepared in methanol. A volume of 100 µL of each concentration or methanol (control blank) was added to 2 mL of the previously prepared ABTS solution. After 1 min of incubation at room temperature, the absorbance was recorded at 734 nm using a spectrophotometer. The % of inhibition was calculated in the same way as for DPPH and the average concentration responsible for 50% inhibition (IC_{50}) was determined.

FRAP method

The assay was performed according to the standard method (21) with some modifications. A volume of 0.2 mL of each fraction was mixed with 1.25 mL of phosphate buffer (0.2 M, pH 6.6) and 1.25 mL of 1% aqueous potassium ferricyanide solution [$K_3Fe(CN)_6$]. The mixture was incubated in water bath at 50°C for 20 min. After incubation, 1.25 mL of 10% trichloroacetic acid was added to stop the reaction and the prepared solution was left for 3 min. Subsequently, an aliquot of 1.25 mL of the supernatant was combined with 1.25 mL of distilled water and 0.25 mL of a 0.1% ferric chloride ($FeCl_3$) aqueous solution. The absorbance was measured at 700 nm using a spectrophotometer. The results were expressed as the average concentration responsible for 50% inhibition (IC_{50}).

Phosphomolybdate Assay

The phosphomolybdate assay (PMA) of the fractions was evaluated according to the standard procedure (22). For each fraction, 0.2 mL was combined with 2 mL of the reagent solution (0.6 M sulfuric acid, 4 mM ammonium molybdate and 28 mM sodium phosphate). The reaction mixtures were incubated at 95 °C for 90 min. Following incubation, the absorbance of the reaction mixtures was measured at 695 nm using a spectrophotometer against a blank after cooling to room temperature. An ascorbic acid standard curve was used to determine the antioxidant capacity of the fractions. The results were expressed as mg ascorbic acid equivalent per g of extract (AAE mg/g dw STD).

In vitro antimicrobial activity

Sensitivity test to crude plant fractions, inhibition test

The evaluation of antimicrobial activity was conducted using the diffusion method on Mueller-Hinton (MH) agar media for bacteria and Sabouraud (SB) agar media for yeast, following the standard method (23), with some modifications. MH and SB agar media were uniformly inoculated by flooding (or swabbing) with 10 µL of a 0.9% saline suspension of the strain under study, previously adjusted using the McFarland 1 standard. Sterile discs (OxoidTM, CT0998B) with a diameter of 6 mm were impregnated with 10 µL of each fraction concentration. After 24 h of incubation at 37 °C for bacteria and 30 °C for yeast, the diameter of the inhibition zone of (DIZ) was measured in millimeters using a ruler, including the diameter of the disc. Negative controls were prepared in isolated petri dishes, replacing the test samples with 30% DMSO and sterile distilled water. Positive controls, including Penicillin (10 µg/mL), Tetracycline (30 µg/mL) and Ampicillin (10 µg/mL) (24).

Determination of minimum inhibitory, bactericidal and fungicidal concentrations

The minimum inhibitory concentration (MIC) of each fraction was defined as the lowest concentration inhibiting bacterial growth. The MIC determination against tested microbial strains employed the microtitration technique (25), utilizing 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) as a viability indicator. In each well of the microplate, 80 µL of liquid culture medium (BHI for bacteria and SB liquid for yeast) was added, followed by 10 µL of the test material. Successive 1/2 dilutions were made from one well to another in the same row. Each well was inoculated with 10 µL of the suspension of the tested strain. The negative control was prepared in isolated wells by adding 10 µL of sterile distilled water, 10 µL of DMSO and 10 µL of the microbial suspension to the culture medium. The microplate wells were incubated for 24 h at 37 °C. Then, 10 µL of an extemporaneously prepared 0.4 mg/mL MTT solution in sterile physiological water was added to each well as an indicator of microbial growth. The prepared microplate was re-incubated for 10 to 30 min at 37 °C. Wells where microbial growth occurred showed a blue-violet color. The minimum concentration at which there was no visible color change from yellow to dark purple after incubation was recorded as the minimum inhibitory concentration.

The determination of minimum bactericidal concentration (MBC) involved subculturing with plating using a loop on MH medium, specifically targeting the wells without visible microbial growth. Following incubation, the plates with no bacterial growth corresponded to the concentrations representing the MBCs. Similarly, the determination of minimum fungicidal concentration (MFC) followed the same method.

Statistical analysis

The determination of total antioxidant capacity, DPPH, ABTS, FRAP and PMA, as well as the measurements of total polyphenols, total flavonoids and total tannins, were carried out in triplicate. The reported values represent the mean ± SD (n= 3) of three measurements. Data analysis was conducted using Microsoft Excel Office 2016, and statistical comparison of means were performed with Graph Pad Prism v8 software using one-way analysis of variance (ANOVA). Results were considered statistically significant at $p<0.05$.

Results

Yield of Extraction

The extraction yield is the ratio of the amount of substances extracted through the solvent's extractive action to the amount of plant material. The yields of the aqueous, ethanolic, dichloromethane and hexane fractions of *L. inermis* seeds are presented in (Table 1).

Table 1. The yield of different fractions from seeds of *L. inermis*.

Fraction with extraction solvent	Yield %
F_1 He	10.38[a]
F_2 DM	14.50[b]
F_3 ETOH	22.27[c]
F_4 Aq	10.78[a]

F-Fraction; **He**- Hexane; **DM** - Dichloromethane; **ETOH** - Ethanolic; **Aq**-aqueous. The letters [a-c] are used to indicate a statistically significant difference ($p<0.05$) between the samples that were tested.

The results indicate significant variations in the extraction yields among the different solvents. The ethanolic fraction exhibited the highest extraction yield with a value of 22.27%, followed by the dichloromethane fraction with 14.50%. The aqueous and hexane fractions had lower extraction yields of 10.78% and 10.38% respectively.

The higher extraction yield obtained with the ethanolic fraction suggests that ethanol was an effective solvent for extracting a wide range of bioactive compounds from *L. inermis* seeds. Ethanol is known for its ability to dissolve polar compounds, making it suitable for extracting a diverse array of phytochemicals. The dichloromethane fraction demonstrated a moderate extraction yield, indicating its ability to selectively extract lipophilic compounds from the seeds of *L. inermis*. The relatively lower extraction yields of the aqueous and hexane fractions suggest that the bioactive compounds present in *L. inermis* seeds may have limited solubility in water and hexane respectively.

The yield rates of the different fractions studied are relatively higher than those cited in the literature. Certainly, the seeds of *L. inermis* allowed the extraction of 12.11% for the aqueous fraction, 21.72% for the ethanolic fraction and 14.92% for the dichloromethane fraction. In contrast, it was reported yields of 7.6%, 10.6% and 2.32% respectively (26). *L. inermis* seeds yielded a hexane fraction with a yield of 13.24%, which is higher than the 6.31% reported (27).

Phytochemical screening

Phytochemical tests, also known as screening tests, are qualitative assays employed to identify various families of secondary metabolites within a specific part of the plant. These tests entail staining and precipitation reactions utilizing specific reagents for each compound family. The intensity of the precipitate and the resulting coloration directly correlate with the amount of the targeted substance. The outcomes of these tests are presented in (Table 2).

Table 2. Phytochemical characterization of different fractions of *L. inermis* seeds.

Fractions/Compounds	F_1 He	F_2 DM	F_3 ETOH	F_4 Aq
Alkaloids	-	-	-	-
Coumarins	-	-	+	+
Flavonoids	-	-	+	+
Saponins	-	-	-	+
Tannins	-	-	+	+
Terpenes	+	+	+	+

He-Hexane; **DM**-Dichloromethane; **ETOH**-Ethanolic; **Aq**-Aqueous, Positive sign (**+**) = Present; Negative sign (**-**) = Absent.

The aqueous and ethanolic fractions of *L. inermis* seeds revealed the presence of flavonoids, tannins, coumarins and terpenes. The dichloromethane and hexane fractions showed the presence of terpenes and the absence of other compounds. However, no positive results were obtained for alkaloids in any of the fractions. These finding align with those reported, where the phytochemical screening of *L. inermis* seeds using dichloromethane indicated the presence of terpenes, while tannins, saponins and flavonoids were present in the aqueous and ethanolic extracts, with the absence of alkaloids in all extracts (26). Identifying various classes of secondary metabolites in plants offers insights into their pharmacological potential. Plant polyphenols, especially flavonoids, tannins and coumarins, find widespread use in therapeutics as anti-inflammatory, antibacterial and antioxidant agents (28-30).

Total polyphenols content, Total flavonoids content and Total tannins content

Table 3 displays the content of total polyphenols, total flavonoids and total tannins in the various fractions of *L. inermis* seeds. The 4 fractions tested exhibited a statistically significant difference ($p<0.05$).

The quantities of polyphenols vary from 594.01±6.07 to 17.2±0.7 mg GAE/g dw. The content of total polyphenols varies depending on the fraction of the studied plant. The ethanolic and aqueous fractions exhibit high quantities of polyphenols, with values of 594.01±6.07 and 336.2±0.8 mg GAE/g dw respectively. These findings are consistent with the results obtained (31), who found a high polyphenol content of 786.54±0.00 mg GAE/g dw, for the methanolic extract of *L. inermis* seeds, followed by 526.48±0.40 mg GAE/g dw for the aqueous extract. Similar results were reported (27), suggesting that the highest amount of polyphenol compounds was obtained from the methanolic extract, with a value of 457.5 g GAE/kg dry mass. A study conducted on another species of henna, *Lawsonia alba* from Algeria, also demonstrated rich polyphenol content in the three selected regions (32).

Table 3. Assay of polyphenols, flavonoids, and tannins in different fractions of *L. inermis* seeds.

Fractions	TPC mg GAE/g dw	TFC mg QE/g dw	TTC mg CE/g dw
F_1 He	17.2±0.7^{d}	46.3±0.4^{d}	56.4±0.6^{d}
F_2 DM	40.8±1.1^{c}	63.0±0.4^{c}	38.3±0.9^{c}
F_3 ETOH	594.0±6.1^{a}	28.3±0.1^{a}	242.4± 1.6^{a}
F_4 Aq	336.2±0.8^{b}	47.3±0.3^{b}	68.1± 1.6^{b}

TPC-Total polyphenols content; **TFC**-Total flavonoids content; **TTC**-Total tannins content; **GAE**-gallic acid equivalent; **QE**-quercetin equivalent; **CE**-catechin equivalent; **EX**-extract. The letters $^{a-d}$ are used to indicate a statistically significant difference (p<0.05) between the samples that were tested.

According to these results, the polar fraction contains a higher concentration of polyphenols compared to non-polar fractions. This variation can be attributed to the better solubility of polyphenols in polar solvents. The 3 parts of *L. inermis*, including leaves, seeds and flowers, exhibited varying polyphenol contents. The aqueous extract of *L. inermis* seeds showed the highest amount of polyphenols at 56.81±1.76 mg GAE/g (33).

Dichloromethane and hexane fractions show moderate values of polyphenols, with 40.8±1.1 and 17.2±0.7 mg GAE/g dw respectively. In contrast, reports are on low levels of polyphenols, with values of 7.36±0.00 mg GAE/g dw and 3.5±1.4 g GAE/kg dry mass respectively (27, 31) .

The total flavonoids contents determined from the fractions of *L. inermis* seeds ranged from 28.3±0.1 to 63±0.4 mg QE/g dw. Among the fractions, the dichloromethane fraction exhibited the highest content of 63±0.4 mg QE/g dw. The aqueous and hexane fractions followed with values of 47.3 ± 0.3 and 46.3 ± 0.4 mg QE/g dw respectively. The ethanolic fraction showed a moderate content of 28.3 ± 0.1 mg QE/g dw. When comparing these results with those obtained by other researchers, differences in the levels of values can be observed. It was reported that the methanolic extract of henna seeds exhibited the highest flavonoids content of 199.95 ± 2.10 mg QE/kg dry mass, followed by the chloroform extract (120.7 ± 3.9 mg QE/kg dry mass) and the hexane extract (21.6 ± 2.4 mg QE/kg dry mass) (27). Reports are found quantities of 31.59 ± 1.00 mg QE/g dw for the ethanolic extract and 6.86 ± 0.23 mg QE/g dw for the aqueous extract(26) . Additionally, reports are on a flavonoid content of 1.045 ± 0.0035 mg QE/g dw in the acetone-water extract (32). According to another report, the aqueous extract of seeds exhibited the highest flavonoid content compared to leaves and flowers, with a value of 10.463 ± 0.141 mg QE/kg dry mass (33).

The determination of total tannins content revealed that the ethanolic fraction had the highest content among the four fractions, with a value of 242.4 ± 1.6 mg CE/g dw. In contrast, the aqueous fraction showed a lower quantity of 68.1 ± 1.6 mg CE/g dw. In a study conducted earlier, it was found that the aqueous extract had the highest tannin value of 101.66 ± 0.01 mg CE/g dw, followed by the methanolic extract with a content of 59.43 ± 0.58 mg CE/g dw (31). Nevertheless, the hexane fraction in our study revealed moderate tannin content of 56.4 ± 0.6 mg CE/g dw. This value is higher than the ones obtained, which reported tannin contents of 0.93 ± 0.00 mg CE/g dw and 50.0 ± 4.5 mg/kg dry mass respectively (27, 31). Lastly, the dichloromethane fraction showed a tannin content of 38.3±0.9 mg CE/g dw.

Antioxidant capacity

The antioxidant capacity of *L. inermis* seed fractions was assessed using four different methods: the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical trapping technique, the ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) method, the FRAP (ferric reducing antioxidant

Table 4. Antioxidant capacity of *L. inermis* seed fractions.

	DPPH IC_{50} µg/mL	ABTS IC_{50} µg/mL	FRAP IC_{50} µg/mL	PMA mg AAE/g dw
F_1 He	1166.20±0.02^{c}	1593.80±0.01^{c}	713.80±0.01^{c}	15.20±0.01^{d}
F_2 DM	488.90±0.10^{b}	623.90±0.01^{b}	195.60±0.01^{b}	67.50±0.01^{c}
F_3 ETOH	3.06±0.01^{a}	9.07±0.01^{a}	14.40±0.01^{a}	406.70±0.01^{a}
F_4 Aq	6.30±0.01^{a}	9.40±0.01^{a}	18.60±0.01^{a}	180.80±0.01^{b}
Quercetin	5.30±0.02^{a}	-	-	-
Ascorbic acid	-	2.50±0.02^{a}		-
Catechin	-	-	13.90±0.03^{a}	-

DPPH-DPPH Free Radical-Scavenging; **ABTS**-ABTS Radical Scavenging Assay; **FRAP**-Ferric Reducing Antioxidant Power; **PMA**-Phosphomolybdate Assay; **AAE**-ascorbic acid equivalent; **EX**-extract. The letters $^{a-d}$ are used to indicate a statistically significant difference (p<0.05) between the samples that were tested. All uncertainty values less than 0.01 are rounded to 0.01.

power) method, and the PMA (phosphomolybdate assay) method. The results of these analyses are presented in (Table 4).

2,2′-Diphenyl-picrylhydrazyl hydrate method

In this study, the antioxidant capacity was assessed by measuring the percentage of DPPH free radical scavenging or inhibition. As there is no absolute measure of antioxidant capacity, the results were compared to a reference antioxidant, Quercetin. The concentration of the fractions or Quercetin required to reduce 50% of the DPPH radical, known as IC_{50}, inversely reflects the antioxidant capacity of the fractions. A lower IC_{50} value indicates a higher antioxidant capacity. The results presented in Table 4 shows that the standard (Quercetin) exhibits an antioxidant capacity reflected by an inhibitory concentration of 5.30±0.02 µg/mL.

The results obtained for the fractions of *L. inermis* seeds reveals significant antioxidant activity in all fractions, except for the dichloromethane and hexane fractions. The ethanolic fraction stands out as the most active, boasting a low IC_{50} value of 3.06±0.01 µg/Ml, surpassing the antioxidant capacity of quercetin. The high antioxidant capacity of the ethanolic fraction can be attributed to its rich content of various polyphenolic compounds, as observed during the assay. Similarly, the aqueous fraction also demonstrates significant antioxidant capacity, with an IC_{50} value of 6.30±0.01 µg/mL. This fraction not only shows positive responses to all phytochemical tests related to polyphenolic compounds but also exhibits an interesting content in terms of dosage. In contrast, the dichloromethane and hexane fractions display weaker antioxidant capacity. In conclusion, the fractions of *L. inermis* seeds exhibit potent antioxidant capacity, likely due to the presence of antioxidant molecules such as flavonoids and tannins, known for their for their ability to reduce the DPPH radical by releasing a hydrogen atom.

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid method

The cationic radical $ABTS^{\cdot+}$ was employed to assess the free radical scavenging capacity of the fractions derived from *L. inermis* seeds. This method employs spectrophotometry to measure the reduction of $ABTS^{\cdot+}$ to $ABTSH^{+}$. Ascorbic acid served as the reference substance in this experiment. The obtained results were used to construct curves illustrating the % of ABTS inhibition at various concentrations (Fig. 2).

The assessment of the antioxidant capacity of the fractions obtained from *L. inermis* seeds, as determined by the ABTS test, discovered that the ethanolic fraction exhibited a potent capacity of 9.07±0.01 µg/mL. Similarly, the aqueous fraction demonstrated an antioxidant capacity of

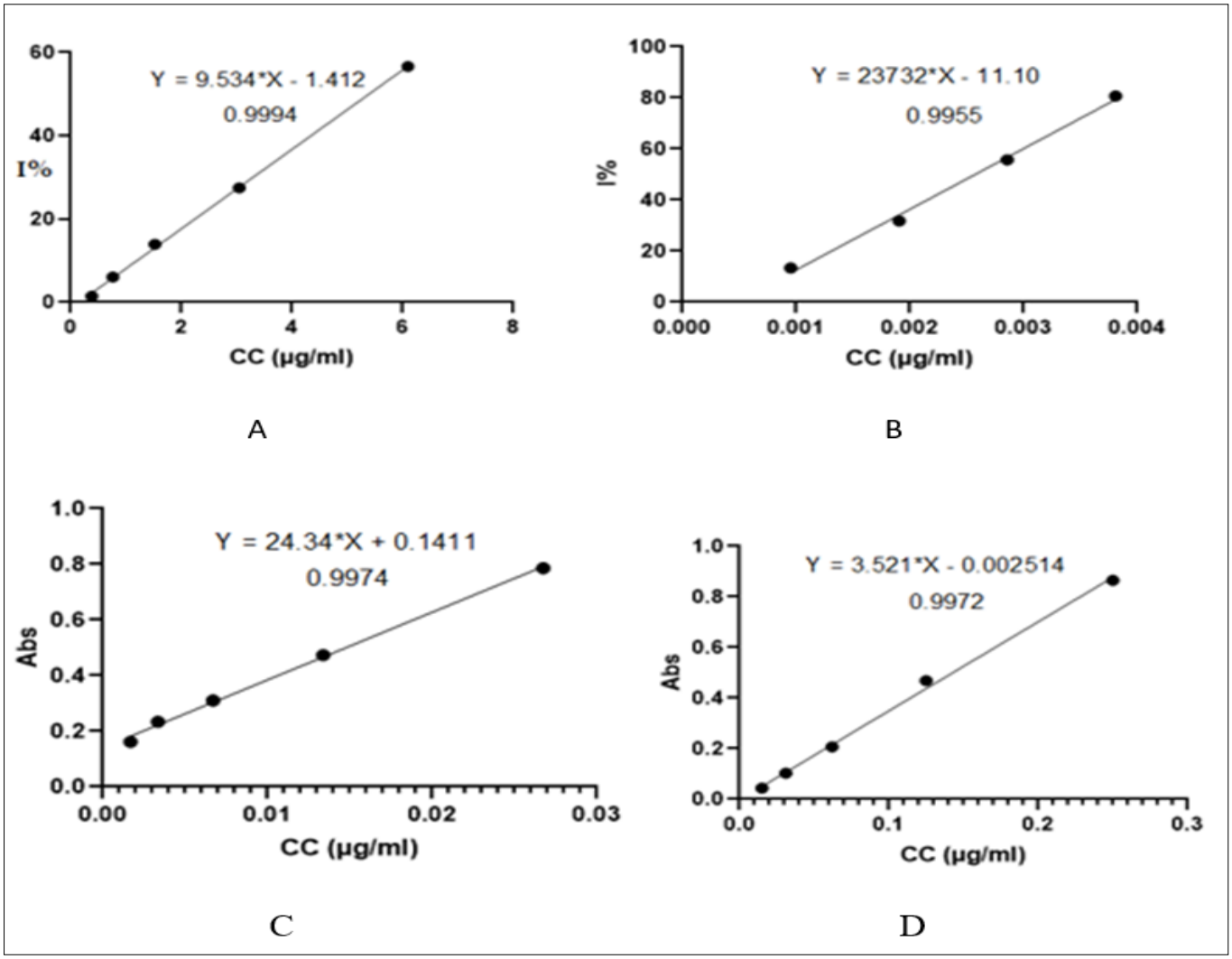

Fig. 2. Reference curve of antioxidant capacity by: (**A**) Quercetin DPPH; (**B**) Acide ascorbique ABTS; (**C**) Catechin FRAP; and (**D**) Acide ascorbique PMA.

9.40±0.01 µg/mL. In contrast, the dichloromethane and hexane fractions displayed lower antioxidant capacities, with values of 623.90±0.01 µg/mL and 1593.80±0.01 µg/mL respectively.

FRAP method

The antioxidant capacity of the fractions from *L. inermis* seeds was assessed using the FRAP (Ferric Reducing Antioxidant Power) method, which measures the ability of the fractions to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). This reduction reaction led to an increase in absorbance at 700 nm. Catechin served as a reference antioxidant in this assay.

The results indicate that the fractions of *L. inermis* seeds exhibit antioxidant capacities. The absorbance curves of the fractions, as a function of their concentrations in the reaction medium, show a similar logarithmic trend to that of the control (Catechin) (Fig. 2). This observation suggests a proportional relationship between the concentration increase and the reduction in absor-bance in the tested samples.

The results of the iron-reducing ability of *L. inermis* seed fractions are presented in Table 4. The ethanolic fraction exhibited the highest antioxidant capacity with an IC_{50} value of 14.40±0.01 µg/mL, which is comparable to the standard Catechin with an IC_{50} of 13.9±0.03 µg/mL. The aqueous fraction also displayed significant antioxidant capacity with an IC_{50} value of 18.60±0.01 µg/mL. On the other hand, the dichloromethane and hexane fractions showed lower antioxidant capacities with IC_{50} values of 195.60±0.01 and 713.8±0.01 µg/mL respectively. The observed reducing power of the fractions can be attributed to the presence of hydroxyl groups in the polyphenolic compounds present in these fractions, which act as electron donors. Previous studies have also indicated that the reducing power of a compound can serve as a valuable indicator of its potential antioxidant activity (31).

Phosphomolybdate assay

The antioxidant capacity of the 4 fractions obtained from *L. inermis* seeds was assessed using the phosphomolybdate assay (PMA). This method relies on the reduction of molybdenum Mo (VI) ions, present in the form of molybdate ions MoO_4^{2-}, to molybdenum Mo (V) ions MoO^{2+} in the presence of the fractions or an antioxidant agent. This reduction process leads to the formation of a greenish complex known as phosphate/Mo (V) at an acidic pH. The intensity of the color produced by the molybdenum (VI) complex is measured in the presence of an antioxidant. The overall antioxidant capacity of the studied fractions is quantified in terms of ascorbic acid equivalents using a calibration curve (Fig. 2).

From (Table 4), the total antioxidant capacity of the fractions depends on the solvent used for extraction (ethanolic, aqueous, dichloromethane and hexane). It was observed that the ethanolic fraction exhibits the highest total antioxidant capacity with a value of 406.7±0.007 mg AAE/g dw. The aqueous fraction demonstrates a moderate antioxidant capacity of 180.80±0.01 mg AAE/g dw. In contrast, the dichloromethane fraction shows a significant antioxidant capacity of approximately 67.50±0.01 mg AAE/g dw, while the hexane fraction exhibits a lower capacity of 15.20±0.01 mg AAE/g dw.

All the tested fractions exhibited antioxidant capacity with variable efficiency, except for hexane and dichloromethane, which demonstrated weak activity. Our results aligns with reported earlier (26). The ethanol extract showed good activity (0.083 µg), followed by the aqueous extract (19.186 µg), while the dichloromethane extract's activity was negligible (44.509 µg). Similar findings were observed where , *L. inermis* exhibited the highest capacity in both DPPH and ABTS radical scavenging activities, with IC_{50} values of 7.95±0.31 µg/µL and 0.29±0.01 µg/µL respectively (27, 31). It was suggested that *Lawsonia alba* seed extracts have a strong antioxidant capacity (32). Antioxidants combat free radicals and protect against various diseases. Similarly, it was found that the aqueous extract presented a robust capacity against DPPH with an IC_{50} of 0.024±0.002 mg/mL, lower than the standard, which had an IC_{50} of 0.089±0.002 mg/mL (33). The results presented in (Table 4) clearly show that the polar fractions (ethanol and aqueous) were effective and powerful scavengers. In the literature, flavonoids, polyphenols and tannins have been shown to possess DPPH and ABTS radical scavenging properties. Polyphenol compounds have been extensively studied for their ability to react with DPPH or ABTS free radicals by electron transfer or donation of a hydrogen atom to neutralize them.

Thus, the high free radical scavenging capacity observed in polar fractions, such as ethanolic and aqueous fractions, may be attributed to the elevated levels of polyphenolic compounds, flavonoids and tannins. The FRAP and PMA assays demonstrated that the ethanolic fractions exhibited the highest reducing power (14.40±0.01 µg/mL and 406.70±0.01 mg AAE/g dw respectively). In contrast, dichloromethane and hexane fractions were found to be inactive in both tests. These results align with the earlier findings (26), indicating that the ethanolic extract showed the best reducing capacity. According to one report, the PMA test revealed superior antioxidant capacity in the seeds compared to that of the flower extracts (33).

Our study breaks new ground by evaluating the antioxidant capacity of different fractions from *L. inermis* seeds. The novelty of our findings lies in the remarkable antioxidant potential observed within these fractions, suggesting that they may exceed the antioxidant activity of the reference standard, Quercetin.

Correlation

To validate the relationship between antioxidant capacity of *L. inermis* seed fractions and their polyphenolic compositions, we aimed to establish a linear correlation between the antioxidant capacity values, determined using the 4 methods (DPPH, ABTS, FRAP and PMA) and the levels of polyphenols, flavonoids and tannins.

Table 5 presents the correlation coefficients between Total Polyphenolic Content (TPC), Total Flavonoid

Content (TFC), Total Tannin Content (TTC), as well as the antioxidant assays (DPPH, ABTS, FRAP and PMA). Notably high coefficients, exceeding 0.8, were observed, particularly in the cases of TPC and PMA as well as TTC and PMA. The correlation between TFC and DPPH, TFC and ABTS, TFC and FRAP was moderate. The negative correlation between TPC, TFC, TTC and antioxidant activities indicates that the activity increases with higher content of these compounds.

Table 5. Pearson's correlation coefficients among TPC, TFC, TTC, DPPH, ABTS, FRAP, and PMA.

Test	TPC	TFC	TTC	DPPH	ABTS	FRAP	PMA
TPC	1						
TFC	-0.8260	1					
TTC	0.8919	-0.8978	1				
DPPH	-0.8156	0.3522	-0.5302	1			
ABTS	-0.8011	0.3276	-0.5161	0.9995	1		
FRAP	-0.7320	0.2206	-0.4577	0.9857	0.9904	1	
PMA	0.9821	-0.8061	0.9392	-0.7879	-0.7767	-0.7249	1

TPC-Total polyphenolic content; **TFC**-Total flavonoids content; **TTC**-Total tannins content; **DPPH**-DPPH Free Radical-Scavenging; **ABTS**-ABTS Radical Scavenging Assay; **FRAP**-Ferric Reducing Antioxidant Power; **PMA**-Phosphomolybdate Assay.

According to one report (26), the correlation analysis revealed strong relationship, with a coefficient of determination (r^2) of 0.991 between TPC (Total Polyphenolic Content) and TFC (Total Flavonoid Content), 0.534 between TPC and DPPH, 0.966 between TPC and PMA, 0.503 between TFC and DPPH, 0.992 between TFC and PMA, and 0.464 between DPPH and PMA.

Antimicrobial activity

Two different methods were employed to assess the antimicrobial activity of *L. inermis* seed fractions. This evaluation followed a two-step procedure: In the initial step, a sensitivity test was conducted using the disk method at various concentrations. The reported values represent the average of 2 measurements. Bacteriostatic action was indicated by the presence of a zone of inhibition around the paper disc impregnated with the tested raw fraction. Subsequently, a second step involved determining the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC).

Among the studied fractions, positive reactions were observed in at least one of the tested microbial strains. Furthermore, there was a noteworthy variation in the diameters of the inhibition zones (DIZ), ranging from 7.0 to 15.5 mm. The inhibition zones of the fractions are detailed in Table 6. It is important to note that the diameter of the inhibition zone can vary between different strains and fractions.

The most substantial inhibition zones were observed in three strains: *S. aureus*, *B. cereus*, and *E. faecalis*, in the ethanolic extract, with zones measuring 15.5±0.7 mm, 14.0±0.0 mm and 14.5±0.7 mm respectively, at a concentration of 500 mg/mL. Subsequently, in the aqueous extract at a concentration of 333 mg/mL, *S. aureus*, *B. cereus* and *E. faecalis* exhibited inhibition zones of 11.5±0.7 mm, 9.5±0.7 mm and 9.5±0.7 mm respectively. The least significant inhibition zones were observed in the *B. cereus* and *P. aeruginosa* strains, with sizes of 8.5±0.7 mm and 7.5±0.7 mm respectively, in the dichloromethane extract at a concentration of 333 mg/mL. Statistical analysis of the diameters of the inhibition zone of

Table 6. The inhibitory effect of *L. inermis* seeds of different fractions concentrations on bacterial pathogens, expressed as Diameter of the inhibition zone in mm.

	CC*	*S. aureus*	*B. cereus*	*E. faecalis*	*P. aeruginosa*	*E. coli*	*C. albicans*
F_1 He	333	NE	NE	NE	NE	NE	NE
	166.5	NE	NE	NE	NE	NE	NE
F_2 DM	333	NE	8.5±0.7^{c}	NE	7.5±0.7^{a}	NE	NE
	166.5	NE	7.5±0.7^{c}	NE	7.0±0.0^{a}	NE	NE
F_3 ETOH	500	15.5±0.7^{e}	14.0±0.0^{a}	14.5±0.7^{a}	NE	NE	NE
	250	14.5±0.7^{e}	13.5±0.7^{a}	12.5±0.7^{a}	NE	NE	NE
F_4 Aq	333	11.5±0.7^{a}	9.5±0.7^{b}	9.5±0.7^{b}	NE	NE	NE
	166.5	9.0±0.0^{a}	8.5±0.7^{b}	8.5±0.7^{b}	NE	NE	NE
Penicillin	10	8.0±0.0^{a}	7.5±0.5^{d}	17.5± 0.5^{c}	NE	NE	_
Ampicillin	10	8.5±0.5^{a}	9.0±0.0^{e}	20.5± 0.5^{d}	NE	11.5±0.5^{a}	_
Tetracycline	30	27.5±0.5^{b}	20.5±0.5^{f}	24.5± 0.5^{e}	NE	7.0±0.0^{b}	_

***CC**-Concentration of extracts in mg/ml, Concentration of Penicillin, Ampicillin, Tetracycline in µg/ml. Diameter of the inhibition zone in mm. **NE**-NO EFFECT The letters $^{a\text{-}d}$ are used to indicate a statistically significant difference (p<0.05) between the samples that were tested.

L. inermis seed fractions showed significance (p<0.05) against *S. aureus*, *B. cereus* and *E. faecalis*. In comparison with the standard antibiotics: penicillin, ampicillin and tetracycline, *S. aureus*, *B. cereus* and *E. faecalis* recorded inhibition zones of 27.5±0.5 mm, 20.5±0.5 mm and 24.5±0.5 mm in the presence of tetracycline. Notably, *P. aeruginosa* exhibited resistance to streptomycin, penicillin and ampicillin.

Comparing our results with other studies, it was demonstrated antibacterial activity of the ethanolic extract of *L. inermis* seeds exhibits against the standard strain of *P. aeruginosa* (34). However, none of our fractions exhibited activity against *C. albicans*, a result consistent with the findings in another study, where none of the samples of fresh and dry seeds showed detectable activity against *C. albicans* (34).

The determination of the minimum inhibitory concentration (MIC) for the fractions was exclusively performed for the microorganisms that exhibited positive results in the inhibition test. According to the finding in Table 7, the MIC recorded in this study ranged from 1.563 to 33.3 mg/mL. Specifically, the MIC value required to inhibit the growth of *S. aureus* and *B. cereus* was 1.563 mg/mL in the ethanol fraction and 8.325 mg/mL in the aqueous fraction. *E. faecalis* was inhibited at a minimum concentration of 3.125 mg/mL in the ethanol fraction and 8.325 mg/mL in the aqueous fraction. The *P. aeruginosa* strain, on the other hand, was suppressed at a minimum dose of 33.3 mg/mL.

The MBC, defined as the minimum antibacterial concentration resulting in microbial death, is characterized by the inability to re-culture the killed bacteria. Table 8 displays the MBC of each fraction studied, revealing values ranging from 3.125 to 33.3 mg/mL. Specifically, the MBC of the ethanolic fraction against *S. aureus*, *B. cereus* and *E. faecalis* strains was 12.5, 3.125 and 6.25 mg/mL respectively. For the aqueous fraction, the MBC against *S. aureus*, *B. cereus* and *E. faecalis* strains was 16.65, 33.3 and 33.3 mg/mL, respectively. Notably, based on our knowledge and research on Scopus, Web of Science and PubMed databases, no study has been done on MIC and MBC.

This discovery further enhances the pharmaceutical value of *L. inermis* seeds and validates the traditional use of *L. inermis*. While most previous studies focused on leaf and flower extracts, our present study demonstrates the bioactivity of *L. inermis* seeds.

Previous research has suggested that the most potent antimicrobial effect may be associated with phenolic compounds, specifically the presence of hydroxyl-phenolic structural groups, which enhances the antimicrobial impact (35). Subsequent studies have further confirmed the antimicrobial activity exhibited by polyphenols and flavonoids (36). Polyphenols exert their antimicrobial activity through various mechanisms, including structural modification of the cell membrane, regulation of membrane permeability, modulation of cellular interactions through hydrogen bonding, reduction of lipid content, and ultimately, inhibition of microbial growth.

Our study significantly expands our understanding of the antibacterial potential of *L. inermis* seeds and provides valuable data on MIC and MBC, which have not

Table 7. Minimum inhibitory concentration of the different fractions of *L. inermis* seed against microorganisms.

Strains	MIC*					
	S. aureus	***B. cereus***	***E. faecalis***	***P. aeruginosa***	***E. coli***	***C. albicans***
F_1 He	-	-	-	-	-	-
F_2 DM	-	8.325^{d}	-	33.3^{c}	-	-
F_3 ETOH	1.563^{a}	1.563^{c}	3.125^{a}	-	-	-
F_4 Aq	8.325^{b}	8.325^{d}	8.325^{b}	-	-	-
Ampicillin	0.063^{a}	>2^{a}	0.04^{c}	0.5^{b}	0.063^{a}	-
Tetracycline	0.012^{a}	0.012^{b}	0.012^{d}	0.012^{a}	0.012^{b}	-

*****Concentration of extracts expressed in mg/mL and antibiotics in µg/mL; **MIC**-Minimum inhibitory concentration. The letters **$^{a-d}$** are used to indicate a statistically significant difference (p<0.05) between the samples that were tested.

Table 8. Minimum bactericidal concentration of the different fractions of *L. inermis* seed against microorganisms.

Strains	MBC*					
	S. aureus	***B. cereus***	***E. faecalis***	***P. aeruginosa***	***E. coli***	***C. albicans***
F_1 He	-	-	-	-	-	-
F_2 DM	-	8.325^{c}	-	≥33.3^{a}	-	-
F_3 ETOH	12.5^{a}	3.125^{a}	6.25^{a}	-	-	-
F_4 Aq	16.65^{b}	33.3^{b}	33.3^{b}	-	-	-
Ampicillin	0.5^{c}	>2^{d}	2^{c}	>2^{b}	0.063^{a}	-
Tetracycline	0.047^{d}	0.012^{e}	0.75^{d}	0.094^{c}	0.012^{b}	-

*****Concentration of extracts expressed in mg/mL and antibiotics in µg/mL; MBC- Minimum bactericidal concentration; The letters **$^{a-d}$** are used to indicate a statistically significant difference (p<0.05) between the samples that were tested.

been previously reported. These findings strongly support the traditional use of *L. inermis* and underscores the potential application of seed fractions in pharmaceutical and medical contexts. Furthermore, our research highlights the crucial role of phenolic compounds in the observed antimicrobial effects.

Conclusion

In summary, this study underscores the remarkable antioxidant and antimicrobial properties of the investigated fractions. The demonstrated antioxidant potential suggests their efficacy in addressing health issues related to oxidative stress. Concurrently, the noteworthy antimicrobial efficacy against specific pathogens indicates a promising avenue for developing treatments to combat infectious diseases.

The insights derived from this research not only contribute to our understanding of the involved bioactive molecules but also underscore the potential of these compounds to be valuable resources for pharmaceutical and therapeutic applications. Further investigations, including *in vivo* studies and dose optimization, are warranted to fully unlock the therapeutic potential and mechanism of action of these compounds. This work paves the way for the development of novel pharmaceutical products and treatments that leverage the antioxidant and antimicrobial prowess of these bioactive agents.

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Authors contributions

AM designed the study, conducted the statistical analysis, and drafted the manuscript. FZ.B, LO, MF, HB and AD. contributed to the statistical analysis. AZ ensured the formal analysis and supervised the process. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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