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PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT ACTIVITY AND ANTIBACTERIAL ACTIVITY OF *ILEX DIPYRENA*

RABIA SHAMS AND JEHAN BAKHT*

Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar Pakistan
**Corresponding author's email: jehanbakht@yahoo.co.uk*

Abstract

The utilization of botanical flora and plant-derived therapeutics is extensively prevalent within indigenous communities globally. Furthermore, in contemporary society, there is a growing inclination to embrace these natural sources as supplements or alternatives to synthetic pharmaceutical agents. This ongoing investigation is concerned with the comprehensive examination of phytochemical constituents, molecular attributes, and antibacterial efficacy inherent to the *Ilex dipyrena* plant. The investigation revealed that the plant's butanol fraction exhibited good antibacterial activity, particularly against *E. faecalis*, when compared to various other extracts and bacterial strains under scrutiny, presenting a zone of inhibition of 26.00±0.33 mm. The botanical specimen demonstrated substantial composition of flavonoids, saponins, carbohydrates, and tannins. Additionally, minute quantities of alkaloids, proteins, and lipids were discerned. Commendable antioxidant activity has been shown by the butanol fraction both in DPPH and ABTS methods. In DPPH assay, the butanol fraction has displayed the highest antioxidant activity as 91.36% inhibition while in ABTS test, the inhibition noted was 88.88% at 1000 µg/ml with the IC_{50} value of 20.91 µg/ml. Gas chromatography-mass spectrometry (GC-MS) analysis has revealed the presence of significant bioactive compounds within the samples. These compounds include coumaran, 2-Hexyl hydroperoxide, Proline, 5-0xo, methyl ester, 4-((1E)-3-Hydroxyl-1-propnyl)-2-methoxyphenol, Cinnamic acid p-hydroxy-methyl ester, Tetradecanoic acid, 1-Octadecyne, n-Hexadecanoic acid, 3-(2,5-dimethoxyphenyl) propionic acid, Linoleic acid, methyl ester, Phytol, 9-12,15-octadecatrienoic acid and Octadecanoic acid.

Key words: Phytochemical, Antioxidant activity, Molecular profiling, Antibacterial activity, *Ilex dipyrena*.

Introduction

The potential of medicinal plants to produce antimicrobial agents holds promise in restricting the growth of pathogenic microbes. This phenomenon involves the rapid propagation of bacteria, fungi, viruses, and protozoa, utilizing mechanisms that are divergent from those elucidated in the context of established antimicrobial agents. This avenue presents a potential strategy to address the challenge posed by resistant microbial strains. Certain bioactive compounds sourced from these plants exhibit intrinsic antibacterial properties alongside the ability to modify antibiotic resistance, and in some cases, their synergistic application with antibiotics enhances their efficacy against bacterial antibiotic resistance (Vaou *et al*., 2021). The prevalence of bacterial strains susceptible to conventional therapeutics and their inclination to develop resistance to alternative treatments underscore the capacity of specific bacterial species to acquire and propagate resistance traits against existing antibacterial agents. This necessitates strategies such as pharmaceutical companies modifying molecular configurations of current drugs, bolstering their efficacy, or reinstating compromised functionalities due to evolving bacterial resistance mechanisms. Such approaches are vital for the introduction of innovative antimicrobial pharmaceuticals (Silva & Júnior, 2010).

The high levels of antioxidants found in medicinal plants are also known to control oxidation of lipids or other molecules. Since these lipid oxidation products interact with biological materials posing to cellular damage and ultimately associated with life threatening diseases like cancer, so the consumption of foods containing significant amount of antioxidants is also highly recommended (Skrovankova, 2012).

Medicinal plants harbor a plethora of secondary metabolites, which although non-essential for plant growth and development, are believed to serve as defensive chemicals, facilitating interaction with the environment and adaptation (Pant *et al*., 2021). Unintentionally, numerous contemporary medications have origins in therapeutic plants, making substantial contributions to medical treatment. The advancement, modernization, and quality control of herbal medicines hinge on meticulous analysis and extraction of plant constituents. The study of medicinal plants contributes to comprehending plant toxicity, safeguarding humans and animals against environmental toxins (Gomathi *et al*., 2015). The global botanical landscape encompasses approximately 258,650 identified plant species, with over 10% employed for therapeutic purposes. The northwestern region of Pakistan, characterized by diverse topography and ecology, hosts a rich array of medicinal plants, embedded within the cultural heritage of the area, utilized for a spectrum of treatments (Khan *et al*., 2019).

Ilex dipyrena predominantly thrives in damp habitats within mild temperate and subtropical zones, with altitudinal distribution ranging from 1,500 to 3,350 meters, extending into tropical zones. The present research employs a comprehensive approach encompassing conventional phytochemical screening, antimicrobial analysis, and qualitative assessment. This multifaceted strategy aims to elucidate the intricate processes involving the extraction, isolation, and subsequent characterization of bioactive compounds derived from plant extracts of *Ilex dipyrena*.

Material and Methods

Plant collection and identification: The Department of Botany at the University of Malakand in Pakistan validated

the plant *Ilex dipyrena* that was utilized in this study, which was gathered from the Malakand division of KPK, Pakistan. The experimental procedure involved the systematic preparation of the leaves and stem sections of the plant. These botanical segments were meticulously divided, diced into suitable dimensions, and subjected to a controlled drying regimen under shaded conditions spanning a period of two to three weeks. Subsequent to the drying process, the individual components of each plant specimen were meticulously pulverized into a finely textured powder using a cutter mill, ensuring uniformity and homogeneity.

This powdered plant material was then subjected to a maceration process involving an 80% methanol solution, allowing for a period of two weeks for efficient extraction of bioactive constituents. Following this steeping phase, the resultant mixtures were subjected to filtration using Whattman filter paper (Whatman no. 1) to separate solid residues from the liquid extracts. To facilitate further concentration and purification, the filtrates underwent evaporation using a rotary evaporator operating at a controlled temperature of 40°C and reduced pressure conditions, thereby ensuring the removal of the solvent.

These meticulously processed procedures adhered to the methodologies outlined by Zeb *et al*., (2014b) and Ahmad *et al*., (2015), aiming to maintain methodological consistency and integrity. The outcome of these systematic steps led to the production of methanolic extracts from *Ilex dipyrena* leaves, manifesting as semi-solid mass with an approximate mass of 500 g indicative of the successful extraction and concentration of bioactive constituents from the plant material.

Extraction and Fractionation: The botanical specimens under investigation underwent a systematic fractionation process employing the sequential solvent-solvent extraction technique, a method meticulously designed to methodically isolate distinct bioactive components. Specifically, the initial step involved the preparation of crude methanolic extracts derived from the leaves of *Ilex dipyrena*, each weighing approximately 300 g. These crude extracts were subjected to a precise procedural protocol, wherein they were individually suspended in 500 ml of distilled water contained within separating funnels. Subsequently, a controlled dilution with 500 ml of n-hexane was performed, aimed at enhancing the partitioning of the bioactive constituents. Upon introducing the n-hexane solvent, the extract underwent vigorous agitation to ensure a thorough mixing of the constituents. The subsequent phase separation facilitated the development of two distinctive layers within the solvent mixture. The uppermost layer, characterized by its colorless appearance, constituted the n-hexane phase, which was meticulously collected. This collection process was then iterated to guarantee the procurement of a purified n-hexane layer.

To proceed with the comprehensive fractionation, each collected extract was subjected to a series of solvent-based partitioning steps with solvents of increasing polarity. Both butanol and ethyl acetate were sequentially employed for this purpose, enabling the segregation of varying groups of compounds based on their differential solubility profiles. Ultimately, the final aqueous fraction was carefully isolated as the terminal step of this fractionation process. These well-defined fractionation procedures were executed in accordance with the protocols delineated by Zeb *et al*., (2014a) and Shah *et al*., (2015), assuring methodological rigor and accuracy in the pursuit of isolating and characterizing distinct bioactive components from the plant materials.

Antibacterial potential of *Ilex dipyrena*

Bacterial strains: All the extracted samples (the crude methanol, ethyl acetate, butanol and aqueous extract) from the plant were tested for their antibacterial properties against *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Bacillus subtilis*. The Quaid-e-Azam University in Islamabad, Pakistan, Department of Microbiology generously contributed all of the bacterial strains. These were identified using a variety of biochemical tests and stored on agar slants under freeze-dried conditions at 4°C for subsequent use (Cowan, 1993).

Preparation and standardization of bacterial strains: Bacterial strains were cultured by an incubation period of 24 hours at a temperature of 37°C. The bacterial suspensions were prepared by adjusting the cell density of the bacterial strains to 1 × 108 CFU/ml using the McFarland standard No 2. Subsequently, the suspensions were diluted to a cell density of 1 × 106 CFU/ml using a double beam UV-visible spectrophotometer (Thermo electron corporation USA) at a wavelength of 625 nm (Petrikkou *et al*., 2001).

Antibacterial assay: The assessment of antibacterial efficacy of plant samples was conducted using the well-diffusion method, as described by Imran *et al*., (2014). Nutrient agar plates were prepared, properly labeled, and placed under aseptic conditions within a laminar flow hood. The plates were then inoculated with the target microorganisms. Using a sterilized cork borer, wells with a diameter of 5 mm were carefully created in the agar plate. Concentrated extracts of the plant, prepared at a concentration of 10 mg/ml. In each petri dish, four wells were arranged along the sides and one well in the center. The central well contained ciprofloxacin at a concentration of 1 mg/ml, serving as the positive control. A micropipette was used to transfer each plant extract, which had a volume of 100 µl, into the respective well of a petri dish. The petri dishes were subsequently incubated at 37°C within a BOD incubator for a period of 24 hours. Following incubation, the zone of inhibition surrounding each sample was measured in millimeters. To ensure accuracy, all samples were analyzed in triplicate and the obtained data was expressed as the mean ± standard error of the mean.

Phytochemistry: The verification of bioactive chemical constituents within plant extracts was accomplished through a series of distinct chemical assays. All the extracts, the crude methanol, butanol, ethyl acetate and aqueous were subjected to tests to ascertain the presence of steroids, alkaloids, carbohydrates, tannins, saponins, proteins, lipids, and flavonoids. The confirmation of alkaloids was achieved

using Mayer's test, wherein Mayer's reagent was applied to the filtrate within a test tube, yielding a white or creamy precipitate as an indication of alkaloid presence (Salhan *et al*., 2011). The assessment of proteins and amino acids relied on Millon's test. Here, a filtrate, resulting from dissolving 100 milligrams of the extract in ten milliliters of distilled water, was mixed with Millon's reagent. The emergence of a white precipitate signified the existence of proteins (Chulet *et al*., 2010). Carbohydrate content was ascertained through Fehling's test, wherein the manifestation of a red precipitate denoted the presence of sugars. The determination of phytosterols employed Libermann-Burchard's test, characterized by a spectrum of colors indicative of phytosterol presence (Salhan *et al*., 2011). The identification of fixed oil was executed via the spot test. A minute quantity of the extract was pressed between filter papers, and the appearance of an oil stain confirmed the presence of fixed oil. For oils and lipids, a small portion of the extract was mixed with a drop of phenolphthalein and a few drops of 0.5 N alcoholic potassium hydroxide, and then subjected to a 2-hour heat treatment in a water bath. The presence of fixed oil and lipids was determined by the formation of soap or partial alkali neutralization (Salhan *et al*., 2011). The evaluation of flavonoids involved warming a 4-milliliter extract solution with one and a half milliliters of 50 percent methanol solution, along with magnesium and strong hydrochloric acid. The emergence of red and orange colors denoted the presence of flavonoids and flavones, respectively (Singh *et al*., 1997). Gallic tannins displayed a blue color upon adding 1-2 drops of ferric chloride solution to 0.5 milliliters of the extract solution, while catecholic tannins exhibited a greenish-black hue. To confirm the presence of saponins, a half-gram plant extract was agitated for 5 minutes in a test tube, with a positive outcome indicated by persistent foam formation (Salhan *et al*., 2011).

DPPH radical scavenging activity: The plant extract's DPPH radical scavenging activity was evaluated as described in Mensor *et al*., (2001). The sample stock solutions were diluted in methanol to achieve final concentrations of 1000, 500, 250, 125, 62.5 µg/ml. One ml of a 0.3 mM DPPH methanol solution was added to a 2.5 ml extract or standard solution and allowed to react for 30 minutes at room temperature. The absorbance of the resultant mixture was measured at 518 nm, and the percentage antioxidant activity (AA percent) was determined by using the formula:

$$AA\% = [(A_B - A_E)/A_B] \times 100$$

Where;
A_B = Absorbance of the blank sample
A_E = Absorbance of the plant extract
Positive control = Solution of Ascorbic acid (vitamin C) served as positive control

ABTS radical scavenging activity: The ABTS radical cation decolorization assay was also employed to investigate the ability of plant extracts to scavenge free radicals (Ahmad *et al*., 2016). A 7 mM solution of ABTS was prepared using deionized water. By mixing ABTS solution with 2.45 mM potassium persulfate (final concentration) and letting the mixture stand in the dark at room temperature for 12–16 hours before use, ABTS radical cation (ABTS+•) was created. Following the incubation period, 50% methanol was added to the solution to bring its absorbance at 745 nm down to 0.7. A test tube containing 300 µl of test sample was filled with 3 mL of ABTS solution. A double beam spectrophotometer was used to measure the absorbance values for six minutes after the solution was moved into the cuvette. As a positive control, ascorbic acid was employed. Following a triple run of each sample, the following formula was used to calculate the % ABTS radical scavenging potential:

$$AA\% = [(A_B - A_E)/A_B] \times 100$$

Where;
A_B = Absorbance of the blank sample
A_E = Absorbance of the plant extract
Positive control = Solution of Ascorbic acid (vitamin C) served as positive control.

GC/MS analysis: The gas chromatography-mass spectrometry (GC/MS) analysis was meticulously carried out at the well-established Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories, situated in Peshawar, Khyber Pakhtunkhwa, known for its rigorous scientific endeavors. The analytical procedure embraced the utilization of a sophisticated GC/MS system, specifically the Shimadzu QP-2010+, which was meticulously equipped with a BD-5 column characterized by dimensions of 30 meters in length, 0.25 millimeters in internal diameter, and a film thickness of 0.25 micrometers. For the controlled flow of analytes, helium gas was chosen as the carrier gas, maintained at a steady flow rate of 1.22 milliliters per minute, a parameter carefully selected and optimized, operating under the splitless injection mode to enhance sample introduction efficiency.

A precisely calibrated temperature regimen was established within the analytical setup. The oven temperature was initially set at 80ºC and maintained for duration of one minute, subsequently sustained at this isothermal condition for an additional minute, ensuring optimal thermal conditions for the separation of the analytes. Meanwhile, the injector port was maintained at a temperature of 250ºC, meticulously controlled to facilitate efficient volatilization and injection of the samples. The detector module was set to operate at 280ºC, promoting sensitive and accurate detection of the separated analytes. The process of sample injection was executed with precision, employing a split ratio of 1:0, ensuring the entirety of the injected sample entered the analytical column. The applied pressure during injection was precisely regulated at 82.7 kilopascals, and the injected volume was maintained at 1 microliter, ensuring consistency in the analytical process. The electron ionization (EI) energy, a critical parameter impacting ionization efficiency, was optimized at 70 electron volts (eV), and the ensuing ionization events were subjected to scan duration of 1.5 seconds. Spanning a comprehensive mass range, the spectrometric analysis encompassed masses ranging from 40 to 800 atomic mass units (amu), thereby capturing a wide spectrum of ionized species generated from

the analytes, contributing to a thorough characterization of the sample constituents. The described analytical protocol underscores the meticulous attention to detail and precision applied during the GC/MS analysis, culminating in a comprehensive and scientifically valid outcome. Data processing, encompassing mass spectra and chromatographs, was conducted using the Chemstation software. This analytical process provided valuable insights into the composition and characteristics of the studied sample.

Results

Antibacterial potential of *Ilex dipyrena*: The *Ilex dipyrena* fractions were tested against six different bacterial strains (Fig. 1). Among the test strains, *E. faecalis* was found most susceptible for *Ilex dipyrena* leaves in its butanol fraction with a zone of inhibition of 26.00±0.33 mm. The plant was also found active against *S. aureus* and *K. pneumonia* with zone of inhibitions of 21.33±0.88 and 18.33±0.33 mm respectively in the butanol fractions. Against *E. coli*, the plant was found more active in the ethyl acetate fraction rather with a zone of inhibition of 23.66±0.88 mm. None of the extracts were found active against *B. subtilis*.

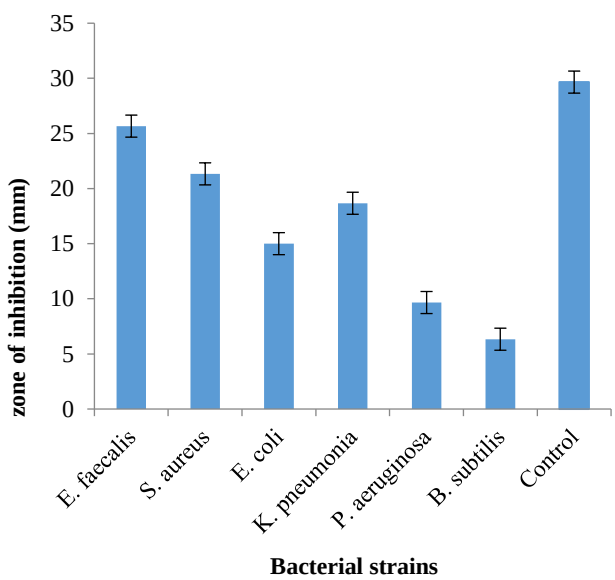

Fig. 1. Antibacterial potential of *Ilex dipyrena* against six different bacterial strains (Bar shows ± SE).

Phytochemistry of solvent fractions of *Ilex dipyrena*: In the context of the present investigation, diverse solvent fractions derived from *Ilex dipyrena* were subjected to comprehensive screening for the presence of various phytochemical constituents, including steroids, alkaloids, carbohydrates, tannins, saponins, proteins, lipids, and flavonoids, as detailed in (Table 1). Initial scrutiny of the crude fraction exhibited minimal indications of alkaloids and, conversely, revealed an absence of sterols. The crude specimen displayed scant levels of proteins and lipids, whereas noteworthy quantities of carbohydrates, tannins, and flavonoids were discerned. Upon closer examination, the investigation unveiled that the butanol fraction displayed affirmative indications for sterols, proteins, lipids, and a substantial abundance of flavonoids. Moreover, appreciable levels of carbohydrates, tannins, and saponins were identified within the butanol fraction, accompanied by marginal traces of alkaloids. The ethyl acetate-derived sample from *I. dipyrena* leaves exhibited pronounced tannin content and yielded negative results for sterols, alkaloids, and proteins. This particular sample contained carbohydrates and saponins in appreciable amounts.

Contrarily, the n-hexane fraction demonstrated negative outcomes for sterols, alkaloids, and proteins. Nevertheless, the n-hexane extract featured flavonoids and saponins in substantial quantities, while trace quantities of carbohydrates and lipids were also ascertained. The aqueous fraction extracted from *I. dipyrena* leaves indicated negative results for sterols and proteins. Notably, this fraction exhibited significant content of saponins, flavonoids, and tannins, accompanied by subtle traces of alkaloids.

Free radicals scavenging activity of *Ilex dipyrena* leaves extracts against DPPH: The results of antioxidant activity of various samples of *Ilex dipyrena* leaves against DPPH have been summarized in (Fig. 2). The test samples employed against DPPH were n-hexane, ethyl acetate, butanol, methanol and aqueous fraction. The standard compound was also used as positive control among the test samples, i.e., Ascorbic acid (vitamin C). All the test samples were found active against DPPH. The butanol fraction has displayed the highest antioxidant activity as 91.36, 85.34, 78.39, 72.47 and 65.44 per cent inhibition at the concentrations of 1000, 500, 250, 125, 62.5 µg/ml, respectively with the IC_{50} value of 20.91 µg/ml. The positive control exhibited percent inhibition of 94.58, 88.68, 84.46, 79.50, 74.47 at the concentrations of 1000, 500, 250, 125 and 62.5 µg/ml respectively with the IC_{50} value of 02 µg/ml. Ascorbic acid and butanol outcomes are parallel to one another. The lowest activity has been calculated for the aqueous fraction, which showed 76.7, 71.3, 65.5, 57.2, 49.65% antioxidant activity at the concentrations of 1000, 500, 250, 125, 62.5 µg/ml, respectively with the IC_{50} value of 61.50 µg/ml. The rest of the samples showed moderate antioxidant activity comparatively.

Free radicals scavenging activity of *Ilex dipyrena* leaves extracts against ABTS: The antioxidant activity of various samples of *Ilex dipyrena* leaves against ABTS are displayed in (Fig. 3). Ethyl acetate, n-hexane, butanol, methanol and aqueous fractions of the plant were used to verify the antioxidant activity of the said plant. Ascorbic acid (vitamin C), the reference chemical, was also utilized as a positive control in the test samples. Of all the fractions, butanol has shown the highest activity. i.e., 88.88, 83.54, 75.01, 67.68 and 59.82 per cent inhibition at the concentrations of 1000, 500, 250, 125, 62.5 µg/ml, respectively with the IC_{50} value of 32.84µg/ml. The positive control exhibited percent inhibition of 91.51, 86.65, 81.25, 77.37 and 72.72 at the concentrations of 1000, 500, 250, 125, 62.5 µg/ml respectively with the IC_{50} value of 02 µg/ml. The results of ascorbic and butanol goes parallel with each other. The aqueous fraction exhibits the lowest activity, with an IC_{50} value of 78.12 µg/ml and antioxidant activity values of 74.4, 66.2, 61.0, 56.4, and 46.9% at doses of 1000, 500, 250, 125, and 62.5 µg/ml respectively. Comparatively, the remaining samples displayed modest antioxidant activity.

Table 1. Different phytochemicals present in the extracts of *Ilex dipyrena.*

Phytochemicals	E. acetate	Butanol	Aqueous	n-Hexane	Crude
Flavonoids	+	+++	++	+++	+++
Proteins	-	+	-	-	+
Carbohydrates	++	++	++	+	++
Alkaloids	-	+	+	-	+
Tannins	++	++	++	++	++
Sterols	-	+	-	-	-
Lipids	+	+	+	+	+
Saponins	+++	+++	+++	+++	+++

*+++ Most abundant, - Not present, + Trace amount, ++ Abundant

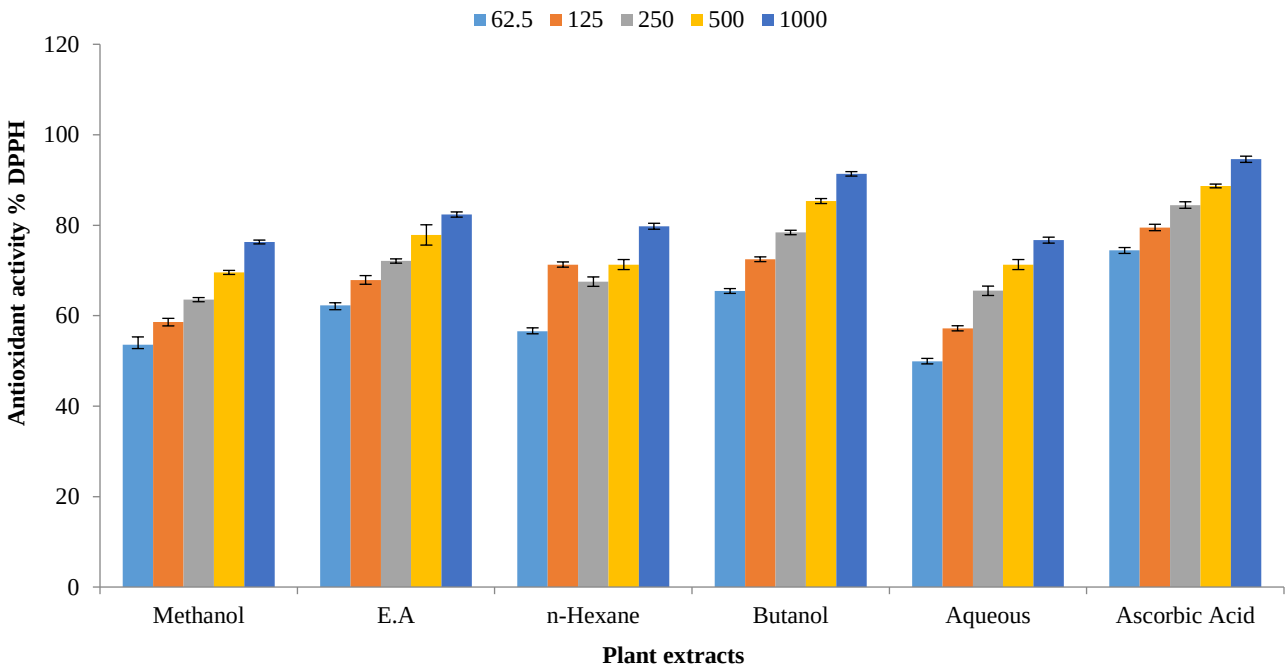

Fig. 2. Radicals scavenging activity of *Ilex dipyrena* against DPPH (Bar shows ± SE).

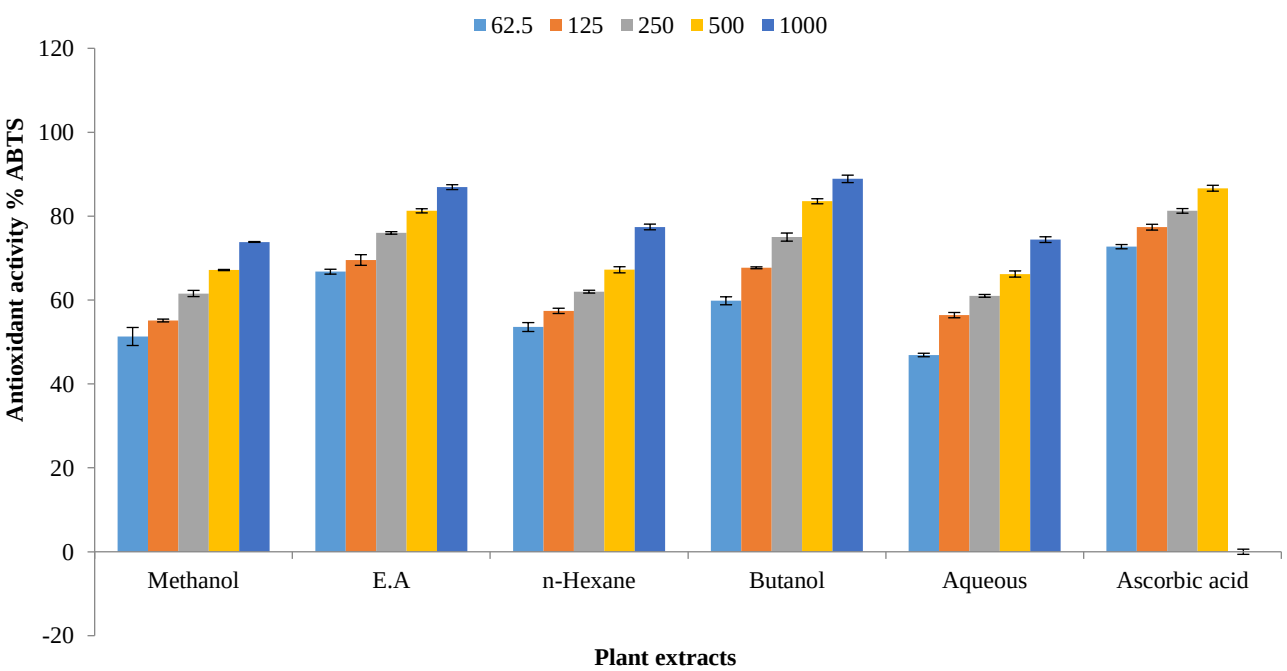

Fig. 3. Radicals scavenging activity of *Ilex dipyrena* extracts against ABTS (Bar shows ± SE).

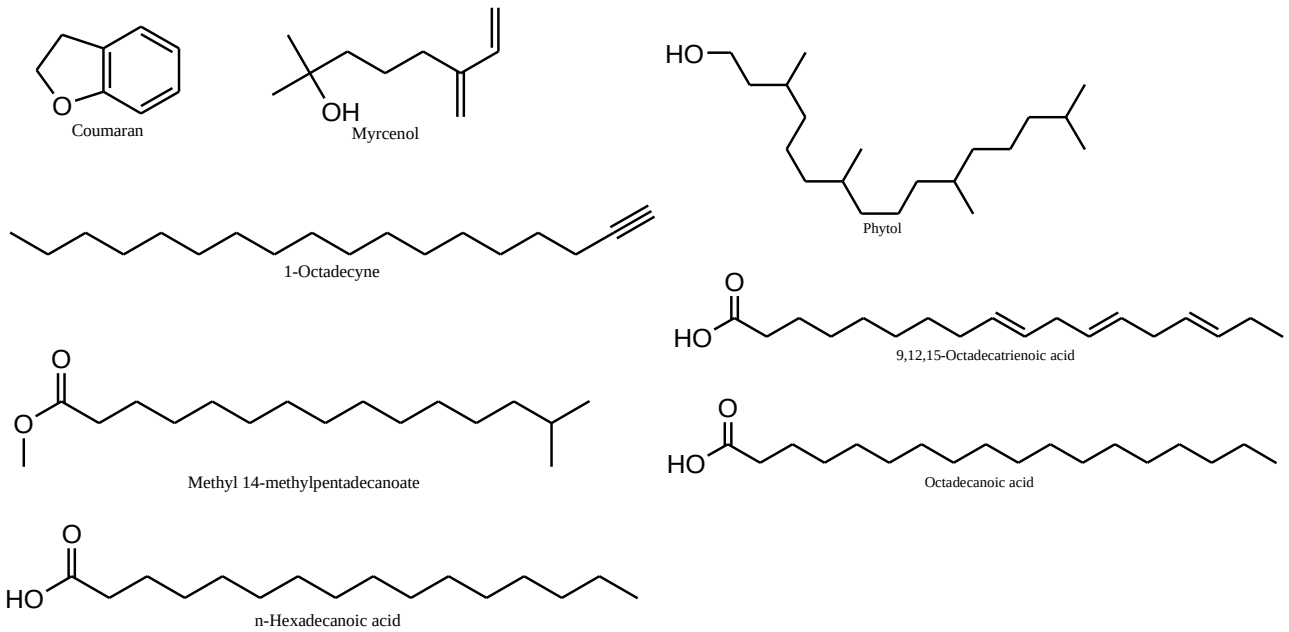

Fig. 4. Important bioactive compounds in *I. dipyrena* leaves.

GCMS analysis of crude methanolic extract of *Ilex dipyrena* leaves: The methanolic extract obtained from *Ilex dipyrena* leaves was subjected to GCMS analysis, resulting in the identification of a total of 16 distinct compounds. The analysis provided crucial information including the retention time, percentage area under the peaks, and concentrations of the identified compounds. The spectral data presented a set of 16 discernible peaks, each corresponding to a distinct compound. These compounds exhibited retention times of 8.545, 14.276, 21.308, 22.418, 23.031, 23.138, 23.338, 24.760, 25.466, 25.999, 27.320, 27.986, 28.105, 28.353, 28.848, and 29.270. Among these, the compound with the highest concentration was identified as phytol, constituting 29.29% of the total, followed by n-Hexadecanoic acid at 20.80%. In contrast, the compound with the lowest concentration was Heptadecanoic acid, detected at 0.65%. The list of bioactive compounds identified through this analysis encompassed Coumaran, 1-Octadecyne, Myrcenol, Methyl 14-methylpentadecanoate, n-Hexadecanoic acid, Phytol, 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-, and Octadecanoic acid. This analysis offers valuable insights into the chemical composition of the methanolic extract of *Ilex dipyrena* leaves and contributes to our understanding of its potential bioactive constituents (Fig. 4).

Discussion

When utilized as medicine, medicinal plants exhibit a variety of traits. Each plant has a variety of substances that may interact simultaneously to either enhance or impair the activities of one another or to counteract any potential negative effects. To obtain the desired result, chemical products might be combined with plant-based components. Some plant-based ingredients have been shown to be useful in avoiding or lowering the chance of contracting specific diseases (e.g, flu) which can assist relieve the burden and expense of employing pharmaceutical treatments (Mohammed, 2019). In this research endeavor, the potential antibacterial effects of *Ilex dipyrena* leaves were explored by evaluating their impact on six distinct bacterial strains, including *E. faecalis*, *S. aureus*, *E. coli*, *K. pneumonia*, *P. aeruginosa*, and *B. subtilis*. The initial focus centered on the assessment of the leaves' influence on *E. faecalis*, a commensal bacterium residing in the human gastrointestinal tract, oral cavity, and vagina. Notably, this bacterium has the capacity to instigate various infections across multiple body sites, resulting in a diverse range of medical conditions. Notably, enterococci have raised to prominence as key nosocomial pathogens, with *E. faecalis* being responsible for the majority of enterococcal infections in humans (Kayaoglu & Ørstavik, 2004).

Among the distinct extracts evaluated, the butanol fraction exhibited substantial activity against *E. faecalis*, while the aqueous fraction displayed no discernible inhibitory effect on the same bacterial strain. The results are in agreement with the findings of Ait-Yahia *et al*., (2018). The investigation extended to *S. aureus*, where the butanol fraction also emerged as the most potent, whereas the aqueous extract failed to elicit any inhibitory activity. The substantial activity of the butanol fraction against *S. aureus* is consistent with the research findings published by Zhou *et al*., (2019). In the context of *E. coli*, the ethyl acetate fraction displayed significant activity, whereas the aqueous extract showed no such effect against this strain. Exploring the effect on *P. aeruginosa*, the ethyl acetate fraction manifested inhibitory action against this strain, whereas the other extracts displayed relatively milder activity. However, *B. subtilis* exhibited resistance to the plant fractions, with only marginal inhibitory zones observed in response to the n-hexane and ethyl acetate fractions, while the methanol, butanol, and aqueous extracts remained ineffective. The stem extracts displayed no activity against this strain.

Medicinal plants serve as a valuable reservoir of potential bioactive chemical compounds, presenting avenues for novel drug development. Notably, alkaloids, tannins, flavonoids, and phenolic compounds are prominent bioactive elements present in plants, holding promise for addressing various health conditions and chronic ailments. The interplay between phyto-constituents and plant bioactivity assumes significance in crafting substances with specific therapeutic properties (Yadav *et al*., 2014). Qualitative analysis of *I. dipyrena* leaves unveiled the prevalence of saponins across all fractions, along with the widespread occurrence of flavonoids, with the exception of the ethyl acetate fraction. Abundant presence of tannins and carbohydrates was evident, except within the n-hexane portion, where trace carbohydrates were detected. A minimal trace of sterols emerged exclusively in the butanol fraction, and a slight presence of alkaloids was noted in the crude, butanol, and aqueous fractions. Lipids were detected in trace amounts across all five extracts, while proteins were confined to the crude and butanol fractions.

Activity of *I. dipyrena* against DPPH proved its great radical scavenging potential. The highest activity being shown by the butanol fraction goes in line with the studies on *Albizia adianthifolia* (Borar *et al*., 2011). Radical scavenging activities using ABTS also manifested great antioxidant potential of the plant. In this experiment also, butanol fraction was the most potent one among the different extracts (Zhou *et al*., 2011).

The application of GCMS analysis yielded valuable insights into the composition of compounds within the samples, providing data on retention times, percentage areas, and concentrations of the identified constituents. Noteworthy bioactive entities present encompass coumaran (Solanki & Shekhawat, 2012), 2-Hexyl hydroperoxide, Proline, 5-0xo, methyl ester, and 4-((1E)-3-Hydroxyl-1-propnyl)-2-methoxyphenol (Shareef *et al*., 2016). Additional compounds include Cinnamic acid p-hydroxy- methylester, Tetradecanoic acid (Sivakumar *et al*., 2011), 1-Octadecyne (Zayed & Samling, 2016), n-Hexadecanoic acid (Aparna *et al*., 2012), 3-(2,5-dimethoxyphenyl) propionic acid (Jeong *et al*., 2015), Linoleic acid, methyl ester (Subbarao *et al*., 2008), Phytol (Santos *et al*., 2013), 9-12,15-octadecatrienoic acid (Dong *et al*., 2000) and Octadecanoic acid (Pu *et al*., 2010). This comprehensive identification of constituents contributes substantially to the broader comprehension of the chemical makeup and potential biological activity associated with *Ilex dipyrena* leaves.

Conclusion

According to the results of our recent investigations, *Ilex dipyrena* has significant and adequate antibacterial potentials. The current investigation revealed that *I. dipyrena* leaf extracts exhibited encouraging antioxidant and radical scavenging properties. The biomolecules present, as seen in the GC MS pattern of the crude methanolic fractions, and their biological functions in *I. dipyrena*, support the ethnobotanical assertions of the plant's therapeutic efficacy.

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