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Research Article

EVALUATION OF PHYTOCHEMICAL CHARACTERISTICS AND ANTIMICROBIAL ACTIVITY OF *PLEUROTUS FLORIDA* MUSHROOM

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ABSTRACT

Mushrooms are white rot fungi regarded as one of the well known food and possessing various kinds of biopharmaceuticals compounds. Mushrooms are superior to many vegetables based on their nutritional value and it contains 40 - 49% of proteins. The present study consolidates in the aspects of cultivation of *Pleurotus florida* mushroom with nutrient supplement, biochemical, qualitative and quantitative phytochemical analysis and the antibacterial activity. *Pleurotus florida* mushroom was cultivated by using *Macrotyloma uniflorum* (horse gram) as a nutrient supplement substrate; adopted by the "layer spawning" method and the effect of mushroom yield were determined. The fresh fruit bodies of mushroom were harvested, dried, powdered and extracted (by using methanol, ethanol, aqueous, ethylacetate and hexane) using soxhlet apparatus. Then the physico-chemical and antibacterial properties of the extracts were carried out using standard bioassay methods. The horse gram nutrient supplemented batch showed better yield i.e. 23% than control batch. Aqueous, ethanolic and methanolic extracts contains high amount of phenolic compounds, glycosides and flavonoids based on the phytochemical analysis and also strongly inhibited the growth of the both Gram-positive and Gram negative bacterium. Whereas, ethyl acetate and hexane extracts shows low reaction in phytochemical analysis and low antimicrobial activity. Bioactive compounds from *Pleurotus florida* mushroom extracts could be used as an alternate therapeutics as antibiotics.

Keywords: *Pleurotus florida,* Phytochemicals, Antibacterial activity

INTRODUCTION

Mushroom is a nutritious source of vegetarian delicacy and a suitable substitute for meat and eggs. It is easily digestible and very popular in most of the developed countries and being accepted in many developing countries like India. Oyster mushrooms are easy to cultivate and process and do not require huge investment. Mushroom farming is being practiced in more than 100 countries and its production is increasing at the rate of 7 per cent per annum. Production of mushroom has already crossed 5 million metric tons annually in the world and is expected to reach around 7 million metric ton in next ten years. India had been known world over for its exotic mushrooms. Total mushroom production in India was 48,000.00 tons in 2005. Oyster mushroom cultivation has increased during the last decade [1, 2]. International demand for oyster mushrooms has remained steady (at about 900,000 t annually) over the last decade. This mushroom accounted for 14.2% of the total world production of edible mushrooms produced in 1997[1]. Although commonly grown on pasteurized rice (*Oryza sativa L.*) straw, oyster mushroom can be cultivated on a wide variety of substrates containing lignin and cellulose. Cultivation of oyster mushroom is becoming popular throughout the world because of their abilities to grow at a wide range of temperatures and to utilize various lignocelluloses[3]. Oyster mushroom cultivation can play an important role in managing and recycling of organic wastes as an alternative to other methods of disposal[4]. The genus *Pleurotus* comprises a group of edible ligninolytic mushrooms with medicinal properties and important biotechnological and environmental applications. The cultivation of *Pleurotus sp.* is economically important in food industry worldwide which has expanded in the past few years. *Pleurotus sp,* is the third most important cultivated mushroom for food purposes. Nutritionally, it has unique flavor and aromatic properties, and it is considered to be rich in protein, fiber, carbohydrates, vitamins and minerals and cholesterol free. *Pleurotus sp.* is promising as medicinal mushrooms, exhibiting hematological, antiviral, antitumor, antibiotic, antibacterial, hypocholesterolic and immunomodulation activities[5]. The protein content of mushrooms has been reported to be twice that of vegetables and four times that of oranges and significantly higher than wheat[6]. So the mushrooms are in increased demand and it could be dependent upon the phenomenal rise in the unit costs of the conventional sources of animal proteins such as beef, pork, chicken and fish[7].

Mushroom species have been shown to possess antioxidant capacity in *in vitro* systems[8]. Like other matrices containing antioxidant compounds, e.g. phenolics[9], organic acids[10] and alkaloids[11] from mushrooms can be used both as a food supplement and in the pharmaceutical industry. The public is becoming increasingly aware of problems with the over prescription and misuse of traditional antibiotics. Worldwide spending on finding new anti-infective agents is increasing. The use of other alternative forms of medical treatments is being investigated by researchers.

Keeping in view the importance of mushroom in India, the study was conducted to study the effect of *Macrotyloma uniflorum* (horse gram) on the yield of *Pleurotus florida.* To further examine the proximate analysis, crude phytochemical constituents and their effect of antimicrobial activity against human pathogens were determined.

MATERIALS AND METHODS

Spawn collection

The spawn packets of *Pleurotus florida* were obtained from Tamilnadu Agriculture University (TNAU) Coimbatore, Tamilnadu, India.

Test microorganisms

A total of 13 bacterial cultures (*Bacillus sp., Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Vibrio sp., Streptococcus sp., Campylobacter sp., Salmonella typhi, Shigella sp., Cornybacterium sp., Pseudomonas sp., Proteus sp.* and *Shigella sonnei*) were used in this study. Microorganisms were obtained from Microbiology laboratory (Periyar University, Salem).

Substrates and preparation

The chopped rice straw substrates were soaked in clean water overnight. Enough water was added to raise the substrate moisture to 75%. The soaked substrate was then boiled for three hours to increase temperature of the substrate to 95ºC. The same pasteurization process was applied to the supplements. Horse gram (*Macrotyloma uniflorum*) powder was used as a nutrient supplement[12].

Spawning

The substrates from the pasteurization drum were cooled to a temperature of 24ºC under clean and sterile conditions inside the

growing room and mixed with supplements before combining with spawn, based on wet weight. The spawn was thoroughly mixed with the substrate; some being placed underneath the substrate surface and a small amount of spawn sprinkled uniformly on the surface. The packed bags were weighed before keeping in cultivation room.

Cultivation conditions and harvesting

The inoculated bags were incubated in a cultivation room and maintained at 25-30ºC with relative humidity of 85 ± 5%, for ramification of the mushroom mycelia. Growth of mushroom was recorded daily for all the treatments. When the bags covered with full of mycelium and pin-heads started appearing, the bags were mouth opened to facilitate the development of fruiting bodies. As soon as the fruiting bodies developed and attained their full size, they were cut just above surface of the substrate with sterile sharp knife or blade. The harvesting was done in 4 flushes of 1 week intervals. After the 2nd flush, the substrate was turned upside down and regularly watered to harvest the 3rd and 4th flushes. The yields of mushroom were recorded.

Proximate analysis

Protein, fat, moisture, ash and total carbohydrate were determined with the procedure recommended by AOAC[13] and Crisan *et al.*, [14].

Preparation of the mushroom extract

Freshly-harvested whole mushrooms were shade dried and finely powdered. Twenty five grams of the powder were extracted with 250 ml of 95% solvents like methanol, ethanol, ethyl acetate, hexane and aqueous using Soxhlet apparatus. The residue was filtered and concentrated to a dry mass by vacuum distillation; the filtrate thus obtained was used as mushroom extract[15].

Preliminary phytochemical characteristics

Preliminary biochemical tests such as carbohydrates, tannins, phlobatannins, saponins, proteins, phenols, flavonoids, steroids, terpenoids, alkaloids, glycosides, cardiac glycosides, resins and fixed oil were carried out on the crude aqueous, methanolic, ethanolic, ethyl acetate and hexane extract using standard procedures described by Trease and Evans[16] and Harborne[17]. Based on the colour variation they were classified to high (+++), moderate (++), low (++) and no reaction (-).

Quantitative phytochemical analysis

Determination of total phenolic contents

The total phenolic content was determined according to the method described by Siddhuraju and Becker[18]. Aliquots (0.1ml) of each extracts were taken in test tubes and made up to the volume of 1 ml with distilled water. Then 0.5 ml of folin-ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. The analysis was performed in triplicate and the results were expressed as the catechol equivalents.

Estimation of total flavonoid content

The total flavonoid content of sample extracts was determined by the use of a slightly modified method described by Zhishen *et al.* [19]. A 0.5 ml extract was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a 5% sodium nitrate solution. After 6 min, 0.15 ml of a 10% aluminium chloride solution was added and allowed to stand for 6 min, then 2 ml of 4% sodium hydroxide solution was added to the mixture. Immediately distilled water was added to bring the final volume of 5 ml, and then the mixture is thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was determined at 510 nm. Catechol was used as a standard compound for the quantification of total flavonoid content.

Antimicrobial activity

Antimicrobial activity of the different extracts of *Pleurotus florida* mushroom was evaluated using agar diffusion technique in petriplates[20]. Briefly 25 µl of each extract (10mg/ml) was loaded on a sterile filter paper disc 6 mm in diameter and air dried. Indicator organisms were spread on Mueller-Hinton agar plates with sterile effusion and the discs were placed on agar plates. After incubation for 24 hours at 30°C, a clear zone around a disc was evidence of antibacterial activity. Diameter of the zones of inhibition was measured in millimeters. Each test was prepared in triplicates and each solvent was used as a negative control.

Statistical analysis

All experiments were conducted in triplicates and the parameters were given as means ± standard error. Both mean and standard deviation were performed where appropriate, using the statistical package within Microsoft® Excel Version 2010.

RESULTS

Pleurotus florida mushroom was cultivated on paddy straw substrate in two different batches. One batch with substrate mixed with horse gram nutrient supplement and the control batch with substrate alone cultivated for about 35days, during which four fleshings were made. The nutrient supplemented batch showed a faster rate of mycelial growth and pin head formation was observed on the 19th day, where as 23rd day in control batch. Time taken for first flush in control batch was also longest in about 34 days while the second flush was harvested in about 44 days and no third and fourth flush were obtained. In case of horse gram supplemented batch first flush was harvested in about 29 days, second flush in 37 days and also third and fourth flush were obtained (Figure 1, Table 1).

Table 1: Growth of *Pleurotus florida* mushroom

S. No	Parameters	Nutrient supplementation batch (days)	Control Batch (days)
1	Primordia initiation	19	23
2	First flush	26	34
3	Second flush	33	44
4	Third flush	37	0
5	Fourth flush	44	0

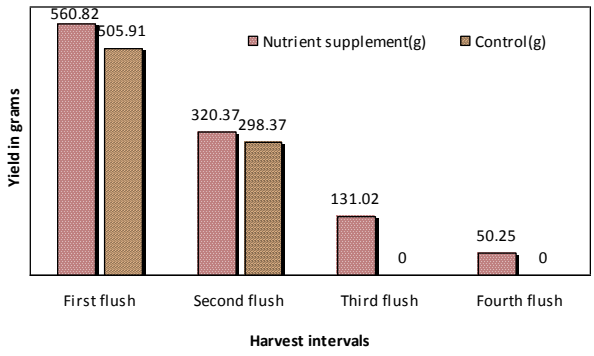

Fig 1: *Pleurotus florida* mushroom yield in two different batches

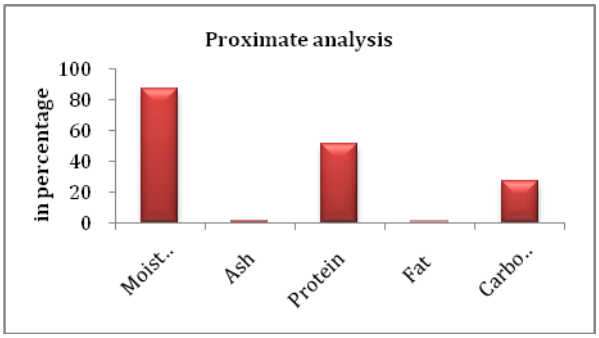

Fig 2 : Proximate analysis of *Pleurotus florida* mushroom

Moisture content for fresh mushroom values was about 87.3%. The fat level is quite low and it is about 0.6 % where as the protein content is 50.7%, on high range. The ash content is around 1.2% while the total carbohydrate concentration is about 26.6% (Figure 2).

Preliminary phytochemical analysis revealed the presence of carbohydrates, glycosides, phenolic compounds, proteins, terpenoids, steroids, tannins, fixed oils, saponin and flavonoids in aqueous and methanolic extracts of *Pleurotus florida* mushroom (Table 2). Ethanol, methanol and aqueous extract are rich in almost all the phytochemical compounds assayed for, though at varying levels. Alkaloids where detected only in ethanol extract at very low level where as resins were not detected in all the extract. In methanol, ethanol and aqueous extracts revealed high levels of proteins and phenolic compounds where as medium level in hexane and ethyl acetate extract. For many of the tests showed no reactions in ethyl acetate and hexane extract.

Table 2: Composition of bioactive compounds extracted from *P. florida*

Extracts	Carbohydrates	Glycosides	Alkaloids	Saponins	Tannins	Steroids	Terpenoids	Resins	Fixed oils	Protein	Phenols	Flavonoids
Methanol	++	++	-	+	+	+	+	-	++	+++	+++	++
Ethanol	++	++	+	+	+	+	+	-	++	+++	+++	++
Aqueous	++	+++	-	+	+	+	++	-	+	+++	+++	++
Hexane	+	+	-	+	-	-	-	-	-	++	+	-
Ethyl acetate	+	++	-	+	+	+	+	-	+	++	++	-

The total phenolic content of *Pleurotus florida* was estimated by the Folin ciocalteau method as 100ml (10mg/ml) contain 62.82mg catechol equivalent. The content of total phenols was higher in methanol extract followed by ethanol and aqueous extract contain 59.5mg and 58.1mg respectively (Figure 3). The total flavonoid content of the selected mushroom was estimated by using aluminium chloride technique in terms of catechol equivalent per as 17.71mg of catechol equivalent in 100ml of extract (10mg/ml). Hexane and ethylacetate contain quit low flavonoid content as 7mg and 9.1mg respectively where as ethanol and aqueous extract contain 15.89mg and 15.32mg (Figure 4).

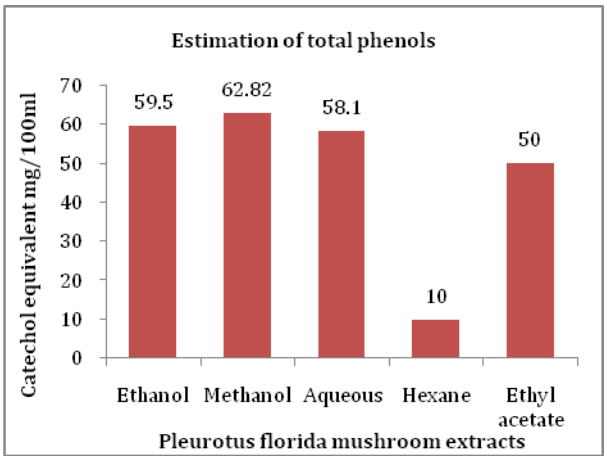

Fig 3: Estimation of total phenolic content

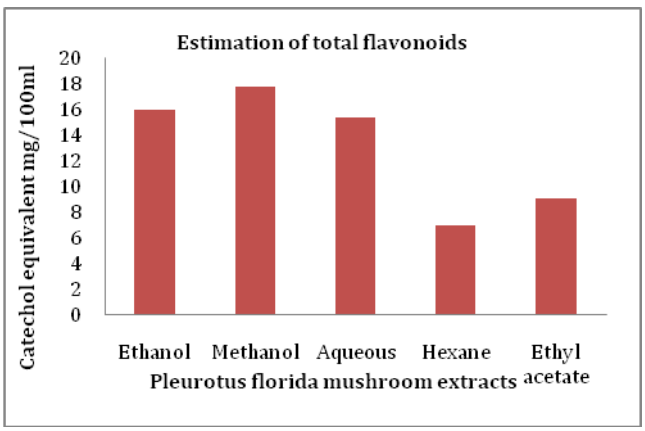

Fig 4: Estimation of total flavonoid content

The results of the present study revealed that the ethanol and methanol extracts demonstrated highest antibacterial activity followed by aqueous extract. Ethanol extract exhibited highest activity against *Pseudomonas sp.* and *Campylobacter sp.* where as in methanol extract activity was high in *E.coli, Salmonella typhi, Staphylococcus aureus, Camphylobacter sp.,* and *Vibrio sp.* The activity of aqueous extract was more potent and revealed high zone (24±1.5 mm) formation against *Vibrio sp.* whereas ethyl acetate and hexane extract showed less antibacterial activity and no activity against most of the test bacterial pathogens, but indicating highest potency against *Staphylococcus aureus* and *Pseudomonas sp.,* respectively. *Proteus sp.,* was resistant to all the five extracts of *Pleurotus florida* at the tested concentration (10mg/ ml). As summarized in Figure 5, *Pleurotus florida* had a narrow antibacterial spectrum and strongly inhibited the growth of the Gram-positive and Gram negative bacteria tested, including *Pseudomonas species* and *Vibrio cholerae.* In conclusion, bioactive compounds from *Pleurotus florida* mushroom extracts could be used as an alternate to antibiotics, considering the side effects and escalating levels of antibiotic resistance among microorganisms.

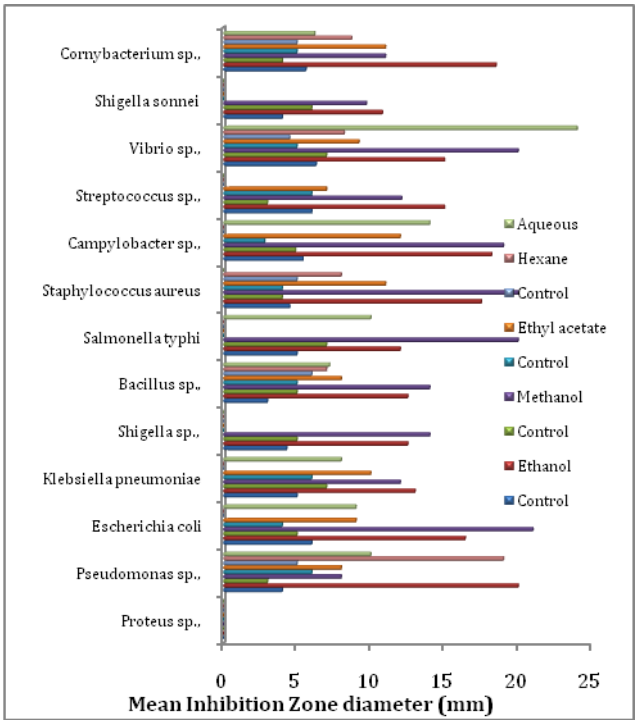

Fig 5: Mean inhibition zone diameter (mm) of the *P. florida* crude extracts against bacterial pathogens

DISCUSSION

In *Pleurotus spp*. the primordial initiation was generally observed on the 24 to 30 days. Ragunathan *et al.*[21] reported the day of primordial initiation was 22 to 27 days but in the present study, it was observed on the 19th day on horse gram supplemented batch. The horse gram supplemented batch resulted in 23% higher mushroom yield than the control batch. According to supplementation with horse gram had a greater potential to improve the accumulation of protein in *Pleurotus florida.* The addition of protein-rich supplements is common practice for nitrogen-deficient composts in the cultivation of mushrooms. Various researchers have used supplements from animal and plant origins, including protein, carbohydrate or oil-rich substances, *Agaricus bisporus*[22] and *Pleurotus sp.,* [23]. Earlier studies showed that horse gram is a good source of protein (17.9–25.3%), carbohydrates (51.9– 60.9%), essential amino acids, energy and a low content of lipid (0.58–2.06%) and is an excellent source of iron and molybdenum[24].

Freshly cultivated *Pleurotus florida* mushroom contain high moisture content about 87.3%. Variation in water contents among the mushroom samples could be caused by the nature of the mushrooms and the different environmental growth factors such as temperature and relative humidity of the metabolic water[25]. The elimination of water content of the sample to dry state will increase the concentration of nutrient relatively. Thus, drying mushrooms is one method that would extend the shelf life of mushrooms by reducing unnecessary biochemical reaction such as enzymatic browning and lipid oxidation that may lead to quality deterioration. The variations of the protein contents among edible mushrooms are affected by a number of factors, namely the type of mushrooms, the stage of development, level of nitrogen available, and the location[26]. Mushrooms proved to have good quality and higher protein content as compared to legumes[27].

The total phenolic content in methanol extract was higher than that of ethanol, aqueous, hexane and ethylacetate extract. The total phenolic content per 1 g of dry extract was higher than that reported for garlic extract (0.98 mg GAE per gram of extract) [28]. Flavonoids are important for human health because of their high pharmacological activities as radical scavengers. Flavonoids are a group of more than 4000 polyphenolic compounds that occur naturally in foods of plant origin. Flavonoids are a large class of phytochemicals which are present in human diets, which possesses a number of beneficial effects on human health, such as antioxidant, anti-inflammatory, antiallergic, antiviral, and anticarcinogenic activities[29].

In the present study methanol extract showed activity against *E.coli* (21 ± 0.9 mm), *Salmonella typhi* (20 ± 0.5 mm), *Staphylococcus aureus* (20 ± 0.4 mm), *Camphylobacter sp.* (19 ± 0.8 mm), *Bacillus sp.* (14 ± 0.5 mm), *Pseudomonas sp.* (8 ± 0.5 mm), *Klebsiella sp.* (12 ± 0.6 mm) and *Vibrio sp.* (20 ± 0.9 mm) (Figure 5). In the previous study the sporophore methanol extract of *Pleurotus florida* showed activity in *E.coli* (13 mm), *Klebsiella sp.* (20 mm) and no activity against *Bacillus sp., Pseudomonas* and *Proteus sp.* [30]. In the present study it revealed zone formation in *Pseudomonas sp.,* (20 ± 0.6 mm), *Salmonella sp.,* (20 ± 0.5 mm) and *Klebsiella pneumonia* (13 ± 0.8 mm) whereas mycelial ethanol extract showed zone formation in *Staphylococcus aureus* (16 mm), *Streptococcus mutans* (14 mm), *Escherichia coli* (12 mm), *Micrococcus luteus* (16 mm), *Bacillus subtilis* (9 mm) and no zone formation against *Pseudomonas aeruginosa*, *Salmonella abony*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Candida albicans*. These results confirm that bioactive components of mushroom may differ in their solubility depending on the extractive solvents. Antimicrobial activity in natural source extracts depends not only on the presence of phenolic compounds but also on the presence of various secondary metabolites[31].

The World Health Organization (WHO) estimates globally that about 1500 people die each hour from infectious diseases; half of these are children less than five years of age[32]. The economic worldwide crisis, high cost of industrialized medicines, inefficient public access to medical and pharmaceutical care, and side effects caused by synthetic drugs are some of the factors contributing to the central role that medicinal plants have in health care[33].

Siddhuraju *et al*., [34] reported that horse gram contained relatively high levels of total phenolics and tannins and it showed higher superoxide anion radical scavenging and higher radical scavenging activity. These tannins and phenolic compounds are responsible for antibacterial activities. Flavonoids have been referred to as nature's biological response modifiers, because of their inherent ability to modify the body's reaction to allergies and virus and they showed their anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activities[35, 36]. Phytoconstituents such as saponins, phenolic compounds and glycosides have been reported to inhibit bacterial growth and to be protective to plants against bacterial infections[37]. The terpenoids and tannins may elicit the antibacterial properties by cell membrane lysis, inhibition of protein synthesis, proteolytic enzymes and microbial adhesions[38]. Saponins are known for their medicinal properties as a natural blood cleanser, expectorant and antibiotics[39]. Among the five solvents tested most of the test pathogens were highly sensitive to methanol and ethanol extract followed by aqueous, ethyl acetate and hexane extracts.

Of the five solvent tested, methanol was determined to be the best solvent for isolation of bioactive secondary metabolites from dried *Pleurotus florida* mushroom followed by ethanol and aqueous extract. However, hexane and ethyl acetate extract of *Pleurotus florida* showed less activity and contain low phenolic compound content. This may be due to the high lipophilic nature of bioactive metabolites. These results indicate that the extraction method had definite effects on the isolation of bioactive principles.

CONCLUSION

Horse gram can be used as nutrient supplement for mushroom cultivation due to the low cost, increased higher mushroom yield (23%) and high phenolic compounds than the control batch. The presence of biologically active secondary metabolite constituents might be responsible for the bacterial activity observed in the present study. Thus from the findings, it was concluded that the methanol is the best solvent for extracting bioactive compounds. These bioactive principles are responsible for the antimicrobial activities against these tested microorganisms.

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