



Phytochemicals and Biological Activities of *Froriepia subpinnata* (Ledeb.) Baill. Extracts

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Abstract

Nowadays, the use of herbal extracts for preparing various drugs is increasing because of the compatibility of the natural compounds present in the extracts with the human body. To this end, in this project, we studied *Froriepia subpinnata* (Ledeb.) Baill. in terms of its natural constituents and biological activities. The chemical composition of the hexane extract of *F. subpinnata* aerial parts was evaluated by the GC/MS analysis and 21 compounds (80.60%) of the total, including phytosterols and hydrocarbons, were identified. Also besides, a significant amount of flavonoids in the methanol extract of *F. subpinnata* ($27.235 \pm 0.048 \mu\text{g/mL}$) was estimated by the AlCl_3 colorimetric method. Two flavonoids: rutin and catechin were identified in the methanol extract of *F. subpinnata* by HPLC analysis. The concentration of the flavonoids was evaluated (23.61 and 6.95) mg/g respectively. Antioxidant activity of the methanol extract of the species was also evaluated by the DPPH (1,1-diphenyl-2-picryl-hydrazyl) assay. We also investigated the antibacterial activity of methanol extract of *F. subpinnata* aerial parts against two bacteria *Staphylococcus aureus* and *Escherichia coli* by the MIC (Minimum Inhibition Concentration) method. The MIC values for *S. aureus* and *E. coli* were evaluated (0.031 and 1.0) mg/mL respectively. Our study shows that *F. subpinnata* possesses a variety of useful natural constituents, which makes it a promising plant for agricultural and medicinal uses.

Keywords: *Froriepia subpinnata*, Phytosterol, Rutin, Antioxidant, *Staphylococcus aureus*.

Introduction

Currently, the use of natural ingredients in herbal extracts has attracted much attention in the medical field. As the natural compounds are more compatible with the human body, pharmaceutical scientists have become interested in the use of herbal extracts as a source of phytochemicals that could be developed as safer new drugs [1]. The Phytochemicals fall into two categories, namely primary and secondary metabolites. Flavonoids and phytosterols are secondary metabolites, which naturally accrue in plants. The most common phytosterols, β -sitosterol and stigmasterol are known as beneficial compounds in the treatment of various diseases and play an important role in the defense function of the body as nutrients [2]. Flavonoids, are another beneficial compounds which are capable to display different biological activities [3]. The

most important role of flavonoids is their antioxidant activity by protecting against oxidative stress and free radical damage. Antibacterial, antiviral, anti-inflammatory, anti-allergic and anti-tumor activities are also known for these useful compounds [4,5].

The family Apiaceae also called Umbelliferae (Parsley family), includes comprising about 400 genera of plants distributed principally in temperate zones of the world. *Froriepia* is one of the genera including three species: *F. subpinnata*, *F. nuda* K. Koch and *F. gracillima* Leute, from which *F. subpinnata* (Ledeb.) Baill. is the only species growing naturally in the north of Iran (The provinces: Gilan, Mazandaran, and Golestan)[6,7]. It is an edible biennial plant which grows up to 150 cm and is known as a diuretic, antispasmodic, carminative and sedative agent in folk medicine [8]. Despite wide applications of this plant in folk medicine, there are only

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a few reports on chemicals and biological activities of this beneficial plant in the literature [9-16].

In continuous of our works on medicinal plants, herein, we focused on *F. subpinnata*. for phytochemical and biological investigations. Chemical composition of the hexane extract were evaluated for the first time in this project. HPLC analysis of flavonoids, antioxidant, and antibacterial activities of the methanol extract of *F. subpinnata* have not been reported before.

Material and Methods

Chemicals: All the solvents which were used for the extraction, HPLC-grade solvents; the chemicals: quercetin, aluminum chloride, sodium acetate and butylated hydroxyl toluene (BHT) were purchased from Merck Co. (Germany); the standard chemicals like 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and the standard flavonoids were purchased from Sigma Chemical Co. (USA); MHA (Muller-Hinton agar) and Resazurin were purchased from Merck Co. (Germany) and the standard antibiotic Cephaxime was provided from Sigma-Aldrich (USA).

Plant Material

The plant material was collected before the flowering stage from Roudsar (Gilan, Iran) in May 2018. The voucher specimen (107710 TARI) is deposited in the Herbarium of Research Institute of Forests & Rangelands (TARI). The aerial parts of the plant were dried in the shade, powdered, and then were used for extraction.

Extraction: The powdered plant (10 gr) was soaked in 100 mL n-hexane for a week at room temperature with occasional stirring. Then was filtered through Whatman filter paper No.1 and finally centrifuged (EBA20, Hettich, Germany) for removing the remained plant residues. The extract was dried by anhydrous Na₂SO₄ and subjected to a GC/MS apparatus. The above procedure was also followed to prepare the methanol extract. The as prepared extract was concentrated and then freeze-dried to lose all the remained solvent. Then the required concentrations were prepared for further uses.

GC/MS analysis: GC/MS analysis was performed on an Agilent/HP 6890/5973MSD (USA), equipped with a split-splitless injector system. Helium was the carrier gas (1 mL/min) and the capillary column TRB-5MS (30 m × 0.25 mm, film thickness 0.25 μm) was used. The column temperature was kept at 35 °C for 3 min. and then increased to 200 °C with a rate of 4 °C/min and kept constant 4 min. A mixture of n-alkanes were injected to the GC as internal standard under the same temperature programming. The temperature at mass analyzer adjusted at 150 °C and Ion source temperature was kept at 230 °C.

The compounds were identified by comparison of the retention times and mass spectra of each compound with those reported in the literature and/or stored on the Wiley library. GC peak area by the percentage of each compound was computed from the Agilent Chem. Station Software without any correction factor and was calculated relative to it.

Total Flavonoids Content

We applied the aluminum chloride colorimetric method to estimate the total flavonoids content in methanol extract (5.2 mg/mL) of *F. subpinnata* aerial parts (previously reported)[17]. This technique is based on the formation of a flavonoid-AlCl₃ complex, which shows the maximum absorption at 415 nm. UV-Visible spectrophotometer (CECIL, CE 7800, UK) was used in this study. A calibration curve was plotted for the standard solutions of quercetin (0-100 ppm), and by using the standard curve equation: ($Y = 0.0673X + 0.0051$, $R^2 = 0.9961$), the flavonoids content of the extract in terms of quercetin equivalent (μg/mL) was calculated. The experiment was repeated three times and expressed as the mean ± standard deviation (SD).

HPLC Analysis

A Waters liquid chromatography apparatus (USA) consisting of a separations module (Waters 2695) and a UV-Visible detector (Waters 996, λ=256 nm) was used for the HPLC analysis. The injection was done by an auto sampler injector and the chromatographic assay was performed on a 15 cm×4.6 mm Eurosphere 100-5 C₁₈ analytical column (Knauer, Germany). The elution was carried out in a gradient system with methanol and distilled water with the flow-rate of 1mL/min. The peaks were monitored at 256 nm, injection volume was 20 μL and the temperature was kept constant at 25 °C. A mixture of available standard flavonoids was also injected to the HPLC in a similar condition and the flavonoids were identified by comparing their retention times with the standard compounds.

Antioxidant Activity

To determine the radical scavenging ability of the methanol extract of *F. subpinnata* aerial parts, we used the DPPH assay reported previously by Mensor *et al.* [18]. Briefly, 2.5 μL of freshly prepared DPPH solution in methanol (40 μg/mL), was added to 10 μL of the five different dilutions of the extract (5.2, 2.6, 1.3, 0.65 and 0.325) mg/mL separately. After 30 min incubation in dark, the absorbance of the test tubes was taken at 517 nm by using a spectrophotometer (CECIL, CE 7800, UK). The same procedure was repeated for BHT as a standard antioxidant. By using the equation: %I =

$100 \left[\frac{A_c - A_s}{A_c} \right]$, the percentage of scavenged DPPH was calculated. In the above equation, A_s represents the absorbance of the sample and A_c represents the absorbance of the control.

Antibacterial Assay

Antibacterial activity of the methanol extract (5.2 mg/mL) of *F. subpinnata* aerial parts was studied by the MIC (Minimum Inhibitory Concentration) method. The extract was tested against the Gram-positive bacterium *Staphylococcus aureus* (ATCC=25923) and the Gram-negative bacterium *Escherichia coli* (ATCC=25922). Microorganisms were identified by Research Center of Biotechnology and Industrial center of fungi and bacteria collections, Iran. To determine the MIC, a high-throughput 96-well microplate bioassay was used according to the method which has been reported before [19]. In this method, first, two-fold dilutions of the methanol extract in Muller Hinton agar (32-0.015 mg/mL; final volume 100 μ L) were prepared. Then, a standardized microbial suspension in normal saline adjusted to 0.5 McFarland scale was made (18-20 h.) and diluted (1:100) by MHA. Afterward, 100 μ L of the as-prepared solution was added to each well. Next, the 96-well micro titration plate was incubated under 37 °C for 20 h. Finally, the wells were visualized and the lowest concentration of the antimicrobial agent (the extract) that completely inhibited the growth of the bacteria was detected and reported (mg/mL). In order to detect the samples which caused turbidity in the media, a colorimetric assay by the reagent Resazurin was used. For this purpose, a stock solution of the reagent in distilled water (4 mg/mL) was prepared and then sterilized by a filter (0.2 μ m-rated membrane). Next, 5 μ L of the solution was added to each well and also to the positive control well, while the plates were shaking. The MIC was determined after observation the color change of the control well (change from violet to pink). The test was done in duplicate and the average was reported as the result. Cefixime was used as the standard antibiotic.

Qualitative control in MIC test: The negative control (the well including 200 μ L of liquid inoculum); the positive control (the well including 100 μ L of liquid inoculum and 100 μ L of a dilute suspension of the bacterium) were used. The absolute number of CFU (Colony Forming Unit) in the positive control well was measured. For this purpose, before incubation of the 96-well plate in the microplate bioassay, a hundred fold-diluted solution of the positive control in normal saline was prepared and 100 μ L of the solution was inoculated on nutrient agar. After incubation (37 °C, 24 h.), the colony-forming units were counted. (This part of project was conducted according to the

Clinical and Laboratory Standards Institute (CLSI) guidelines).

Results and Discussion

Twenty-one compounds (80.60 %) were identified by GC/MS analysis in the hexane extract of *F. subpinnata* aerial parts. The main components were found as follow: 26, 27-Di (nor)-cholest-5,7,23-trien-22-ol, 3-methoxymethoxy (17.74%); β -sitosterol (9.03%); stigmasterol (8.60%); docosane (5.58%) and tetracosane (4.54%) (Fig. 1 & Table 1). The crude extract of plants include natural compounds with special therapeutic properties. Phytosterols which structurally resemble cholesterol are suggested as reducing agents of the cholesterol levels in the human body [20]. β -Sitosterol and stigmasterol, the phytosterols which were found in the hexane extract of *F. subpinnata*, are of utmost importance and have been isolated from a number of plant species [21]. These natural compounds are reported as potent risk-reducing agents in cardiovascular diseases [22]. In addition, anti-cancer and anti-angiogenic activities have been evaluated for the above-mentioned phytosterols [23-25]. The other identified compound: 26, 27-Di (nor)-cholest-5,7,23-trien-22-ol, 3-methoxymethoxy, is also a phytosterol and naturally occurs in plants extracts. It have been isolated from *Acanthus ilicifolius* leaves and its potential chemo protective activity against hepatocellular carcinoma has been evaluated by In-silico study [26]. The mentioned compound was also identified in *Avicennia officinalis* L., *Acanthus ilicifolius* L. and *Morinda tinctoria* Noronha extracts by GC-MS analysis [27,28].

Total flavonoids content of methanol extract of *F. subpinnata* aerial parts was found (27.235 \pm 0.048 μ g/mL). HPLC analysis showed a number of compounds from which, the flavonoids rutin and catechin were identified by comparing their retention times (R_t) with the retention times of the standard available flavonoids (Fig. 2). Using the standard plot of rutin and catechin (Fig. 3), the concentration of each flavonoid in the extract was calculated (122.77 and 36.14) ppm respectively. The initial concentration of the extract was 5200 ppm (by measuring the peak area). Given these results, the concentration of rutin and catechin in the extract was calculated (23.61 and 6.95) mg/g respectively (mg of flavonoid/g of dried extract). Previously, total flavonoids content of the aqueous extract of *F. subpinnata* was reported (39.62 \pm 2.4) mg/g by Ebrahimzadeh *et al.*, which shows higher amounts of flavonoids than our study [13]. It may be due to the used solvent in the extraction process.

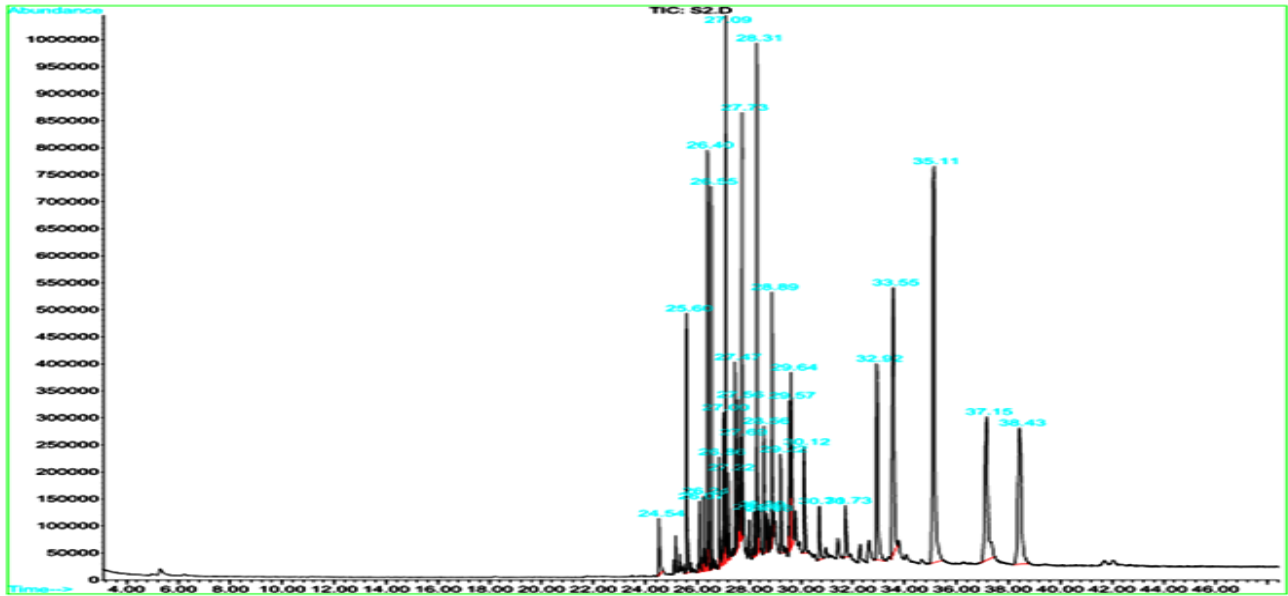


Fig. 1 GC chromatogram of the hexane extract of *Froriepia subpinnata* L. aerial parts.

Table 1 GC/MS analysis of the hexane extract of *Froriepia subpinnata* L. aerial parts.

| No. | Compounds | Retention time (min) | Peak area (%) | Molecular Formula |
|-----|---|----------------------|---------------|--|
| 1 | n-Tetradecane | 24.54 | 0.94 | C ₁₄ H ₃₀ |
| 2 | n- Hexadecane | 25.60 | 2.85 | C ₁₆ H ₃₄ |
| 3 | n-Heptadecane | 26.07 | 0.87 | C ₁₇ H ₃₆ |
| 4 | Unknown | 26.24 | 0.67 | - |
| 5 | n-Octadecane | 26.40 | 3.51 | C ₁₈ H ₃₈ |
| 6 | Neo-phytadiene | 26.55 | 2.54 | C ₂₀ H ₃₈ |
| 7 | Phytol | 27.47 | 2.06 | C ₂₀ H ₄₀ O |
| 8 | Unknown | 27.52 | 1.25 | - |
| 9 | n-Eicosane | 27.60 | 2.41 | C ₂₀ H ₄₂ |
| 10 | 2-methyl-Eicosane | 27.69 | 3.55 | C ₂₁ H ₄₄ |
| 11 | 1-Docosane | 27.73 | 5.58 | C ₂₂ H ₄₆ |
| 12 | n-Tricosane | 28.20 | 0.72 | C ₂₃ H ₄₈ |
| 13 | n-Tetracosane | 28.31 | 4.54 | C ₂₄ H ₅₀ |
| 14 | Hexacosane | 28.49 | 2.57 | C ₂₆ H ₅₄ |
| 15 | 1-Eicosanol | 28.56 | 1.78 | C ₂₀ H ₄₂ O |
| 16 | Unknown | 28.68 | 0.64 | - |
| 17 | Unknown | 29.23 | 1.64 | - |
| 18 | Docos-13-enamide | 29.57 | 2.81 | C ₂₂ H ₄₃ NO |
| 19 | Octacosane | 29.64 | 2.84 | C ₂₈ H ₅₈ |
| 20 | Nonacosane | 30.12 | 2.22 | C ₂₉ H ₆₀ |
| 21 | Triacotane | 30.70 | 1.25 | C ₃₀ H ₆₂ |
| 22 | Vitamin-E | 31.72 | 1.91 | C ₂₉ H ₅₀ O ₂ |
| 23 | Stigmasterol | 32.93 | 8.60 | C ₂₉ H ₄₈ O |
| 24 | β-Sitosterol | 33.55 | 9.03 | C ₂₉ H ₅₀ O |
| 25 | 26, 27-Di (nor)-cholest-5,7,23- trien-22-ol, 3-methoxymethoxy | 35.11 | 17.74 | C ₂₇ H ₄₂ O ₃ |
| 26 | Unknown | 37.15 | 8.63 | - |
| 27 | Unknown | 38.43 | 6.57 | - |
| - | Total identified | - | 80.60 | - |

While the previous study is based on aqueous extract, we evaluated the methanol extract of *F. subpinnata*. It can be concluded that since flavonoid-glycosides are more soluble in water than flavonoid aglycones, the plant probably contains higher amounts of flavonoid-o-glycosides [29].

The HPLC analysis revealed the flavonoids rutin and catechin which are well known for their beneficial effects on health. Rutin is documented for several pharmacological activities such as anti-inflammatory, anti-oxidant, anti-diabetic, etc. [30]. Catechin is also known as a beneficial natural product and it is proposed that catechin-containing extracts may affect the cholesterol level and can be useful in treating heart diseases [31]. Previously, Jorkesh *et al.*, evaluated the phenolic compounds in the methanol/acetic acid (85:15) extract of *F. subpinnata*, by HPLC analysis. They reported coumarin, ferulic acid, and chlorogenic acid as the main compounds [16].

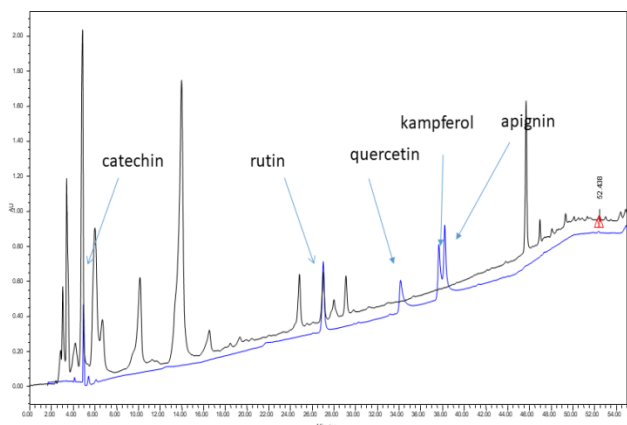


Fig. 2 HPLC analysis of the methanol extract of *Froriepia subpinnata* (Ledeb.) Baill. aerial parts in comparison with the available standard flavonoids.

Antioxidant activity of the methanol extract of *F. subpinnata* aerial parts (5.2 mg/mL) was evaluated by DPPH method and was compared to BHT as a standard antioxidant. The IC_{50} values for the extract and BHT, was calculated (2.7 ± 0.04 and 1.8 ± 0.02) mg/mL respectively. The extract showed significant antioxidant activity in comparison with BHT (Fig. 4). Flavonoids are valuable antioxidants which can reduce the damages induced by oxidative stress which is conducted by free radicals in cells [32]. Free radicals play an important role in the development of various physiological and pathological diseases [33-37]. Antioxidants through their scavenging power are beneficial for the management of those diseases [38, 39]. The methanol extract (5.2 mg/mL) of *F. subpinnata* in our study showed a good radical scavenging effect 73% in comparison with BHT (92%).

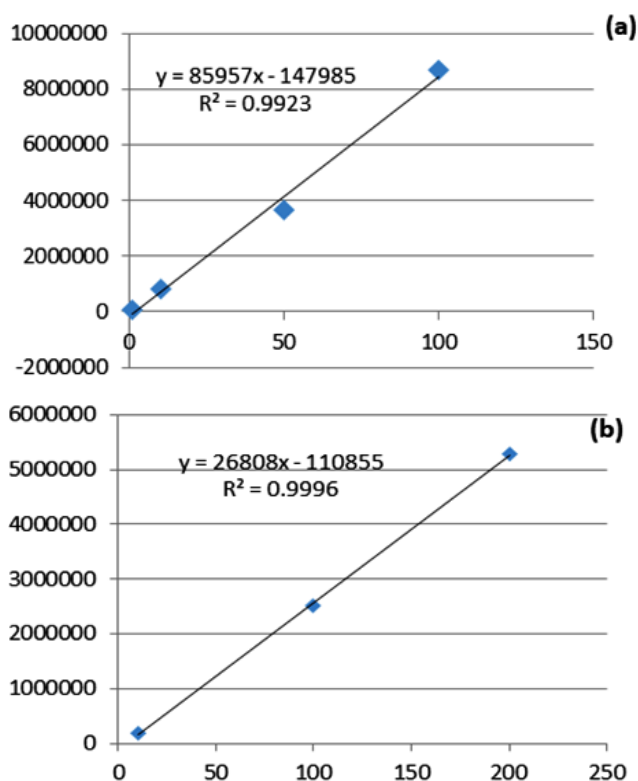


Fig. 3 Calibration plots for rutin (a) and catechin (b).

In the previous study on the aqueous extract of *F. subpinnata* collected from Sari (Mazandaran Province), the IC_{50} value in DPPH assay was reported (0.14 ± 0.06) mg/mL for the aqueous extract of the plant [13]. In another study, Jorkesh *et al.* reported the antioxidant capacity in the range of (31.36-81.82) (DPPH%) for 52 accessions of *F. subpinnata* [16]. The result of our study (73.4 DPPH %), shows a similarity with the best previously reported results by Jorkesh *et al.*

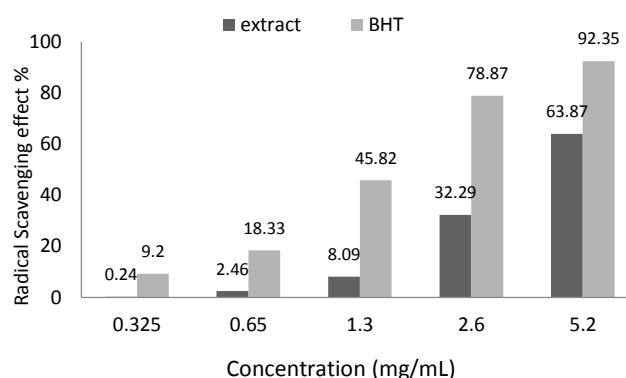


Fig. 4 Radical scavenging capacity of the methanol extract of *Froriepia subpinnata* (Ledeb.) Baill. in comparison with BHT.

Antibacterial activity of the methanol extract of *F. subpinnata* was tested by the MIC method. The lowest concentration of the extract which prevented visible growth of *S. aureus* and *E. coli* were evaluated (0.03 and

1.0) mg/mL respectively. While the MIC values of Cefixime as a standard antibiotic for the mentioned bacteria were found (1.0 and 0.25) µg/mL respectively. Cefixime minimal inhibitory concentration interpretive criteria for *S. aureus* and *E-coli* was obtained by the given standards of Clinical Laboratory Standard Institute (CLSI). Plants extracts are rich in a wide range of secondary metabolites that can bind to the bacterial cell wall and disrupt the microbial membranes [40]. Previous studies on the antibacterial properties of *F. subpinnata*, have been carried out on the essential oil of this plant. Mohamadzadeh *et al.*, assessed the antibacterial effects of *F. subpinnata* essential oils against *Pseudomonas aeruginosa*, by disk diffusion and micro dilution methods [11]. In another study, the antibacterial activity of the essential oil of *F. subpinnata* was tested against six bacterial and fungal strains and *S. aureus* showed the best result (MIC values 1-2 µg/mL) [12]. The MIC is one of the most basic anti-microbial susceptibility testing methods due to reproducibility and economic advantages [12]. Our results confirms the effectiveness of *F. subpinnata* methanol extract against two bacteria *S. aureus* and *E. coli* which are beneficial on humane health. In conclusion, *F. subpinnata* as a medicinal plant, possesses valuable biologically active components, which makes it a promising plant for agricultural and medicinal uses. More investigations at cellular levels and in-vivo studies are necessary to elucidate antimicrobial and antioxidant activities.

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