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Original Research Article

In vitro evaluation of the antioxidant, anti-Propioni bacterium acne and antityrosinase effects of *Equisetum ramosissimum* (Jordanian horsetail)

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Abstract

Purpose: *The investigation of Equisetum ramosissimum Desf. (Equisetaceae) extracts for total phenolic content, potential antioxidant properties, and antibacterial activity against Propioni bacterium acne and antityrosinase effects.*
Methods: *The aerial parts of the E. ramosissimum from Jordan were extracted by maceration and Soxhlet methods, using solvents of different polarities. The composition of the extracts were qualitatively screened using standard phytochemical tests. Quantitatively, total phenolic content (mg/mL, equivalent to gallic acid), ABTS free radical scavenging activity (IC_{50}), anti-P. acne (MIC and MBC), and tyrosinase inhibitory effects (IC_{50}) were also determined.*
Results: *The aqueous-methanol Soxhlet extract contained the highest total phenolic content (0.675 mg/mL gallic acid equivalents). Besides, phytochemical screening tests revealed the presence of phenols, flavonoids, tannins, alkaloids and saponins in the aqueous methanol Soxhlet extract, contributing to the antioxidant (IC_{50} = 0.125) and antityrosinase (IC_{50} = 1.125) effects. This extract also showed potent antimicrobial effects against P. acne (MIC = 6.250 mg/mL; MBC = 12.500 mg/mL). Other extracts, including ethanol, water and ethyl acetate, showed lower total phenolic content with moderate and weak biological activity.*
Conclusion: *E. ramosissimum is a promising plant species to be considered for antioxidant, antiacne, and antityrosinase effects. However, further testing (including invivo, histological examination, and high-performance liquid chromatography (HPLC) analysis) is necessary to understand more about its mechanisms of action.*

Keywords: *Antioxidant, Anti-Propioni bacterium acne, Antityrosinase, Equisetum ramosissimum, Total phenolic content, Jordanian horsetail*

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INTRODUCTION

Herbal products have been widely used to treat different skin problems [1]. Globally and through generations, most patients with common skin problems prefer to use medicinal plants as their first choice due to their relative safety and low cost [2]. Therefore, plant extracts and compounds derived from natural products are

widely used as antioxidant, antiseptic, antimicrobial, and anti-hyperpigmentation agents in dermatology [3]. Still, there is a need to test the effects of plant extract for different biological properties, with special emphasis on natural products possessing potential skin healing characteristics [4]. *Equisetum ramosissimum*, commonly known as branched horsetail, belongs to the family Equisetaceae and is an erect evergreen plant with well-branched scabrous stems that are widely distributed worldwide [5]. A previous study showed that aerial parts *E. ramosissimum* collected from China contain phenols and flavonoids, mainly kaempferol and quercetin [6].

Traditionally, the aerial parts of *E. ramosissimum*, considered a crude drug, are used to treat several diseases, especially among rural and tribal people. It is usually used as a diuretic, an antitussive, and an astringent. It is additionally used for treating swellings, pain in the eye, diarrhea, jaundice, hepatitis, renal lithiasis, and gonorrhea. It also acts as a protective agent against melanoma and melanogenesis [7-9]. Methanolic extract of the plant species grown in Nepal also displayed moderate antimicrobial activity against selected species of *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, and *Klebsiella pneumonia* [10]. Moreover, an ethanolic extract prepared using the species *E. ramosissimum* grown in northern Iraq has also shown antifungal activity against two types of yeast: *Candida albicans* and *Cryptococcus neoformans* [11].

To the best of our knowledge, a study conducted by Alebous and coworkers [12] has investigated the chemical composition of the essential oil in *E. ramosissimum* species obtained from the Jordanian local market, the study revealed the presence of sesquiterpenoids (36.4%) and monoterpenoids (41.7%), of which the major two compounds are α-bisabolol (12.3%) and cuminaldehyde (9.8%). This study aims to prepare different extracts of *E. ramosissimum* collected from its natural habitat in Jordan, and to investigate the total phenolic content of each extract, to correlate their phytochemical content with their potential antioxidant property and antityrosinase activity, as well as their antibacterial effect against *P. acne*.

EXPERIMENTAL

Collection of plant material

E. ramosissimum samples were collected from Ain Khnezerah – Fifa natural reserve, which is located in the south western part of Jordan, in June, 2017. The species was authenticated by Mr. Ibrahem Mahasneh, a professional taxonomist at the Nature Conservation Monitoring Centre, the *Royal Society for the Conservation of Nature* (RSCN). A voucher specimen is available at the RSCN herbarium (number: E.r-5/7/2017), as it is considered the official authority for plants identification in Jordan

The aerial part of the *E. ramosissimum* sample was washed, dried at room temperature and then ground using a blender (Waring 8011S/G). Finally, ground material was kept in airtight glass containers at room temperature until use.

Extraction of plant material

One hundred grams of the dried, powdered aerial parts of the *E. ramosissimum* plant were extracted by maceration in 98% ethanol or 95% ethyl acetate, and allowed to stand at 25°C with frequent agitation for 5 days. Soxhlet extraction was carried with water or aqueous methanol (80:20) at a constant temperature of 40°C.

The extracts were then filtered and completely dried, and then kept in a deep freezer at -20°C in a dark and airtight glass container until use.

Preliminary phytochemical screening tests

These tests were performed to qualitatively identify the possible presence of different phytochemical compounds in the extracts using standard procedures [13, 14]. Results were identified based on detected color intensity as +: weak presence, ++: moderate presence, +++: strong presence, -: negative (absence). Except for saponins, these were determined by measuring the foam layer height.

Determination of total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu (FC) method as described by Saqallah *et al* [15]. Stock solution for each extract was prepared at a concentration of 10 mg/mL in distilled water and 1% dimethyl sulfoxide (DMSO). Furthermore, 4 different dilutions were made from each stock as 1.0, 0.5, 0.25, and 0.125 mg/mL. An aliquot of 12.5 µL of each dilution was treated with 250 µL of 2% sodium carbonate solution in 96-well microplates, and left to react for 5 min at room temperature. Then, 12.5 µL of 50 % FC phenol reagent was added and allowed to stand for 30 min at room temperature. The absorbance of the reaction mixture was measured at 630 nm using a microtiter plate reader. A calibration curve was

prepared using a Gallic acid standard solution in the concentration range of 0.1-1.0 mg/mL. Data is expressed as an equivalent of Gallic acid (mg) for each milliliter of extract.

Determination of antioxidant property by ABTS assay

Based on the method described previously by Re *et al* [16], $ABTS^{\bullet+}$ was produced by reacting 7 mM (3.6 mg/mL) ABTS with 2.45 mM (662.28 mg/mL) potassium sulfate in aqueous solution, and then the mixture was kept in the dark at 25 °C for 24 h before use. Then, the $ABTS^{\bullet+}$ solution was diluted in 80 % ethanol to achieve the desired absorbance before use.

Stock solutions of 10 mg/mL of each extract in 80 % ethanol were prepared. A volume of 3.9 mL of $ABTS^{\bullet+}$ solution was added to 0.1 mL of each extract concentration and vigorously mixed. After reaction at room temperature for 5 min, the absorbance was measured at 734 nm. The control used for this test was the ABTS radical + ethanol. A calibration curve was prepared using the Trolox standard solution at various concentrations. Data are expressed as $ABTS^{+}$ scavenging activity (B) as in Eq 1 [16].

B (%) = {(Ac – At)/Ac}100 …… (1)

where Ac and As are the absorbance of control and test samples, respectively.

Determination of antimicrobial activity

The method described previously by Talib and Saleh [17] was used to evaluate the extracts antibacterial activity against *P. acne* (ATCC 11827), provided by Hamdi Mango Centre for Scientific Research at The University of Jordan (Amman-Jordan). Bacteria were grown for 48 h in reinforced clostridial broth at 37 °C under anaerobic conditions using anaerobic jars containing AnaeroGen Sachets.

The turbidity of the bacterial culture was adjusted to 0.5 McFarland standard (1×10^8 CFU/ml), prepared by mixing 0.05 ml of 1 % barium chloride with 9.95 ml of 1 % anhydrous sulfuric acid. The absorbance was measured spectrophotometrically in the range of 0.08 - 0.10 at 625 nm.

The method described previously by Talib and Mahasneh [18] was used to define the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for each extract using the sterile 96-well microplate assay method. A stock solution of 100 mg/ml in reinforced clostridial broth (CB) was prepared for each extract.

All wells except the first were occupied with CB (100 µL) and then was filled with a serial two-fold dilution of the stock extracts, and mixed. The plates were then inoculated with bacterial suspension (100 µL/well) and incubated at 37 °C for 48 h using an anaerobic jar. Then the turbidity was measured using a micro-plate reader (Tecan, Austria) at 620 nm wavelength. Plant extract (200 µL) was added to the first well and used as a blank. Gentamycin (2 mg/mL) was used as the positive control, while CB was used as the negative control. MIC was calculated using Eq 2 [18].

MIC (%) = 100 – {(At – Ab)/Ac} …… (2)

where At, Ab and Ac are the absorbance of test, blank and negative control samples, respectively.

An aliquot of 10 µL from each well without visible growth was added to a sterile Eppendorf tube, with 2 mL of CB and incubated for 48 h at 37 °C. The visual turbidity of the incubated Eppendorf tubes was examined. Eppendorf tubes containing the lowest concentration of the extract that remained clear (prevent the growth of bacteria after sub-culturing on CB) were marked as the MBC.

Determination of tyrosinase inhibition activity

This test was performed using the method described by Samy *et al*, utilizing a tyrosinase inhibitor screening kit [20]. For each extract, a 100 mg/mL stock solution was prepared and dissolved in 1% dimethyl sulfoxide (DMSO) and then diluted 5-fold with tyrosinase assay buffer (25 µL of the stock solution diluted with 475 µL of buffer) before use. Using a 96-well plate, 40 µL of the sample test solution was added to the first well, followed by serial two-fold dilutions for each tested extract, and addition of 50 µL of tyrosinase enzyme solution, mixing and incubation for 10 min at room temperature. Thirty microliters of the tyrosinase substrate solution were added to each well and protected from light with vigorous mixing for 30-60 min. The absorbance of each solution was measured at 490 nm using a microtiter plate reader. The control used for this test was the tyrosinase assay buffer. A calibration curve was developed using a Kojic acid standard solution. The results are expressed as tyrosinase inhibition (T) as shown in Eq 3 [20].

T (%) = {(N – S)/N}100 …… (3)

where N and S are the slope of negative control and sample, respectively.

Statistical analysis

Statistical analyses were performed using ANOVA with SPSS (Statistical Package for the Social Science, Chicago, Illinois). A *P*-value < 0.05 was considered significant. Furthermore, all IC_{50} values measured in this study were calculated for each *E. ramosissimum* extract using nonlinear regression in SPSS (version 21).

RESULTS

Phytochemical profile

The aqueous methanol and ethanol extracts were the richest extracts regarding their contents of alkaloids, saponins, phenols, flavonoids, and tannins (Table 1). Steroids, terpenoids,and glycosides compounds were not detected in any of the tested extracts.

Total phenolic content

The aqueous methanol extract showed the highest total phenolic content at 1 mg/ml, followed by the ethanol and water extracts. The ethyl acetate extract showed the lowest phenolic content (Figure 1). All measured data showed a concentration-dependent trend.

Antioxidant property

The highest antioxidant activity was observed for the aqueous methanol extract, followed by the ethanol and water extracts. The lowest antioxidant activity was observed for the ethyl acetate extract (Table 2).

Antibacterial activity against *P. acne*

The highest antibacterial activity against *P. acne* was obtained with the ethanol extract, followed by the aqueous methanol extract. Ethyl acetate and water extracts showed very weak activities against *P. acne* (Table 3).

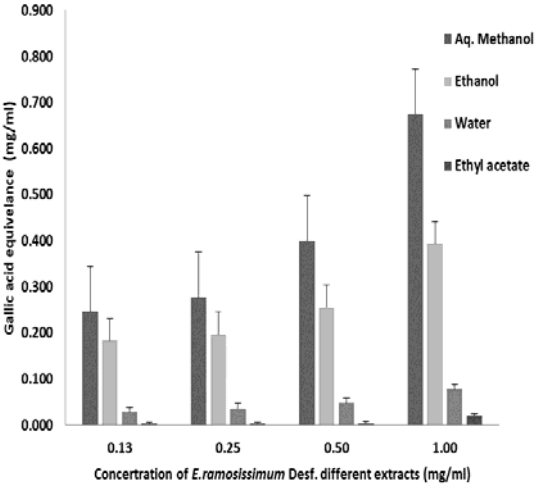

Figure 1: Total phenolic content (mg GAE/ml) at different concentrations of *E. ramosissimum* extracts

Table 2: IC_{50} values (mg/mL) of the *E. ramosissimum* extracts obtained by ABTS radical scavenging test

Extract solvent	IC_{50} (mg/mL)
Trolox	11.826
Aqueous methanol	0.125
Ethanol	0.125
Water	0.250
Ethyl acetate	1.000

Table 3: The MIC and MBC values (mg/mL) of *E. ramosissimum* extracts against *P. acne*

Extract solvent	MIC (mg/mL)	MBC (mg/mL)
Gentamycin	3.125	3.125
Aqueous methanol	6.250	12.500
Ethanol	3.125	6.250
Water	25.000	50.000
Ethyl acetate	50.000	50.000

Tyrosinase inhibitory effect

The highest antityrosinase activity was observed with the aqueous methanol extract, followed by the ethanol extract. The lowest activities were observed with water and ethyl acetate extracts (Table 4).

Table 1: Phytochemical composition of *E. ramosissimum* extracts

Test	Aqueous methanol	Ethanol	Water	Ethyl acetate
Tannins	++	++	+	+
Saponins	++	+++	+	-
Alkaloids	+	+++	+	-
Flavonoids	+++	++	+	+
Phenols	+++	++	++	+
Glycosides	-	-	-	-
Terpenoids	-	-	-	-
Steroids	-	-	-	-

+: weakly presence, ++: moderately presence, +++: strongly presence, -: negative (absence)

Table 4: The IC_{50} values (mg/mL) for the *E. ramosissimum* extracts using the tyrosinase inhibition test

Extract solvent	IC_{50} (mg/mL)
Kojic acid	2.132
Aqueous methanol	1.125
Ethanol	2.500
Water	20.000
Ethyl acetate	>20.000

DISCUSSION

Phenolic compounds are a group of plant secondary metabolites that appear in different plant extracts [20]. As expected, phenols were revealed when the aqueous methanol solvent was used for extraction, as they possess moderate to high polarity. Moreover, using qualitative phytochemical screening tests, this extract also showed the presence of flavonoids and tannins. The ethanol and water extracts showed lower total phenol contents, followed by the less polar ethyl acetate extract.

A previous study by Paulsamy and coworkers revealed that the methanol extract of the Indian species of *E. ramosissimum* possesses moderate antioxidant activity [6]. Similarly, in the current study, the highest antioxidant effect was observed for the aqueous methanol and ethanol extracts, followed by the water and ethyl acetate extracts. Also, the ethanolic extract showed strong positive results for the presence of alkaloids and saponins, which have been previously shown to contribute to good antioxidant effect from another plant extracts [21,22].

Despite the high phenolic content of the aqueous methanol extract, it showed a lower antimicrobial effect against *P. acne* relative to the ethanolic extract. The latter was able to show an antimicrobial effect against *P. acne* similar to that of Gentamycin effect. These findings may be attributed to the possible synergistic antimicrobial effect of phenols, alkaloids, and saponins against *P. acne*. The same findings were previously reported using the ethanolic extracts of *Glycyrrhiza glabra* and *Calendula officinalis*, both of which showed antibacterial properties against *S. aureus* and *P. acnes*, which were attributed to the presence of alkaloids, saponins, and flavonoids [23].

Previous studies showed that saponin extracted from the nutshell of *Xanthoceras sorbifolia* was useful in the treatment of skin hyperpigmentation disorders [24]. Another study indicated that the bark ethanolic extract of *Berberis aristata* contains a considerable amount of alkaloids compared with the other constituents and possess a potential anti-hyperpigmentation effect on human skin [25]. The phytochemical screening tests revealed the presence of saponins and alkaloids, with high and moderate content in the ethanolic and aqueous methanol extracts, respectively. The ethanolic extract has shown similar anti-tyrosinase activity to kojic acid, while the aqueous methanol extract was superior to Kojic acid activity, with a two-fold lower concentration required to obtain the same anti-tyrosinase effect, possibly due to its higher total phenolic content compares to the other extracts.

CONCLUSION

E. ramosissimum collected from Jordan's natural reservoir contains secondary metabolites tannins, flavonids, phenols, saponin, and alkaloids in varying abundance. Aqueous methanol and ethanol extracts exhibited the strongest anti-oxidant and anti-tyrosinase activities. Further studies, especially on the polar fractions of the plant extracts is recommended.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Reem Issa: She is the corresponding author, and she participated in the drafting of the manuscript and giving final approval of the version to be published. Noor Sabah Abed Savaya: She participated in the main design, analysis and interpretation of the data in addition to drafting the manuscript. Wamidh Hadi Talib: He participated in the experimental design and interpretation of the data.

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