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Evaluation of antioxidant potential, total phenolic content and phytochemical screening of aerial parts of a folkloric medicine, *Haplophyllum tuberculatum* (Forssk) A. Juss

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ABSTRACT

Objective: To quantify total phenols, flavonoids and to investigate the *in vitro* antioxidant power of *Haplophyllum tuberculatum* (*H. tuberculatum*) leaves extracts of varying polarities.

Methods: The authenticated sample of *H. tuberculatum* (50 g) leaves was dried under shade, powdered and extracted exhaustively with ethanol by cold percolation method. The alcoholic extract was further partitioned into petroleum ether, acetone, chloroform and methanol to obtain the fractions of varying polarities which were subjected to qualitative phytochemical testing. Total phenolics and flavonoids content in the acetone, chloroform and methanol extracts were quantified by using standard colorimetric methods. Petroleum ether extract was omitted because it did not show the presence of either tannins or flavonoids. *In vitro* antioxidant activity and total antioxidant capacity were determined by using 1,1 diphenyl-2-picrylhydrazyl radical and phosphomolybdenum reagent. Ascorbic acid was used as a reference antioxidant for comparison purpose.

Results: Qualitative phytochemical results of leaves extracts confirmed the presence of major secondary plant metabolites. The extraction of phenolic compounds varied considerably according to the polarity of solvent. The most polar fractions *i.e.* methanol were observed to have the highest phenolic content (561.22 mg/g of gallic acid equivalent) and flavonoids (165.54 mg/g of quercetin equivalent). Although the free radical scavenging activity of leaves fractions was noted to be slightly lower than the reference compound, a direct relationship was observed between phenolic content and *in vitro* antioxidant activity. On the other hand, leaves fractions exhibited significant total antioxidant capacity as ascorbic acid equivalent.

Conclusions: The aerial part of *H. tuberculatum* is rich in phenolic compounds which might play a vital role in the discovery of natural antioxidants.

1. Introduction

Natural products have been used by the mankind since prehistoric time to fight against the diseases. A number of therapeutic agents used today are obtained from the plants. Therefore, the plant products or their derivatives are considered to be of paramount importance in drug discovery and drug development[1]. The useful pharmacological effects of medicinal plants are attributed to the presence of phytoconstituents which are primarily secondary plant

metabolites[2]. These medicinal plants are widely used all over the world as traditional medicines.

In a worldwide review on herbal medicines by World Health Organization, it has been reported that approximately three-fourth of the populations in developing countries use traditional and folkloric herbal medicines for their common ailments[3]. Therefore, a systematic approach should be made to unlock the hidden potential of these ethnomedicinal plants. Isolation of bioactive compounds from the traditional medicinal plants can be used as a basis for further ethnopharmacological studies[4].

Herbal medicines are very popular in the Arab world including Oman[5]. The flora in Northern and Central Oman consists of about 600 species, the majority of which occur in the mountains and foothills[6]. *Haplophyllum tuberculatum* (*H. tuberculatum*), commonly known as "Tafr al tays" in Arabic, is the most widespread

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species in the genus *Haplophyllum* occurring from North Africa to the Middle East and is the only species recorded from Oman. This plant is widely used by traditional medicinal practitioners in Sudan, Saudi Arabia, Qatar and Oman. In Sudan, herbalists use it to treat allergic rhinitis, asthma and gynecological disorders and as an antispasmodic[7]. It is also a widely grown perennial herb in Northern Oman. Its aerial parts are commonly used as a household remedy to treat dizziness, constipation, arthritis, headache *etc.*, especially by aged people in the villages. The juice expressed from the leaves is externally applied as a remedy for headaches and arthritis. Pulverized leaves added to ghee are used as a liniment to treat painful and swollen joints. An extract of the plant is also used to relieve knee pain in the knee and symptoms of gout[8].

The volatile oil isolated from the fresh flowering aerial parts of *H. tuberculatum* showed repellent activity against *Aedes aegypti* mosquito which causes yellow fever and weak antibacterial activity against several human pathogens[9]. Eissa *et al.* evaluated the aerial parts of *H. tuberculatum* for antioxidant and cytoprotective activity[10]. They reported that high performance liquid chromatography-characterized ethanol extract of the plant was rich in phenolic compounds and its antioxidant activity evaluated by oxygen radical absorbance capacity assay method was quite significant (1.283 $\mu\text{mol/L TE/mg}$ sample). In cell system model of oxidative stress, pretreatments with ethanol extract at the concentrations of 2.5, 0.25 and 0.025 $\mu\text{g/mL}$ significantly attenuated hydrogen peroxide-induced loss in viability by 13.5%, 17% and 20.5%, respectively, which demonstrated beneficial effects of *H. tuberculatum*[10].

An extensive literature survey indicated that very few pharmacological studies have been conducted so far on *H. tuberculatum* species either grown in Oman or elsewhere. Because this plant is reported to contain coumarins, flavonoids, glycosides and alkaloids and it might possess beneficial biological activities including antioxidant activity. Therefore, the present study aimed to quantify total phenolics, flavonoids and to investigate *in vitro* antioxidant potential of *H. tuberculatum* leaves extracts of varying polarities to discover natural antioxidants and to provide justification for utilizing this plant as traditional medicine in Oman.

2. Materials and methods

2.1. Plant material

2.1.1. Collection and identification

The aerial parts (500 g leaves) of *H. tuberculatum* were collected from Al-Sharqiya region of Sultanate of Oman in February-March 2015. The collected plant sample was identified at Department of Pharmacy, Oman Medical College, where a voucher specimen was preserved. The leaves were dried under shade for 3 days, and then

powdered using kitchen grinder.

2.1.2. Preparation of crude plant extract by cold maceration

The dried and powdered leaves (50 g) were immersed in 1000 mL of 95% ethanol and kept for 1 week for extensive extraction in a dark room at room temperature. The ethanolic extract was removed by vacuum filtration and the plant material was re-extracted two more times until it was exhausted. The collected alcoholic extracts were pooled and concentrated in a vacuum hood at low temperature to obtain the crude viscous extract.

2.1.3. Partitioning of the crude ethanolic extract

The ethanolic extract of leaves was fractionated into petroleum ether, acetone, chloroform and methanol fractions by using Kupchan partitioning method. Briefly, extract was suspended in 50% aqueous alcohol and extracted twice with petroleum ether, chloroform and acetone. All solvents were removed using rotary vacuum evaporator to obtain corresponding extracts. The yield of the viscous masses was recorded and was kept in a refrigerator at 2–8 °C until it was used in experiment.

2.2. Preliminary phytochemical analysis

The various organic fractions of leaves extracts of *H. tuberculatum* were screened for the identification of common classes of bioactive plant metabolites as per the standard chemical tests[11].

2.3. Estimation of total phenolic content (TPC)

Total phenolics were estimated by a well established colorimetric method[12]. An external standard plot was constructed using gallic acid (GA) to calculate TPC in organic partitionates. Briefly, an accurately measured 250 μL of the organic fractionation was mixed with previously diluted (10 fold with distilled water) 1.5 mL Folin-Ciocalteu reagent. The mixture was maintained at 60 °C for 5 min on a water bath followed by the addition of NaHCO_3 solution (1.5 mL, 7.5%). The resulting mixture was then incubated for 1 h and 30 min at room temperature for the color development. A double beam UV spectrophotometer was used to measure the absorbance at 765 nm. A typical blank solution was prepared in the similar manner using 250 μL of the respective solvent in place of the sample.

2.4. Estimation of total flavonoids content (TFC)

AlCl_3 colorimetric method was employed to quantify the level of total flavonoids[13]. Briefly, 5% (w/v) NaNO_2 solution (75 μL) was mixed with 50 μg organic extracts or standard solution of quercetin (20–100 $\mu\text{g/mL}$) in a test tube. The reaction mixture was allowed

to stand for 6 min at room temperature. Subsequently, 150 μ L of a 10% (w/v) $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ solution was gradually added to the above mixture and kept at room temperature for further 5 min for reaction to occur. It was followed by the addition of 0.5 mL of 1 mol/L NaOH. Sufficient quantity of the distilled water was added to make the final volume of the mixture to 2.5 mL. The contents were mixed well to measure the absorbance at 510 nm. The results of TFC were presented as milligram of quercetin equivalents (QE) in each gram of the dry extract.

2.5. Evaluation of antioxidant activity

In vitro antioxidant activity of leaves of *H. tuberculatum* medicinal plant in different solvent extracts was evaluated by using 1,1 diphenyl-2-picrylhydrazyl (DPPH) assay and total antioxidant capacity method [14,15].

2.5.1. DPPH radical scavenging assay

The working standard solution of DPPH (100 μ mol/L) was prepared in methanol. About 2 mL of DPPH solution and 1 mL of organic crude extracts or standard ascorbic acid in methanol of different concentrations (5–80 μ g/mL) were mixed and kept in dark place for 45 min at room temperature for reaction to occur. The optical densities of these solutions were recorded at 517 nm. A control blank was prepared in the same manner using only DPPH and methanol.

2.5.2. Determination of total antioxidant capacity

About 0.3 mL organic extracts and 2.7 mL of the phosphomolybdenum reagent were mixed in a test tube, so that the final concentration of the extract in the reaction mixture was 50 μ g/mL. The absorbance of the developed color was measured at 695 nm. Ascorbic acid was used as reference compound for comparison purpose. For the blank, sample was replaced with ethanol. Total antioxidant capacity of the plant extracts was expressed as ascorbic acid equivalent (mg/g of dry extract).

2.6. Statistical analysis

All the experiments were performed in triplicate and the results were presented as mean \pm SD. Statistical significance between groups was analyzed by applying Student's *t*-test and One-way ANOVA. Values of *P* less than 0.05 and 0.01 were considered statistically significant.

3. Results

3.1. Percentage yield of fractionation

A total of 50 g of powdered leaves of *H. tuberculatum* produced approximately 5.674 g (11.348%) of viscous mass by cold percolation method in ethanol. Crude alcoholic extract of leaves upon Kupchan partitioning method yielded different amounts of petroleum ether, chloroform, acetone and methanol extract (Table 1).

Table 1

Percentage yield (w/w) of organic fractions obtained from *H. tuberculatum* leaves.

Fractions	Amount (g)	Each extract (%)	Percentage of yield (w/w)
Petroleum ether	2.283	40.24	4.566
Chloroform	0.780	13.74	1.560
Acetone	0.961	16.94	1.922
Methanol	1.650	29.08	3.300

3.2. Preliminary phytochemical analysis

The results of qualitative phytochemical testing revealed the presence of secondary plant metabolites in leaves extracts of varying polarities (Table 2). Petroleum ether, the least non-polar solvent was found to contain only steroidal compounds. Methanol and acetone extracts showed the presence of polyphenols (tannins and flavonoids), carbohydrates, steroids, alkaloids except proteins and amino acids. Alkaloids and proteins were absent in chloroform extract. Since FeCl_3 test was negative for petroleum ether extract. We, therefore, estimated the TPC and TFC in methanol, acetone and chloroform extracts only.

Table 2

Results of preliminary phytochemical testing of *H. tuberculatum* leaves extracts.

Phytochemical classes	Test	Petroleum ether extract	Chloroform extract	Acetone extract	Methanol extract
Tannins	FeCl_3 test	-	+	+	+
Steroids	Salkowski test	+	+	+	+
Flavonoids	Ammonia test	-	+	+	+
Proteins and amino acids	Ninhydrin test	-	-	-	-
Alkaloids	Wagner's test	-	-	+	+
Carbohydrates	Molisch's test	-	+	+	+

+: Detected; -: Not detected.

3.3. TPCs

The TPC in the leaves extracts was expressed in terms of milligram of gallic acid equivalents (GAE)/g of dry extract (Table 3). The line equation obtained from the standard calibration curve of GA was used to calculate the TPC.

$$Y = 0.0024X + 0.0006, r^2 = 0.9985$$

where, *Y*: absorbance; *X*: amount of GA in μ g.

TPC of organic extracts showed considerable variation and was found to be in the following order (from the highest to the lowest), methanol > chloroform > acetone. TPC was in the range of 182–561 mg of GAE/g of the extract. ANOVA single factor test also indicated a significant difference ($P < 0.001$) in the TPC of organic fractionations.

Table 3

TPC and TFC in organic extracts of *H. tuberculatum* leaves.

Organic extracts	Total phenolics (mg of GAE/g of dry extract)	Total flavonoids (mg of QE/g of dry extract)
Petroleum ether	-	-
Chloroform	254.00 \pm 30.42	49.27 \pm 11.34
Acetone	182.89 \pm 15.50	52.84 \pm 5.13
Methanol	561.22 \pm 83.18	165.54 \pm 29.12
<i>P</i> value (ANOVA)	< 0.001*	< 0.001*

Total phenol and flavonoid contents were expressed as mean \pm SD ($n = 3$). -: Not detected; *: Significant values, $P < 0.01$ determined using ANOVA single factor test.

3.4. TFCs

TFCs in the leaves extracts were expressed in terms of milligram of QE/g of dry extract and were compiled in Table 3. The line of equation obtained from the standard plot of quercetin was used to calculate the TFC.

$$Y = 0.0084X - 0.0076, r^2 = 0.9973$$

where, Y : absorbance; X : amount of quercetin in μg .

TFCs of three organic extracts were found in the following order (from the highest to the lowest), methanol > acetone > chloroform. TFC was in the range of 49–165 mg of QE/g of the extract. ANOVA single factor test also indicated a significant difference ($P < 0.001$) in the TFC of organic fractions.

3.5. In vitro antioxidant activity

The ability of the organic extract of *H. tuberculatum* leaves to scavenge free radicals was examined by DPPH assay. The percent inhibition of DPPH radicals of leaves extracts was found to be much lower than the positive control, however all the extracts exhibited dose-dependent antioxidant activity. The antioxidant activity of fractions was observed in the order, methanol > chloroform > acetone extract (Table 4). The IC_{50} value for the extracts was in the range of 111.34–142.11 $\mu\text{g/mL}$ and was much higher than the ascorbic acid (13.68 $\mu\text{g/mL}$) indicating them to be of lower potency.

Table 4

In vitro antioxidant activity of organic extracts of *H. tuberculatum* leaves or ascorbic acid by DPPH assay. %.

Concentrations ($\mu\text{g/mL}$)	Ascorbic acid	Chloroform	Acetone	Methanol
5	25.68 \pm 0.86	10.45 \pm 4.37 [*]	1.97 \pm 1.30 ^{***}	8.95 \pm 1.68 [*]
10	39.44 \pm 1.07	17.32 \pm 5.43 [*]	7.46 \pm 0.82 ^{***}	15.38 \pm 1.30 [*]
20	51.83 \pm 1.86	18.97 \pm 4.87 [*]	16.25 \pm 1.40 ^{**}	22.92 \pm 2.46 [*]
40	66.82 \pm 0.71	27.33 \pm 2.69 [*]	22.99 \pm 3.21 [*]	29.82 \pm 1.74 [*]
80	84.69 \pm 0.53	32.86 \pm 4.16 [*]	27.42 \pm 2.24 ^{**}	36.73 \pm 0.83 [*]
IC_{50} value	25.58	136.20	142.11	111.34

Values were expressed as mean \pm SD ($n = 3$). ^{*}: $P < 0.05$ when compared with ascorbic acid; ^{**}: $P < 0.05$ when compared with methanol; ^{***}: $P < 0.05$ when compared with chloroform by Student's *t*-test.

3.6. Total antioxidant capacity

Phosphomolybdenum reagent prepared by mixing equal amounts of 0.6 mol/L sulfuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate was used to investigate the total antioxidant capacity of leaves fractions by colorimetric method. A standard calibration curve was constructed by using l-ascorbic acid in order to obtain the line equation for calculation of total antioxidant activity as milligram of ascorbic acid equivalent (AAE)/mg of the dried extract. The absorbance of sample/test was measured at 695 nm and the total antioxidant capacity was computed from the following linear regression equation.

$$Y = 0.0012X + 0.0392$$

where, Y = absorbance and X = total antioxidant capacity in AAE.

The total antioxidant capacity calculated in the crude organic fractions of *H. tuberculatum* (leaves) was observed in the following order, chloroform < acetone < methanol extract. Antioxidant capacity of methanol was found to be 2007.87 mg of AAE/g of dry extract

and was approximately 21 times more than the chloroform extract (Table 5).

Table 5

Results of total antioxidant capacity of the organic extracts of *H. tuberculatum* leaves.

Crude extracts	Antioxidant capacity (mg of AAE/g of dry extract)
Chloroform	92.95 \pm 22.51
Acetone	522.87 \pm 90.63
Methanol	2007.87 \pm 122.65
<i>P</i> value (ANOVA)	< 0.001 [*]

Results were expressed as mean \pm SD ($n = 3$). ^{*}: $P < 0.05$ by ANOVA single factor test.

4. Discussion

The results of qualitative chemical analysis revealed the occurrence of major classes of biologically active phytochemicals in acetone and alcoholic extracts of *H. tuberculatum* leaves including polyphenols (tannins and flavonoids), steroids, alkaloids and carbohydrates. Polyphenols and other secondary plant metabolites are considered as source of natural antioxidants. Results of several other studies have shown that the natural antioxidants are much safer than synthetic antioxidants. The antioxidant activity of phenolic compounds such as flavonoids and tannins is attributed to their reducing properties by virtue, of which these compounds can scavenge, neutralize or inhibit the formation of free radicals [16]. Phenolic compounds were not detected in non-polar petroleum ether extract, hence, quantification of total phenolic and flavonoid contents was done for acetone, chloroform and methanol extracts of *H. tuberculatum* leaves only. Similarly, these three organic fractions were also evaluated for their free radical scavenging activity by *in vitro* DPPH method and total antioxidant capacity by phosphomolybdenum method.

It was observed that methanol is significantly better solvent than acetone or chloroform in extracting phenolic compounds from the leaves extracts of *H. tuberculatum*. The maximum level of phenols (561.22 mg/g) and flavonoids (165.29 mg/g) was observed in the most polar extract. The TPC and TFC of methanol extract were significantly higher than the acetone/chloroform extracts (182.89/52.84 and 49.27/52.84 mg/g respectively). Results of several studies have shown the occurrence of phenolic compounds in non-polar extracts such as hexane, petroleum ether but no phenolic compounds were detected in the current study [17]. Thus, it could be postulated that useful biological actions of *H. tuberculatum* might be owing to its high flavonoids and phenolics content.

The antioxidant power of the leaves extracts of *H. tuberculatum* was evaluated by reliable and well established *in vitro* colorimetric models of testing *viz.* DPPH assay and phosphomolybdenum method. DPPH is a free stable radical in methanol solution and it discolors upon reaction with reducing agent. The scavenging potential of the sample antioxidant is evaluated by its ability to discolor or decrease the absorbance of the DPPH solution which depends upon number of electrons that donates to DPPH to convert it into a neutral molecule. The organic extracts of *H. tuberculatum* leaves showed the presence of biologically active secondary plant metabolites. Bioactive phytochemicals such as flavonoids, triterpenes, alkaloids, tannins *etc.* are known to possess significant antioxidant activity.

These compounds have hydrogen donating ability which leads to discoloration of DPPH solution[18]. Although the tested extracts displayed mild antioxidant activity in comparison to ascorbic acid, it was observed to be concentration dependent. The maximum activity was observed at 80 µg/mL. Polar solvent *i.e.* methanol was better than the other two organic extracts in exhibiting antioxidant activity. It was interesting to note that a direct correlation exists between TPC and antioxidant activity of the organic extracts ($r^2 = 0.9906$).

Total antioxidant capacity of polar fraction *i.e.* methanol extract of *H. tuberculatum* was found to be quite significant in comparison to acetone and chloroform extracts. It can be concluded that the methanol is the ideal solvent for the extraction of TPC (561.22 GAE), and it also contains the maximum number of bioactive chemicals which could be responsible for its antioxidant and total antioxidant capacity. Thus, selective extraction of antioxidants by using appropriate polar organic solvent from natural sources of appropriate solvent could be proved useful in isolation of bioactive compounds in high yield.

The finding of this pilot project revealed that leaves of *H. tuberculatum* species grown in Oman contain high content of phenolic compounds which might be responsible for its biological actions and its use in traditional medicine. Further studies are recommended to isolate and quantify the phenolic compounds present in *H. tuberculatum* leaves which might be used as natural antioxidants.

Conflict of interest statement

We declare that we have no conflict of interest.

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