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Determination of total phenol, *in-vitro* antioxidant and anti-inflammatory activity of seeds and fruits of *Zizyphus spina-christi* grown in Oman

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ABSTRACT

Objective: To perform phytochemical screening and to evaluate the *in-vitro* antioxidant and anti-inflammatory activities of ethanolic extract of seeds and fruits of *Zizyphus spina-christi* (ZSC) growing in Oman.

Methods: Alcoholic extract of the dry powdered seeds and fruits of ZSC was obtained by cold maceration method and was subjected to preliminary phytochemical screening. Total phenolic content were estimated by using Folin-Ciocalteu reagent and 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used to determine *in-vitro* antioxidant activity of plant extracts. Anti-inflammatory activity was investigated by protein denaturation method.

Results: Phytochemical analysis of both the extracts revealed the presence of major classes of phytochemicals such as tannins, alkaloids, flavonoids, cardiac glycosides *etc*.. ZSC seeds were found to contain the highest total phenolics but ZSC fruits exhibited the maximum antioxidant activity. The anti-inflammatory activity of both parts of the plant extract was significant and comparable with the standard anti-inflammatory drug, diclofenac.

Conclusions: Based on the results of this pilot study, it can be concluded that ZSC is a good source of natural antioxidants which can be used to prevent progression of many chronic diseases. Further detailed phytochemical studies are needed to identify the chemical compounds responsible for exhibiting potent anti-inflammatory activity.

1. Introduction

Medicinal plants have been known for millennia and are considered as potential sources of pharmaceutical agents and/or as sources of lead compounds in drug development[1]. According to the World Health Organization, more than 80% of the populations within developing countries rely on the use of herbal and other traditional medicines for their primary healthcare[2]. The beneficial effects of plant materials are due to the presence of secondary plant metabolites[3]. Nature has bestowed Oman with an enormous wealth of medicinal plants[4]. Oman has approximately 1 204 terrestrial plants, many of which are reported to be used by herbalist as traditional medicines[5]. Therefore, there is a need to screen and explore these traditional medicinal plants for bioactive compounds as a basis for further pharmacological studies[6]. Scientific examination and validation of traditional therapeutic use of the plant medicines may lead to the development of new and effective drugs as has occurred in the past[7].

Ziziphus spina-christi (L.) Willd. (ZSC) commonly known as Christ's thorn or Jerusalem-thorn in English and Sidr in Arabic belongs to family Rhamnaceae. It is a deciduous tree, native to the warm-temperate and subtropical regions, including south and east of Asia and Middle East[8]. It is a wild tree, with spiny branches and small, orange-yellow fruits that grows throughout Middle Eastern region including Oman[4]. ZSC is a very popular traditional medicine across gulf region and is widely used in the management of pain and inflammatory related problems[9-11]. ZSC has also been reported to have activity against bacterial and fungal pathogens that are normally

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quite resistant to modern medications[12]. Its fruits are very nutritious and are usually eaten fresh. Fruits of ZSC are used to treat pulmonary ailments such as tuberculosis, cough, bronchitis, *etc.*, fever, dysentery and to promote the healing of fresh wounds[13]. The seeds are sedative and are taken sometime with buttermilk to halt nausea, vomiting and abdominal pains associated with pregnancy[11,14]. The root bark infusion is used traditionally in Africa as a remedy for stomach pain and other gastrointestinal tract ailments. It has been used in folk medicine as a demulcent, a stomachic, as astringent for toothaches and as mouth wash[15].

Pharmacological studies have demonstrated that ZSC were known to possess hypoglycemic, hypotensive, antimicrobial, hepatoprotective, antioxidant, antitumor and immune stimulatory activities[16,17]. These biological activities could be attributed to the presence of secondary plant metabolites present in ZSC. Various photochemicals previously isolated from ZSC include cyclopeptide alkaloids, flavonoids, sterols, tannins and saponin glycosides[14,18-21].

The present study was conducted with the objectives (i) to identify the major classes of phytochemicals present in the fruits and seeds of ZSC variety grown in Oman, (ii) to compare total phenolic content (TPC) and radical scavenging activity in fruits and seeds of ZSC and (iii) to evaluate anti-inflammatory activity of ZSC fruits and seeds against denaturation of protein to validate its use in traditional system of medicine.

2. Materials and methods

2.1. Drugs and chemicals

Diclofenac sodium was a kind of gift from National Pharmaceutical Industries LLC, Muscat, Oman. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and gallic acid were purchased from Sigma-Aldrich USA. Folin-Ciocalteu reagent was obtained from Merck, Germany. All other chemicals used in the study were of analytical grade procured locally.

2.2. Collection of plant material

Ripened fruits of ZSC plant were collected from Muscat, Oman in the month of November-December, 2012. The plant material was identified and authenticated by a botanist of Department of Natural Science, Oman Medical College. A voucher specimen (PHAR-425-12) was deposited at the herbarium unit of the pharmacy department for future reference. The fruits were cleaned and washed under tap water. The edible parts of ZSC fruits were separated from seeds and dried under shade. The dried samples were powdered in a heavy duty grinder and kept in air tight containers until use.

2.3. Extraction of the plant material

The dried powdered fruits and seeds (100 g each) were extracted by maceration with 1000 mL 70% ethanol for 3 d at room temperature with occasional shaking. The extract was filtered and the marc was re-extracted by the same process until plant materials were exhausted. The collected filtrates were pooled and evaporated to dryness under reduced pressure to yield the dry extracts (10.34% and 7.93 % for yield of fruits and seeds respectively) and were stored at 4 °C until used.

2.4. Phytochemical screening of ethanolic extracts

The freshly prepared crude extracts of fruits and seeds of ZSC were subjected to qualitative phytochemical analysis for the presence of various classes of active chemical constituents such as tannins, saponins, glycosides, flavonoids, alkaloids, terpenes, steroids, *etc.* using standard procedures[22,23].

2.5. Determination of TPC

The TPC of the two ethanolic extracts were determined by using Folin-Ciocalteu reagent following a reported method[24]. Gallic acid was used as a reference standard for plotting calibration curve. A volume of 0.5 mL of the plant extract (100 μg/mL) was mixed with 2 mL of the Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and were neutralized with 4 mL of sodium carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature for 30 min with intermittent shaking for color development. The absorbance of the resulting blue color was measured at 765 nm using double beam UV-vis spectrophotometer (UV Analyst- CT 8200). The TPCs were determined from the linear equation of a standard curve prepared with gallic acid. The content of total phenolic compounds was calculated as mean±SD (n=3) and expressed as mg/gm gallic acid equivalent (GAE) of dry extract.

2.6. Determination of antioxidant activity by DPPH-scavenging assay

The free radical scavenging activity of the fruits and seeds extract of ZSC and of standard solution (ascorbic acid) were evaluated by using DPPH radical scavenging method as described by Alhakmani *et al*[24]. The assay mixture contained 2 mL of 1.0 mmol/L DPPH radical solution prepared in methanol and 1 mL of standard or extract solution of different concentrations (10-500 μg/mL) in ethanol. The solution was rapidly mixed and incubated in dark at 37 °C for 20 min. The decrease in absorbance of each solution was measured at 517 nm using UV/V which is Spectro photomer. Ascorbic acid was used as positive control while DPPH radical solution with

1 mL methanol was taken as blank. The percent (%) radical scavenging was calculated by the following formula:

$$\text{Free radical scavenging activity (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

Where A_c is absorbance of control at 517 nm; A_s is absorbance of sample.

The concentration of sample required to scavenge 50% of the DPPH free radical (IC_{50}) was determined from the curve of percent inhibitions plotted against the respective concentration.

2.7. Evaluation of in-vitro anti-inflammatory activity

Anti-inflammatory activity of the ZSC fruits and seeds extract was evaluated by protein denaturation method[24]. Diclofenac sodium, a powerful non steroidal anti-inflammatory drug was used as a standard drug. The reaction mixture consisting of 2 mL of different concentrations of ZSC fruits/seeds extract (100-500 μg/mL) or standard diclofenac sodium (100 and 200 μg/mL) and 2.8 mL of phosphate buffered saline (pH 6.4) was mixed with 2 mL of egg albumin (from fresh hen's egg) and incubated at (27±1) °C for 15 min. Denaturation was induced by keeping the reaction mixture at 70 °C in a water bath for 10 min. After cooling, the absorbance was measured at 660 nm by using double distilled water as blank. Each experiment was done in triplicate and the average was taken. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\text{Inhibition (\%)} = \frac{A_t - A_c}{A_c} \times 100$$

Where A_t is absorbance of test sample; A_c is absorbance of control.

2.8. Data analysis

The results are expressed as mean±SD. Student's *t*-test and One-way analysis of variance (ANOVA) where applicable, were used to analyze level of statistical significance between groups. *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Phytochemical screening

Preliminary phytochemical analysis showed the presence of major classes of secondary metabolites such as tannins, alkaloids, flavonoids, cardiac glycosides, *etc.* in both of the extracts (Table 1). Saponins, protein and amino acids were absent in both the extracts. Seed extract showed the absence of steroids and terpenoids while their presence was revealed in fruit extract.

Table 1

Results of phytochemical analysis of ZSC fruit and seed extract.

Phytochemical test	Name of the test	ZSC fruit extract	ZSC seed extract
Tannins	$FeCl_3$ test, Lead acetate test	+	+
Steroids	Salkowski test	+	−
Flavonoids	Ammonia test, Alkaline reagent test	+	+
Saponins	Frothing test	−	−
Proteins and amino acids	Ninhydrin test	−	−
Alkaloids	Hager's, Meyer's and Wagner's test	+	+
Carbohydrates	Molisch's test	+	+
Glycosides	Nitroprusside test	+	+
Cardiac glycosides	Keller Killiani test	+	+
Terpenoids	Salkowski test (modified)	+	−

+: present, −: absent.

3.2. TPC

The TPC of the ZSC fruit and seed extracts expressed in terms of gallic acid and yield (%) (w/w) are presented in Table 2. The TPCs were calculated using the following linear regression equation based on the calibration curve of gallic acid:

$A = 0.008\,0x + 0.072\,7$; $R^2 = 0.996\,7$

Where A is absorbance and x is amount of gallic acid in μg. Seed extract was found to contain higher amounts of phenolic compounds as compared to fruit extract. However, an inverse relationship was observed in yield (%).

Table 2

Yield (%) (w/w) and TPCs of ZSC fruit and seed extract expressed in terms of GAE.

Extract	Yield (%) (w/w)	mg of GA/g of extract	*P*-value (*t*-test)
Seed	7.93	34.53±1.43	0.0013*
Fruit	10.34	24.62±0.83	

TPC values are expressed as mean±SD, n=3. *Significant values, $P<0.05$, using student's *t*-test.

3.3. DPPH free radical scavenging activity

The scavenging effect of different concentration of ZSC fruit and seed extract on the DPPH free radical was compared with standard antioxdiant, ascorbic acid. The results are expressed as % inhibition and are shown in Table 3. Both the extracts showed a dose dependent scavenging activity of which fruit exhibited 54.10% inhibition of free radicals at 200 μg/mL whereas at the same concentration seed extract showed only 42.59% inhibition. However, their scavenging ability was found to be non significant ($P>0.05$) in comparison to ascorbic acid as evident by the IC_{50} value of fruit extract.

Table 3

Percentage inhibition of DPPH free radical by ZSC extract/ ascorbic acid at 517nm.

Concentration (μg/mL)	Inhibition of DPPH (%)		
	Fruit extract	Seed extract	Ascorbic acid
10	4.74±0.50	1.84±0.10	21.58±12.00
20	10.30±0.43	6.05±0.11	89.68±35.00
50	13.63±1.10	10.08±1.40	90.71±1.23
100	26.00±85.00	23.68±0.95	92.32±2.05
200	54.10±24.00	42.59±0.10	94.21±1.01
IC_{50} value	187.91	−	14.57

Values are mean±SD, n=3.

3.4. In-vitro anti-inflammatory activity

The inhibitory effect of different concentrations of ZSC fruit and seed extract on protein denaturation are summarized in Table 4. Both the extracts (100-500 μg/mL) showed significant inhibition of denaturation of egg albumin in a dose dependent manner. The *in-vitro* anti-inflammatory activity of the extracts was comparable to the diclofenac sodium, a reference drug at dose level of 100 and 200 μg/mL. A significant difference in the inhibition of thermally induced protein denaturation was observed in case of seed extract when compared with fruit extract at concentrations of 100 and 500 μg/mL.

Table 4

In-vitro anti-inflammatory effect of ZSC fruit and seed extract by protein denaturation method.

Treatment	Concentration (μg/mL)	Inhibition of protein denaturation (%)
Fruit extract	100	44.10±1.20
	200	95.15±0.84
	500	98.98±2.30
Seed extract	100	67.09±1.43*
	200	98.98±1.67
	500	118.07±2.87*
Diclofenac sodium	100	84.95±1.46
	200	120.12±2.76

Values are mean±SD, n=3. *Significant values, P<0.05, using student's t-test, fruit extract *v.s.* seed extract.

4. Discussion

Mankind is using medicinal plants or natural products obtained from them since ancient times to treat their acute or chronic diseases. These medicinal plants are reported to possess diverse pharmacological actions which are attributed to the presence of phytochemicals such as alkaloids, flavonoids, glycosides, tannins, steroids, *etc*. Some of these plants are important sources of natural antioxidants[25] that have been shown to reduce the risk and progression of many acute and chronic diseases including cancer, cardiovascular diseases and neurodegenerative diseases by scavenging free radicals which are implicated in the pathogenesis of these diseases[26].

The results of preliminary phytochemical screening confirmed the presence of various classes of secondary metabolites in the fruit and seed extract of ZSC including poly phenols (tannins and flavonoids). Plant polyphenols are important dietary antioxidants because they possess an ideal structural chemistry for free radical scavenging activity. Numerous *in-vitro* studies have conclusively shown their antioxidant potential in protecting against many diseases[27]. The present study indicated that fruits and seeds of ZSC were rich in polyphenols (24.62 and 34.53 mg/g of GAE of dry extract respectively), which was significantly higher than the previously reported TPC in the fruits of ZSC in Oman as well as from other gulf countries[17]. Antioxidant activity of natural products is partially attributed to the presence of phenolic, thus the high TPC in fruits and seeds of ZSC could be considered as a rationale for this commonly used traditional medicine in many acute and chronic ailments of different etiology.

DPPH free radical scavenging method is commonly used to test *in-vitro* antioxidant activity of plant extracts[28]. DPPH is a stable free radical at room temperature and its reduction capability to accept an electron or a hydrogen radical from antioxidants is determined by measuring decrease in its absorbance values at 517 nm. DPPH radical scavenging activity of fruit and seed extract of ZSC was compared with standard ascorbic acid. Although ascorbic acid showed better scavenging activity at all tested concentrations than the ZSC fruit and seed extract, the extracts still showed good free radical scavenging activity. The antioxidant activity might be due to the presence of poly phenols in seed and fruit extracts. The free radical scavenging property of ZSC may be one of the mechanisms by which this plant is effective as a traditional medicine. The consumption of the fruits of ZSC can be beneficial in preventing oxidative stress related degenerative diseases.

Non-steroidal anti-inflammatory drugs are routinely prescribed for the management of inflammatory conditions, but their use is associated with many undesirable side effects such as gastric irritation, ulcer, *etc*[29]. Medicinal plants used in traditional medicine to treat anti-inflammatory conditions seem a viable and logical alternative in search of safe, potent and effective anti-inflammatory agents. ZSC is commonly used as a traditional medicine across gulf region to treat inflammatory conditions, hence, to validate its use scientifically, protein denaturation bioassay method was selected to evaluate its potential as an anti-inflammatory drug. It is a well known fact that denaturation of tissue proteins lead to inflammatory and arthritic diseases[30]. Plant products that can prevent protein denaturation, therefore, would be worthwhile for research and development of anti-inflammatory drug therapy.

ZSC extracts and standard drug diclofenac sodium exhibited dose dependent percentage inhibition of heat induced protein denaturation in fresh egg albumin. Percent inhibition of protein denaturation with respect to control is a measure of protein stabilization[31]. Though ZSC seeds extract showed a moderate free radical scavenging activity, its effect on inhibition of protein denaturation was found to be better than the ZSC fruit extract and was comparable with the standard non-steroidal anti-inflammatory drug, diclofenac sodium.

The results of this study suggest that ZSC is rich in phenolic compounds and has a good antioxidant activity. It can be used as a natural source of antioxidants to prevent the progression of many diseases. ZSC extracts also produced marked *in-vitro* anti-inflammatory activity that justifies its use in traditional system of medicine in Oman as well as in other gulf countries. However, further detailed investigations are needed to isolate the phytoconstituents responsible for its anti-inflammatory actions.

Conflict of interest statement

We declare that we have no conflict of interest.

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