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Phytochemical analysis, cytotoxic, antioxidant and anti-diabetic activities of the aerial parts of *Sorghum halepense*

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Abstract

Phytochemical screening, cytotoxic activity, total phenolic content, anti-oxidant and anti-diabetic activities of the *Sorghum halepense* methanolic extract and its different fractions were evaluated. Methanolic extract and its various fractions revealed the presence of reducing sugars, tannins, steroids, glycosides, flavonoids in the methanolic extract where they were absent in n-hexane fraction except flavonoids. Gums and saponins were absent in all the samples. Methanolic extract indicated the highest cytotoxic (80.7 ± 1.3%) and anti-diabetic (62.5%) activities. The maximum total phenolic contents (28.7 ± 1.4 mg/mL) were found in the chloroform fraction. An aqueous fraction expressed the highest antioxidant activity 74.1 and 97.1% free radical scavenging properties in DPPH and ABTS assays respectively whereas, in the case of H_2O_2, methanolic extract indicated maximum (36.9%) activity. In conclusion, the extract of aerial parts of *S. halepense* is a source of compounds against cancer, diabetic and free radical associated disorders.

Introduction

The damage caused by free radicals is the crucial etiological factor concerned with several chronic diseases such as cancer, atherosclerosis, diabetes mellitus, arthritis, and neurodegenerative diseases and also in the aging process. The epidemiological studies have revealed that the use of diets rich in fruits and vegetables and those containing selected natural antioxidants such as plant polyphenols, vitamin C and flavonoids are linked to reduced prevalence of cardiovascular and some cancerous chronic diseases (Gheldof and Engeseth, 2002; Liu et al., 2000; Siddhuraju and Becker, 2003; Zuo et al., 2002).

The anti-diabetic activities of *Achillea santolina* (Grover et al., 2002), *Adiantum capillus-veneris* (Al-Snafi, 2013), *Agrimony eupatoria* (Al-Snafi, 2015), *Aristolochia longa* (El Omari et al., 2019), *Atractylis gummifera* (Bouabid et al., 2019), *Eruca sativa* (Kishore et al., 2017), *Gaultheria trichophylla* (Alam and Saqib, 2017), *Leucas aspera* (Annapandian and Sundaram, 2017), *Myrica gale*, *Rhodiola rosea*, *Rumex acetosa*, *Taraxacum officinale* (Sekhon-Loodu and Rupasinghe, 2019), *Trixis angustifolia* (Salazar-Gómez et al., 2019) and *Urtica dioica* (Ahangarpour et al., 2012) have been reported.

The antidiabetic effect of *Sorghum bicolor* (Chung et al., 2011) has been reported. However, the antidiabetic effect of *S. halepense* is not known.

The aim of this study was to investigate the phytochemicals, cytotoxic properties, total phenolic content, antioxidant and anti-diabetic properties of crude methanolic extract of aerial parts of *S. halepense* and its *n*-hexane, chloroform and aqueous fractions through brine shrimp lethality, DPPH, ABTS and H_2O_2 assays and quantification of total phenolic content.

Materials and Methods

Chemicals for biological activities

Chemical reagents sodium phosphate monobasic, sodi-

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um phosphate dibasic, DPPH (2, 2-diphenyl-1-picryl-hydrazyl), ABTS•+ (2,2-azinobis (3-ethyl-benzothiazoline)-6-sulfonic acid disodium salt), hydrogen peroxide, amylase, DNS (dinitrosalicylic) acid, sea salts, glucophage, potassium persulfate were of analytical grade. Methanolic extract and its *n*-hexane, chloroform and aqueous fractions of the aerial part of *S. halepense*. Biotechnology Medical Services (BMS, UV-1602) spectrophotometer was used for measuring absorbance.

Plant material

The aerial parts of *S. halepense* were collected in March 2017 from the District Bannu, Pakistan. It was identified by Prof. Abdur Rehman, Govt. Postgraduate College, Bannu, Khyber Pakhtoon Khwa, Pakistan.

Preparation of crude extract

The aerial parts of young *S. halepense* were collected, washed with tap water, dried under shade and ground into fine powder with the help of pestle and mortar. The powder (600 g) was extracted in 1.5 L 70% methanol by keeping the powder in mentioned solvents at room temperature with frequent agitation for 72 hours and the resultant liquid was filtered (Whatman No. 3 filter paper, Whatman Ltd., England). The filtrate was sited at room temperature to vanish the solvent content. The resultant gummy methanolic extract (26.8 g) was collected and stored for future use.

Preparation of fractions

Methanolic crude extract (20 g) of *S. halepense* was sequentially extracted each with 300 mL *n*-hexane, chloroform and water using separating funnel to avoid any sort of damages to the filtrate. The respective solvents were evaporated completely at room temperature. The resulting extracts of *n*-hexane (2 g), chloroform (3.2 g) and water (7.9 g) were stored for further designed assays.

Phytochemicals screening

The methanolic extract of *S. halepense* and its different fractions were subjected to phytochemicals screening by using standard methods to investigate the presence of cardiac glycosides, saponins, flavonoids, tannins, steroids, reducing sugar and gums (Rice-Evans et al., 1996; Trease and Evans, 1989).

Cytotoxic assay

Methanolic extract and its various fractions of *S. halepense* were subjected to brine shrimp lethality bioassay to find out their cytotoxic activities (Meyer et al., 1982). A small amount of brine shrimp eggs (1 mg) were put in 4% artificial seawater contained in a cabin covered by aluminum foil and placed for 24 hours. After hatching, the shrimps crossed the porous central wall of the cabins and came into an illuminated one. The working solution of methanolic extract and its different fractions were prepared in methanol with different concentrations i.e. 100, 250, 500 and 1000 µg/mL. Test tubes containing test samples were placed at room temperature for complete evaporation of methanol followed by inoculation with 10 shrimps to each test tube and incubation for 24 hours. Then counted live shrimps in each test tube, calculated %lethality with the help of Abbot's formula and compared the results (lethality) of control with experimental.

%Death = [(Sample - control)/control] × 100

Total phenolic content

The total phenolic content present in the methanolic extract and its fractions of *S. halepense* was determined by the method described elsewhere (Singleton and Rossi, 1965) with slight modification. The sample solution (250 µL; 1–5 mg/mL) was added to 2.5 mL Folin–Ciocalteu reagent (10 x diluted in distilled water) and incubated at room temperature for 5 min. A saturated solution of Na_2CO_3 (2.5 mL, 60 mg/mL) was mixed to the reaction mixture and placed again for 2 hours at the mentioned temperature. Gallic acid was used as the standard. Measured the absorbance at 725 nm spectrophotometrically and articulated the results as gallic acid equivalent. Gallic acid solutions (1-5 mg/mL) were used to prepare the calibration curve and expressed the results as gallic acid equivalents (GAE).

Antioxidant assays

ABTS radical cation assay

The capability of methanolic extract and its various fractions of *S. halepense* to scavenge ABTS•+ (2, 20-azinobis (3-ethyl-benzothiazoline)-6-sulfonic acid disodium salt) free radicals was determined by making slight changes in (Re et al., 1999) protocol. Prepared ABTS (7 mM) and potassium persulfate solutions (2.45 mM), mixed together and placed for eight hours in dark. The mentioned stock solution was diluted with the relevant solvent (50%) and adjusted its absorbance of about 0.900 (± 0.02) at 745 nm at 30°C. 300 µL (125-2000 µg/mL in a respective solvent) of each sample was mixed with ABTS working solution and measured the absorbance. The percentage scavenging property of samples applied and the ascorbic acid was calculated using the following formula:

Scavenging effect (%) = [(control absorbance (ABTS)-sample absorbance) / (control absorbance)] × 100

Hydrogen peroxide scavenging (H_2O_2) assay

The hydrogen peroxide scavenging activities of *S. halepense* methanolic extract and its different fractions were estimated according to the protocol mentioned elsewhere (Wettasinghe and Shahidi, 2000) protocol. The prepared sample solutions (125–2000 µg/mL), phosphate buffer (100 mM, pH 7.4) and hydrogen peroxide solution (43 mM) in the phosphate buffer. The

Box 1: DPPH Method

Principle

A hydrogen donor is an anti-oxidant. It measures the compounds that are radical scavengers. DPPH• accepts hydrogen from an anti-oxidant. DPPH• is one of the few stable and commercially available organic nitrogen radicals. The anti-oxidant effect is proportional to the disappearance of DPPH• in the test sample.

Requirements

Ascorbic acid, DPPH (1,1-diphenyl-2-picrylhydrazyl), Extract of *S. halepense*,

Preparation of solutions

Ascorbic acid: Working solutions (125, 250, 500, 1000, 1500 and 2000 µg/mL) of ascorbic acid were prepared.

Extract: Working solutions (125, 250, 500, 1000, 1500 and 2000 µg/mL) of methanolic extract and its different fractions were prepared.

DPPH solution: The DPPH solution was prepared by dissolving 3 mg of it in 100 mL methanol.

Procedure

Step 1: The DPPH solution was incubated for about 30 min at 25°C in the dark.

Step 2: The absorbance was measured at 517 nm (purple color) spectrophotometrically and adjusted it to less than one. The color turns from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from an anti-oxidant.

Step 3: Then mixed sample/standard and DPPH solutions and recorded absorbance.

Calculation

The potential of plant extracts to scavenge DPPH free radicals was calculated by using the following equation.

%DPPH free radicals scavenging effect = $(A_1-A_2/A_1) \times 100$

Where, A_1= the absorbance of DPPH (control) and

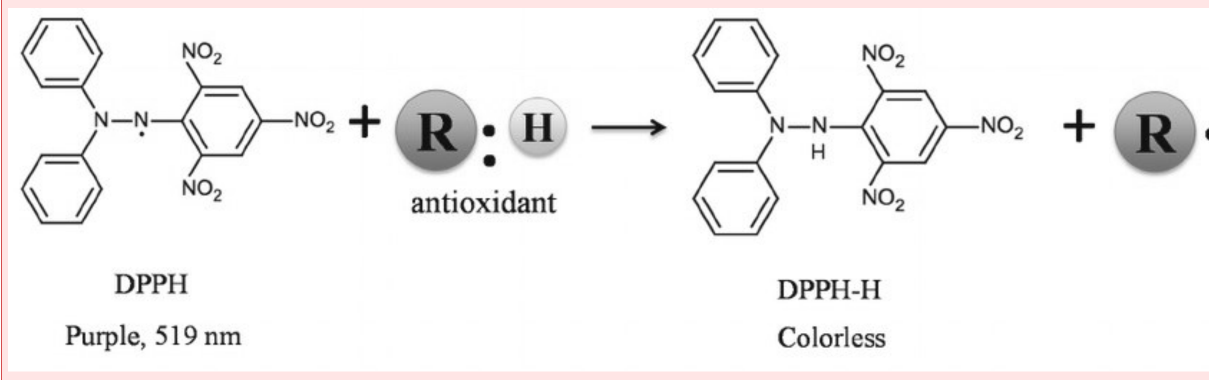
A_2= the absorbance in the presence of samples

Notes

1. Wear gloves while handling DPPH solution
2. Lab coat and goggles should be used
3. Work in fume hood
4. DPPH is stored in the freezer. It should be protected from light and the time out of the freezer should be minimized

References

Gyamfi et al., 1999; MacDonald-Wicks et al., 2006; Moon and Shibamoto, 2009



absorbance of the reaction mixture was measured at 230 nm against a blank containing H_2O instead of H_2O_2 after its incubation for 40 min at room temperature. The following formula was to calculate the percentage of scavenging activities of plant extract.

Scavenging effect (%) = [(control absorbance (H_2O_2)-sample absorbance) / (control absorbance)] × 100

All tests were conducted in triplicate and articulated the results as means ± SD.

a-Amylase inhibition

The inhibitory properties of plant extract against α-amylase were investigated by using Worthington Enzyme Manual Worthington (Kwon et al., 2007) guideline. Plant extract (300 µL), starch (500 µL; 1%) and α-amylase solutions (0.5 mg/mL) were mixed and pre-incubated at 25°C for 10 min. The enzymatic reaction was stopped by the addition of 1.0 mL of dinitrosalicylic (DNS) acid, a color reagent, incubated in the boiling water for 5 min and then cooled to room temperature. The starch, α-amylase and DNS acid solutions were prepared in sodium phosphate buffer (20 mM, pH 6.9 with 6 mM NaCl). The reaction mixture was diluted by adding 3 mL distilled water to each test tube and measured the absorbance at 540 nm spectrophotometrically. The following formula was used to calculate the percentage inhibition of α-amylase.

Amylase inhibition (%) = [(control absorbance (blank)-sample absorbance) / (control absorbance)] × 100

Statistical analysis

The statistical analyses of results were carried out by using GraphPad Prism software. All the experiments were conducted in triplicate and articulated the results as mean ± SD (standard deviation). Pearson correlation

coefficient was determined in further analyses of results between total phenolic contents and different antioxidant and anti-diabetic activities. The $p<0.05$ was considered to be statistically significant.

Results

Phytochemical screening

The phytochemical screening of *S. halepense* methanolic extract and its various fractions revealed the presence of reducing sugars, tannins, steroids, glycosides, flavonoids in the methanolic extract whereas they were absent in the *n*-hexane fraction except flavonoids (Table I). Gums and saponins were absent in all the samples.

Cytotoxic assay

The cytotoxic activities using brine shrimps lethality assay showed 80.7 ± 1.3, 60.3 ± 1.1, 50.4 ± 1.6 and 40.6 ± 1.5% dead brine shrimp in crude methanolic extract and its chloroform, aqueous and *n*-hexane fractions respectively at the concentration of 1,000 µg/mL (Table II).

Total phenolic contents

The maximum amount of phenolic contents was observed in chloroform fraction (28.7 ± 1.4 mg GAE/g) followed by methanol (22.5 ± 1.3 mg GAE/g), aqueous (15.1 ± 1.3 mg GAE/g) and *n*-hexane fraction (5.6 ± 1.4 mg GAE/g).

Antioxidant assays

DPPH (1,1-diphenyl-2-picrylhydrazyl) method

The aqueous fraction indicated highest antioxidant activity (74.1%) followed by crude methanolic extract (73.5%) and hexane fraction (32.9%) whereas chloroform revealed lowest activity (24.6%) at the concentrations of 2 mg/mL. Ascorbic acid expressed 86.4% antioxidant activity at the same concentration. The antioxidant characteristics of *S. halepense* methanolic extract and its different fractions were concentration-dependent.

ABTS-radical cation assay

The highest antioxidant activity was revealed by an aqueous fraction (97.1%) followed by crude methanolic extract (96.7%) and chloroform fraction (79.0%) while the said activity was not observed in *n*-hexane fraction. The mentioned activity of aqueous fraction (97.1%) was comparable with ascorbic acid (96.2%) (Figure 1). An indirect correlation was observed between phenolic contents and antioxidant activities of the applied samples, the chloroform fraction exhibiting maximum phenolic contents (28.7 ± 1.4 mg/mL) expressed minimum antioxidant activity (79.0%), the methanolic extract had moderate phenolic contents (22.5 ± 1.3 mg/mL) had shown moderate antioxidant activity (96.7%).

Hydrogen peroxide scavenging (H_2O_2) capacity

Table I

Phytochemical screening of *S. halepense* methanolic extract and its chloroform and aqueous fractions

		Crude methanolic extract and its fractions			
Phytochemical	Tests	Aqueous	Methanol	Chloroform	*n*-Hexane
Saponins	Alkaline reagent test	-	-	-	-
Flavonoids	Ferric chlorides test	+	+	-	+
Glycosides	Fehling test	+	+	+	-
steroids	Millon's test	+	+	+	-
Reducing sugar	Benedict's test	+	+	+	-
Tannins	Ferric chloride test	+	+	+	-
Gums	Molish's reagent test	-	-	-	-

Table II

%Lethality of brine shrimps caused by crude methanolic extract and its various fractions of *S. halepense*

Concentration (µg/mL)	%Lethality of crude methanolic extract and its fractions				
	Methanolic extract	Chloroform	Aqueous	*n*-Hexane	Control
100	40.7 ± 1.3	30.6 ± 1.2	20.3 ± 1.5	10.2 ± 1.7	00 ± 00
250	50.8 ± 1.5	30.8 ± 1.1	30.9 ± 1.3	10.3 ± 1.6	00 ± 00
500	70.6 ± 1.7	50.4 ± 1.8	30.1 ± 1.5	30.7 ± 1.9	10.2 ± 1.1
1000	80.7 ± 1.3	60.3 ± 1.1	50.4 ± 1.6	40.6 ± 1.5	00 ± 00

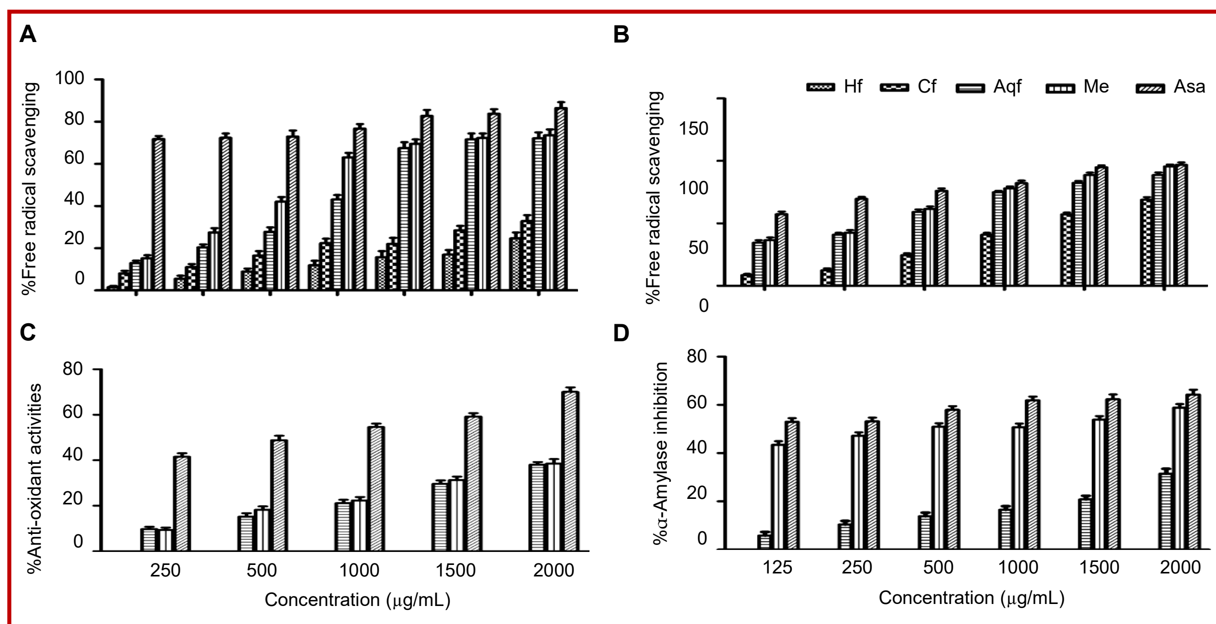


Figure 1: DPPH free radical scavenging capability (A), ABTS free radical scavenging capability (B), H_2O_2 free radical scavenging capability (C) and anti-diabetic activity (D) of the *S. halepense* methanolic extract and its fractions;

Hf: n-Hexane fraction, Cf: chloroform fraction, Me: methanolic extract, Aqf: aqueous fraction and Asa: ascorbic acid; n-hexane fraction did not show anti-oxidant activity in B; hexane fraction and Cf: chloroform fraction did not show anti-oxidant activities in C; hexane fraction and Aqf: aqueous fraction did not express anti-diabetic activities in D

Table III

Correlation between phenolics and *S. halepense* extract soluble fractions

Assays	Correlation R^2 phenolics	Significance
%DPPH radical scavenging ability	0.5	ns
%ABTS scavenging ability	0.6	ns
%H_2O_2 scavenging	0.9	ns
%Alpha-amylase inhibition	0.8	ns

The applied extract sample of *S. halepense* expressed scavenging of H_2O_2 in a concentration-dependent manner. The crude methanolic extract and its water fraction indicated 36.9 and 36.5% scavenging activity respectively on hydrogen peroxide while ascorbic acid showed 51.0% at the concentration of 2000 μg/mL. The chloroform and *n*-hexane fraction did not express H_2O_2 scavenging activities (Figure 1).

Anti-diabetic assay

Crude methanolic extract and its different fractions of *S. halepense* were subjected to an anti-diabetic assay to assess their anti-diabetic properties (Figure 1). The said characteristic of glucophage (commercially available medicine used as a standard) and crude methanolic extract were observed (48.9%) and (62.5%) respectively.

Correlation among total phenolic contents, anti-oxidant and anti-diabetic activities

Chloroform fraction exhibited maximum phenolic content (28.7 ± 1.4 mg GAE), with minimum DPPH and ABTS free radical scavenging properties while did not show anti-diabetic and hydrogen peroxide radicals scavenging activities (Table III).

Discussion

The phytochemical assessment of aerial parts of young *S. halepense* extract and its diverse fractions showed the existence of reducing sugars, tannins, steroids, glycosides, flavonoids in the methanolic extract where they were absent in *n*-hexane fraction except flavonoids. Gums and saponins were absent in all samples. Aerial parts of young *S. halepense* methanolic extract and its chloroform, aqueous and n-hexane fractions showed brine shrimp lethality up to 80.7 ± 1.3%, 60.3 ± 1.1%, 50.4 ± 1.6% and 40.6 ± 1.5% respectively at a concentration of 1000 μg/mL. Different results were observed in different antioxidant assays i.e. aqueous fraction expressed highest antioxidant activity 74.1% and 97.1% free radical scavenging properties in DPPH and ABTS respectively whereas in case of H_2O_2 methanolic extract indicated maximum (36.9%) activity. It is found that plant extracts with higher total phenolic contents have lower antioxidant properties. Aerial parts of young *S. halepense* methanolic extract and its different fractions were subjected to amylase inhibition assay to find out

their anti-diabetic properties. The anti-diabetic activities of glucophage (used as a standard) and methanolic extract of aerial parts of young *S. halepense* were recorded (48.9%) and (62.5%) respectively. The higher anti-diabetic behavior of methanolic extract than glucophage indicates that the mentioned extract is an effective source of anti-diabetic compounds. Dose-dependent percentage inhibition of alpha amylase was observed.

The major chemical constituents of *S. halepense* are sorgoleone and dihydrosorgoleone (Baerson et al., 2008).

Comparable phytochemicals constituents were observed in various extracts of leaves of *T. chebula* (Kathirvel and Sujatha, 2012). The reported cytotoxic activities of *Calligonum polygonoides (*Khan et al., *2015)* and *Solanum surrantance burm* (Shah et al., 2013). *Arcangelisia flava, Coscinium blumeanum* and *Fibraurea tinctoria* (Keawpradub et al., 2005) are harmonious with our results. Previously, unlike antioxidant results have reported in different antioxidant assays (Hagerman et al., 1998). Our results are congruent with earlier studies which reported indirect relations between phenolic content and antioxidant activities exhibited by plant extracts (Kahkonen et al., 1999; Ismail et al., 2004). Our results resemble with curry tree and petai that contained high total phenolic content but lower anti-oxidant activities (Wong et al., 2006). Many previous studies have reported anti-diabetic properties and antioxidant activities of phenolic compounds in different parts of medicinal plants (Krings and Berger, 2001; Samal, 2013). Resembling dose-dependent and higher anti-diabetic results were revealed by the extract of *P. guajava* leaves *in vitro (*Manikandan et al., 2016).

Phytochemical assessment of plant extract and its different fractions revealed the presence of various phyto constituents that demonstrated specific therapeutic effects. Further study of plant extract is dependent on the presence of these compounds. Phenolic compounds, like flavonoids and terpenoids are the major antioxidants that exercise scavenging of free radicals and hence neutralization of reactive oxygen species (Hossain et al., 2011). Flavonoids use several mechanisms such as scavenging of free radicals and the inhibitions of enzymes involved in the production of free radicals and thus prevent the development of oxidative stress (Benavente-García et al., 1997). Usually the phytochemicals are extracted in different solvents where each one has specific influence on the extraction of said constituents depending on the nature of solvent and its interface with desired compounds. Therefore, the extracted compounds and their dry weight will be variable extracted in various solvents (Li et al. 2006; Pereira et al., 2008; Zhang et al., 2009). The cytotoxic findings imply that the assessed extracts are probable potential sources of antimicrobial and anti-cancer compounds. The differences observed in scavenging behavior of test sample and its fractions against various free radicals system may depict that different oxidation reduction mechanisms are involved in the different assays. The higher free radical scavenging activity of aerial parts of young *S. halepense* aqueous fraction and methanolic extract to forage more free radicals (ABTS·+) might be owing to higher molecular weight phenolics (tannins) and their convenience depends on molecular weight, the number of aromatic rings and nature of hydroxyl group's exchange than the explicit functional groups. Various antioxidant compounds such as β-carotene, tocopherol, selenium, vitamin C or phenolic compounds perform in different ways with divers mechanisms (Ismail et al., 2004). Serious health problems like obesity and diabetic results from metabolic disorder of carbohydrates. Problems in insulin secretion or deterioration in its function decreases the breakdown of disaccharides and polysaccharides and eventually cause the onset of diabetes mellitus (Funke and Melzig, 2006). Inhibition of alpha amylase, an enzyme responsible for digestion of carbohydrate slows down the process of carbohydrate digestion and hence reduce the uptake of glucose by intestines (Cheng and Fantus, 2005). Searching of natural inhibitor of amylase can be conducted by *in vitro* amylase inhibition assay and hence ultimate estimation of anti-diabetic characteristics of plant extracts.

Conclusion

The aerial parts of young *S. halepense* contain considerable cytotoxic, antioxidant and anti-diabetic properties. The chloroform fraction exhibited maximum phenolic contents but minimum antioxidant activities.

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Conflict of Interest

Authors declare no conflict of interest

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