PHYTOCHEMICAL ASSESSMENT, TOTAL PHENOLIC CONTENT, CYTOTOXIC, ANTIOXIDANT AND ANTIDIABETIC ACTIVITIES OF *HYOSCYAMUS INSANUS*

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ABSTRACT

Hyoscyamus insanus (family Solanaceae) was traditionally used for treatment of asthma, relieving pain and increasing female body weight. The present project was intended to assess the phytochemicals, total phenolic content, cytotoxic, antioxidant and antidiabetic activities of *Hyoscyamus insanus* leaves methanolic extract and its special fractions. After extraction, the methanolic extract was sequentially fractionated with *n*-hexane, chloroform and water followed by phytochemicals analysis of each sample. Brine shrimp lethality bioassay, Folin-Ciocalteu phenol reagent, DPPH, $ABTS^{\bullet+}$, H_2O_2 and alpha-amylase inhibition assays were employed for the assessment of cytotoxic property, total phenolic content, antioxidant and anti-diabetic potency of methanolic extract and its fractions. Amino acids and protein, carbohydrates, flavonoids and saponins were found in the methanolic extract and aqueous fraction while chloroform fraction exhibited carbohydrates, flavonoids and saponins. The highest cytotoxic activity (80.6±1.2%) was exhibited by methanolic extract while maximum total phenolic content (21.93±1.17 mg GAE/g) were found in the chloroform fraction. The employed antioxidant assays expressed different results i.e. methanolic extract demonstrated highest; 83.99%, 74.19% and 51.15% free radical scavenging characteristics in ABTS, DPPH and H_2O_2 assays respectively. The methanolic extract showed significant (53.44%) antidiabetic properties. The correlation of total phenolic content with percentage antioxidant and anti-diabetic capabilities of methanolic extract and its special fractions was observed to be non-significant ($P > 0.05$). It is concluded that the *Hyoscyamus insanus* is a potential source of phenolic, cytotoxic, antioxidant and antidiabetic compounds.

Keywords: Antidiabetic, antioxidant, cytotoxic, *Hyoscyamus insanus.*

INTRODUCTION

Plants are the principal source of medicinal compounds and roundabout in the world, 25% prescribed drugs are plant based (Rates 2001). In developing countries, the limited availability of health facilities has increased the importance of folk medicines, mostly based on plants, both urban and rural areas. This is because of its easy availability, inexpensiveness, safety and effectiveness (Katewa et al. 2004).

Plants contain many biologically active compounds, it is mandatory to evaluate the presence of the desired compound through various standard assays. The commercial utilization and expansion of plant based therapeutic compounds to improve health and food preservation are of great interest in the current era (Rice-Evans et al. 1996). Plants possess many biologically active compounds including various antioxidants related to potential health benefits (Arnous et al. 2001).

Antioxidants are the substances that defuse free radicals produced during metabolism and are involved in damaging various components of cells, tissues, and organs. Free radicals possess one or more unpaired electrons that on reaction with other molecules results in pathological conditions (Pal et al. 2009). The antioxidant may be natural or synthetic. Man-made antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are often utilized in foods but they have side effects and are carcinogenic (Branen 1975). The natural sources of antioxidants are phytochemicals primarily phenolics that may present in almost all products and parts of a plant such as leaves, stems, fruits, roots, vegetables, nuts, seeds, and bark. The potential antioxidant characteristics of plant phenols and polyphenols perform significant functions in the avoidance of different pathological conditions such as cardiovascular, cancer, and neurodegenerative diseases supposed to be related with oxidative stress (Losso et al. 2007).

Diabetes mellitus usually was known as diabetics and it suffers 2.8% of the population throughout the world (Mukesh & Namita 2013). It is a cluster of metabolic disorders distinguished by improper hyperglycemia which is caused by a comparative or complete shortage of insulin or by a resistance to the action of insulin at the cellular level. Increased thirst, hunger, and frequent urination are the symptoms of hyperglycemia i.e. diabetics (Agarwal et al. 2009; Fiore et al. 2005) results in chronic hyperglycemia and may lead to several complications like coma, diabetic ketoacidosis, or damages to kidneys, eyes, heart, nerves and blood vessels (Malviya et al. 2010) or death if it is not diagnosed and treated well in time (Shibata 1994). A number of synthetic drugs are present to reduce hyperglycemia but their use is limited by their side effects. Traditionally used Plant materials for the treatment of diabetes are considered one of the good natural sources for the development of a new drug against the mentioned disease (Umashanker & Shruti 2011).

One of the therapeutic advances which include lowering hyperglycemia intends at inhibiting the enzyme alpha-amylase. The hyperglycemia in type 2 diabetes mellitus can be restricted up to a greater extent by employing the inhibitors of alpha-amylase and alpha-glucosidase (Nair et al. 2013). The inhibition of alpha-amylase results in low availability of glucose to be taken by the intestine and in turn low blood glucose level and hence decreased diabetes. Thus it becomes a matter of high importance to search for new drug/compound to control diabetic problems which are still a great challenge to the medical community (Shafi & Tabassum 2013).

Hyoscyamus belongs to Solanaceae, which is very important from a scientific, economic and ethnic point of view. It has four species in Pakistan, eight species in former USSR and six species in Turkey (Hajrasouliha et al. 2014; Khan 2008) while in the Iranica flora it has two subgenera and 18 species (Hajrasouliha et al. 2014) where a resident species of Pakistan, *Hyoscyamus insanus* (subgenus Dendrotrichon) with chromosome number 2n = 28 is one of its key species (Sheidai et al. 1999).

Traditionally, *Hyoscyamus insanus* has been utilized for different purposes, such as feed, firewood, and health improvement (Hamayun et al. 2006). Its seeds were utilized by women to make heavier their bodies, while its smoke was inhaled for the healing of asthma. This plant has an analgesic property. The main alkaloids, Hyoscyamine (atropine), hyoscine, and apoatropine were found in it (Tafaghodi & Rahimizadeh 2003). The existence of some enzymes, peroxidase, superoxide dismutase, and malate dehydrogenase in it has been documented (Sharifi et al. 2006).

The current project was designed to evaluate the phytochemicals, total phenolic content, cytotoxic, antioxidant and antidiabetic capacities of methanolic extract of *Hyoscyamus insanus* leaves and its different fractions.

MATERIAL AND METHODS

Chemical reagents for biological activities

The chemical reagents, DPPH (2,2-diphenyl-1-picrylhydrazyl), $ABTS^{\bullet+}$ (2, 2-Azinobis (3-ethyl-benzothiazoline)-6- sulfonic acid disodium salt), H_2O_2 (hydrogen peroxide), gallic acid, ascorbic acid, sodium phosphate monobasic, Folin–Ciocalteu reagents, sodium phosphate dibasic, potassium persulfate and all other chemicals were of analytical grade. Methanolic extract of *Hyoscyamus insanus* leaves and its fractions extracted in *n*-hexane, chloroform and aqueous.

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Plant material

In March 2017, *Hyoscyamus insanus* fresh leaves were collected from Tehsil Mir Ali, North Waziristan Agency (NWA), Pakistan. Its taxonomic status was confirmed by Prof. Abdur Rehman, Govt. Post Graduate College Bannu, Khyber Pakhtunkhwa (KPK) Pakistan. The voucher specimen G-331 was deposited to the department of Biotechnology, University of Science and Technology Bannu.

Preparation of methanolic extract

The leaves of *Hyoscyamus insanus* were collected and after collection cleaned with tap water, dried in the shade for 15 days, pulverized into fine powder using a pestle and mortar, immersed the powder in methanol (70%) and kept for 72 hours with frequent stirring. The solution was filtered, dried the filtrate at 25 ºC and the resinous extract (26.37 g) was put into falcon tubes for future use.

Preparation of fractions

The prepared methanolic extract (20 g) was serially extracted with 300 ml *n*-hexane, chloroform and aqueous each using separating funnel by the process of fractionation. The filtrates of *n*-hexane (2.89 g), chloroform (5.93 g) and aqueous (9.13 g) fractions were dried completely at 25 ºC. Finally, the resultant extract of each fraction was stored for further use.

Phytochemicals screening

Phytochemicals analysis of methanolic extract of *Hyoscyamus insanus* leaves and its various fractions were carried out by adopting standard assays to explore the availability of saponins, flavonoids, alkaloids, cardiac glycosides, carbohydrates, tannins and protein and amino acids (Rice-Evans et al. 1996; Trease & Evans 1989).

Cytotoxic assay

Cytotoxic properties of methanolic extract of leaves of *Hyoscyamus insanus* and its special fractions were found during brine shrimp lethality bioassay (Meyer et al. 1982). 1mg shrimp eggs were cultured in non-natural seawater (4% w/v) in dark cabin. Followed by hatching, the shrimps came into the illuminated chamber via a central porous wall. Working solutions of all samples with different concentrations were prepared in methanol i.e. 100 µg/ ml, 250 µg/ ml, 500 µg/ ml, 1000 µg/ ml. The said concentrations of each sample were practiced in triplicate in experimental groups. For the complete evaporation of methanol, the test tubes were kept at 25 ºC, then added 10 shrimps into every test tube and kept for 24 hours at 25 ºC. Thereafter, counted the shrimps in each test tube, % mortality was calculated according to Abbot's formula and compared the results (mortality) of experimental with control.

% Mortality = (Experimental-control/control) × 100

Total phenolic content

Folin–Ciocalteu reagents was practiced to find out the total phenolic content in plant extract and its special fractions using the method of Singelton and Rossi (Singleton & Rossi 1965). The working solution of Folin–Ciocalteu reagents (10X diluted in distilled water) was prepared. 250µl sample solution (1–5mg/ml) was added to 2.5ml of working solution and incubated at 25 ºC for 5 minutes. Then after mixing 2.5ml solution of Na_2CO_3 (60mg/ml), the reaction mixture was incubated for 2 hours at 25 ºC. Gallic acid solution of the same concentration was set and exercised as a standard. At 725nm the absorbance was measured and articulated the results as a gallic acid equivalent.

Antioxidant assays

DPPH (1, 1-diphenyle -2- picryle hydrazyl) method

The free radical foraging properties of plant extracts was measured by opting (Gyamfi et al. 1999) procedure where the DPPH is used to produce free radicals. Prepared working solution of samples having different concentration i.e. 62.5µg/ml, 125µg/ml, 250µg/ml, 500µg/ml, 1000µg/ml, 1500µg/ml and 2000µg/ml. DPPH (3 mg) was dissolved in distilled water (100 ml) , incubated for 30 minutes at 25°C in the dark and afterward measured its absorbance at 517nm spectrophotometrically. 200µl from each working solution was added with 1800µl of DPPH while in case of standard ascorbic acid solution was mixed with DPPH instead of sample solution. The capability of samples to forage free radicals was calculated by the following equation.

DPPH free radicals scavenging capacity (%) = (Control-Experimental/Control) ×100

Where control = absorbance of DPPH solution

Experimental = absorbance of DPPH solution containing the sample.

ABTS radical cation assay

ABTS free radical scavenging assay was used to determine free radical foraging ability of plant extract by following the standard procedure (Re et al. 1999). Potassium persulfate solution (2.45 mM) and ABTS•+ solution (7 mM) were assorted and incubated overnight in the dark as it is sensitive to light. The relevant solvent was prepared by diluting (50%) the stock solution and then adjusted its absorbance of about 0.900 (±0.02) at 745 nm at 30 °C. 300 µl (62.5 µg/ml -2000 µg/ml in relevant solvent) extract solution was put together with the diluted ABTS solution (3ml) and measured the absorbance. In standard, ascorbic acid solution was opted as a substitute for a sample solution. The percent scavenging properties of plant extracts and ascorbic acid were calculated by using the formula;

Scavenging effect (%) = [(CA (ABTS) - SA) / (CA)] × 100.

Where CA= control absorbance

SA= sample absorbance

Hydrogen peroxide scavenging assay

H_2O_2 foraging assay was adopted to detect the hydrogen peroxide scavenging capabilities of plant extracts (Wettasinghe and Shahidi 2000). 300µl of sample solution (125–2000µg/ml), 1.4 ml phosphate buffer (100 mM, pH 7.4) and 300µl of a 43mM H_2O_2 solution were mixed and incubated for 40 min at 25 ºC. The optical density was measured at 230 nm against a blank solution having H_2O as a substitute of sample solution and calculated the percentage scavenging characteristics of applied samples as follows:

Scavenging effect (%) = [(CA (H_2O_2) - SA) / (CA)] × 100.

Where CA= control absorbance

SA= sample absorbance

All tests were carried out in triplicate and the results were presented as means ± SD.

Alpha-amylase inhibition

The inhibitory effect of methanolic extract and its specific fractions on alpha-amylase was studied using the Worthington Enzyme Manual protocol (KWON et al. 2007). A sample solution (300 µL) was mixed with 500 µL of starch solution (1%) in sodium phosphate buffer (20mM, pH 6.9 with 6 mM NaCl), and then 500 µL of sodium phosphate buffer (20mM, pH 6.9 with 6mM NaCl) having 0.5 mg/ml of alpha-amylase. In control, instead of the sample solution, 300 µL distilled water was added with the reaction mixture. Glucophage (commercially existing medicine) was set as a positive control at the same concentration as the sample. The reaction mixture was agitated gently and initially incubated at 25ºC for 10 minutes.

1.0 ml color reagent, dinitrosalicylic (DNS) acid was added to each test tube to stop the reaction and subsequently incubated for 5 mints in boiling water, cooled to 25 ºC and then diluted by pouring distilled water (3 ml) to each tube. The optical density at 540 nm was measured spectrophotometrically and the percent inhibition of alpha-amylase was calculated as follows.

Alpha-amylase inhibition (%) = [(CA (Blank)-SA) / (CA)] × 100

Where CA= control absorbance

SA= sample absorbance

Statistical analysis

Statistical analysis was conducted by GraphPad Prism software. The results of triplicate experiments were recorded as a mean ± SD (standard deviation). Moreover, the results were analyzed to determine the Pearson correlation coefficient between total phenolic content and various antioxidant and anti-diabetic assays. The $p < 0.05$ was deliberated to be significant statistically.

RESULTS AND DISCUSSION

For the sustainable health, it is very much necessary to explore the natural therapeutic substances which are supposed to be safer than synthetic ones. These therapeutic substances are extracted from plants materials in special sorts of solvents depending upon the nature of the desired substances and the interaction of desired components with solvent also significantly manipulates the quantity of extract. Hence, the quantity of extracted medicinal compounds and their dry weight in various solvents will be different (Pereira et al. 2008; Zhang et al. 2009).

Phytochemical analysis

Phytochemical evaluation of the crude extract is the initial requirement for further study. The crude methanolic extract of *Hyoscyamus insanus* leaves and its special fractions were assessed and determined the existence of amino acids and protein, carbohydrates, glycosides, alkaloids, saponins, tannins and flavonoids in the methanolic extract and aqueous fraction where they were absent in *n*-hexane fraction except saponins, tannins and alkaloids. Carbohydrates, flavonoids, tannins and saponins were also found in chloroform fraction (Table 1). Similar results were observed in various extracts of *T. chebula* leaves and fruits (Kumar 2006). It is mandatory to identify the phytochemical compounds within the extract as it provides the base whether or not to continue the study of plant extract.

Table 1. Phytochemical screening of leaves of *Hyoscyamus insanus* methanolic extract and its chloroform, aqueous and *n*-hexane fractions.

	Phytochemicals and tests		Methanolic extract and its fractions			
S#	Phytochemical	Tests	Aqueous	Methanol	Chloroform	*n*-Hexane
1	Saponins	Foam test	+	+	+	+
2	Flavonoids	Alkaline reagent test	+	+	+	-
3	Alkaloids	Wagner's reagent test	+	+	+	+
4	Glycosides	Fehling test	-	+	-	-
5	Carbohydrates	Milisch's test	+	+	+	-
6	Tannins	Ferric chloride test	+	+	+	+
7	Protein and amino acids	Biuret test	+	+	-	-

Cytotoxic activity

Cancer is a cluster of life threatening ailments in the current era, and the search for effective new anti-cancer medicinal compounds derived from plants is one of the most promising branches of research on natural products. The methanolic extract, the aqueous, chloroform and *n*-hexane fractions showed the death of brine shrimps larvae with up to 80.6 ± 1.2%, 60.2 ± 1.3%, 30.5 ± 1.5% and 30.3 ± 1.2% at a concentration of 1000 µg / ml during bioassay. This suggests that methanolic extract and its aqueous fraction are a possible potential source of cytotoxic compounds.

The previous study of cytotoxic potential of Coscinium blumeanum, Fibraurea tinctoria and Arcangelisia Flava has been shown similar results (Keawpradub et al. 2005). The results are shown in Table 2. Alkaloids have analgesic and cytotoxic/ antitumor characteristics and, therefore, are depolymerized and prevent the formation of protein microtubules in the mitotic spindle during cell division. This practice helps to prevent the partition or splitting up of tumor cells and, as a result, leads to a decrease in cancer levels (Ogunwenmo et al. 2007; Ngoci et al. 2011).

Table 2. Percentage lethality of brine shrimps caused by methanolic extract and its different fractions of *Hyoscyamus insanus* leaves.

Concentration (µg/ml)	Methanolic extract and its fractions % age lethality				
	Methanolic extract	Aqueous	Chloroform	*n*-hexane	Control
100	40.5±1.5	30.3±1.7	10.3±1.7	10.3±1.7	00±00
250	50.4±1.6	30.7±1.3	10.9±1.1	10.4±1.6	00±00
500	70.6±1.4	50.4±1.6	20.1±1.9	30.2±1.8	10.2±1.8
1000	80.6±1.2	60.2±1.3	30.5±1.5	30.3±1.2	00±00

Total phenolic content

Medicinal plants possess a wider range of phenol concentrations ranging from 2.34-152.32 mg GAE/g (Tupe et al. 2013). In the current study, the chloroform fraction contained the maximum quantity of total phenolic content (21.93±1.43 mg GAE/g) whilst the lowest (5.56±1.28mg GAE/g) was found in *n*-hexane fraction. The gallic acid solutions (1 to 5 mg/ml) were used to establish the calibration curve and represented the results as gallic acid equivalents (GAE). The results are presented in Table 3. A number of phenolic compounds in the applied extract react none specifically with phosphotungstic and phosphomolybdic acids present in Folin-Ciocalteu phenol reagent via complex oxidation-reduction reactions (Escarpa & González 2001; Singleton et al. 1999). Different phenolic compounds react in a different way to the Folin–Ciocalteu based on the number of phenolic groups in phenolic compounds (Singleton et al. 1999). Thus it may explain the results in *Hyoscyamus insanus* leaves where the highest quantity of phenolic contents was found in chloroform fraction (21.93±1.17 mg GAE/g) whereas the lowest (5.56±1.28 mg GAE/g) in *n*-hexane fraction.

Table 3. Phenolic content (mg/g gallic acid equivalent) of methanolic extract and its various fractions of *Hyoscyamus insanus* leaves.

Extracts	Methanolic extract	Chloroform fraction	Aqueous fraction	*n*-Hexane fraction
Total phenolic contents	17.36±1.13	21.93±1.43	13.2±1.27	5.67±1.28

Antioxidant activity

Owing to the increasing tendency of oxidative stress-related diseases, many in *vitro* and *in vivo* methods have been adopted by researchers to investigate antioxidant activities of plant-based naturally occurring antioxidants. The literature study revealed that 19 different in vitro techniques are used for antioxidant assessment (Wong et al. 2006). To achieve more confirmed results, it is mandatory to implement more than one method (Kazazic et al. 2016) and hence in the present study, we opted for the most frequently used three assays (DPPH, ABTS and H_2O_2) for the estimation of antioxidant aptitude of plant extract and its special fractions. In the mentioned assays, ascorbic acid was exercised as a standard and compared the antioxidant properties of standard and samples.

DPPH assay

In DPPH assay, the ascorbic acid, methanolic extract, aqueous fraction, *n*-hexane and chloroform fractions exhibited 86%, 55.52%, 40.75%, 38.22% and 22.92% antioxidant activities respectively at the concentration of 2mg/ml (Figure 1). The antioxidant properties of the *Hyoscyamus insanus* leave methanolic extract and its special fractions were found to be concentration dependant. Comparable results were found during the previous study of antioxidant characteristics of *Barleria longiflora* leaves extracts (Kalpana et al. 2016).

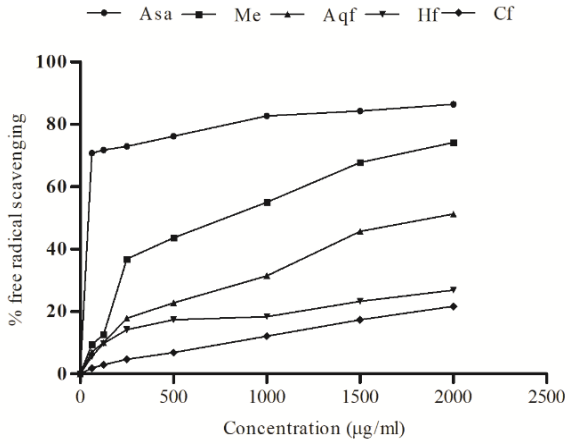

Figure 1. DPPH free radical scavenging capability of *Hyoscyamus insanus* leaves methanolic extract and its fractions. Cf: chloroform fraction, Me: methanolic extract, Aqf: aqueous fraction, Hf: *n*-hexane fraction and Asa: ascorbic acid.

ABTS free radical scavenging assay

It is very attractive as its analysis is easy and appropriate for the estimation of both lipophilic and hydrophilic antioxidants. In the said assay, crud methanolic extract and it's aqueous, chloroform and *n*-hexane fractions expressed 97.82%, 59.35%, 51.67% and 24.35% antioxidant activities respectively. The results are articulated in figure 2. An indirect association between antioxidant activities and total phenolic content was observed, the *n*-hexane fraction exhibiting maximum phenolic contents (26.66 mg GAE/g) expressed minimum 24.35% antioxidant activities while the aqueous fraction with lowest phenolic contents (6.33 mg GAE/g) have moderate antioxidant activities (51.67%). A similar relationship between antioxidant activities and phenolic contents were observed during the study of and *Acalypha indica* (Miliauskas et al. 2004).

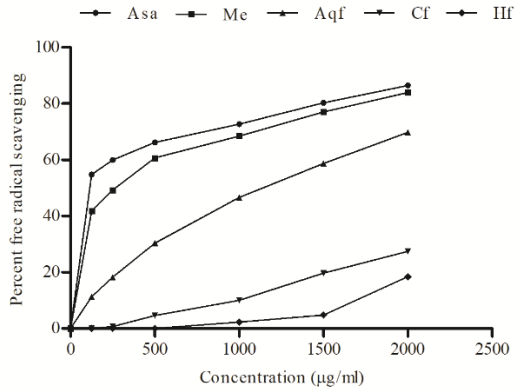

Figure 2. ABTS free radical scavenging capability of *Hyoscyamus insanus* leaves methanolic extract and its fractions. Cf: chloroform fraction, Me: methanolic extract, Aqf: aqueous fraction, Hf: *n*-hexane fraction and Asa: ascorbic acid.

Hydrogen peroxide assay

In this assay, the applied extracts showed forage of H_2O_2 in a concentration-dependent way. 2000 µg/ml of ascorbic acid, methanol extract and its aqueous fraction expressed 61.04%, 46.16%, and 46.11% free radical scavenging respectively while chloroform and *n*-hexane fractions did not show antioxidant potential (Figure 3). In the presence of transition metal ions, H_2O_2 converts to OH radical and singlet oxygen which is toxic to cells and food systems (Karadag et al. 2009).

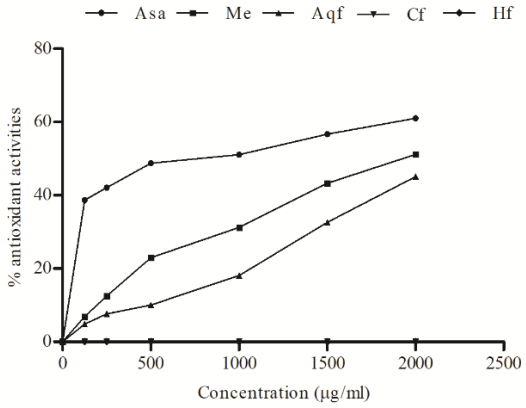

Figure 3. H_2O_2free radical scavenging capability of *Hyoscyamus insanus* leaves methanolic extract and its fractions. Cf: chloroform fraction, Me: methanolic extract, Aqf: aqueous fraction, Hf: *n*-hexane fraction and Asa: ascorbic acid.

Plants exhibit a number of metabolites with greater diversity in their structures; the mentioned diversity oscillates the antioxidant profiles greatly from plant to plant and their response to various assays. The employed three methods expressed different results i.e. methanolic extract demonstrated the highest 83.99%, 74.19% and 51.15% free radical scavenging capacities in ABTS, DPPH and H2O2 assays respectively. Differences in the results suggest the existence of various antioxidants in the extracts used and the participation of various mechanisms of antioxidant reactions adopted in various analyzes. The observed higher free radicals neutralization characteristics of *Hyoscyamus insanus* leaves methanolic to remove further free radicals (ABTS • +) can be associated with higher molecular mass phenolic compounds (tannins), and their efficacy is more reliant on the number of aromatic rings, molecular weight and OH substitution than particular functional groups. Hangerman and colleagues reported congruent observations working on high molecular weight plant polyphenolics (tannins) as biological antioxidants (Hagerman et al. 1998). The chloroform fraction containing the highest total phenol (TPC) has a lower antioxidant activity.

Similar results were obtained for petai and Curry trees, which have elevated levels of TPC but lesser antioxidant activity (Wong et al. 2006). Similarly, an indirect relationship between phenolic contents and antioxidant activity was observed by scientists (Ismail et al. 2004) during their studies of phenolic compounds in plant extracts. Different antioxidant substances like b-carotene, vitamin C, tocopherol, selenium or phenolic compounds have different antioxidant activities (Ismail et al. 2004). Flavonoids are also phenolic in nature and fulfill their role as anti-oxidants and, therefore reduce the oxidative stress (Ngoci et al. 2011). They also act as; 'nature's biological modifiers' as anti-inflammatory, anti-allergens and provocative second-phase enzymes that remove carcinogens and mutagens (Ogunwenmo et al. 2007; Ngoci et al. 2011). In addition, the presence of superoxide dismutase and peroxidase in *Hyoscyamus insanus* (Sharifi et al. 2006) can also contribute to its effectiveness as an antioxidant.

Antidiabetic activity

Methanolic extract of *Hyoscyamus insanus* and its various fractions were scrutinized by alpha-amylase inhibition assay and estimated their inhibitory potencies against alpha-amylase. The mentioned characteristics of Glucophage (standard), methanolic extract and aqueous fraction were found as 49.48%, 48.92%, and 15.19% respectively. Figure 4 represents the results. Diabetes mellitus results from a metabolic disorder of carbohydrates where the alteration in the secretion of insulin or deterioration in its function causes a decline in the catabolism of disaccharides and polysaccharides (Funke & Melzig 2006). The glucose uptake by intestines can be lessened by increasing the time of carbohydrate digestion by inhibiting alpha-amylase, carbohydrate digestive enzyme (Cheng & Fantus 2005). Thus, in *vitro* alpha-amylase inhibition assay can be opted to investigate anti-diabetic activities of plant extract.

The anti-diabetic potential of *Hyoscyamus insanus* leaves methanolic extract and its derived special fractions were uncovered via alpha-amylase inhibition assay. The cited properties of methanolic extract, its water fraction and Glucophage were found (15.48%), (53.44%) and (58.92%) respectively. The methanolic extract showed so far comparable anti-diabetic activities with the standard. It proposes that the plant extract is a valuable source of important antidiabetic compounds and needs its isolation and purification. The percentage inhibition of alpha-amylase was found to be dose-dependent. Alike results were documented in the *in vitro* study of *P. guajava* leaves (Manikandan et al. 2016), Banana peel (Vasu et al. 2017).

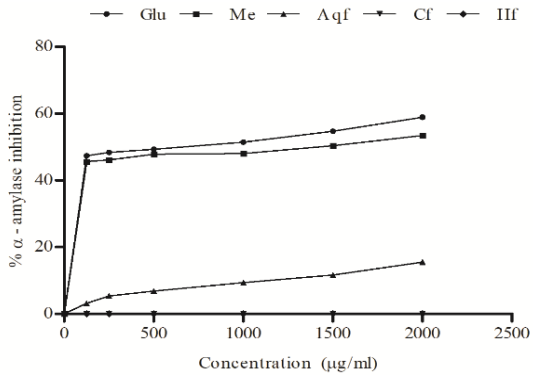

Figure 4. Anti-diabetic activity of *Hyoscyamus insanus* leaves methanolic extract and its fractions, Cf: chloroform fraction, Aqf: aqueous fraction, Hf: *n*-hexane fraction, Me: methanolic extract and Glu: glucophage *(Cf: chloroform fraction and Hf: *n*-hexane fraction did not show anti-diabetic activities).

The correlation among the percentage antioxidant and anti-diabetic behavior of crude methanolic extract and its special fractions and total phenolic content were analyzed (Pearson, two-tailed P-value) and uncovered the fact that it is non-significant in all cases (P> 0.05). Table 4 represents the results. In previous studies, comparable results were reported (Sahreen et al. 2017).

Table 4. *Hyoscyamus insanus* leaves methanolic extract and its different soluble fractions were used in the correlation, P value (two tailed) and ns: Non significant.

S. No	Assays	Correlation R^2 Phenolics	Significance
1	% DPPH radical scavenging ability	0.0158	ns
2	$ABTS^{\bullet+}$ % scavenging ability	0.02105	ns
3	% H_2O_2 scavenging	0.02105	ns
4	% Alpha-amylase inhibition	0.06189	ns

CONCLUSION

The crude methanolic extract indicated higher cytotoxic, antioxidant and antidiabetic as compared to its aqueous, chloroform and *n*-hexane fractions. But the chloroform fraction expressed the highest phenolic contents (21.93±1.17 mg GAE/g). This might be due to the greater diversity within the structures of plants metabolites and their solubility in different solvents. With the current findings, it is difficult to conclude the cytotoxic, antioxidant and antidiabetic activities of an individual compound as all of them are not known. However, it is evident that that the compounds extracted in 70% methanol (higher to intermediate polarity) revealed significant cytotoxic, antioxidant and antidiabetic activities. Moreover, purification and characterizations of compounds from methanolic extract will sharpen its pharmacological significance.

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