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Phytochemical Constituents and *in vitro* Antioxidant and Cytotoxic Activities of Different Extracts from the Aerial Parts of *Heliotrophium hirsutissimum* GRAUER

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Abstract

The aim of this study is to detect the active phytochemicals in six different extracts namaly (diethyl ether, petroleum ether, ethyl acetate, methanol and, water (infusion and decoction) of the aerial parts of the *Heliotropium hirsutissimum* and to identify *in vitro* antioxidant and cytotoxic activities of these extracts. The phytochemicals present in the plant were assessed by standard methods. Six different extracts of *H. hirsutissimum* arial parts were tested for antioxidant activity using DPPH radical scavenging, H_2O_2 scavenging and metal chelating assays, cytotoxic activity using Brine Shrimp Lethality Assay. Phytochemicals found in the plant showed differences according to the extract type. As a result of the phytochemical screening of the aerial parts of the *H. hirsutissimum*, alkaloids, phenol, saponin, tannins and anthraquinones were detected. Flavonoid was not found in any of the extracts. The highest radical scavenging activity was found in the infusion extract, while the other extracts had low-free radical scavenging activity and H_2O_2 scavenging and metal chelating activities. It was determined that the different extracts obtained from *H. hirsutissimum* have no cytotoxic effects on *Artemia salina* nauplii compared with umbelliferone, which is a positive control at the applied concentration range (100 µg / mL-1000 µg / mL). The results demonstrated that the antioxidant properties of the *H. hirsutissimum* aerial-part extracts showed differentiation in the extract type and that none of the extracts have cytotoxic effects.

KeyWords: *Artemia salina*, Cytotoxic effect, DPPH scavenging activity, *Heliotropium hirsutissimum*, Phytochemical screening

1. Introductıon

The importance of medicinal plants and traditional health systems in solving health care problems around the world has gained much attention. Historically, all medicinal preparations were derived from plants, whether in the simple form of raw plant materials, or in the refined form of crude extracts, mixtures, etc. Recent estimates suggest that several thousands of plants have been known with medicinal applications in various crops (Farnsworth and Soejarto, 1991).

Natural products are the substances which originate from plants, animals, microbial and marine sources. Because of their vast availability in nature, they also play a significant role in the discovery of new therapeutic agents leading to the identification of bioactive molecules which allow the development of new pharmaceutical agents. İn addition they are used as a tool involved in the clarification of complex cellular and molecular mechanisms of actions of many biological and pathological processes (Ghori *et al.*, 2016). Some plants have been subjected to the isolation of the active ingredients (chemical compounds) and their subsequent modification. These chemical compounds are able to perform physiological action in the body and are known as phytochemicals.–These have important health benefits, and can also be used to treat infections especially of microbial origin (Ranjaragan and Sathiyavani, 2014). A lot of plants and natural components have demonstrated antibacterial and wound-healing properties as well as anticancer activity, which confirms the potential for novel agents to be identified from uncharacterized natural plant resources (Holetz *et al.*, 2002; Aridogan *et al.*, 2002; Kaileh *et al.*, 2007; Gonçalvez *et al.*, 2008; Martins *et al.*, 2009) .

The family Boraginaceae is comprised of one-hundred genera and about two thousand species. The plants of this family are widely distributed in temperate, especially Mediterranean and tropical regions. *Heliotropium* is an important medicinal plant and is a large genus of the family Boraginaceae which consists of about 250-300 species around the whole World. Some of the taxa of this genus are *H. Bacciferum* Forssk., *H. europium* L., *H. baluchistanicum* K., *H. gillianum* R., *H. biannulatum* B., *H. Ovalifolium* Forssk., *H. Strigosum* Willd., *H. Eichwaldi* Steud., *H. indicum* L., *H.glutinosum* Phil., *H. sclerocarpum* Phil., *H. Sinuatum* Miers., *H. Subulatum* Hochst., *H. foertherianum* D. and *H. ovalifolium* Forssk, and *H, hirsutissimum Grauer* (Ghori et al., 2016). Plants of the genus *Heliotropium* display a wide range of pharmacological activities. Different biological activities of extracts and their bioactive constituents provide a basis

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for a better understanding of the underlying mechanisms involved (Singh *et al*., 2013). *Heliotropium* species have been used for their chloretic, antipyretic and cicatrizing activities (Baytop, 1999) and have been traditionally used for the treatment of gout, various inflammations, rheumatism, poisonous bites and skin diseases as a healing agent in various countries around the world. *Heliotropium* species are highly valued for antimicrobial and antioxidant activities due to the isolation of secondary metabolites like alkaloids, flavonoids and terpenoids. Because of these properties, *Heliotropium* can be used for the treatment of various bacterial and fungal infections in modern medicine as confirmed by folk medicinal studies. In addition, the anti-inflammatory, antiviral, antitumor, antidiabetic and antihyperlipidemic as well as the gastroprotective activities also enhance the medicinal value of *Heliotropium* in the future (Ghori *et al.*, 2016). Due to the broad range importance of the ethno-pharmacological flora, this study was arranged to collect ethno-medicinal knowledge about *H. hirsutissimum.*

They are known to contain pyrrolizidin alkaloids (heliotrine, lasiocarpine, europine, supinine) (Güner, 1986). The Pyrrolizidine alkaloids that are abundantly found in *Heliotropium* are responsible for its poisonous nature such as hepatotoxicity, mutagenicity and hepatocarcinogenicity. The toxic nature of pyrrolizidine alkaloids can be attributed to different reasons. The plants which are the main source of these alkaloids are consumed in food, and are sometimes used in the form of herbal medicines (Ghori *et al.*, 2016). However, no toxicological or pharmacological studies have been carried out in detail so far on this plant, with the exception of a recent study which explored its antioxidant properties.

Brine Shrimp (*Artemia salina* L.) bioassay is considered as a preliminary screening for the presence of antitumor compounds and is used to determine the plant extract toxicity (Meyer *et al*., 1982). Using Brine Shrimp larvae, pharmacognosists and natural-product chemists were able to detect and isolate the plant constituents and its active compounds with a variety of pharmaceutical activities (Alali *et al*., 2008). *Brine shrimp* is considered a rapid, inexpensive, and in-house bioassay for screening and fractionation monitoring of physiologically active plant extracts (Jayasuriya *et al*., 1989). *Brine shrimp* is utilized previously in various bioassay systems (Meyer *et al*., 1982; Ratnayake *et al*., 1992). According to Meyer *et al*. (1982) several extracts derived from naturall products which had LC50 ≤ 1000 µg/ml using *Brine shrimp* bioassay were known to contain physiological active principles (Meyer *et al*., 1982).

The present study is aimed at evaluating the phytochemical constituents, antioxidant potential and free radical-scavenging capacity of different extracts of the *Heliotropium hirsutissimum* Grauer (Boraginaceae) and the cytotoxic activities of these extracts on developing brine shrimp nauplii. The activities have been selected because of their great medicinal relevance. Over the last years, interest in the antioxidant activity of plant extracts has become larger and very important due to the fact that free radicals (e.g. reactive oxygen species (ROS) can be responsible for various diseases including heart diseases, strokes, arteriosclerosis and cancer, as well as the aging process.

2. Materials and Methods

2.1. Plant Collection and Identification

The fresh aerial parts of *Heliotropium hirsutissimum* were collected from the surrounding areas of Adnan Menderes University Central Campus, Aydın, Turkey, during Agust of 2012. The plant was recognized by its local name and was then clarified by Dr. Özkan Eren of Adnan Menderes University, Art and Science Faculty, Department of Biology, Aydın, Turkey. A voucher specimen of the plant has been deposited (AYDN-2266) in the herbarium for further reference. Fresh healthy flowers, leaves and stems, thoroughly washed (2-3 times) with water and were dried in the shade room at the ambient temperature for two to three weeks. The dried plants were finely ground with a mixer and were stored in the dark at room temperature in closed containers until further use.

2.2. Extraction of the Plant Material

Dried ground whole plants of *H. hirsutissimum* were extracted with a solvent series of increasing polarity (diethyl ether, petroleum ether, ethyl acetate, methanol, and water (infusion and decoction) extracts). For extractions, 60 g of plant materials were used. 600 mL of solvent was added to 60 g of the plant material. After completing the first soxhlet extraction with diethyl ether (at 40°C for approximately twelve hours, until the solvent became colorless) and filtration, the plant material was dried and subjected to the second extraction with petroleum ether, the third extraction with ethyl acetate, and the fourth extraction with methanol (Goffin *et al*., 2003; Lee *et al*., 2003; Miliauskas *et al*., 2004; Avcı *et al*., 2006). The extracts were evaporated and yielded 1.162, 0.954, 1.678 and 4.920 g dried mass, respectively. After the methanol extraction, the plant material was dried and subjected to the water (infusion) extraction. For water (infusion) extraction, 600 ml distilled water at 80°C was added to the plant material for ten minutes and the extract was filtered. For the second water extraction (decoction), 600 ml of distilled water was added to 60 g of dried plant material and boiled for ten minutes, and extract was filtered (Ljubuncic *et al*., 2005). The filtered extracts were lyophilized and yielded 1.811 and 2.507 g dried mass, respectively. The extracts were sealed in glass bottles and stored at -20°C until use.

2.3. Phytochemical Screening for Six Different Extracts

Phytochemical tests of the six different extracts were carried out to detect the presence of particular compounds using a standard procedure. These concentrations were selected according to the data obtained from the preliminary experiments. The extracts were subjected to preliminary phytochemical testing for the detection of major chemical groups (Table 1). The details of the tests as are follows:

2.3.1. Detection of Phenols

Ethyl acetate and methanol extracts prepared in ethanol were spotted on a filter paper. A drop of phosphomolybdic acid reagent was added to the spots and was exposed to ammonia vapors. Blue coloration of the spots indicates the presence of phenols (Ravishankara *et al*., 2002).

2.3.2. Detection of Tannins

To 2-3 mL of methanolic extract, 10 % alcoholic ferric chloride solution was added. Dark blue or greenish grey coloration of the solution indicates the presence of tannins in the extracts (Ravishankara *et al*., 2002).

2.3.3. Detection of Alkaloids

A drop of extracts prepared in methanol was spotted in a small piece of precoated TLC plate, and the plate was sprayed with Dragendorff's reagent. Orange coloration of the spot indicates the presence of alkaloids (Ravishankara *et al*., 2002).

2.3.4. Detection of Anthraquinones

About 50 mg of the extracts was heated with 10 % ferric chloride solution and 1 mL of concentrated hydrochloric acid. The extracts were cooled, filtered, and the filtrates were shaken with diethyl ether. The ether extracts were further extracted with strong ammonia. Pink or deep red coloration of aqueous layer indicates the presence of anthraquinones (Ravishankara *et al*., 2002).

2.3.5. Detection of Saponins

10 mg of the extracts were mixed with hot water, and the mixtures were shaken for thirty seconds. The formation of a stable foam indicates the presence of saponins (Dominguez, 1973).

2.3.6. Detection of Flavonoids

To 2-3 mL of the extracts prepared in methanol, a piece of magnesium ribbon and 1 mL of concentrated hydrochloric acid were added. Pink-red or red coloration of the solution indicates the presence of flavonoids (Ravishankara *et al*., 2002).

2.4. Determination of Total Phenolic Content

The total phenolic compound contents in the extracts were determined by the Folin-Ciocalteu method (Singleton *et al*., 1999). 100 μL of the properly diluted extract solutions were mixed with 1 mL of FC reagent. The reagent was pre-diluted, ten times, with distilled water. After standing for three minutes at room temperature, 3 mL of (2 % w/v) a sodium carbonate solution was added. The solutions were mixed and allowed to stand for two hours at the room temperature. Then, the absorbance was measured at 760 nm, using a UV–visible spectrophotometer (Shimadzu PharmaSpec UV-1700, Japan). A calibration curve was prepared using a standard solution of gallic acid (20, 40, 60, 80 and 100 mg/L). Results were expressed as mg gallic acid equivalents/g of sample.

2.5. Determination of Antioxidant Activity

2.5.1. DPPH Free Radical Scavenging Assay

The assay for DPPH free radical scavenging potential is based on the scavenging activity of stable 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH). The free radical scavenging activity of diethyl ether, petroleum ether, ethyl acetate, methanol and water (infusion and decoction extracts) extracts from *H. hirsutissimum* was tested for their ability to bleach the stable 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) (Brand-Williams *et al*., 1995). One milliliter of 0.1 mM DPPH methanol solution was added to 3 mL of various concentrations of the extracts in methanol. The mixture was shaken vigorously and was left at room temperature. After thirty minutes, the absorbance of mixture was measured at λ=517 nm. Tests were carried out in triplicate. Rutin (100 ppm), a citrus flavonoid glycoside, was used as a standard, and the Shimadzu PharmaSpec UV–1700 UV-Visible spectrophotometer was used for measurement. Finally, the DPPH radical scavenging activity of the extracts was calculated using the following equation:

Scavenging capacity % = 100 - [(Ab of sample-Ab of blank) × 100/Ab of control]

where Ab control is the absorbance of $DPPH^{\bullet}$ solution without extracts.

The extract concentration providing 50 % inhibition (IC_{50}) was calculated from the plot of inhibition percentage against extract concentration.

2.5.2. H_2O_2 Scavenging Assay

Hydrogen peroxide scavenging activities of the extracts from *H. hirsutissimum* were determined by the method described by Ruch *et al.* (1989). A solution of H_2O_2 (40mM) was prepared in phosphate buffer (pH 7.4). The reaction mixtures contained 40 mM of H_2O_2 and different concentrations of the extracts, and the absorbance values were measured after ten minutes using wavelength of 230 nm. Ascorbic acid was used as the standard.

2.5.3. Metal Ion Chelating Ability

The chelation of ferrous ions by extracts was estimated by method of Dinis *et al.,* (1994). Briefly, 50 µL of 2 mM $FeCl_2$ was added to 1 mL of different concentrations of the extracts. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for ten minutes. The absorbance of the solution was thereafter measured at 562 nm. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated according to the following formula:

$$[(A_0 - As)/ As] \times 100$$

where A_0 is the absorbance of the control, and As is the absorbance of the extract/ standard. Na_2EDTA is used as positive control (standard).

2.6. Cytotoxic Activity

The cytotoxicity of the *H. hirsutissimum* extracts was evaluated by *Artemia salina* lethality test according to the procedure described by Solis *et al.* (1993). Brine shrimp eggs were hatched in artificial sea water prepared from commercial sea salt 40 g/L. The compartments plastic chamber are used, the eggs were sprinkled into the compartment which was darkening. After forty-eight hours of incubation at room temperature (25-29°C), nauplii were collected by pipette from the lighted side of the chamber, and were exposed to a 60-W lamp, pH 8.8.

The extracts were dissolved in DMSO (up to 2 % of final dosage), and were diluted with sea water. Serial dilutions were made in the wells of 96-well microplates in triplicate in 500 µL sea water. Control wells with DMSO were included in each experiment. A suspension of nauplii containing 10-15 organisms (100 µL) was added to each well. The plates were covered and incubated at room temperature (25-29°C) for five and twety-four hours.

From 20 mg of each extract, 1, 10, 100 and 1000 ppm solutions were prepared in triplicate. Then, ten specimens

with forty-eight hours of hatching in sea water and distilled water (1:1) were placed in each tube and three negative control tubes (saline solution and DMSO 1 %). Appropriate volumes of the saline solution in tubes were added until 5 mL of the saline solution containing 10 nauplii each to obtain the final sample concentrations. After twenty-four hours, the plates were then examined under the binocular stereomicroscope, and the numbers of dead (non-motile) nauplii in each well were counted. The percentage of lethality of the nauplii for each concentration and control was calculated. Umbelliferon was used as positive control and salt water was used as negative control. For each plate, the number of the dead and of live nauplii, was counted and % death was determined:

$$\%Death = \frac{\text{Number of dead nauplii}}{\text{Number of dead nauplii + Number of live nauplii}} \times 100$$

2.7. Statistical Analysis

The number of deaths was counted and the results were tabulated and submitted to probit analysis in SPSS® 11.5 software (IBM® Corp., NY, USA), obtaining the value of LC_{50} with a 95 % confidence interval. Each experiment was carried out triplicate. Statistically significant difference was considered at the level of $p<0.05$.

3. Results

3.1. Phytochemical screening

Results of phytochemical screening are presented in Table 1. Phenols were detected in the petroleum ether and water extracts (infusion and decoction) but not in diethyl ether extract, ethyl acetate and methanol extract of *H. hirsutissimum*. Tannins were present only in the methanol extract. A very small amount of Tamins was determined in the diethyl ether extract but not in the other extracts. Alkaloids were detected in the diethyl ether, petroleum ether, ethyl acetate and decoction extracts, but not in the methanol extract. Alkaloids were determined with a very small amount in the infusion extract. Anthraquinones were detected in the ethyl acetate and methanol extracts. Saponins were present in the ethyl acetate and methanol extracts. Flavonoids were not detected in any of the extracts.

Table 1. Qualitative analysis of the phytochemicals of different extracts of *H. hirsutissimum*

Phytochemicals	Extracts					
	Diethyl ether extract	Petroleum ether extract	Ethyl acetate extract	Methanol extract	Aqueous extract (Infusion)	Aqueous Extract (Decoction)
Phenols	-	+	-	-	+	+
Tannins	*	-	-	+	-	-
Alkaloids	+	+	+	-	*	+
Anthraquinones	-	-	+	+	-	-
Saponins	-	-	-	+	+	+
Flavonoids	-	-	-	-	-	-

+ present; *: slightly present; -: absent

3.2. Determination of Total Phenolic Content

The total phenolic content in the examined plant extracts is expressed in terms of gallic acid equivalent (the standard curve equation: $y = 0.003x + 0.001$, $r2 = 0.9975$). The total phenolic content values are expressed as mg of GA/g of extract (Table 2).

Table 2. Total phenolic contents of *H. hirsutissimum* extracts expressed in terms of gallic acid equivalent (mg of GA/g of extract)

Extracts	mg of GA/g of extract
Diethyl ether	27.6
Petroleum ether	27.7
Ethyl acetate	47.7
Methanol	31.0
Infusion	23.3
Decoction	30.0

Each value is the mean of three analyses

3.3. Antioxidant Activity

3.3.1. DPPH Scavenging Activity

The antioxidant activities of six different extracts are expressed in terms of the percentage of inhibition (%) and IC_{50} values (µg/mL) (Table 3). Parallel to the examination of the antioxidant activity of the plant extracts, the values for standard compounds were obtained and compared to the values of the antioxidant activity. The standard substance was rutin. A lower value of EC_{50} indicates a higher antioxidant activity. Antioxidant activities of the extracts showed different values. Extracts showed a concentration-dependent radical scavenging activity. The highest activity to neutralize DPPH radicals was found for infusion extract, which neutralized 50 % of free radicals at the increased concentration (30.5 ± 0.019 for 25 ppm; 52.8 ± 0.00 for 50 ppm and 74.4±0.16 for 100 ppm, repectively). A moderate activity was found for methanol, and decoction extracts. Due to the low activity of diethyl ether, petroleum ether, ethyl acetate, and decoction extracts, IC_{50} are not calculated for these extracts.

Table 3. Antioxidant (DPPH scavenging) activity of six different extracts of *H. hirsutissimum*

Extracts	Concentrations (ppm)	DPPH scavenging activity (%±SD)
Diethyl ether	25	0.00±0.0025
	50	0.00±0.0025
	100	15.8±0.0025
Petroleum ether	25	0.00 ±13.27
	50	0.00 ±0.005
	100	11.0±0.030
Ethyl acetate	25	0.00 ±0.0076
	50	0.00 ±0.0023
	100	12.1±0.025
Methanol	25	21.2±0.021
	50	30.5±0.0005
	100	43.6±0.000
Infusion	25	30.5±0.0190
	50	52.8±0.0005*
	100	74.4±0.1646*
Decoction	25	10.7±0.0076
	50	14.6±0.010
	100	19.6±0.0020
Rutin	100 ppm	89.1±0.0015*

**p* <0.05

3.4. H_2O_2 Radical Scavenging Activity

H_2O_2 scavenging activities (%) of different concentrations of different extracts are shown in Table 4. H_2O_2 scavenging activity of diethyl ether extract was observed at 5 and 10 ppm concentrations (33.1 % and 14.19 %, respectively), but was not observed at 20 ppm concentration. Petroleum ether and methanol extracts showed moderate H_2O_2 scavenging activity. Other extracts showed low H_2O_2 scavenging activity at tested concentrations (5, 10 and 20 ppm).

Table 4. H_2O_2 scavenging and metal chelating activity of six different extracts of *H. hirsutissimum*

Extracts	Concentrations (ppm)	H_2O_2 scavenging activity (%±SD)	Metal chelating activity (%±SD)
Diethyl ether	5	33.1±0.0016	0.00±0.0215
	10	14.19±0.0023	0.00±0.0015
	20	0.00±0.0008	0.00±0.0090
Petroleum ether	5	44.9±0.0043	0.00±0.0120
	10	41.5±0.0012	0.00±0.0045
	20	23.7±0.0043	23.4±0.0020
Ethyl acetate	5	28.3±0.0043	0.00±0.0010
	10	14.0 ±0.0019	0.00±0.0000
	20	0.00±0.085	0.00±0.0010
Methanol	5	41.3 ±0.0071	23.4±0.0020
	10	20.9±0.0005	0.00±0.0055
	20	14.3±0.0009	10.1±0.0005
Infusion	5	31.7±0.0008	0.00±0.0090
	10	16.1±0.0012	0.00±0.0000
	20	27.8±0.0019	36.1±0.0010
Decoction	5	31.2±0.0022	0.00±0.0050
	10	22.2±0.014	0.00±0.0045
	20	15.3±0.0015	0.00±0.0025
Ascorbic acid	2μg/ml	71.8±0.0069*	
EDTA	2μg/ml		100.0±0.0000*

**p*<0.05

3.5. Metal Ion Chelating Activity

The metal ion chelating activity of six different extracts of *H. hirsutissimum* extracts was determined at 5, 10 and 20 ppm concentrations, and the results are depicted in Table 4. Although standard agent EDTA, showed a high ion metal chelating potential (100 %), the extracts showed a low chelating activity. The metal chelating activity was 36.1 % (20 ppm) in the infusion extract, 23.4 % (5 ppm) in the methanol extract and 23.4 % (20 ppm) in the petroleum ether extract. Other extracts did not show metal chelating activity.

3.6. Brine Shrimp Lethality Assay

The results of lethal effects of the extracts to brine shrimp larvae (% mortality at different concentrations and LC_{50} values) were shown in Table 5. An approximate linear correlation was observed when logarithm of concentration versus percentage of mortality was plotted on the graph paper, and the values of LC_{50} were calculated using Microsoft Excel 2003. According to Nguta *et al.* (2011), crude extracts with LC_{50} values less than 100 ppm are considered highly toxic. Those with LC_{50} values between 100 ppm and 500 ppm are moderately toxic and the ones with LC_{50} values between 500 ppm and 1000 ppm, are considered mildly toxic. Those with LC_{50} values above 1000 ppm are considered non-toxic.

As a result of evaluating the extracts' data by Probit analysis; it was found that the tested extracts did not reach the LC_{50} values at the determined concentration ranges (10, 25, 50, 100, 250, 500 and 1000 ppm). Of the six tested extracts, those with LC_{50} between 10 and 1000 ppm showed weak toxicity. LC_{10} value results are: (p<0.05) showed no significant difference in the other extracts except methanol (LC_{10} = 258.610 ppm) and diethyl ether (LC_{10} = 93.344 ppm). The extracts showed weak cytotoxic activity with LC_{50} value compared with the activity of standard with LC_{50} value (Table 5).

Table 5. Effects of the six different extracts of *H. hirsutissimum* on the *Brine Shrimp* nauplii (*Aretmia salina)* after a 24-hr exposure

Extracts	Time (h)	LC_{10} (ppm)	LC_{50} (ppm)
Diethyl ether	24	93.344	>1000
Petroleum ether	24	>1000	>1000
Ethyl acetate	24	>1000	>1000
Methanol	24	258.610	>1000
Infusion (water)	24	>1000	>1000
Decoction (water)	24	>1000	>1000
Umbelliferon (positive control)	24	53.773	170.836

4. Discussion

Herbal products are considered an important source of potentially useful compounds for the development of new phytotherapeutic agents. *Heliotropium* has been traditionally used for the treatment of gout, various inflammations, rheumatism, poisonous bites, and skin diseases as a healing agent in various countries around the

World (Ghori, 2016). The *Heliotropium* species grow in arid regions with extreme environmental conditions and produce a resinous exudate from the trichomes that cover its foliar surface and stems. Phytochemical research revealed that this exudate is constituted by a mixture of different compounds, mainly flavonoids and aromatic geranyl derivatives (Villarroel, 1991; Tores et al., 1994; Urzua et al., 2000; Modak, 2003). A variety of constituents are identified and isolated from different species of the genus *Heliotropium* which are phytochemically active and have significant therapeutic effects. Many classes of organic compounds such as pyrrolizidine alkaloids (PAs), phenolic compounds, terpenoids, and quinones are very abundantly present in *Heliotropium*. *Heliotropium* species are highly valued for their antimicrobial and antioxidant activities due to the isolation of secondary metabolites such as alkaloids, flavonoids and terpenoids (Ghori et al., 2016).

The phytochemical screening in the present study, has revealed the presence of phenols, tannins, alkaloids, flavonoids, anthraquinones, and saponins in six different extracts of *H. hirsutissimum* (Table 1). Phenols were detected in the petroleum ether and water extracts (infusion and decoction) but not in diethyl ether, ethyl acetate and methanol extracts of *H. hirsutissimum*. Tannins were present in the methanol extract and were determined as of a very small amount in the diethyl ether extracts, but not in the other extracts. Alkaloids were detected in the diethyl ether, petroleum ether, ethyl asetate, and the water extracts (decoction) and with very small amounts in the infusion extract, but not in the methanol extract. The inability to observe alkaloids in the methanol extract may be due to the fact that the alkaloids are not soluble in methanol or may be exposed to the antagonistic effect of any substance contained therein. Alkaloids play some metabolic role and control development in the living system. They are also involved in protective functions in animals, and are used as medicine especially the steroidal alkaloids (Lalitha *et al*., 2012). Phenolics and alkaloids detected in the extracts are compounds that have been documented to possess medicinal properties (Salah *et al*., 1995; Obdulio *et al*., 1997; Okwu, 2004; Liu, 2004; Harini *et al*., 2014). The flavonoids and phenolic compounds in plants have been reported to exert multiple biological effects including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic etc. (Lalitha *et al*., 2012). The presence of alkaloids has also been identified in other *Heliotropium* species (Reina *et al*., 1998; Souza et al., 2005; Osungunna and Adedeji, 2011). Previous studies have reported that pyrrolizidine alkaloids, which are a class of alkaloids, are present in almost all species belonging to the Boraginaceae family. Anthraquinones were detected in the ethyl acetate and methanol extracts. Saponins are present in the ethyl acetate and methanol extracts (Table 1). In the current study, saponins were found to be the most common content observed after alkaloids in the plant extracts. Previous studies have also found saponins in the methanol extract of the *H. indicum* (Sharma and Alexander, 2011). The results of this study are also in accordance with the literature.

The total phenolic contents in the *H. hirsutissimum* extracts depend on the type of the extract, i.e. the polarity of the solvent used in the extraction. The high solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction (Zhou and Yu, 2004; Mohsen and Ammar, 2008). The variation may be due to environmental conditions, which can modify the constituents of the plant.

Comparatively, the stable organic radical DPPH has been broadly utilized in the determination of the antioxidant activity of different plant extracts as well as purified compounds (Yen and Duh, 1994; Brand-Williams, *et al.,* 1995). The ability of antioxidants for DPPH radical scavenging is supposed to be due to their hydrogen donating property (Soares *et al.,* 1997). After acceptance of an electron or a hydrogen atom, a stable diamagnetic molecule will emerge which results in the vanishing of the absorption band at 517 nm. The radical scavenging activity of the samples corresponds to the remaining DPPH in an inverse manner. Numerous antioxidant methods have been proposed to evaluate the antioxidant activity. Of these, the total antioxidant activity, reducing power, DPPH assay, metal chelating, active oxygen species such as H_2O_2, O^- and OH^- quencing assays are the most commonly used in the evalution of antioxidant activities of plant extracts.

Different extracts of *H. hirsutissimum* showed low DPPH scavenging activity whereas other *Helioptrium* species showed strong antioxidant activity. The plant*, H. strigosum,* showed excellent DPPH scavenging activity. The antioxidant activity was shown by other plants of the genus, *Heliotropium* (Begüm, 2014). *H. taltalense* and flavonoids islolated exhibited antioxidant activity which suggest that *H. strigosum* may possess flavonoids responsible for the antioxidant activity (Modak *et al*., 2009). Similarly, the ethyl acetate fraction also showed DPPH scavenging activity and is consistent with the antioxidant activity shown by *H.sinuatum.* Modak *et al*., (2003) islolated, 4-(3',5'-dihydroxynona-decyl) phenol 1, and eight flavonoids from *H. sinuatum* (Modak *et al*., 2003), and *H. strigosum* (Hussain *et al*, 2010) reported the antioxidant activity of these compounds. The aqueous and organic extracts from the same plant showed different activities, the organic extracts showed the same or greater activity than the aqueous extracts, these results suggest that the interesting active compounds in this plant have a limited solubility in water, and are expected to be non-polar hydrophobic organic compounds (Jadarat *et al.,* 2014).

Hydrogen peroxide is a weak oxidizing agent, and can inactivate a few enzymes directly, usually by the oxidation of essential thiol (-SH) groups. Hydrogen peroxide itself is not very reactive, but sometimes it can be toxic to cells because of the increase in the hydroxyl radicals in the cells. It can cross cell membrane rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radical, and this may be the origin of many of its toxic effects (Halliwell, 1991; Mohan *et al*., 2012). When the results of this study were compared with ascorbic acid, six different extracts of *H. hirsutissimum* did not show H_2O_2 scavenging activity except the petroleum ether extract. (Table 4). Another *Helioptrium* species, *H. ramosissium,* has a strong H_2O_2 scavenging ability (Shatat *et al*., 2015). Also, the extract of *H. indicum* leaves exhibits the greatest antioxidant activity through the scavenging of free radicals.

The iron-chelating capacity test measures the ability of antioxidants to compete with ferrozine in chelating ferrous ion (Elmastas *et al*., 2006). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of samples possessing chelating activity, the formation of complexes is decreased. Therefore, measurement of the rate of color reduction helps to estimate the chelating activity of the samples. *H. indicum* leaves exhibited the greatest iron chelator and iron reducing power. Phytochemicals of *H. indicum* leaves exhibited the greatest antioxidant activity DPPH, superoxide anion scavenging and metal chelator (iron chelator and iron reducing power). When the results of this study were compared with other *Heliotropium* species, the antioxidant activity, phenolic content, H_2O_2 scavenging and metal cheathing activity of the *H. hirsutissimum* extracts were different. This may be due mainly to the extract of *H. hirsutissimum* being a crude extract with active compounds that are not purified. However, the active compounds of the *H. Hirsutissimum* extracts will be purified and identified in the future in an ongoing research in the laboratory.

The brine shrimp lethality assay is a general bioassay that seems to be capable of detecting a wide spectrum of bioactivity present in the crude extracts. The commercial availability of inexpensive brine shrimp eggs, the low cost, the safety and ease of performing the assay, as well as the requirement of no special technology all make this a very helpful bench-top tool for the phytochemistry laboratory. The lethality to brine shrimp is recommended as an effective prescreen to the existing cytotoxicity and antitumor assays. A number of studies have established the use of the brine shrimp assay to screen plants commonly used as pesticides, anticancer, and with molluscicidal, larvicidal, fungicidal, and cytotoxic activity (Khalighi-Sigaroodi *et al*., 2012). In order to study the toxicity of these medicinal ponctuation, we performed brine shrimp lethality bioassay based on the ability to kill *Artemia naupli* in laboratory condition. Substances submitted to this bioassay, which lead to the death of half of the specimens at a lethal concentration of up to 1000 ppm, are considered active. They were subsequently tested in these trials, obtaining a good correlation (McLaughlin and Rogers, 1988; Arcanjo *et al*., 2012).

The results showed no cytotoxic properties of the six different extracts obtained from *H. hirsutissimum* on *Artemia salina* larvae (Table 5). The lethality of the crude extracts (LC_{50} value less than 100 ppm) to brine shrimps indicates the presence of potent cytotoxic compounds which necessitate further investigation (Alam, et al., 2011). Shah *et al.,* (2015) have shown that *H. strigosum* and its organic fractions exhibit cytotoxic and phytotoxic activities against *Artemia salina* Leach. The ethyl acetate and chloroform fractions showed marked cytotoxicity action. Ethyl acetate and chloroform fractions were more potent for the evaulated toxicity effects. The ethyl acetate and chloroform fractions showed marked cytotoxicity action. In the phytotoxicity study, ethyl acetate was the most potent, followed by chloroform. Researchers have suggested that studies should be done to isolate active compounds in the cytotoxic properties of the extract (Shah *et al*., 2015).

5. Conclusion

On the basis of the results obtained this study confirms the cytotoxic activity of different exracts of *H. hirsutissimum* against *Brine shrimp* larvae. When the results of this study were compared with other Heliotropium species, the antioxidant activity, phenolic content, H_2O_2 scavenging and metal cheathing activity of the *H. hirsutissimum* extracts were found different. These differences may be due to the parts of the plant used, climate and soil differences. Furthermore, this study found that the plant extracts have a broad spectrum of activities. Nonetheless, activity-directed assay is necessary on this plant with a view to isolating and characterizing the active metabolite responsible for the observed activity. Finally, this study can be used as a basis for utilizing this plant species for further investigation in drug discovery for potential new natural bioactive compounds.

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Conflıct of Interests

Authors have declared that no conflict of interests exists

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