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Full Length Research Paper

Phytochemical screening and evaluation of *in-vitro* antioxidant potential of *Monotheca buxifolia*

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The present study aimed to investigate the antioxidant potential of *Monotheca buxifolia* leaves (*ML*) by employing some *in vitro* methods. The antioxidant components were initially extracted from the plant in methanol and were further fractionated in solvents of different polarity. The Phytochemical screening showed that the leaves of *Monotheca buxifolia* are naturally enriched with anthraquinones, flavanoids, reducing sugars, terpenoids, tannins, saponins, and cardiac glycosides. The trolox equivalent antioxidant capacity (TEAC) and Ferric reducing antioxidant power values for leave extracts were ranged from 30.3-109.2 mM of Trolox equivalents and 8.9-29.9 mg/L of $FeSO_4$ equivalents respectively. The EC_{50} values of leaves fractions were found to be in range 14.4-172.8 (mg of dried mass/mL). Using Total phenolic contents assay, the amount of total phenolics varies from 39.2-131.8 mg/L of gallic acid equivalents for leaves. The total lavanoids content were found to be 262.75-825.45 mg/L of Quercetin for leaves and fruit fractions. The percentage bound iron for metal chelating activity varies from 69-73%. The percentage scavenging activity of superoxide anions radical was 61-73% for leave extracts. On the basis of these results obtained here *ML* may be considered as a rich source of antioxidant.

Key words: Antioxidant activity, *Monotheca buxifolia*, phytochemical screening, TEAC value, $EC_{50.}$

INTRODUCTION

Almost 80% of the world population depends exclusively on plants for their health and healing. However in developed world reliance on surgery and pharmaceutical products is more usual. But in recent years, their attention is diverting towards the natural supplements. This motivation of people towards herbs is due to their concern about the harmful effects of drugs, which are prepared from synthetic materials (Khalil et al., 2007). There has been a sudden rise of interest in therapeutic potential of plants due to presence of antioxidants which reduces the tissue injuries caused by free radicals. These free radicals are produced as a result of metabolic processes and environmental pollutants, i.e. radiations, toxins, smoking, deep fried and spicy food items as well as physical and emotional stresses, also contribute to it. These free radicals cause depletion of immune system antioxidants which leads to change in gene expression and induce abnormal proteins. These free radicals contribute to several hundred human disorders like atherosclerosis, ischemia, gastritis, cancer, AIDS, reperfusion injury of many tissues, central nervous system injury, diabetes mellitus, and aging processes. However several synthetic antioxidants are commercially available such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), Propyl gallate (PG), these synthetic antioxidants are widely employed in food industry to prevent oxidation and spoilage of food. But these are quite unsafe and their toxicity is a problem of major concern. This upsurges the trend of substituting the synthetic antioxidants with natural antioxidants. Natural antioxidants such as phenolics and flavanoids from fruits, vegetables, spices, tea and wine are already exploited commercially either as nutritional supplements or antioxidant additives (Pourmorad et al., 2006). Such natural antioxidants show

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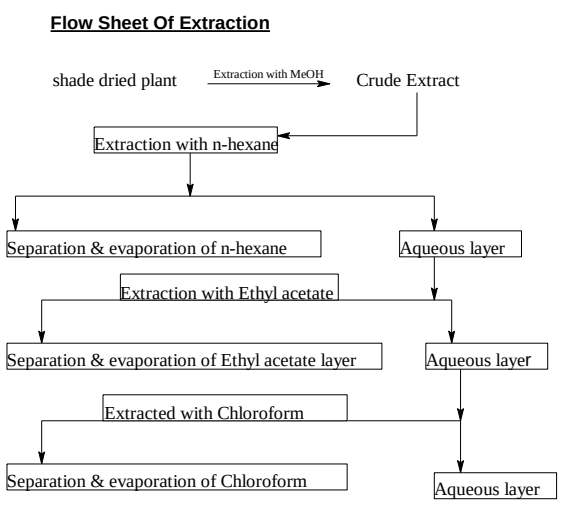

Figure 1. Extraction scheme of *ML* (Monotheca buxifolia leaves) in different solvents

their activity due to their redox properties which makes them reducing agents, hydrogen donors, metal chelators, or singlet oxygen quenchers (Demiray et al., 2009). These exogenous antioxidants assist the body's antioxidant defense system against the free radicals and reduce the oxidative stress (Gan et al., 2010). This prompted us to speculate some novel natural sources of antioxidants. Therefore the objective of present study is to evaluate the radical scavenging potential of *Monotheca buxifolia leaves (ML),* which is a broad leaved evergreen tree, belonging to family *Sapotaceae.* It is a native plant of District Dir Lower, Hindukash Range of Pakistan, locally known as gurguri. Ethanibotanically, it fruit is laxative, digestive and is commonly used in urinary tract diseases (Khan et al., 2010).

MATERIALS AND METHODS

All the chemicals and solvents were of analytical grade. 2,2`-azinobis-(3-ethylbenzo-thiazoline-6-sulphonic) diammonium salt (ABTS), 2,4,6-Tris-2-tripyridyl-s-triazine(TPTZ), 2,4,6-Nitrotetrazolium Blue Chloride (NBT), Nicotinamide Adenine Dinucleotiode (NADH), Phenozinemetho Sulphate (PMS) and Ferrozine were obtained from Fluka chemicals, Switzerland. Trolox (6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), were purchased from Aldrich Chemical Co., Gillingham, Dorset, UK). 2, 2`- Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma chemical company Ltd, USA.

Experiments were performed on UV-1700 Pharma- Spec UV-Visible Spectrophotometer, Shimadzu, Japan equipped with CPS controller. All the experiments were performed three times and results obtained were averaged. Data obtained, except where specifically mentioned, is mean ±SD (n = 3).

Leaves *(ML)* of *Monotheca buxifolia* were obtained from District Dir Lower (DDL) Pakistan. The leaves of plant were subjected to shade-drying and then to grinding. 50 g of finely ground leaves sample was then exhaustively extracted with methanol using Soxhlet apparatus at 45^0C for 12 h. Then resulting extracts were filtered using Whatmann filter paper. Allowed these extracts to concentrate under vacuum at 45^0C using Stuart-RE 300 rotary evaporator and calculated the yield of extract. Dissolved the residues in appropriate volume of distilled water and allowed it to fractionate using organic solvents (25X3 ml) of varying polarity (n-hexane, Ethyl acetate, chloroform), as shown in Figure 1.

These resulting extracts and the water extracts were allowed to concentrate under vacuum using Stuart-RE300 rotary evaporator, at temperature according to the boiling point of the solvent. Then yield of every residue was calculated. These residues were dissolved in appropriate volume of respective solvent to get the stock solutions (3 mg/ml) to be used for antioxidant activity evaluation.

Phytochemical screening

The phyto constituents of leaves of *Monotheca buxifolia* (ML) were identified by performing standard protocols (Ayoola et al., 2008).

Test for Tannins

Took 0.5 ml of plant sample solution in a test tube and allowed it to boil. Then add few drops of 0.1% $FeCl_3$ and waited for blue-black or greenish brown coloration.

Test for Saponins

Added 0.5 ml of sample solution in a test tube and boiled. Then added 5 ml of distilled water and shudder it violently till persistent froth is formed. To this froth, added 3 drop of olive oil and mixed it vigorously till an emulsion is formed.

Test for Anthraquinones

Took a small quantity of sample extract and boiled it with concentrated H_2SO_4 then filtered it and added chloroform and mixed it gently. Separated out the chloroform layer and added dilute ammonia solution to it and waited for the color changes.

Test for Terpenoids

For Terpenoids determination, took 5 ml of sample extract and added 3 ml of chloroform and 2 ml of concentrated sulfuric acid to develop a layer and waited for the red color appearance at the interface.

Test for Flavanoids

For flavanoids determination, took 0.5 ml of sample material and added 5 ml of dilute ammonia solution and 2 ml of

concentrated sulfuric acid. Appearance of yellow color will indicate the +ve test.

Test for reducing sugars.

To 0.5 ml of sample extract, added 0.5ml of Fehling A and 0.5 ml of Fehling B and heat the reaction mixture for a while. Appearance of brick red coloration showed the positive result.

Test for Cardiac glycosides

To 0.5 ml of sample extract added 1 ml of glacial acetic acid having one drop of $FeCl_3$ solution. Then added 1 ml of concentrated sulfuric acid. A violet or brown colored ring formed showing the presence of Cardiac glycosides.

Test for Alkaloids

Put a drop of plant extract solution on the origin of TLC plate, then poured few drops of Dragondorff reagent on this spot and waited for the appearance of red color.

Antioxidant activity

$ABTS^{\bullet+}$ Assay

An improved version of ABTS assay, as devised by (Re et al., 1999), was employed to assess the antioxidant potential of ML extract. $ABTS^{\bullet+}$ radical cation was generated by reacting 7mM ABTS solution with 2.45 mM solution of Potassium persulfate and this mixture was allowed to stand in dark at room temperature for 12-16 hours before use. The $ABTS^{\bullet+}$ stock solution was diluted with PBS buffer of pH 7.4 or methanol to an absorbance of 0.70$\pm$ 0.02 at 734 nm. For the evaluation of antioxidant activity, added 10µl of sample to 2.99ml of diluted solution of $ABTS^{\bullet+}$ (A=0.70$\pm$ 0.02) and noted the change in absorbance after every 1 min interval for 8 mins. Appropriate solvent blank was run in parallel. All the samples were run in triplicate and mean values of absorbance were calculated. Percentage inhibition is calculated by using following formula:

$$\text{percentage inhibition}_{(\text{at } 734\text{nm})} = (1-A_f / A_0) \times 100$$

DPPH radical scavenging activity

DPPH (1, 1-diphenyl-2-dipicryl-hydrazyl) radical cation scavenging activity of *ML* extracts was evaluated as described by (Shimada et al. 1992). To a 2.5 ml of methanolic solution of DPPH (25 mg/l) having an absorbance of 1.0$\pm$0.02 at 515 nm, appropriate volumes of sample extracts were mixed. The reaction progress of the mixture was monitored at 515 nm for 30 minutes. The percentage of DPPH remaining was calculated as:

$$\%DPPH_{rem} = [DPPH]_{t=t} / [DPPH]_{t=0} \times 100$$

Where $[DPPH]_{t=t}$ and $[DPPH]_{t=0}$ were the absorbance of DPPH solution remaining at time t and t =0.

Ferric reducing antioxidant power (FRAP assay)

The ferric reducing ability of plant extracts was evaluated by protocol described by Benzie and Strain (1996). The FRAP reagent contained 300 mM acetate buffer of pH 3.6, 10 mM TPTZ (2, 4, tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffers, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3$. $6H_2O$ solution and then warmed at 37^0C before use. To 3 ml of freshly prepared FRAP reagent added 300 ul of double distilled water and 100 ul of sample extract and noted its absorption at 593 nm for 4 m.

Metal chelating activity

Total phenolic content determination

Total phenolic contents of *ML* extracts were determined using Folin-Ciocalteu (FC) reagent according to the method of (Slinkard and Singleton, 1997). An aliquat of 40 µL was pipetted into separate cuvettes, and to each 3.16 mL of double distilled water was added. Folin–Ciocalteu reagent (200 µL) was added and mixed well. After 8 min, 600 µL of sodium carbonate solution was mixed thoroughly in the solution. The solution was allowed to stand at 40°C for 30 min and absorbance of each solution was determined at 765 nm against the blank. A concentration versus absorbance linear plot was thus obtained. The concentration of total phenolic compounds of each fraction was expressed as Gallic acid equivalents.

Total Flavonoid Content

The total flavonoid content was determined with aluminium chloride ($AlCl_3$) according to a known method described by (Dewanto et al., 2002) using Quercetin as a standard. An aliquot of 250 µL of sample extract or quercetin standard solutions was mixed with 1.25 mL of distilled water in a test tube followed by addition of 75 µL of 5% $NaNO_2$ solution. After 6 min 150 µL of 10% $AlCl_3$.$6H_2O$ solution was added and allowed to stand for 5 m. Added 500 µL of 1 M NaOH and the volume of the mixture was raised to 2.5 mL with distilled water and mixed well. The absorbance was recorded at 510 nm. The amount of total flavonoid was expressed as milligrams of quercetein equivalents per gram of extract.

RESULTS AND DISCUSSION

Phytochemical screening

Phytochemical screening involved the qualitative identification of plant's constituents. This type of screening helped us a lot to recognize the class of plant's constituents and to exploit such important phytochemicals commercially. The results showed that

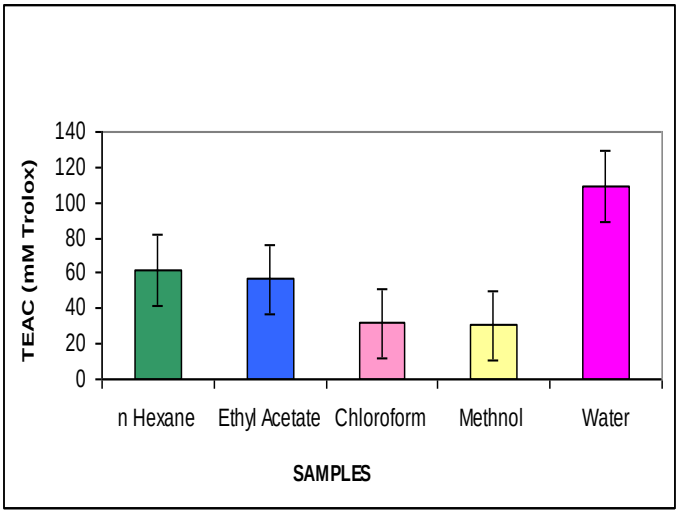

Figure 2. TEAC Value of ML.

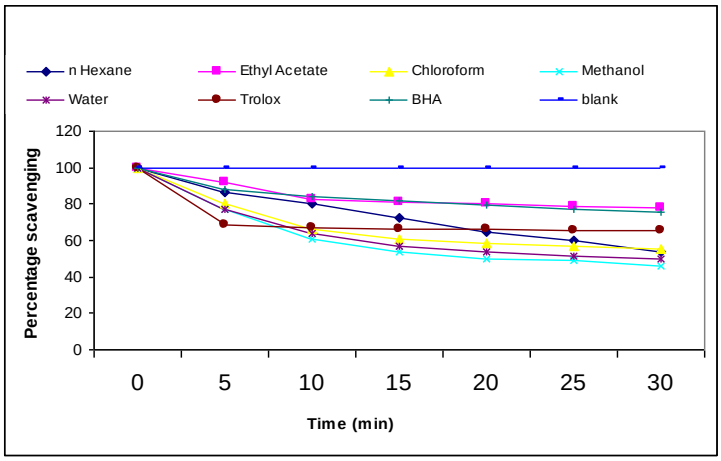

Figure 3. DPPH Assay for ML.

the phyto constituents originally present in methanol extracts segregated in organic solvents of different polarity. The results demonstrated that methanol extract of ML was full of Tannins, Flavanoids, Reducing sugars, anthraquinones, Cardiac glycosides, terpenoids, and saponins. Alkaloids were absent in all fractions (methanol, aqueous, n-hexane, chloroform, ethyl acetate) of ML (Table 1).

Likewise, Saponins, reducing sugars and Flavanoids were present in all extracts of ML. Terpenoids also showed a positive response for methanol, n-hexane, and chloroform extracts of ML but a negative response for n-hexane extract. Cardiac glycosides were only present in methanol, n-hexane, and chloroform extracts of ML. Tannins were present in methanol, chloroform and aqueous extracts of ML. Anthraquinones were present in methanol, chloroform and water extracts of ML. The reducing sugars, flavanoids and saponins were present in all the extracts of leaves.

$ABTS^{\cdot+}$ assay

The $ABTS^{\cdot+}$ assay was calibrated by using an alph Tocopherol analogus to Trolox as standard. All the fractions of leaves of *Monotheca buxifolia* exhibited antioxidant potential as they all scavenged the ABTS radical cation. All the extracts showed a near-linear variance between fall in absorbance and the corresponding concentration of sample extract added. The results of ABTS assay were expressed in terms of TEAC value and shown in Figure 2.

As TEAC is the measure of effective antioxidant activity of the substance. A higher TEAC value referred to a greater antioxidant potential. The TEAC value was calculated for each extract. As all the extracts had a great antioxidant potential, but the aqueous extract of leaves of *Monotheca buxifolia* showed relatively large potential of antioxidant activity having the TEAC value of 109.2 m Mol of Trolox equivalence.While the n-hexane and ethyl acetate extracts of ML had slightly less antioxidant potential in terms of TEAC values.The methanol and chloroform extracts of ML had the least TEAC value. The overall trend of TEAC values of leaves of *Monotheca buxifolia* is following:

Aqueous>nhexane>Ethylacetate>Chloroform>Methanol

DPPH free radical scavenging assay

DPPH is a stable and commercially exploited nitrogen based organic radical having a lambda maximum of 515 nm. On reduction, its original purple color solution fades away, which show the antioxidant activity of the sample. The results of DPPH free radical scavenging activity were represented as percentage DPPH remaining. The results of DPPH assay showed that aqueous extract of *ML* had the least EC_{50} value and a large antioxidant potential. The chloroform and methanol extracts had larger values of EC_{50} and lesser antioxidant potential. While the Ethyl acetate and n-hexane extracts showed comparatively higher values of EC_{50} and least antioxidant potential. The trend of EC_{50} values of *ML* is following:

Aqueous>Chloroform>Methanol>Ethyl acetate>n-hexane

The results of DPPH assay are shown in Figure 3.

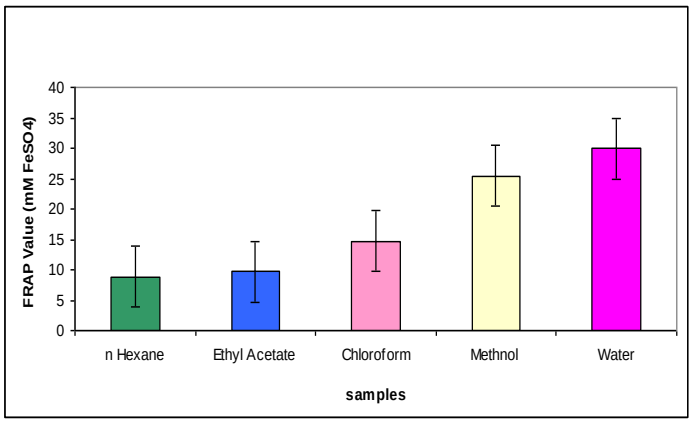

Figure 4. The FRAP values of ML

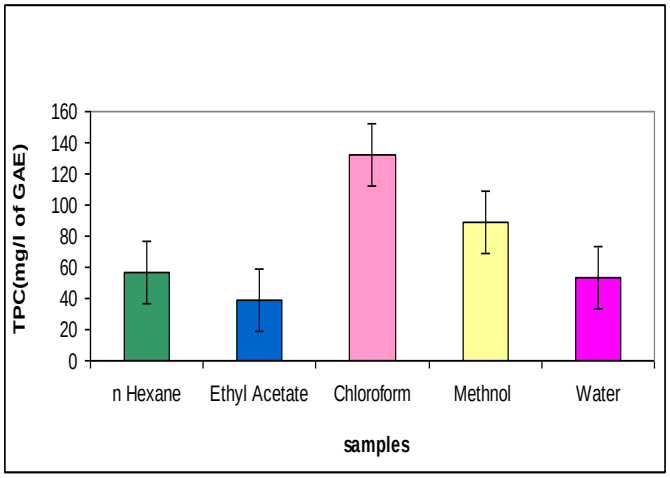

Figure 5. TPC Contents of ML.

FRAP (Ferric reducing antioxidant power)

The FRAP assay employed Fe (III)-$(TPTZ)_2$ Cl_3 (pale yellow in color) as an oxidizing agent, When it came in contact with an antioxidant, it gets reduced to Fe (II)-$(TPTZ)_2$ Cl_3 (Blue in color) and absorbs at 593 nm. The results of FRAP assay are often expressed in Mm of $FeSO_4$. A FRAP unit can be defined as the reduction of ferric (III) to ferrous (II). The aqueous extract of ML showed the highest FRAP value, referring to highest antioxidant potential (Figure 4). Then here came the Methanol and chloroform extract having comparatively less antioxidant potential. The ethyl acetate and n-hexane extracts showed the least antioxidant potential. The order of increasing FRAP value is following:

Aqueous>Methanol>chloroform>ethyl acetate>n-hexane

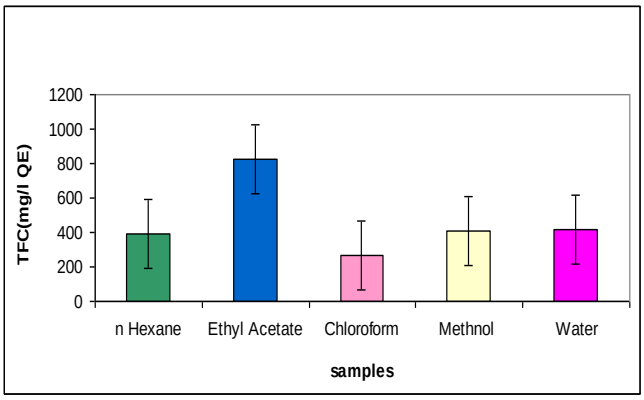

Figure 6. TFC contents of ML.

Total Phenolic Contents determination

The Folin-ciocalteu reagent is employed for the determination of total phenolic contents. Under basic conditions, poly phenols give rise to phenolate anion which has the ability to reduce F.C reagent. A blue colored complex formed for this assay and results are expressed as Gallic acid equivalents. It is thought that Phenolic compounds can attribute to the total antioxidant activity. The chloroform extract of ML showed the highest phenolic contents (Figure 5). The increasing order of phenolic contents of ML is following:

Chloroform>Methanol>n-hexane> Water>Ethyl acetate

Total Flavanoid contents determination

Flavanoids are one of the most important natural products that impart antioxidant potential and other medicinal properties to the plant. The total Flavanoids contents of leaves of *Monotheca buxifolia* were evaluated and the results revealed high concentration level of Flavanoids. The total flavanoid content of plant is expressed in terms of Quercetin equivalents. The ethyl acetate extract of ML showed a high concentration of Flavanoids, other fractions contained significant but slightly less quantities of Flavanoids (Figure 6). The increasing order of flavanoid contents of ML is as follows:

Ethyl acetate>water>methanol>n hexane>chloroform

Superoxide radical scavenging activity

This assay employed an in-vitro generation model [PMS/NADH/O_2] of superoxide radical anions and Nitro

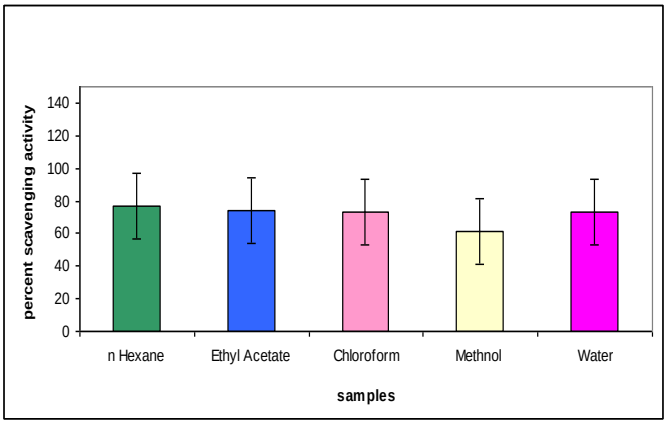

Figure 7. % scavenging of superoxide radical anion of *ML*.

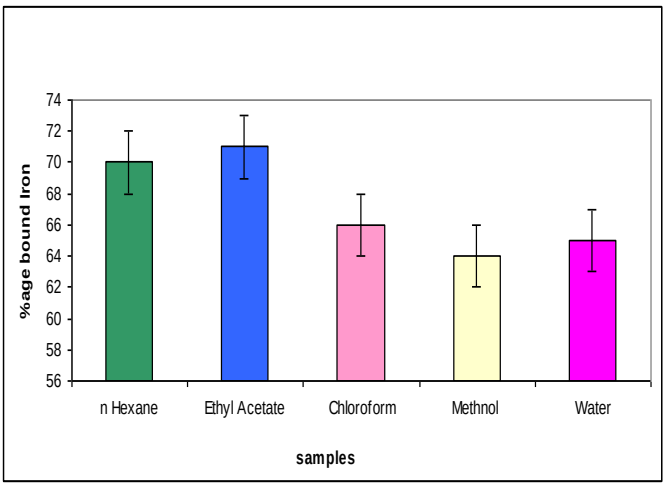

Figure 8. Metal chelating activity of ML.

blue tetrazolium (NBT) as a probe. The results of this assay showed that percentage scavenging of superoxide radical anion is maximum for n-hexane fraction of ML. The ethyl acetate fraction of ML showed a slightly less percentage scavenging of superoxide radical anion, but the water and chloroform fractions show similar scavenging levels. The methanol extract showed a slightly less percentage scavenging of superoxide radical anion (Figure 7). The increasing order of percentage scavenging of superoxide radical anion is following:

n hexane>Ethyl acetate>water> chloroform>methanol

Fraction showed the maximum value of percentage of bound iron. Other fractions also show metal chelating activity but the value of percentage of bound iron is less than the ethyl acetate (Figure 8). The trend representing the order of percentage of bound iron is shown below:
Ethyl acetate>n hexane>chloroform>water>methanol

Metal chelating activity

The chelation of Iron (II) is usually determined by ferrozine assay. The results of this assay are expressed as percentage of bound iron. Iron (II) forms a colored complex with ferrozine which can be determined at 562 nm. The Ethyl acetate fraction of ML showed the maximum value of percentage of bound iron. Other fractions also show metal chelating activity but the value of percentage of bound iron is less than the ethyl acetate (Figure 8 and Table 1). The trend representing the order of percentage of bound iron is shown below:

Ethyl acetate>n hexane>chloroform>water>methanol

Table 1. Results of phytochemical screening.

Name of the sample extract	Reducing sugar	Tannins	Anthraquinones	Saponins	Alkaloids	Flavonoid	Terpenoids	Cardiac glycosides
n-hexane	+	-	-	+	-	-	-	+
Methanol	+	+	+	+	-	+	+	+
Ethyl acetate	+	+	-	+	-	+	+	-
Chloroform	+	+	+	+	-	+	+	+
water	+	-	+	+	-	+	+	-

Conclusion

The results of phytochemical constituents revealed that leaves of *Monotheca buxifolia* are chemically enriched with flavanoids, terpenoids, saponins, anthraquinones, cardiac glycosides, tannins and reducing sugars. The obtained results showed that the aqueous fraction has highest Trolox equivalant antioxidant activity (TEAC value), percent inhibition of DPPH and FRAP value while the ethyl acetate fraction showed highest metal chelating activity and total flavonoid contents. n-hexane and chloroform showed the highest superoxide anion radical scavenging activity and total phenol contents respectively.

Due to the presence of such compounds the aqueous fraction showed excellent antioxidant activity, the ethyl acetate fraction showed moderate activity, methanol fraction has lowest and methanol fraction has very low values of antioxidant activity due to lower amounts of such compounds. Hence, it was concluded that aqueous fraction is rich in strong antioxidants.

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