EVALUATION OF *IN VITRO* ANTI-ARTHRITIC AND ANTIOXIDANT ACTIVITIES OF EXTRACTS OF *COTULA ANTHEMOIDES* L.

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ABSTRACT

Background: *Cotula anthemoides* L. commonly recognized as peeli booti (babuna), is ubiquitous globally particularly in warm regions of the world. This plant has been practiced in rural areas for pain management associated with arthritis. Some of its constituents discovered in previous studies have been speculated to possess antioxidant properties as well.

Aim: Current study manifests the *in vitro* anti-arthritic and antioxidant activities on the plant. Proximate analysis, phytochemical analysis (primary and secondary metabolites) UV visible scanning and FTIR were performed on the powder as well as on the extracts of plant.

Results: The results came out to be as moisture content 5%, total ash 15.23%, acid insoluble ash 0.99%, water soluble ash 9.66%, sulphated ash 16.69%, alcohol soluble extractives 10.25%, and water-soluble extractive values 62.95% all the values came out to be under range of British Pharmacopeia. Different extracts of plant of *C. anthemoides* L. were squeezed out by hot extraction technique. UV visible scanning and FTIR scans demonstrated the

characteristic features and presence of various functional groups. The antioxidant activity was investigated following DPPH, phosphomolybdenum and ABTS models using ascorbic acid as standard while anti-arthritic activity was analyzed by inhibition of protein denaturation method using standards diclofenac sodium and acetyl salicylic acid.

Conclusion: Study results revealed that plant possesses both activities and has the potential to be explored further for its ultimate use in future medications.

Keywords: *Cotula anthemoides* L., DPPH, anti-arthritic,antioxidant, phosphomolybdenum, ABTS

INTRODUCTION

According to the World Health Organization (WHO), about three-quarters of the world population relies upon traditional remedies (mainly herbs) for the health care of its people [1]. Herbal medicines have less adverse effects as compared to synthetic drugs because of combinations of medicinal constituents coupled with minerals and vitamins [2]. About 1% of population is afflicted by rheumatoid arthritis and the ratio is two to three times more common in women than in men [3]. Many synthetic drugs are used for the treatment of arthritis to reduce the inflammation and pain but result in showing significant side effects. Herbal plants, dietary sources and natural compounds are used to reduce the pain and inflammation with fewer side effects [4]. A large number of medicinal plants have been tested and found to contain active principles with curative properties against arthritis [5]. Likewise, free radicals are being produced after the process of oxidation in the body, these free radicals may prove to be damaging for the cells. The first line defense against the free radical specie is

preventing their formation. Present study is carried out to evaluate the anti-arthritic and anti-oxidant potentials of plant *Cotula anthemoides* L. Presence of minerals and trace elements in herbs and medicinal plants has raised the need of finding their levels through different techniques [6]. Thus, standardization verifies the presence of one or more active constituent in the medicinal plant. Herbal products lack the ability to be used actively as therapeutics agents because of lack of standardization. This has proved to be the major resistive factor in uplifting the use of herbal products. There are many factors like collection of material, environmental conditions, and development of plant according to the nature of soil, drying techniques, and handling of the plant which cause the variation in percentage content of active material and phytochemicals in plant. Valuation of primary and secondary metabolites have been suggested for standardization [7].

Cotula anthemoides is found on moist places. There are around 80 species of the genus Cotula [8]. *Cotula* species are traditionally used as anti-inflammatory, antipyretic, antiprotozoal, analgesic, bacteriostatic, or antiseptic agents and in the treatment of digestive disorders [9]. According to the literature review this plant has been used anciently for the treatment of arthritis. The whole plant heated with mustered oil and decoction is applied externally on affected parts 2-3 times a day in rheumatism [10]. There are many biologically active compounds found in the aerial parts of this plant. The whole plant of cotula is hot extracted, cooled and dries. This mixture is then applied to the affected areas for few days. Visible ailment is observed with the usage of this plant According to the Studies which have been conducted on the aerial part of *Cotula anthemoides L.* by using techniques of Gas chromatography and GC-MS. 0.7% yield of Essential oils were obtained which have

intense penetrating odor and are yellow in color. The ratio of essential oil of *C. anthemoides L.* is 98% which includes camphor (27.4%), thujone (12.9%), camphene (10.7%), santolina triene (13.0%), and -curcumene (5.3%). GC-MS was performed, and the obtained concentration came out to be different than the reported concentrations. agarospirol (10.4%), and by 1-eicosanol (17.1%) in stems and by hexacosane (31.7%) in flowers, Roots oil hold the major constituents which are: heptacosane (28.4%) , octacosane (5.4%), 1-eicosanol (14.6%) and-amorphen (5.2%). The bacteria against which essential oils obtained from plant was used were namely *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* (HS), *Escherichia coli* ATCC 25922, *Escherichia coli* (HS), *Enterobacter aerogenes* (HS), *Klebsiella pneumoniae* (HS), *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* (HS), and *Proteus mirabilis* (HS). The growth of most of the bacteria was inhibited by the essential oils and agar dilution method helped in finding the minimum inhibitory concentrations of essential oils [11].

Column chromatography of methanolic extracts from aerial parts of *Cotula anthemoides* L. has led to the isolation of 11 constituents. Four constituents named as coumarin, ursolic acid, α-pinene and gibberlic acid A-3 were discovered from spectral analysis. This plant also contains constituents which possess anticancer and anti-microbial properties [12]. Also many Antiradical potential and anti-tyrosinase activity of compounds were isolated from the aerial parts of *C. anthemoides* L. [13]The major traditional uses when later brought into researchers consideration concluded that these active compounds can be used therapeutically as well in medications. The presence of important phytochemicals in *C. anthemoides* L. have made this plant eligible for bringing it into research. For this reason,

study is conducted to expose the analytical calculations, primary and secondary metabolites, anti-arthritic and antioxidant activities from the extracts of the whole plant.

MATERIALS AND METHODS

Plant material

The whole plant of *Cotula anthemoides* L. was collected from the garden of GCU Lahore, Punjab, in March 2018. The model was submitted in the herbarium of Government College University (GC. Herb. Bot.). The whole plant was shade dried for 15 days under fresh air. Dried plant was abraded and crushed into fine powder, which is stored in airtight container for further testing. Analytical graded solvents and chemicals were used in the whole procedure which were purchased from Merck, Germany and BDH England.

Preparing of extracts

Hot extraction:

The process of hot extraction was carried out very cautiously through the apparatus named as Soxhlet apparatus. Powder weigh about 60g is taken in the thimble which is placed in apparatus. First of all, petroleum ether (B.P 42-62°) was used, second solvent used was chloroform (B.P 61-62°) and lastly methanol (B.P 64-65°). Solvents were used in the volume of 1.5 liters and extraction was proceeded till the siphon was cleared off. This process was repeated with petroleum ether, chloroform, and methanol separately. Extracts were collected separately and dried [14]. For the drying of extract rotary evaporator is used. The temperature of rotary is kept below the boiling point of all the solvents. After drying extract was collected in tared washed and labelled dishes which is kept in oven at 40ºC for further proper drying. The extracts which were in semi solid forms were taken in labeled and tared vials or dishes, which after drying in oven are stored in cool place for investigations.

Proximate analysis

The extractive values such as alcohol soluble and water-soluble extractive values were

figured out using the procedures which are mentioned in USP 2005. Moisture content was expressed as the percent of the air-dried powder. The extractive values shows the of presence of polar compounds like tannins, phenols and glycosides [15]. Total ash, water soluble ash, acid insoluble ash and sulphated ash were determined. The values came out to be under good ranges which are mentioned ahead in results. These extractive values tells the type of constituents present in the crude drug.[16]

Determination of primary and secondary metabolites

Phytochemical screening is important because 80% of population relies on medicinal plant which involves the plant extracts [17]. There are two groups of phytochemicals, primary metabolites, and secondary metabolites. The primary metabolites include total proteins, total lipids, and carbohydrates. Primary metabolites have an important role in the development and growth of the plants while the secondary metabolites has important role in the defense mechanism of plants. They provide protection against various issues. Plant Extracts of *Cotula anthemoides L.* were studied for quantification of metabolites (primary and secondary metabolites) such as total proteins [18], total polyphenols [19], carbohydrates, total lipids [20], total flavonoids [21], and total glycosaponins [16] and total polysaccharides [7]. The results from the various experiments carried out above shows that the plant material contains various quantities of carbohydrates, lipids, and proteins. The secondary metabolites which include total polysaccharides, total polyphenols, total flavonoids, total glycosponins showed presence after performing experiments.

Analytical studies

FTIR scans and UV visible profiling

The presence of various functional groups was known by FTIR scan of powder of plant. KBr disc method was used to obtain spectra. The plant contains useful primary

metabolites like carbohydrates, proteins, and lipids. The UV Visible profiling by using the ultraviolet visible spectrophotometer indicates that lambda maximum of all extracts came out to be in region of 200 nm-400 nm.

Anti-Arthritic activity

Evaluation of In vitro anti-arthritic activities are done by method: Inhibition of protein denaturation and Bovine serum albumin (BSA) denaturation method.

Inhibition of protein denaturation

In this method the test solution is prepared which consisted of 0.45 ml of Bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of different concentrations of all extracts of *C. anthemoides* L. (100 and 250 µg/ml) in DMSO. Next, we will prepare the Test control (0.5ml) consisted of 0.45 ml of bovine serum albumin and 0.05 ml of distilled water.

While the product control (0.5 ml) consist of 0.45 ml of distilled water and 0.05 ml of all the extracts of plant. The standard solution consisted of 0.45 ml of Bovine serum albumin and 0.05 ml of acetyl salicylic. These obtained samples were incubated for 20min at the temperature of 37 °C and later temperature is increased up to 57 °C where samples are kept for time duration of three minutes. After cooling of above samples in tubes, 2.5 ml of phosphate buffer saline (pH 6.3) was added to each tube. The absorbance was measured at 660 nm by using spectrophotometer. The control represented 100% of protein denaturation. The results were compared with acetyl salicylic acid. The percentage inhibition of protein denaturation [22]was calculated as below:

$$\text{Percent inhibition} = \frac{(\text{O.D of test solution} - \text{O.D of product control}) \times 100}{\text{O.D of test control}}$$

(O.D = Optical density)

Bovine serum albumin (BSA) denaturation method

This method was reported by [23] for the investigation of anti-arthritic activity. All the extracts of plants were used for this method

to find the maximum activity holding extract. In this method the test solution (0.5 ml) contained 0.5% w/v aqueous solution of BSA (0.45 ml) and test solution (0.05 ml) of different concentrations. Test control solution (0.5 ml) contained 0.5% w/v aqueous solution of BSA (0.45 ml) and distilled water (0.05 ml). Product control (0.5 ml): 0.45 ml distilled water and test solution (0.05 ml) of different concentrations were used. Standard solution (0.5 ml): 0.5% w/v aqueous solution of BSA (0.45 ml) and diclofenac sodium (0.05 ml) of different concentrations were used. Test solution (0.05 ml) of different concentrations (2, 5, 10, 20, 30 and 60 µg/ml) and standard drug diclofenac sodium (0.05 ml) of different concentrations (2, 5, 10, 20, 30 and 60 µg/ml) were mixed with 0.5% w/v aqueous solution of BSA (0.45 ml). Then the samples were incubated at 37 ℃ for 20 min followed by incubation at 57 ℃ for 3 min. 2.5 ml of phosphate buffer (pH 6.3) was added to all the above samples after cooling. UV-Visible spectrophotometer (Analab) was used to measure the absorbance at 255 nm. The control represents 100% protein denaturation. The percentage inhibition of protein denaturation was calculated by the following formula:

$$\text{Percent inhibition} = \frac{(\text{O.D of test solution} - \text{O.D of product control}) \times 100}{\text{O.D of test control}}$$

O.D = Optical density

Antioxidant activities

DPPH Assay

The extracts of plant were subjected to anti-oxidant activity by using DPPH (2,2-diphenyl-1-picrylhydrazyl) using the technique [24] with few modifications. In this method DPPH solution (1 ml, 0.1mM) was added to the sample solution (1 ml) and 2 ml of methanol. Abatement in the concentration of free radical was monitored after thirty minutes, using uv-visible spectrophotometer at 517 nm in comparison to blank. Absorbance of control and standard which is Ascorbic acid was also measured. All

readings were taken in triplicate and mean values were used for calculations. Percent inhibition can be analyzed by the subsequent equation:

$$\% \text{ Inhibition} = \frac{Ac - As}{Ac} \times 100$$

Ac = Absorbance of control

As = Absorbance of sample

Phosphomolybdenum assay

[25] Prieto performed and described this method for antioxidant activities. In this method Extract solution (0.5 ml, 1mg/ml of methanol) is added to 4.5 ml of reagent solution. This reagent solution comprised of 0.6 M sulfuric acid (1.5 ml), 28 mM sodium phosphate (1.5 ml) and 4 mM ammonium molybdate (1.5 ml). Then the incubation of obtained solution was done for time-period of 90 mins at temperature of 95°C and after this it is cooled to room temperature, the absorbance was measured at 695 nm using uv-visible spectrophotometer against blank. All readings were taken in triplicate and average values were used for calculations while percentage inhibition was analyzed through equation.

ABTS assay

[26] Al-Qudah proposed radical cation 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS•+) decolorization assay method. The ABTS•+ cation radical solution was prepared by reacting quantities of 7 mM of ABTS and 2.4 mM of potassium persulfate (K2 S2 O8) solutions for 16 hour in dark at room temperature. The solution was diluted with methanol. The reaction mixture contains 3 mL of ABTS•+ solution and 1 mL of the sample solutions of methanol extracts at different concentrations (0.10 - 1.00 mg/mL). The absorbance of blank and all prepared solutions is measured using UV spectrophotometer at 734 nm. ABTS scavenging capacity of the sample was compared with that observed for ascorbic acid and three readings were taken and percentage inhibition was analyzed through equation.

RESULTS AND DISCUSSIONS

Physicochemical analysis of C. anthemoides L. plant powdered material.

To evaluate the quality and purity of powder of whole plant different ash values and extractive values are determined. Any foreign material like silica, oxalate carbonates etc. can be identified and determine from ash value tests [27]. The results came out to be as moisture content 5%, total ash 15.23%, acid insoluble ash 0.99%, water soluble ash 9.66%, sulphated ash 16.69%, alcohol soluble extractives 10.25%, water soluble extractive values 62.95%. All the values mentioned under table came out to be under range according to British Pharmacopeia. (Table 1)

Estimation of primary and secondary metabolites

Primary metabolites have an important role in the development and growth of the plants while the secondary metabolites has important role in the defense mechanism of plants.

The results show that the plant material contains various quantities of carbohydrates, lipids, and proteins. Chloroform extract of plant contains the maximum quantity of polyphenols while the petroleum ether extract contains the minimum quantity of polyphenols. The regression line equation for polyphenol is $y= 0.013x+0.0935$, $R^2= 0.985$). Chloroform extract contains the maximum number of total flavonoids and linear regression line equation is ($y = 0.0071x + 0.2209$ $R^2 = 0.9816$). Total polysaccharides are present in maximum amount in the extract of chloroform and its regression equation is ($y = 0.0024x +0.0507$ $R^2 = 0.9569$). Methanol extract contains the maximum amount of glycosponins. it proved from the above study that the estimation of primary and secondary metabolites is useful for standardization of extracts of plant. Physicochemical properties of extracts of plant were determined and analysis shows that they are within specific ranges which are mentioned in the table.

These parameters are very significant because they are used to evaluate the quality, purity, and efficacy of medications (Table 2, Table 3).

FTIR and UV

The spectrum indicates the presence of O-H stretching (H-bonding) at the range of 3000-3500 cm-1. RCOOH, RNH_3^+, $R_2 NH_2^+$, and $R_3 NH^+$ in range of 2500-3000 $cm^{-1.}$ C-H stretching and C=C stretching can be observed at 1500-2000 cm^{-1}Whereas, C-C stretching is visible at the 1403.93 $cm^{-1.}$ The peak in the range of 1000-1200cm-1 clearly shows the presence of polysaccharides, aliphatic phosphate (P-O-C) stretching and organic silicone (Si-O-Si), C-O single bond. This obtained spectrum shows the presence of some important functional groups as well, for example, polyphenols, glycosaponins, amines and flavonoids. While in UV results Chloroform extract of plant. shows maximum peaks at 250nm which is in ultraviolet region. The methanol extract shows second highest peak and petroleum ether shows minimum peak in the region. The maximum ultraviolet absorbing species are present in chloroform extract. The order of highest peaks of these extracts is chloroform > methanol > petroleum ether (Fig. 1.1, Fig. 1.2).

Inhibition of protein denaturation:

According to the procedures described for protein methanol extract of plant *C. anthemoides L.* show maximum anti-arthritic activity with concentrations of product control i.e., at 100 µl. While chloroform showed little activity at 100 µl and petroleum ether extract show least activity (Table 4, Fig. 4.1).

Bovine serum albumin (BSA) denaturation

The results exhibit that methanol extract of plant *Cotula anthemoides L* showed clear activity and anti-arthritic effects. Arthritis is an autoimmune disease which shows the effects of denaturation of proteins. And BSA denaturation was inhibited by methanolic

extract of plant. Hence, it is deduced based on aforementioned in-vitro anti-arthritic activity that methanolic extract of plant showcases the myriad potential to inhibit the protein denaturation and may be envisaged to give us alternate treatment for arthritis among others. Data explicitly demonstrate the direct increase relationship found between percentage inhibition and concentration increase and it also correlates to the standards like diclofenac sodium and acetyl salicylic acid (Fig. 5.1, Table 5).

Antioxidant activity

While *in vitro* activities of DPPH, phosphomolybdenum and ABTS assay proves that methanol extract of plant shows significant antioxidant activity with reference to the standard which is Ascorbic acid. DPPH assay showed the percentage inhibition of methanol extract which is 80.76% and is closer to ascorbic acid calculated as 82.41% After methanol, chloroform possess the activity while petroleum ether showed minimum or zero activity. Phosphomolybdenum assay showed the similar results of maximum percentage inhibition of methanol extract which is 58% and nearest to the standard. ABTS assay was carried on methanol extract only with different concentrations which showed closer inhibition values to the standard with same concentrations and results are expressed in table as well as form of chart ahead. This plant possesses some medicinally active components which can be used therapeutically in future for medications of ailments related to arthritis and can be used as antioxidants as well (Fig. 6.1, Fig. 7.1, Fig. 8.1, Table 6, Table 7, Table 8).

CONCLUSION

Physicochemical analysis of plant *C. anthemoides* L. and estimation of its primary and secondary metabolites showed the presence of polysaccharides, flavonoids, glycosaponins and polyphenols while extractive values are under the ranges required to evaluate the anti-oxidative and anti-arthritic potential of plant. UV analysis

showed the maximum ultraviolet absorbing species are present in chloroform extract while FTIR analysis is carried out and presence of various functional groups is identified. Under this consideration in this study, multiple investigations were performed on the extract of whole plant of *C. anthemoides* L. which gave plausible clues and highlighted the presence of therapeutically active components which ultimately can be used in the ailments and as medicines in future. In vitro anti-arthritic activity carried out by bovine serum albumin showed the visible potential of reducing arthritic inflammation which can be useful in future for the treatment of arthritis. Methanol extracts showed the significant percentage inhibition in close ranges with that of standard. Furthermore, medicinally active components in extracts of plant. *C. anthemoides* L. shows antioxidant activity. DPPH, phosphomolybdenum and ABTS assays were carried out on the extracts of plant which were compared with standard used i.e., ascorbic acid. Methanol extracts showed antioxidant potential which can be used in medicines in future. On one hand this study put forth the enormous anti-oxidative and anti-arthritic potential of *C. anthemoides* L. whereas on the other hand it also set the map for in vivo studies to be carried out by scientific community soon which definitely would be crucial for its practical clinical translation.

The authors declare no conflict of interest.

TABLES:

1. Table for physicochemical analysis of *C. anthemoides* L. plant powdered material.

Physicochemical property	% age content ± SD
Moisture content	5 ± 0.013
Total ash	15.23 ± 0.22
Acid insoluble ash	0.99 ± 0.15
Water soluble ash	9.66 ± 0.34
Sulphated ash	16.69 ± 0.38
Alcohol soluble extractive	10.25 ± 0.33
Water soluble extractive	62.95 ± 0.66

2. Table for the estimation of primary metabolites:

Sr. No.	Primary metabolites	Quantity± SD %w/w
1.	Total proteins	6.5 ± 0.03
2.	Total lipids	18.6 ± 0.1
3.	Total carbohydrates	54.52 0.22

3. Table for the estimation of secondary metabolites

Extracts	Total Polyphenols mg/g	Total Flavonoids mg/g	Total Polysaccharides mg/g	Total Glycosaponins mg/g
Petroleum ether	14.32±0.234	0.812±0.215	21.094±0.637	49.066±1.222
Chloroform	40.88±0.279	64.61±0.357	113.87±1.101	82.4±2
Methanol	27.93±0.347	1.844±0.422	97.76±0.636	95.066±2.44

4. Table Inhibition of protein denaturation

Sample	Concentration µg/ml	Absorbance (mean)	% inhibition ± S.D
Standard 1 (PE)	100	0.56	49.23 ± 0.652
Petroleum ether 1	100	0.051	41.58 ± 1.535
Standard2 (chloroform)	100	0.56	69.23 ± 1.051
Chloroform 2	100	0.052	63.07 ± 1.559
Standard3 (methanol)	100	0.56	93.84 ± 1.08
Methanol 3	100	0.055	92.30 ± 0.386

5. Table for Bovine Serum Albumin (BSA) denaturation

Treatment	Concentration µg/ml	Absorbance (mean)	%Inhibition at 100 µl ± S.D
Control		0.268	
Diclofenac sodium 1	10	0.302	71.64 ± 0.966
Diclofenac sodium 2	20	0.287	77.23 ± 0.850
Diclofenac sodium 3	30	0.272	82.83 ± 1.009
Diclofenac sodium 4	60	0.241	94.40 ± 0.780
Diclofenac sodium 5	80	0.225	100 ± 0.577
Methanol 1	10	0.272	82.83 ± 1.694
Methanol 2	20	0.264	85.82 ± 0.860
Methanol 3	30	0.258	88.05 ± 1.100
Methanol 4	60	0.232	97.76 ± 1.068
Methanol 5	80	0.229	98.88 ± 1.116

6. Table for DPPH model

Extracts	**Absorbance with DPPH (Mean)**	**%age activity Ac – As/Ac × 100 ± S.D**
Test control	0.182	
Petroleum ether	0.181	0.00 ± 0.577
chloroform	0.083	54.39 ± 0.88
Methanol	0.035	80.76 ± 1.051
Ascorbic Acid (standard)	0.032	82.41 ± 1.22

7. Table for Phosphomolybdenum

Sample	**Absorbance (mean)**	**%age activity= Ac– As/Ac × 100 ± S.D**
Control	0.397	
Petroleum ether	0.358	9.82 ± 0.935
Chloroform	0.329	17.12 ± 1.126
Methanol	0.166	58 ± 0.555
Ascorbic acid (standard)	0.121	54.15 ± 1.173

8. Table for ABTS activity

Sample C (mg/mL)	**Absorbance (Mean)**	**%age activity Ac – As/Ac × 100 ± S.D**
T.C	0.503	
0.10	0.298	40.75±1.27
0.20	0.266	47.11±0.98
0.40	0.256	49.10±0.82
0.60	0.249	50.49±0.47
0.80	0.245	51.29±0.60

1.00	0.234	53.47±0.57
Standard Concentration	**Absorbance (Mean)**	**%age activity** **Ac – As/Ac × 100 ± S.D**
0.10	0.22	56.26±1.17
0.20	0.192	61.82±0.93
0.40	0.183	63.61±0.589
0.60	0.176	65.00±0.32
0.80	0.167	66.79±0.89
1.00	0.156	68.98±0.74

Figures:

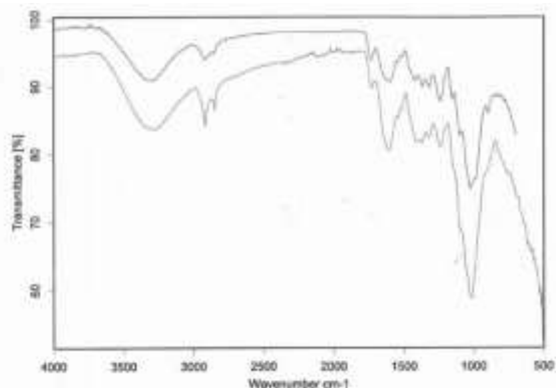

Fig. 1.1 FTIR scans of powder of *C. anthemoides* L.

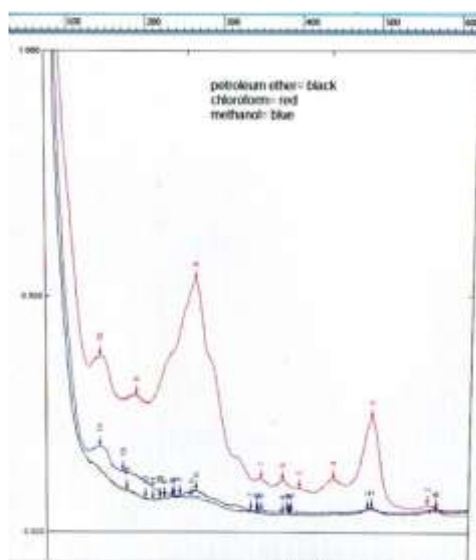

Fig.1.2 UV visible spectrum of extracts of plant *C. anthemoides L.*

Inhibition of protein denaturation at 100ml

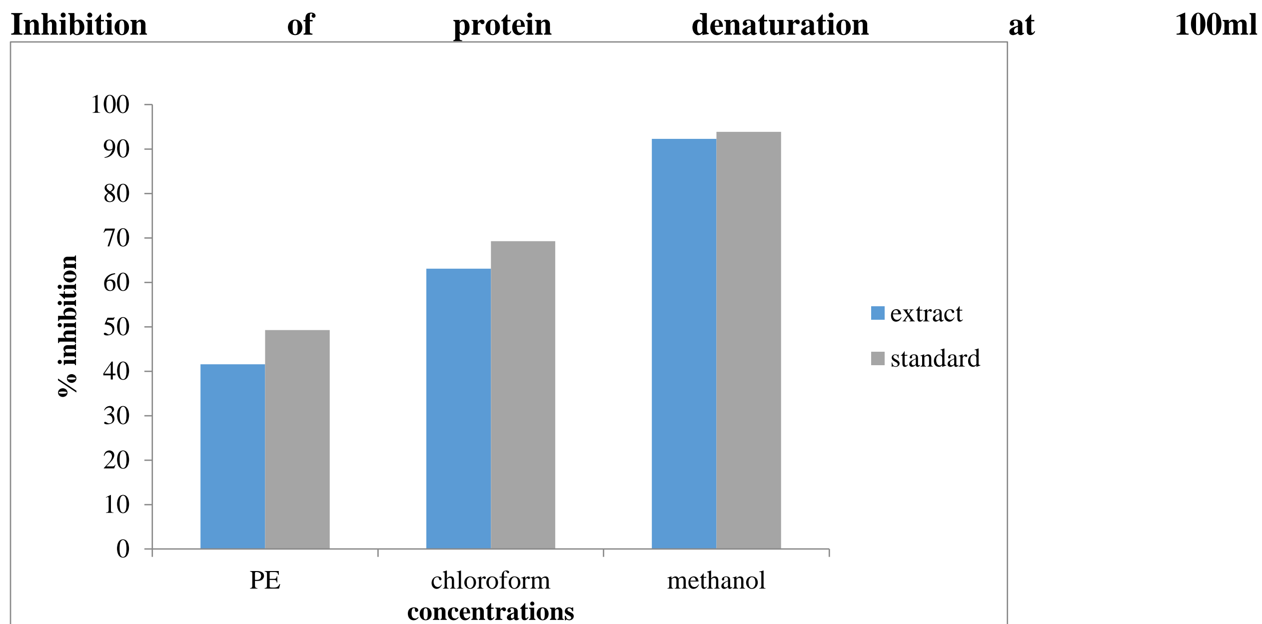

Fig. 4.1 Comparison of standard and extracts possessing inhibition of protein denaturation with concentration of product control as 100 µl

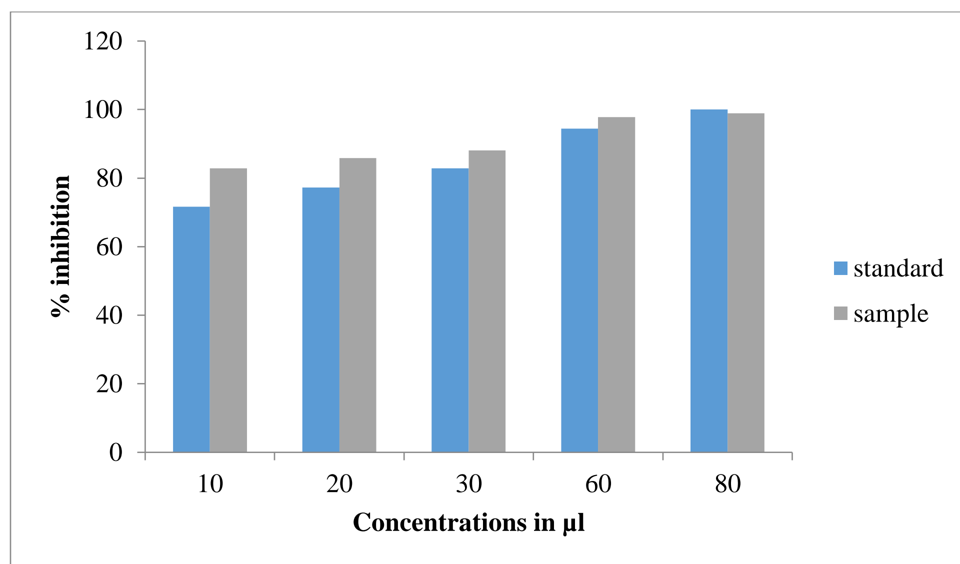

Fig. 5.1 Percentage inhibition of BSA denaturation

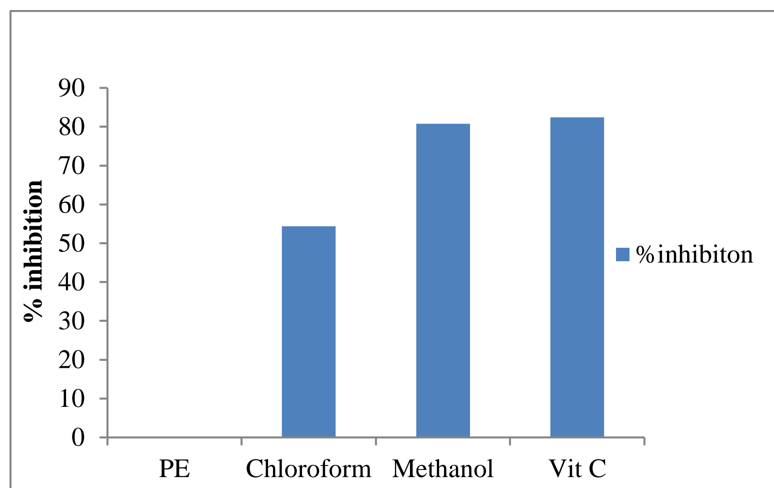

Fig. 6.1 Graph showing DPPH antioxidant assay.

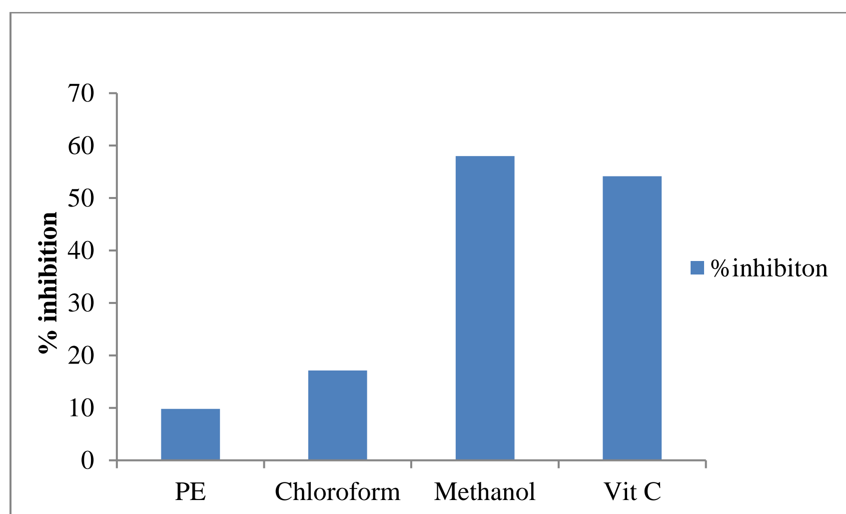

Fig 7.1 Graph showing phosphomolybdenum antioxidant assay.

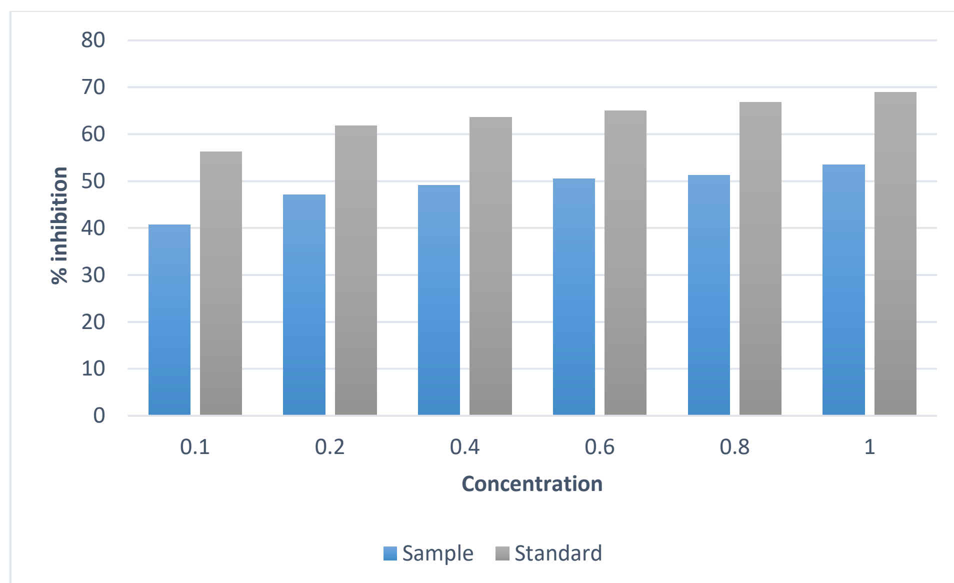

Fig 8.1 Graph showing ABTS antioxidant assay

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