

Evaluation of antioxidant efficacy of different fractions of *Tagetes erecta* L. Flowers

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Abstract: Oxidative stress is an important pathogenesis of numerous chronic diseases. Free radicals and other reactive oxygen species are recognized as agents involved in the pathogenesis of sicknesses. The main characteristic of an antioxidant is its ability to trap free radical. Plants rich in phenolic compounds are generally reported to show good antioxidant activity. *Tagetes erecta* L (Marigold) belongs to the family Asteraceae. The flowers are especially employed to cure eye diseases, colds, conjunctivitis, coughs, ulcer, bleeding piles and to purify blood. The extractive efficiency of phenolic compounds from plant material is greatly dependent on the choice of solvent. In this study, six solvents of different polarity viz., hexane, toluene, ethyl acetate, acetone, methanol and water have been used for the extraction of *Tagetes erecta* L. flower by individual cold percolation method. Phenolic and flavonoid content of extracts were determined using Folin-ciocalteu assays and aluminium chloride colorimetric method respectively. Antioxidant activity was carried out by DPPH radical scavenging activity assay, superoxide free radical scavenging (SO) assay, ABTS radical cation scavenging activity, ferric reducing antioxidant power (FRAP), Reducing capacity assessment. Maximum extractive yield was in water extract followed by methanol extract. There was a direct correlation between phenolic content and antioxidant activity. Methanolic extract had maximum phenolic content and maximum FRAP and SO activity. ABTS and DPPH did not show positive correlation with total phenol content in ethyl acetate extract. Ethyl acetate extract had maximum flavonoid content. *T. erecta* flowers can be used as a natural source of antioxidants to combat the oxidative stress related disorders.

Key word: *Tagetes erecta*, Influence of solvent, Antioxidant activity, positive correlation

I. Introduction

Nature is the best combinational chemist and has cure for all diseases and illness of mankind. Medicinal plants are back bone of several indigenous traditional system of medicine. Traditional used medicinal plants are screened for their antimicrobial efficacy (kalayou et al., 2012). The undesirable effect of the modern medicine has diverted the attention of the people toward herbal medicines.

Oxidative stress is an imbalance between reactive oxygen species (ROS) production and the intracellular capacity for removing ROS. Its leading to excessive damage of all bio molecules like DNA, RNA, lipids, proteins micronutrients like carotenoids, vitamins, etc (Lee et al., 2010) The oxidation induced by free radicals can result in cell membrane destruction and membrane protein disintegration and mutation, which can further initiate the development of many diseases, such as cardiovascular disease, cancer, liver injury, and arthritis (Alok et al., 2014)

Medicinal plants produce many substances that are biologically active and working together catalytically and synergistically to increase the activity (Patwardhan and Gautam, 2005). The plants have effective capacity to scavenge free radicals and represent a source of multifunctional properties. Medicinal plants are rich in secondary metabolites that exhibit a remarkable diversity of both chemical structures and biological activities and are promising source of lead compounds for new drugs targeting neurodegenerative diseases (Dastmalchi et al., 2007).

Plants have different phytochemicals and depending on their type and concentration they will demonstrate different radical scavenging capacities (Dragland et al., 2003). They may exert cell defensive action by more than one biochemical mechanism (Velioglu et al., 1998). Plant antioxidant play a major role to combat against cellular damage and disease, each plant plays a specific role in disease prevention and treatment.

Synthetic phenols such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and propyl gallate are most frequently used as antioxidants. However, the safety of some of these compounds is questioned because they have many side effects and are also reported to be carcinogenic. Therefore much interest has been focused on natural antioxidants. Natural antioxidants occur in all higher plants and in all parts of the plant like wood, bark, stems, pods, leaves, fruits, roots, flowers and seeds (Kim et al., 1997). It is now well recognized that the antioxidant activity of these plants is mainly due to the presence of phenolic compounds (Knezeric et al., 2012).

The extraction yield and the antioxidant activity of the extracts from plants highly depend on the solvent polarity, which determines both quality and quantity of the extracted antioxidant compounds (Franco et al., 2008). The properties of extracting solvents considerably affected the total phenolics content and antioxidant capacity in fruits and vegetables (Michiels et al., 2012). Solvent polarity is very important factor higher the polarity, better the extraction of phenolic compounds (Naczki et al., 2006). The polarity of solvent and its types can influence the mechanisms of hydrogen atom transfer (HAT) and single electron transfer (SET), which are critical for measuring antioxidant properties (Perez-Jimenez and Saura-Calixto, 2006). Different solvents systems have been used for extraction of phenol from plants and their efficiency varies. Commonly used solvents for extracting various substances from plant material are petroleum ether, toluene, ethyl acetate, acetone, and water (Kanerla et al., 2011).

Tagetes erecta L. popularly known as marigold, is grown as an ornamental plant. Flowers of this plant are used in garlands for societal and religious purposes in Eastern countries. The flowers are usually thrown away after their spiritual uses. This plant belongs to the family Asteraceae (Compositae). Different parts of *T. erecta* plant including flower is used in folk medicine. The flowers are especially employed to cure eye diseases, colds, conjunctivitis, coughs, ulcer, bleeding piles and to purify blood (Manjunath 1969; Kirtikar et al. 1994; Ghani, 2003). In the present study, the influence of solvents of various polarity on antioxidant potential of *T. erecta* flowers is evaluated employing various *in vitro* assay systems like DPPH, superoxide free radical scavenging activity, ABTS, FRAP and reducing power.

II. Materials and methods

Chemicals

All the chemicals were obtained from Hi Media Laboratories and Sisco research Laboratories Pvt. Limited, Mumbai, India. Ultra purified water was used for experiment. Solvents hexane(HE), toluene(TE), ethyl acetate(EA), acetone(AC), methanol(ME), and so on were obtained from Merck, India

Plant material

Fresh flowers of *Tagetes erecta* were collected from Rajkot, Gujarat, India. Flower was identified by comparison with specimens at Department of Biosciences (SU/Bio/517Thakrar), Saurashtra University, Rajkot, Gujarat, India. The flowers were washed thoroughly with tap water, petals were separated and shade dried. The dried petals were homogenized to fine powder and stored in air tight bottles which were later used for solvent extraction.

Extraction and Fractionation

The dried powder of the flower petals was extracted individually by the cold percolation method (Parekh and Chanda, 2007). Fractionation of acetone extract of flower was done by solvent-solvent partition method (Tang et al., 2010).

Determination of total phenol and flavonoid content

The amount of total phenol and flavonoid content of different solvent extracts of *T. erecta* and its fraction was determined by Folin- ciocalteu's reagent method (Mc Donald et al., 2001) and aluminium chloride colorimetric method (Chang et al., 2002) respectively.

Antioxidants activities

The antioxidant activity of the different solvent extracts of *T. erecta* flower and its fraction was evaluated by DPPH free radical scavenging activity, superoxide anion radical scavenging activity, ABTS cation free radical scavenging activity, ferric reducing antioxidant power and reducing capacity assessment by the methods described in our earlier work (Kanerla et al., 2012)

III. Results and Discussion

Extractive yield

The extractive yield of *T. erecta* flower using different solvents with increasing polarity is presented in Fig. 1. The extractive yield was maximum in aqueous extract followed by methanol extract. The extraction ability of different solvents from flower for recovering extractable components followed the order: aqueous > methanol > hexane > acetone > ethyl acetate > toluene. Aqueous extract was superior in their ability to extract phytoconstituents from *T. erecta* (18.11%). Extraction with toluene offered the least yield (0.96%). Methanol and acetone both are polar solvents but methanol had more extractive yield than acetone and non polar solvent hexane had more extractive yield than polar solvent acetone. Both less polar solvents ethyl acetate and toluene had minimum extractive yield. It can be concluded that polar compounds are more than non polar compounds. Significant differences of extractive yield among different solvents might be attributed to the varied polarity of

the solvents used as well as the availability of different extractable phytoconstituents present in *T. erecta* flowers. Under the same time and temperature conditions, the solvent used and the chemical property of sample are the two most important factors (Dorta et al., 2012). The choice of solvent has a great influence on the extraction yield but it does not imply that the solvent which had maximum yield will show maximum activity under investigation (Anwar et al., 2013). Maximum extractive yield was in aqueous extract but this may be because it could solubilize large number of compounds which may or may not have antioxidant property. Effect of extraction solvent on antimicrobial and antioxidant activity of medicinal plant extracts is reported by other researchers (Kanerla et al., 2012; Kchaou et al., 2013)

Total Phenol content

Plant phenolic compounds exhibit remarkable antioxidant activity by scavenging dangerous free radicals like super oxide anion, hydrogen peroxide, hydroxyl radical and nitric oxide generated during normal metabolic processes. Phenolic compounds are secondary plant metabolites with diverse beneficial biological activities such as anti-inflammatory, anti allergic, antibacterial, anti atherosclerotic, anti carcinogenic activity, ant mutagenic, antitumor, antiviral effects (Balasundaram et al., 2006).

Folin-Ciocalteu reagent, a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids, is reduced to blue oxides of tungsten (W_8O_{23}) and molybdene (Mo_8O_{23}) during phenol oxidation. This reaction occurs under alkaline condition provided by sodium carbonate. The intensity of blue colour reflects the quantity of phenolic compounds, which can be measured using spectrophotometer (Conforti et al., 2006).

In the present work, the total phenolic content of *T. erecta* extracts and fractions was measured by the Folin-Ciocalteu method which is expressed as gallic acid equivalents (GAE/g extract) in Fig.2. There were significant differences amongst different solvent extracts. Highest polyphenol content was in polar solvent methanol extract (102.34 mg GAE/g) followed by acetone fraction I and II (79 mg GAE/g) and acetone extract (75.91 mg GAE/g). Semi polar solvent ethyl acetate and aqueous extracts had 50 mg GAE/g and 49.98 mg GAE/g total phenol content respectively. Lowest phenol content was in toluene extract (6.81 mg GAE/g). Effect of solvent was clearly envisaged. Both polar solvents methanol and acetone had more Total phenol content than semi polar solvent extracts toluene and ethyl acetate. Even amongst the polar solvents methanol extract had more Total phenol content than acetone and amongst semi polar solvent extracts ethyl acetate had considerable more Total phenol content than toluene. Ghasemzadeh et al. (2011) report maximum phenol content in methanol extract followed by acetone extract which supports the present results that total phenol content is more in polar solvents (Hegazy et al., 2012; Kamaraj et al., 2012; Kanerla et al., 2012). There is a direct correlation between phenolic content and antioxidant activity of medicinal plants as reported by several researchers (Patel et al., 2011; Kanerla and Chanda, 2013). Plants which have more phenolic content showed good antioxidant activity (Kchaou et al., 2013)

Total Flavonoid content

Flavonoids are a group of polyphenolic components synthesized by plants with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes, anti-inflammatory action, antiviral, reduce blood-lipid and glucose, chelation of metal ions and to enhance human immunity (Atoui et al., 2005; Uma Maheshwari and Chatterjee, 2008).

The total flavonoids content of *T. erecta* flowers was also measured spectrophotometrically by using the aluminium chloride colorimetric assay. The amounts of Total Flavonoid content of *T. erecta* flower is shown in Fig.3. Semi polar solvent ethyl acetate and polar solvent acetone extracts shows highest flavonoids content 277.68 and 202.68 mg/g as compared to other organic solvent extracts. Polar solvent methanol showed flavonoids content 104.32 mg/g followed by acetone fraction I and II. Aqueous and toluene extract showed lower flavonoid content. Significant differences were detected amongst various solvent extracts of *T. erecta* flower.

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH assay is a general test used to evaluate the antioxidant capacity of plant extracts. The DPPH radical is commonly used for fast evaluation of the antioxidant property of a given compound (Bozin et al., 2008). The colour change from purple to yellow after reduction can be quantified by decrease of absorbance at wave length 517 nm. It is usually expressed as IC_{50} value, the amount of antioxidant necessary to decrease the initial concentration of DPPH by 50 %. Lower IC_{50} value indicates high antioxidant activity (Molyneux, 2004).

The *T. erecta* flower extracts showed moderate capacity to neutralize this radical; solvent extracts of varied polarity exhibited a wide range of antioxidant capacities. IC_{50} value of *T. erecta* flower extracts ranged from 30 to 153 μ g/ml (Table.1). Strong DPPH radical scavenging activity was found in the ethyl acetate extract and acetone fraction II. The IC_{50} values of ethyl acetate extract was 30 μ g/ml and acetone fraction II was 36

ug/ml and their concentration range was 5 to 30 ug/ml and 10 to 60ug/ml respectively (Fig. 4). However these activities were lower than that of the standard ascorbic acid antioxidant activity whose IC₅₀ value was 11.4 ug/ml (Table.1). Polar solvent acetone and methanol extracts showed IC₅₀ values 47 ug/ml and 74 ug/ml and their concentration range was 10 to 60 ug/ml and 15 to 90 ug/ml respectively. The DPPH free radical scavenging activity of aqueous extract was in the concentration range 30 to 180 ug/ml and it's IC₅₀ value was 153 ug/ml. Lowest activity was shown by toluene extract whose IC₅₀ value was >1000. Among all the solvent extracts, ethyl acetate and acetone fraction II showed moderate DPPH scavenging activity as compared to other solvent extracts. The DPPH radical scavenging of *T. erecta* flower might be attributed to its hydrogen donating ability

Superoxide anion radical scavenging activity (SO)

Superoxide (SO) and hydroxyl radical (OH) are the two most dangerous free radicals constantly produced in living organisms. SO is considered as major biological source of ROS and they are harmful because they may oxidize and reduce other compounds and potentially form more reactive species via secondary reactions (Estevez and Jordan., 2002). Therefore, superoxide scavenging capacity in the human body is very important as the first line of defence against oxidative stress.

Superoxide anion radical scavenging activity of different solvent extracts and its fraction investigated is shown in Fig. 5. All the different solvent extracts showed poor super oxide anion radical scavenging activity. Out of 7 extracts, 5 extracts showed IC₅₀ values more than 1000 ug/ml, while the reaming extracts showed varied level of activity. Gallic acid was used as standard and its IC₅₀ values was 185 ug/ml. Polar solvent methanol extract and FS I showed superoxide anion radical scavenging activity with IC₅₀ values of 216 and 315 ug/ml respectively and its concentration range was from 90 to 540 and 90 to 630 ug/ml respectively (Fig. 5 and Table. 1).

2, 2'-Azino-bis-(3-ethyl) benzothiazoline-6-sulfonic acid (ABTS) radical cation scavenging activity

ABTS assay is better to assess the antiradical capacity of both hydrophilic and lipophilic antioxidant because it can be used in both organic and aqueous solvent system as compared to other antioxidant assay. ABTS oxidized with PPS (absorption maxima at 734 nm) leads to the generation of ABTS free radicals. This method is based on the ability of antioxidants to reduce the ABTS radical cation (Re at al., 1999). In the present work, different solvent extracts and its fraction were evaluated for their ABTS radical cation scavenging activity. Ascorbic acid was used as standard and its IC₅₀ values was 6.5 ug/ml.

Out of 7 extracts investigated, all the extracts showed ABTS radical cation scavenging activity except toluene extracts. IC₅₀ values ranged from 15 to 102 ug/ml (Fig 6. Table. 1). Ethyl acetate extract and FS II showed good ABTS radical cation scavenging activity with IC 50 values of 15 and 19 ug/ml and its concentration range was from 6 to 36 and 8 to 48 ug/ml respectively (Fig.6 A, F). Polar solvents acetone and methanol extracts showed moderate activity and its IC₅₀ values were 26.4 and 33 ug/ml and concentration range was from 8 to 48 and 10 to 60 ug/ml respectively. Aqueous extract, and FS I showed poor ABTS radical cation scavenging activity (Fig.6 D, E). Among all the extracts, ethyl acetate and acetone fraction I showed moderate ABTS scavenging activity as compared to other solvent extracts. Moderate to weak antioxidant activity by ABTS method was shown by some medicinal plant extracts (Simao et al., 2013).

Ferric reducing antioxidant power (FRAP)

FRAP is considered to be rapid and is of a semi-quantitative assay. In FRAP assay, antioxidant capacity is evaluated based on the ability of the sample extracts to reduce ferric tripyridyltriazine (Fe (III) – TPTZ) complexes to ferrous tripyridyltriazine (Fe (II) – TPTZ). This assay is performed using freshly prepared FRAP reagent consisting of 2,4,6-tris (1-pyridyl)-5-triazine (pH 3.6). A blue product (ferrous-TPTZ complex) is formed due to the reduction of ferric iron in FRAP reagent (Benzie and Strain, 1996). The higher the FRAP value, the higher will be the antioxidant activity of sample extracts. FRAP activity of *T. erecta* flower is shown in Fig.7.

Highest antioxidant activity by the FRAP assay was obtained for the extract produced with polar solvent methanol 25.09 M/g followed by acetone extract 14.83 M/g respectively (Table 1). Acetone fraction I and II showed 14.84 M/g and 15.83 M/g FRAP activity. Semi polar solvent ethyl acetate and aqueous extract showed 8.55 M/g and 7.55 M/g FRAP activity and toluene extract showed least FRAP activity (Fig.7). The results suggest that methanol extract had maximum antioxidant capacity while toluene extract had least. The results also suggest that extracting solvents play an important role in exhibiting the antioxidant properties of medicinal plants. Siddhuraju (2007) reported similar results for *Macrotyloma uniflorum* seed extract.

Reducing capacity assessment

The reducing power of a compound is related to the electron transfer ability and may therefore serve as a significant indicator of its potential antioxidant activity. However the antioxidant activity of plant extracts may be because of various mechanisms like prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging capacity (Yildirim et al., 2001).

In the present work, the electron donating capacity was measured at a concentration range of 20 to 180 ug/ ml (Fig.8). The reducing power of all the solvent extracts was dose dependent which showed steady increase with increase in the concentration of the extract. In the present work, the ability of different solvent extracts to reduce Fe+3 to Fe+2 was compared with that of ascorbic acid (standard) and the results are presented in Fig. 8. Methanol extract showed significantly more reducing capacity than the standard. FS II and Acetone extract showed slightly more and FS I, Ethyl acetate and aqueous extracts showed less activity than the standard. Toluene extract showed least, almost negligible reducing capacity.

The reducing properties are generally shown with the presence of reductones, which have the capacity to donate an electron to free radicals and convert them into stable radicals. Further reductones can react directly with peroxides and also with certain precursors and prevent the formation of peroxides (Wang et al. 2008).

Correlation of antioxidant activity with total phenol content

There are several reports that there is direct correlation between the total phenol content and antioxidant activity (Choudhary and Swarnkar, 2011; Kaneria et al., 2012; Zulkefli et al., 2013). But this is not always true; sometimes there is no correlation between total phenol content and antioxidant activity (Kaneria and Chanda et al., 2012; Chanudom et al., 2013), simply because the non phenolic compounds also contribute to antioxidant capacity of a plant extract (Ismail et al., 2004).

There was a direct correlation between total phenol content and antioxidant activity. Total phenol content was maximum in methanol extract and maximum FRAP and SO activity. On the other hand, the other two antioxidant assay i.e. ABTS and DPPH did not show positive correlation. It is not necessary that total phenol content shows positive correlation with all antioxidant assays. Toluene had low total phenol content and it showed poor antioxidant activity in all assays done. ABTS and DPPH did not show positive correlation with total phenol content in ethyl acetate extract; ethyl acetate extract had lower phenol content and it had higher ABTS and SO activity. Other solvent extracts showed varied levels of correlation. The results again prove that before concluding the antioxidant capacity of a plant extract, there need to be done more than one antioxidant assay and more than one solvent.

IV. Conclusion

It can be concluded that the extracting solvent affects significantly the total phenol content and the antioxidant activity measured and therefore it is suggested to use more than one extraction system for better assessment of antioxidant activity. The factors responsible for antioxidant capacity of *T. erecta* flowers are phenolics and non phenolics. On the whole, *T. erecta* flower extracts exhibited good antioxidant activity. *T. erecta* flowers can be used as a natural source of antioxidants for medicinal use.

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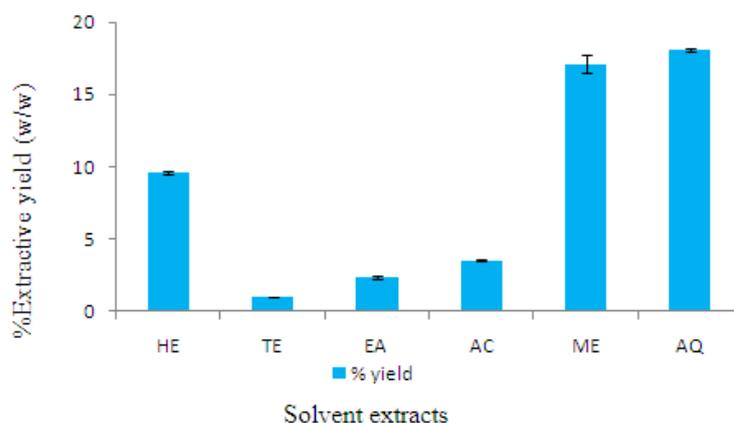


Fig. 1: Extractive yield of *T. erecta* flower in different solvent extracts

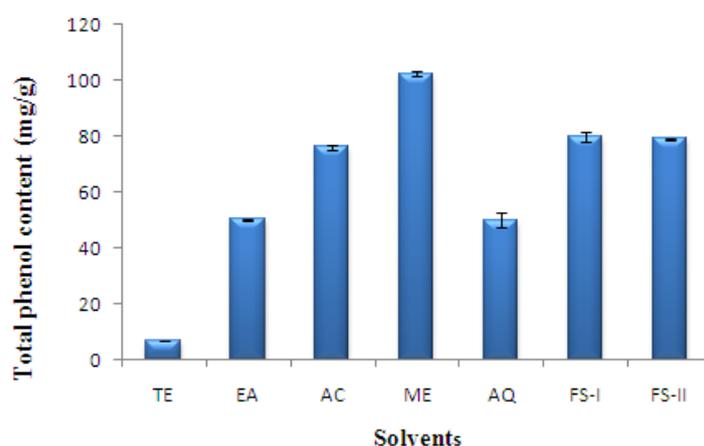


Fig. 2: Total phenol content of different solvent extracts of *T. erecta* flower

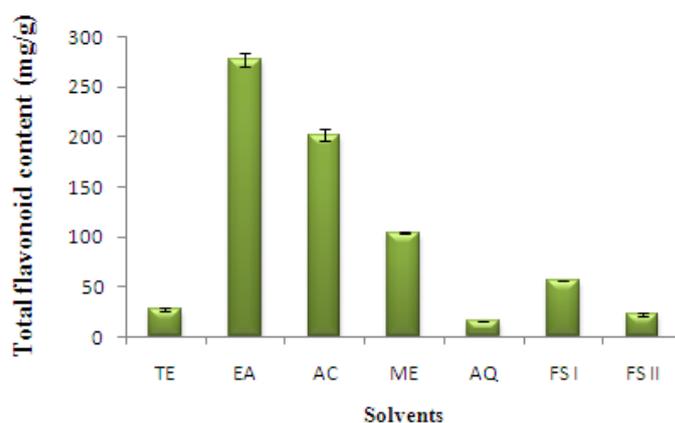


Fig. 3: Total flavonoid content of different solvent extracts of *T. erecta* flower

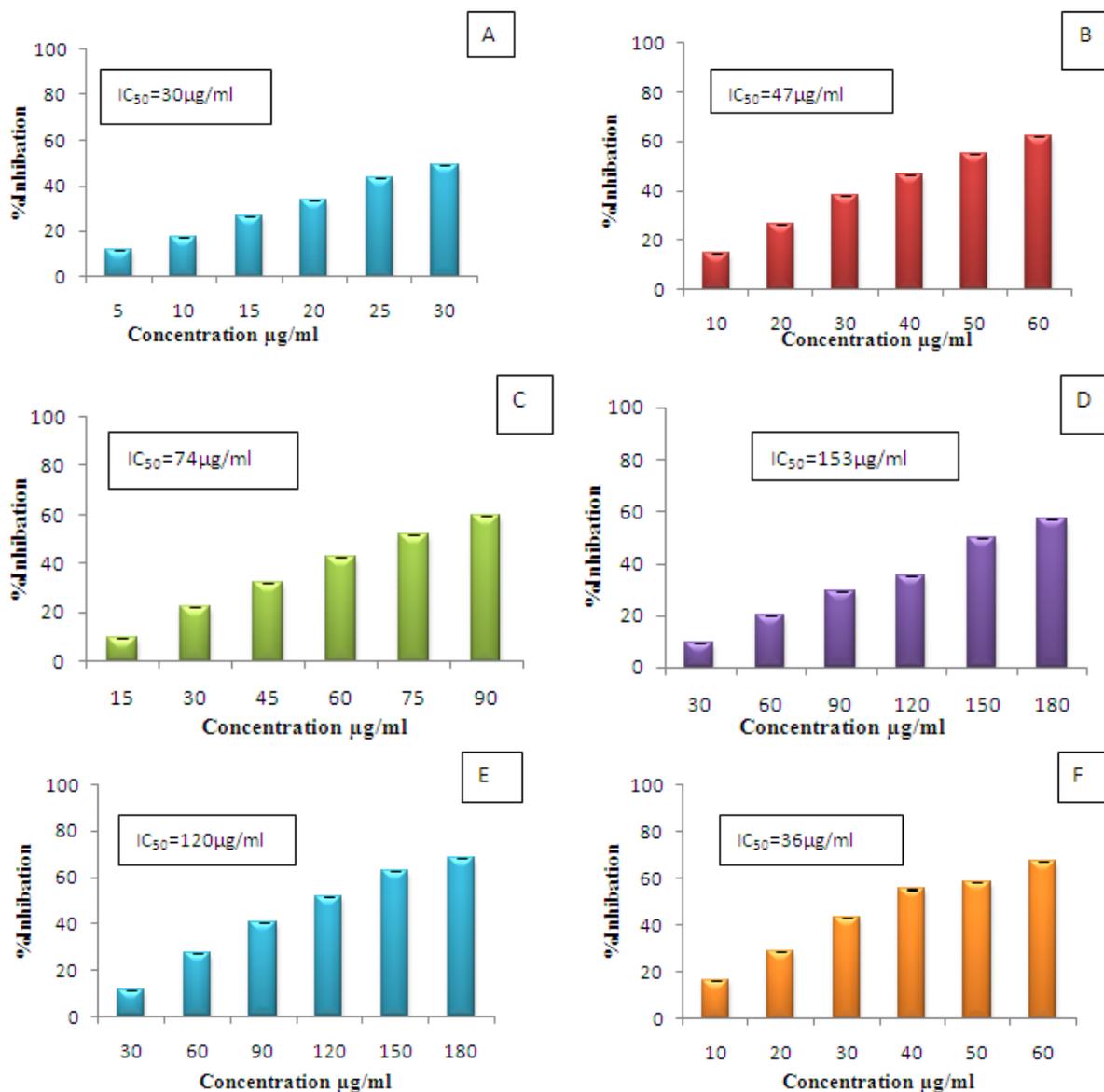
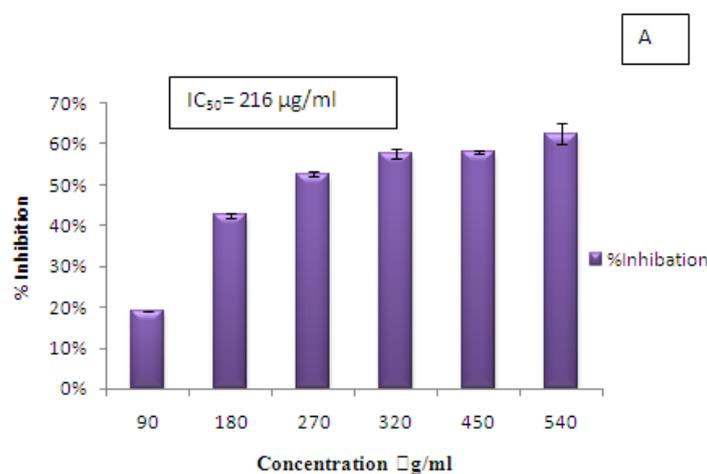


Fig. 4: DPPH free radical scavenging activity of *T. erecta* flower in different solvent extracts (A) Ethyl acetate (B) Acetone (C) methanol (D)Aqueous (E) Acetone Fraction I (F)Acetone Fraction II



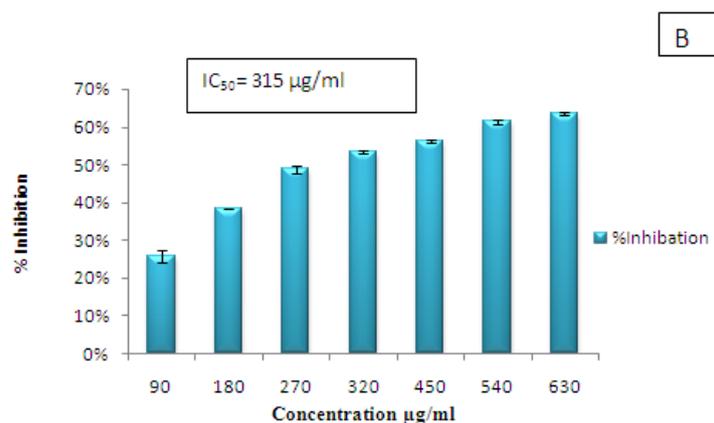


Fig. 5: Super oxide free radical scavenging activity of *T. erecta* flower in different solvent extracts (A) Methanol (B) Acetone Fraction I

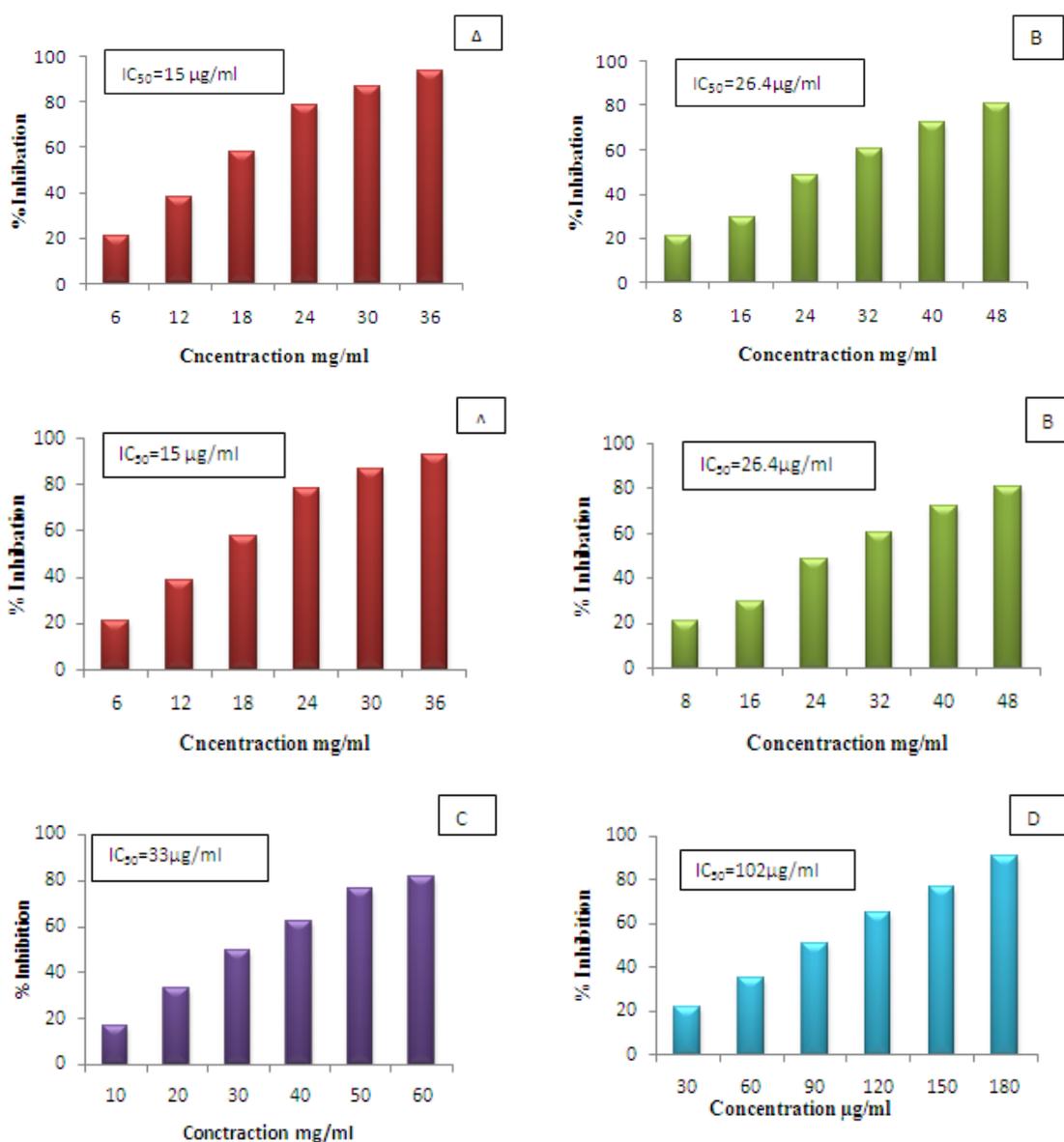


Fig. 6: ABTS radical cation scavenging activity of *T. erecta* flower in different solvent extracts (A) Ethyl acetate (B) Acetone (C) methanol (D)Aqueous (E) Acetone Fraction I (F)Acetone Fraction II

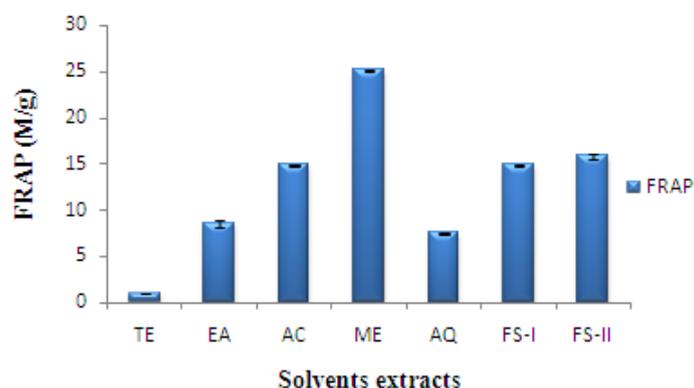


Fig. 7: Ferric reducing antioxidant power (FRAP) of different solvent extracts of *T. erecta* flower

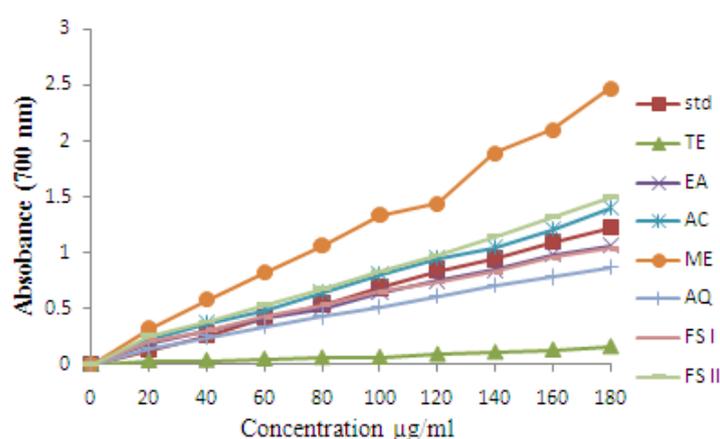


Fig. 8: Reducing capacity assessment of standard ascorbic acid and different solvent extracts of *T. erecta* flower

Table 1: IC₅₀ values of antioxidant activity of different solvent extracts of *T. erecta* flower

No.	Solvent	IC ₅₀ Value (µg/ml)			FRAP activity M/gm
		DPPH activity	SO activity	ABTS activity	
1	Toluene	>1000	>1000	>1000	0.944
2	Ethyl acetate	30	>1000	15	8.55
3	Acetone	47	>1000	26.4	14.83
4	Methanol	74	216	33	25.09
5	Aqueous	153	>1000	102	7.55
6	Acetone FS I	120	315	80	14.84
7	Acetone FS II	36	>1000	19	15.83
8	Standard: Gallic acid		185		
9	Standard: Ascorbic acid	11.4		6.5	

DPPH: 2,2-diphenyl 1-picryl-hydrazyl free radical; SO: superoxide anion radical; 2, 2'-Azino-bis-(3-ethyl) benzothiazoline-6-sulfonic acid (ABTS) radical cation scavenging activity; FRAP: Ferric reducing antioxidant power: