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Evaluation of bioactivity of *Annona muricata, Piper betle* and *Mentha spicata*

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ABSTRACT

Plants produce wide variety of phytochemical compounds which have been known to perform most biological activities such as antimicrobial, anticancer, antioxidant activities etc. Plants which possess these medicinal properties are known to be medicinal plants. In this study, Annona muricata, Piper betle and Mentha spicata were collected from Chennai, Tamil Nadu, India. The crude extracts were taken using various solvents like acetone, ethanol, chloroform, toluene and petroleum ether. All the extracts were subjected for qualitative analysis for the phytochemicals. All the samples were also subjected for antibacterial activity and TLC bioautography analysis for antioxidants. Since they found to possess antioxidant activity, quantitative assay for antioxidant activity like DPPH and FRAP assays were performed.

Keywords: *Annona muricata, Piper betle* and *Mentha spicata*
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INTRODUCTION

Most medicinal plants are native to India and they have been used in various traditional systems to treat diseases [1]. Phytochemicals of plants were found to be a cure for most diseases and this made human to rely on it for treating most diseases [2]. Since these plants are less toxic, economically viable, safe and dependable, it has attracted the world market towards it as well as drawn the attention of researchers and scientists into the unexplored arena of modern drug discovery [3]. There are enough evidences for biological activities exhibited by plants, some are as follows -. *Aegle marmelos* showed antimicrobial activity against *Bacillus subtilis, E.coli, Klebsiella pneumonia* [4]. Raji *et al* [5] found chloroform extract of *Cassia alata* to exhibit antimicrobial activity against *Pseudomonas aeruginosa.* Plants are also proven to have antioxidant activity too [1, 6-8].

Annona muricata belongs to family *Annonaceae* and it is well known for its edible fruit annona, which is slightly acidic taste once ripe and called as sour sop [9]. *Annona muricata* has been proven to possess antibacterial activity [10-11]. Leaves extract of *Piper betle* was found to be active against *E. coli, Streptococcus pyogenes* and *Staphylococcus aureus*[12]. Hydroxychavicol and Methyl chavicol isolated from *Piper betle* has shown anticancer and antioxidant activity respectively [13-14]. The ethanol extract of *Piper betle* was found to scavenge free radicals while analysed by DPPH method [15]. Ethyl acetate and aqueous extract of *Mentha spicata* was found to show antibacterial activity as well as antifungal activity [16-17]. The present study was aimed at investigating the phytochemicals, antibacterial activity and antioxidant activity of three Indian medicinal plants - *Annona muricata, Piper betle* and *Mentha spicata.*

MATERIALS AND METHODS

2.1 Sample collection and extraction

The leaves of *Annona muricata, Piper betle* and *Mentha spicata* were obtained from in and around Chennai, Tamil Nadu, India. The leaves were dried in shade ground into fine particle using mixer grinder. These samples were added with solvents of different polarity like Acetone, Chloroform, Ethanol, Methanol, Petroleum ether and Toluene

in the ratio of 1:10 (ie.10g of leaf powder in 100ml of solvent) in a 250ml conical flask. These conical flasks were kept in the rotatory shaker at 60 rpm for 24h. Samples were filtered using Whatmann No.1 filter paper. Thus obtained extracts were dried, weighed and stored in refrigerator at 4^0C till use. The dried samples were mixed in 1:1 ratio appropriate solvent (1mg/ml) while used for the study [4, 18].

2.2 Qualitative phytochemical analysis

Qualitative phytochemical analysis for flavonoids, phenols, saponins, Cholesterol, Cardiac glycosides etc. were done by following the procedures of Kokate [19] and Edeoga *et al* [20].

2.3 Test for antibacterial activity by determination of zone of inhibition

Using a sterile cotton swab, 24h culture of *Pseudomonas aeruginosa* was swabbed all over the sterile nutrient agar in petriplate, Wells of 3mm diameter were bored and 8 µl of working suspension of different concentrations of plant extracts were added into the well. Positive control (erythomycin) and negative control (solvent used for dissolving the extract) were also kept. Plates were incubated at 37˚C for 24h and the plates were observed and measured for zone of inhibition using scale [4, 21].

2.4 TLC DPPH autography analysis

The extracts were chromatographed on an Aluminium foil-baked normal particle silica gel layer 60F254 TLC plates and was developed with chloroform. Developed plated were dried, sprayed with DPPH (0.004% w/v in 95% methanol) and the band developed bright yellow to pink colour due to the reduction of DPPH were determined as antioxidant molecule [22-23]. The Rf value of the samples were calculated.

2.5 DPPH radical scavenging activity

The free radical scavenging capacity of the extracts was determined using DPPH method [22, 24-25] with slight modification. DPPH (0.004% w/v) was prepared in 95% methanol. 10µl, 20µl, 30µl, 40µl, 50µl concentration of sample were taken in a 96 well plate as duplicates. This was mixed with the freshly prepared 10µl DPPH Solution and made to 60 µl with methanol. 10 µl freshly prepared DPPH added with 50µl methanol was taken as blank control and ascorbic acid was taken as positive control. After 30 minutes of dark incubation, absorbance was taken at 517nm using an ELISA reader. The percentage inhibition was the calculated using the formula.

$$\text{Percentage inhibition} = \frac{\textit{Absorbance of positive control} - \textit{absorbance of sample}}{\textit{Absorbance of positive control}} * 100$$

IC_{50} values were obtained by probit analysis [26].

2.6 Ferric-reducing antioxidant power (FRAP) assay

FRAP assay was performed in accordance to the method followed by Sadeghi *et al* [27]. FRAP reagent was prepared right away before analysis by mixing 25ml 300mM acetate buffer (pH3.6), 2.5 ml TPTZ (10mM) solution, and 2.5 ml $FeCl_3.6H_2O$ (20mM) solution. Plant extracts (1mg/ml) were added with their appropriate solvents. 200µl of extracts were added with 1.8 ml FRAP reagent and incubated at 37 ºC for 30 min in dark. The samples were read at 595 nm having distilled water as blank. $FeSO_4$-$7H_2O$ (100 - 1000 µM) was used for calibration. Ascorbic acid was used as positive control. Results are expressed in the terms of mM Fe^{2+}/ mg sample [28-29].

RESULTS AND DISCUSSION

Various solvents like Acetone, Toluene, Ethanol, Chloroform, Petroleum ether were used to prepare the extracts of the 3 medicinal plants – *Annona muricata, Piper betle* and *Mentha spicata.* Different solvents were extracting out different secondary metabolites which absolutely relies on their polarity nature. All the plants are known to possess these secondary metabolites but the concentration and the form differs. Most of the solvents were found to extract phenolics out of the plants (Table 1). *Annona muricata* was found to be with most of the secondary metabolites like phenols, cardiac glycosides etc. Even Solomon-Wisdom *et al* [11] found steroids, alkaloids, saponnins, tannins, flavonoid and cardiac glycosides in methanolic and aqueous extract of *Annona muricata. Mentha spicata* was found to have phenols, saponins, alkaloids etc. but did not have quinones, whereas *Piper betle* was found to have quinones too. This is on par with the results of Ullah *et al* [16], who found tannin, alkaloids, glycosides, flavonoids, steroids, coumarines, sterols and terpenes but not anthraquinones in various samples of *Mentha spicata* collected in Pakistan. Ullah *et al* [16] also reported the absence of saponin in *Mentha spicata* but in this study *Mentha spicata* showed the presence of saponins (Table 1). Zaidi and Dahiya [30] also found flavonoids, saponins, cardiac glycosides, reducing sugars and steroids in *Mentha spicata*. Datta et al[15] reported *Piper betel* to possess phenolics, carbohydrates, flavonoids.

None of the extract showed antibacterial activity in this study (Table 2). This is absolutely against the results of Solomon-Wisdom *et al* [11], who found *Annona muricata* to have antibacterial activity against *Escherichia coli Streptococcus pyogenes, Bacillus subtilis, Salmonella typhimurium* and *Klebsiella pneumonia.* Even Datta *et al* [15] found *Piper betel* to have antibacterial activity against *Proteus vulgaris, Klebsiella, Pseudomonas* and *Staphylococcus aureus.* Ullah *et al* [16] also reported *Mentha spicata* to exhibit antibacaterial as well as antifungal activity.

TLC bioautography for antioxidant compounds revealed acetone extract and toluene extract of *Annona muricata* to have more antioxidant molecules whereas other extracts too showed antioxidant compounds (Fig.1 and Table 3). Ahalya et al[31] also revealed that ethanol extract of *Annona muricata* to have antioxidant activity. Ethanol extract of *Mentha spicata* showed more compounds with antioxidant property which is followed by other extracts (Fig.2 and Table 4). *Mentha spicata* has been reported to have more phenoloics [30]. Kanatt *et al* [32] found total phenolics of *Mentha spicata* to have resistant to thermal denaturation and retained radical-scavenging activity even after autoclaving and suggested that it could be used as an antioxidant in meat processes involving heating.

In this study, acetone was found to extract more antioxidant compounds than the other solvents used. Using acetone, totally seven compounds were extracted with antioxidant property whose Rf values are 0.16, 0.25, 0.40 and 0.88 (Fig. 3 and Table 5). Annegowda *et al* [33] identified three compounds namely - eugenol, allylpyrocatechol, and eugenyl acetate from *Piper betle* through TLC bioautography for antioxidant followed with HPTLC and GC-MS assisted analysis.

Toluene extract of *Anona muricata* showed potent antioxidant activity than the other solvent extracts (Fig. 4). Baskar *et al* [34] also found *A. muricata* to possess potent in vitro antioxidant activity than *A. squamosa* and *A. reticulate* through DPPH free scavenging activity. In this study, IC_{50} value of toluene extract was found to be ≤10 µg. Ahalya *et al* [31] found IC_{50} value of ethanolic extract of *A. muricata* as 109µg/ml. thus it says toluene extraction is better for the antioxidant separation from *A. muricata*.

Table 1: Phytochemical analysis of various plant extracts

Phytochemicals	*Annona muricata*					*Mentha spicata*					*Piper betle*				
	EE	AE	TE	PE	CE	EE	AE	TE	PE	CE	EE	AE	TE	PE	CE
Carbohydrates	+	-	+	-	-	+	-	+	+	-	+	-	-	-	+
Protein	-	+	-	+	-	-	+	-	-	+	+	+	+	+	-
Phenols	+	-	+	-	+	+	-	+	+	-	+	+	+	-	+
Cholesterol	-	+	-	+	-	-	+	-	-	-	+	-	-	+	-
Cardiac glycosides	-	+	-	-	-	-	+	-	+	-	-	+	+	-	+
Saponins	-	-	-	-	+	-	-	-	+	+	-	+	-	-	+
Quinone	-	-	-	+	+	-	-	-	-	-	+	+	+	-	+
Alkaloids	+	-	+	-	-	+	-	+	+	+	-	-	+	-	-
Flavanoids	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+
Leucoanthocyanids	-	-	+	+	+	-	-	+	-	+	+	-	-	+	+
Anthocyanins	+	+	-	-	-	+	+	-	-	+	+	-	-	-	+
Volatile oil	+	-	+	-	+	+	-	+	-	+	-	-	-	-	+
Terpenoids	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-

AE - Acetone extract, EE – ethanol extract, CE – chloroform extract, TE – toluene extract, PE – petroleum ether extract

Table 2: Antibacterial Activity of plant extracts against *Pseudomonas aeruginosa*

PLANT NAME	ANTIBACTERIAL ACTIVITY				
	EE	AE	TE	PE	CE
Piper betle	-	-	-	-	-
Mentha spicata	-	-	-	-	-
Annona muricata	-	-	-	-	-

AE - Acetone extract, EE – ethanol extract, CE – chloroform extract, TE – toluene extract, PE – petroleum ether extract

Mentha spicata was found to exhibit antioxidant activity. IC_{50} values of ethanol extract, acetone extract, chloroform extract, toluene extract and petroleum extract were found to be ≤ 45µg, ≤10 µg, ≤10 µg, ≤10 µg and ≤15 µg respectively (Fig.5). The antioxidant potential of mints is great since they got more phenolics[30]. The most abundantly found phenolic constituents are rosmarinic acid, flavones, flavanones and their glycosidic forms are also reason for their antioxidant property [35-36]. DPPH scavenging activity of *Piper betle* was found to be more with acetone extract and the IC50 value of the acetone extract was found to be ≤ 20µg, whereas the other extracts did not show significant free radical scavenging activity (Fig.6). Contradictory to this results Arambewela *et al* [37] found ethanolic extract to show potent antioxidant activity than hot water extract.

Acetone extract of *Mentha spicata* was found to show increased FRAP activity it was followed with toluene extract of *Annona muricata* and acetone extract of *Piper betle* (Fig.7). Padmini *et al* [38] FRAP activity of 1,000 ppm *A. muricata* fruit pulp extract was equivalent to that of 24.51± 0.20 ppm of vitamin C. *Piper betle* collected from Banarasi and Calcutta, India also showed highest antioxidant activity which has been confirmed by FRAP and ABTS assay [39]. Neelima *et al* [40] found extract of *Mentha spicata* to release 430 mM Fe^{2+}/ mg.

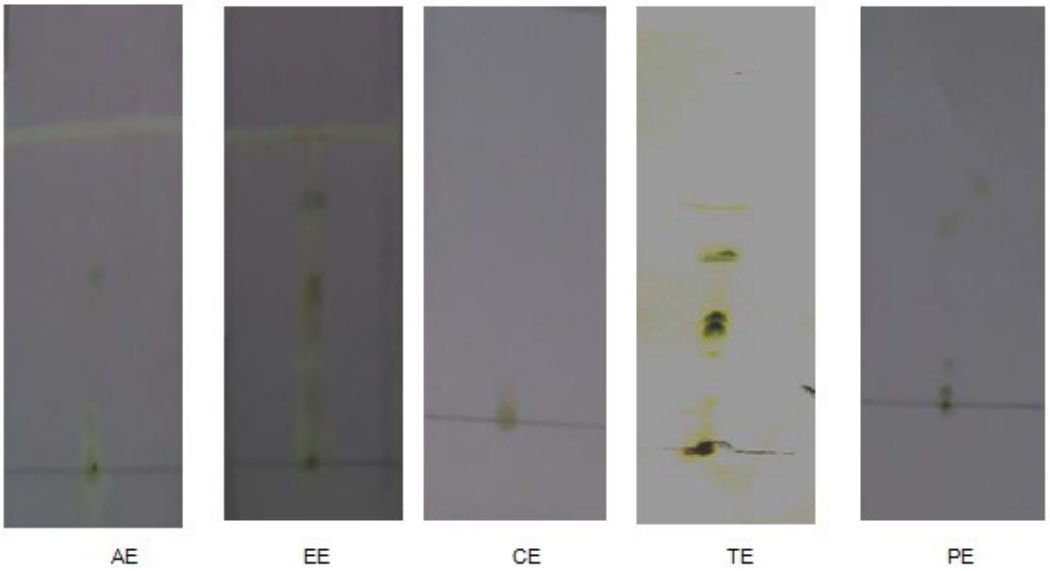

Fig.1: TLC bioautography for Antioxidant activity in *Annona muricata.* AE - Acetone extract, EE – ethanol extract, CE – chloroform extract, TE – toluene extract *Annona muricata*, PE – petroleum ether extract

Table 3: Rf values of antioxidant molecules obtained from *Annona muricata*

S.no	Acetone extract	Ethanol extract	Chloroform extract	Petroleum ether extract	Toluene extract
1	0.16	0.17	0.05	0.05	0.05
2	0.25	0.23	0.14	0.16	0.16
3	0.40	0.32			0.32
4	0.88	0.94			0.43
5	-	-			0.92

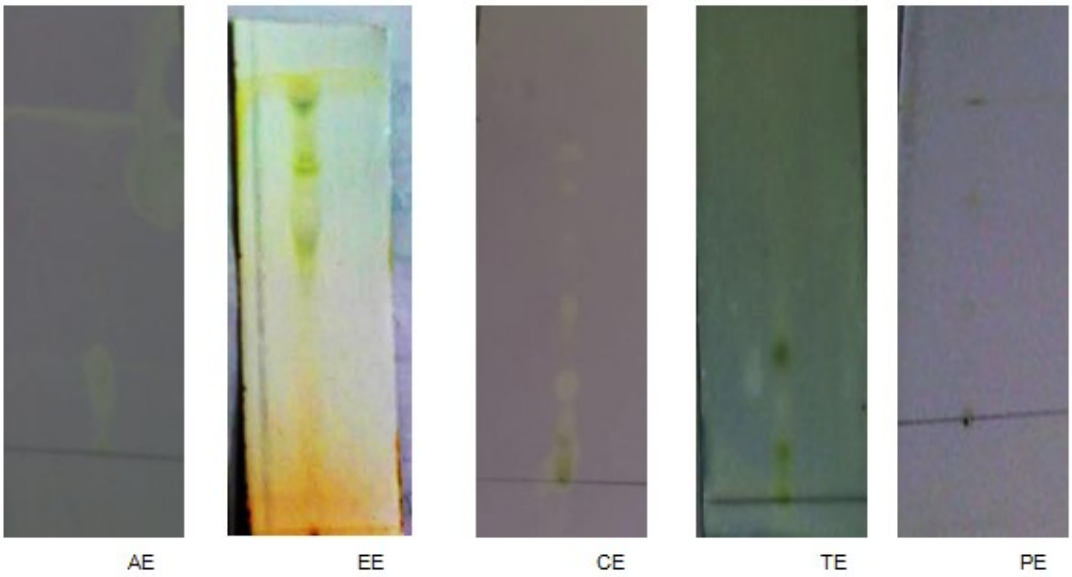

Fig.2: TLC bioautography for Antioxidant activity *Mentha spicata.* AE - Acetone extract, EE – ethanol extract, CE – chloroform extract, TE – toluene extract, PE – petroleum ether extract

Table 4: Rf values of antioxidant molecules obtained from *Mentha spicata*

S.no	Acetone extract	Ethanol extract	Chloroform extract	Petroleum ether extract	Toluene extract
1	0.14	0.52	0.09	0.03	0.09
2	0.17	0.64	0.24	0.04	0.24
3	0.19	0.72	0.61	0.33	0.61
4	0.99	0.78	0.97	0.69	0.97
5		0.84			
6		0.98			

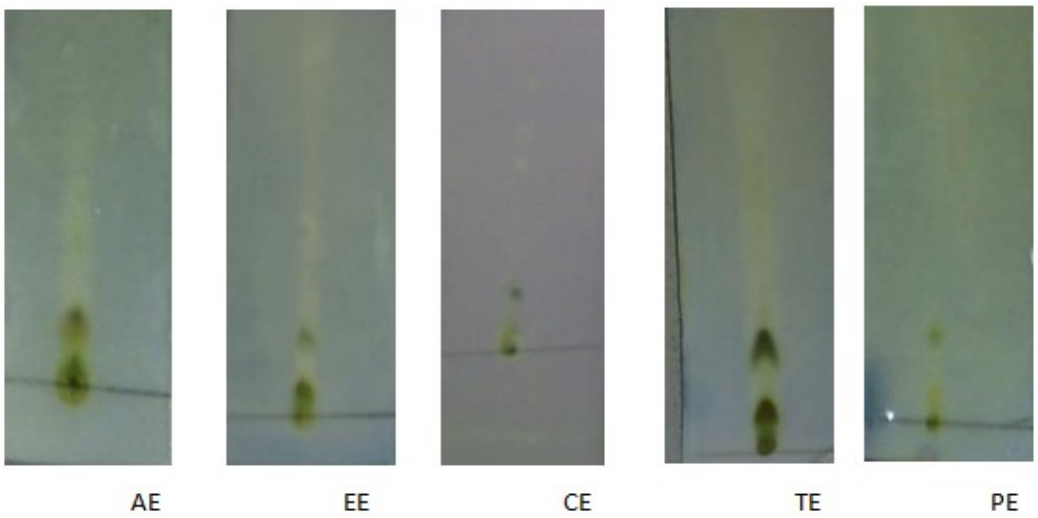

Fig.3: TLC bioautography for Antioxidant activity of *Piper betle.* AE - Acetone extract, EE – ethanol extract, CE – chloroform extract, TE – toluene extract, PE – petroleum ether extract

Table 5: Rf values of antioxidant molecules obtained from *Piper betle*

S.no	Acetone extract	Ethanol extract	Chloroform extract	Petroleum ether extract	Toluene extract
1	0.03	0.67	0.06	0.03	0.04
2	0.16	0.78	0.10	0.09	0.10
3	0.29	0.92	0.13	0.29	0.26
4	0.40	1.00	-	0.33	-
5	0.64				
6	0.76				
7	0.83				

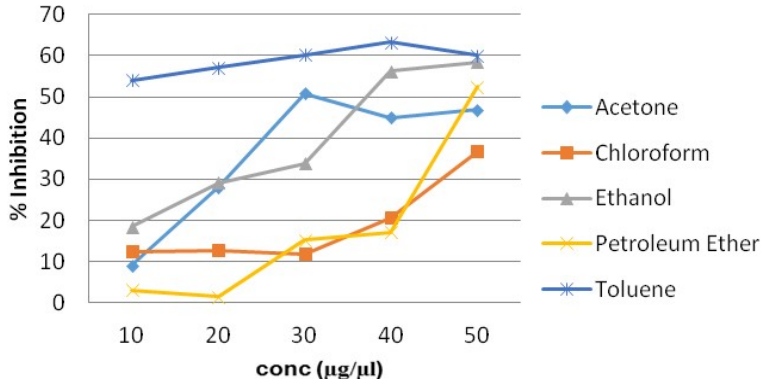

Fig.4: Percentage DPPH inhibition of *Annona muricata*

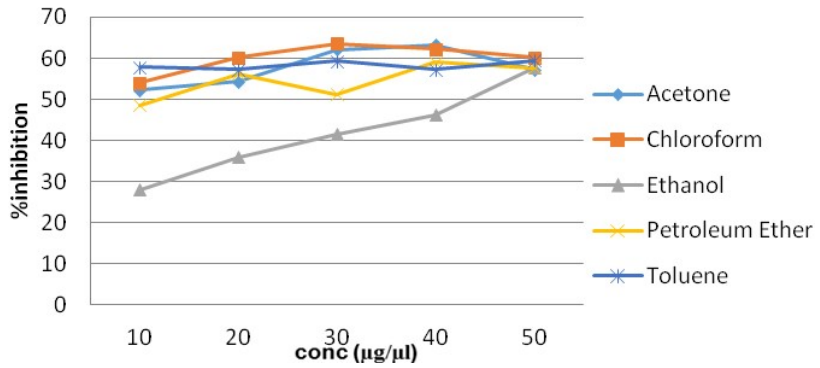

Fig.5: Percentage DPPH inhibition of *Mentha spicata*

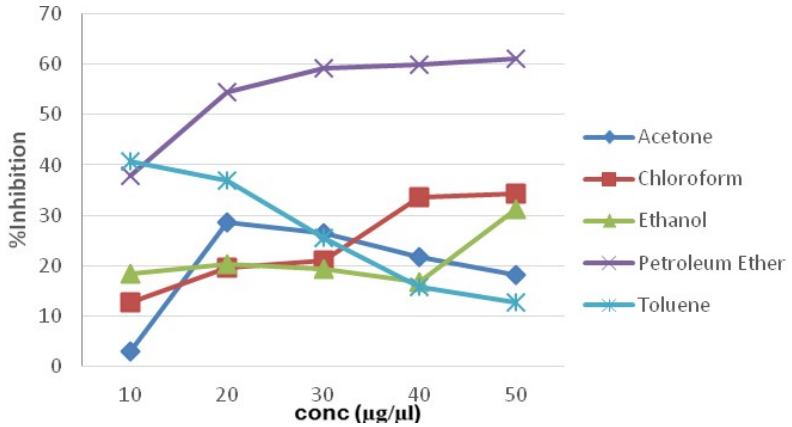

Fig.6: Percentage DPPH inhibition of *Piper betle*

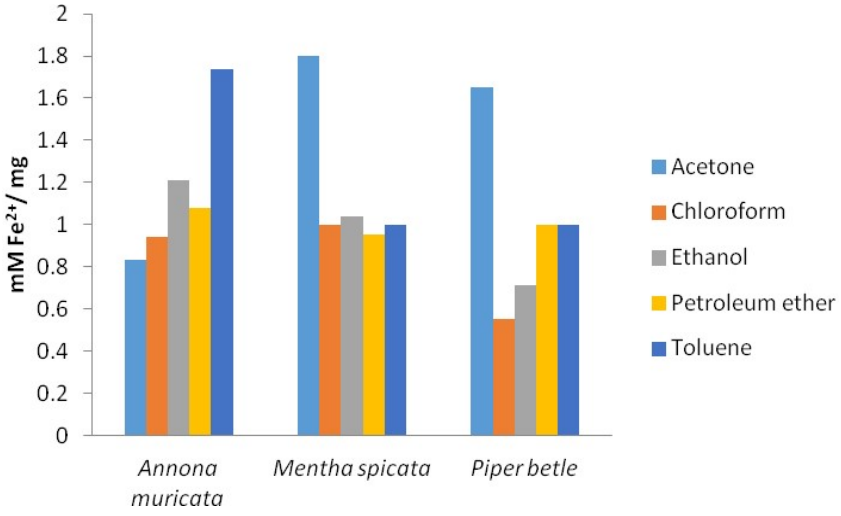

Fig.7: FRAP ASSAY performed for various plant extracts

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