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Effect of solvent extraction in antioxidant activity and RP-HPLC based study of phenolic acids and flavonoids and *Cayratia trifolia* (L.) Domin

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Abstract
Cayratia trifolia (L.) Domin (Vitaceae) is perennial climber with wide array of ethnomedicinal uses. The present study was focussed to evaluate the *in vitro* antioxidant activity of the plant in different solvent extracts *viz*. 70% ethanol, methanol, chloroform and benzene, along with HPLC based identification and quantification of the phenolic acids and flavonoids. The results indicated that 70% ethanol extract was the most potent solvent for extraction of total phenolics which decreased with decreasing polarity. the phenolic content ranged between 42.200 ± 0.196 GAE/ g dry mass in 70% ethanol to 14.872 ± 0.370 GAE/ g dry mass in benzene. ABTS radical scavenging activity of 27.752 ± 0.26%/ g dry mass was observed in 70% ethanol. The HPLC analysis confirmed the presence rutin (6.077 ± 0.001 mg/ 100g dry mass), naringin (1.078 ± 0.005 mg/ 100g dry mass), syringic acid, ferulic acid and others. The present study validates the ethnomedicinal use of the plant and also indicates the need of further study designed for maximum utilization of the same in a more practical and commercial way.

Keywords: Cayratia trifolia, antioxidant, solvent extraction, phenolic acids, RP-HPLC

Introduction
The search of plants with medicinal potential is largely based on ethnobotanical information. Through the ethnobotanical surveys, many of the medicinal plants have been recorded; analysed and preserved for posterity. Indian plants have been proposed for their interesting antioxidant activities and other medicinal properties with no serious side effects. Traditional herbal medicine is still a major source of novel active biological compounds with versatile activities, including anti-inflammatory, anti-diabetic, anti-carcinogenic, anti-viral, anti-bacterial and cardio and gastro protective activities. Antioxidant based formulations are prepared for the prevention and treatment of various complex chronic age related diseases such as atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer that have gained importance in the past few decades (Devasagayam *et al*., 2004) [1]. *Cayratia trifolia* (L.) Domin (*Vitaceae*) is found throughout the hilly regions in India. This perennial climber also grows wildly in Jammu, Rajasthan, Assam, Tripura and West Bengal extending into peninsular India up to 600 m (Gupta and Sharma, 2007) [2]. There has been few ethnomedicinal reports of the plant to be used as diuretic and is applied as poultice on tumors and is useful in neuralgia and splenopathy, leucorrhea (Gupta and Sharma, 2007; Gaur and Sharma, 2010) [2, 3]. Leaves roots and seeds are used as poultice on ulcers and boils (Gaur and Sharma, 2010) [3]. Extract of tuber along with infusion of its seeds is taken orally by diabetic patients to check sugar levels (Swarnkar and Katewa, 2008) [4]. The study reports the antioxidant activities of the aerial part extracts of the plant along with a HPLC based identification of phenolic compounds which can explain and justify their use in traditional medicine in the past as well as in the present.

Material and Methods
Plant Materials
C. trifolia were collected from different locations of Kolkata, India and were identified from Botanical Survey of India, Howrah. Plant material was shade dried, pulverized and kept in an airtight container for further antioxidant analysis.

Extraction of plant material
1 g dry sample was extracted separately with 20 ml of 70% ethanol, methanol, chloroform, benzene with continuous stirring for 18–24 h at ambient temperature, filtered with Whatman

#1 filter paper and diluted to 25 ml with respective solvent and used for analysis of antioxidant attributes.

Estimation of total phenolic content

Total phenolic content in the different solvent extracts was assessed by Folin-Ciocalteu procedure (Singleton and Rossi, 1976) [5], and was expressed as mg/g gallic acid equivalent (GAE)/ g dry mass.

Estimation of total flavonoid content

Total flavonoid content was estimated following method of Ordonez *et al*. (2006) [6] and expressed as mg/ g rutin equivalent (RE)/ g dry mass.

Ferric Reducing Antioxidant Power (FRAP) Assay

Total antioxidant capacity of plant extracts was determined in terms of Ferric Reducing Antioxidant Power (FRAP) using the method of Benzie and Strain (1996) [7] and expressed as µ mole trolox equivalent (TE) mg/ g dry mass.

Measurement of reducing power

the method of Oyaizu (1986) [8] and followed to determine the reducing power of the different extract and was calculated as ascorbic acid equivalent (AAE) in mg/ g of dry mass.

Free radical scavenging assay

Free radical scavenging assay was performed using DPPH (2,2-Diphenyl-1-picryl-hydrazyl) method (Blois, 1958) [9] and ABTS [2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] method (Re *et al*. 1999) [10]. Free radical scavenging activity was represented as % scavenging activity / g dry mass.

Metal chelating ability

Metal chelating property of different extracts was extimated following process of Lin *et al*. (2009) [11] was with slight modifications and represented as % metal scavenging activity/ g dry mass.

Anti-lipid peroxidation assay in linoleic acid system

The method of Amabye (2015) [12] was followed with slight modifications to determine the anti-lipid peroxidation ability of the different solvent extracts and was represented as % inhibition/ g dry mass.

RP-HPLC study for the estimation of Phenolic acids and Flavonoids

RP-HPLC analysis was performed with Dionex Ultimate 3000 liquid chromatogram having a diode array detector (DAD) and with Chromeleon system manager as data processor. Separation of phenolics was achieved by a reversed-phase C18 column (5 micron particle size, 250 × 4.6 mm). Fourteen standard phenolic acids (gallic acid, protocatechuic acid, gentisic acid, chlorogenic acid, p-hydroxy benzoic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, sinapic acid, salicylic acid and ellagic acid) and eight flavonoids (catechin, rutin, myricetin, quercetin, naringin, naringenin, apigenin and kaempferol) was prepared having concentration 1 mg/ml. The standard and working solutions were filtered through 0.45 µm PVDF-syringe filter and the mobile phase was degassed before the injection (20 µL) to the chromatograph.

Chromatographic analysis of phenolic acids and flavonoids

Chromatographic analysis was performed following method Seal *et al*. (2016) [13]. 20 µL of sample was injected into the HPLC column. The mobile phase contains Solvent A (methanol) and Solvent B (0.5% aq. acetic acid) and the column temperature was maintained at 25 ^{0}C. A gradient elution method was performed with a total run time of 105 min. Phenolic compounds were detected at three different wavelengths (272, 280 and 310 nm) using a photo diode array UV detector. Each phenolic compound was identified by its RT value and comparison with standards under the same conditions. The quantification of phenolic acids and flavonoids in the extract was carried out by the measurement of the integrated peak area.

Results and Discussion

Extraction yield, total phenolics and flavonoids content in *C. trifolia*

The extraction yield (Table 1) of C. trifolia ranged from 2.932 ± 0.109% in chloroform to 13.076 ± 0.104% in 70% aq. ethanol. Results suggest that efficient extraction was achieved using hydro-alcohol. Hydro-alcohol has been variously reported to be more efficient for extraction of antioxidant over pure solvent system in wild edibles (Seal *et al*., 2013; Datta *et al*., 2018a) [14, 15]. The polarity of the solvent, nature of antioxidant compounds to be extracted, parts used for extraction is the important factors that affect extraction yield (López *et al*., 2011) [16]. The extraction procedure, time and temperature of extraction are physical parameters that also influence extraction.

Table 1: Extractive capacities (% extractive yield), total phenolic content (mg GAE/ g dm) and flavonoid content (mg RE/ g dm) in *C. trifolia* in different solvent system

Parameters	Extracts of *C. trifolia* in different solvent system			
	70% ethanol	Methanol	Chloroform	Benzene
Extractive Value (% extractive yield)	13.076 ± 0.104^a	7.281 ± 0.162^b	6.469 ± 0.154^c	2.932 ± 0.109^d
Total phenolic content (mg GAE/ g dm)	42.200 ± 0.196^a	33.590 ± 1.958^b	24.084 ± 1.650^c	14.872 ± 0.370^d
Total flavonoid content (mg RE/ g dm)	16.324 ± 0.084^a	13.724 ± 0.186^b	11.663 ± 0.214^c	7.427 ± 0.026^d

GAE: Gallic acid equivalent; RE: Rutin equivalent Each value in the table was obtained by calculating the average of three experiments (n=3) and data are presented as Mean ± SEM. Statistical analysis were carried out by Turkeys test at 95% confidence level and statistical significance were accepted at the $p< 0.05$ level. The superscript letter denotes the significance of various parameters.

The phenolic acid content ranged between 42.200 ± 0.196 mg GAE/ g dry mass to14.872 ± 0.370 mg GAE/ g dry mass. Results indicate that hydro-ethanol extracts had the maximum phenolic content which diminished with polarity. Polar solvents such as aqueous methanol/ethanol are often better solubiliser of phenolic compounds as compared with absolute methanol/ethanol (Sultana *et al*., 2007; Seal *et al*., 2013) [14, 17]. Maximum flavonoid content was also observed in 70% ethanol solution (16.324 ± 0.084mg RE/ g dry mass). The flavonoid content was also observed to decrease with polarity.

Similar results were observed in another medicinal sedge *C. compressus* (Datta *et al*., 2018a) [15].

Reducing property and FRAP of *C. trifolia*

Reducing property and FRAP both the experiments were used to ascertain the reducing ability of Fe^{+3} to Fe^{+2} of the plant extracts, which is the reflection of the antioxidant potential of the sample. Fe^{+3} promote free radical formation by Fenton reaction and also facilitate lipid peroxidation. The results (Table 2) of both reducing property and FRAP of the solvent extracts can be ranked as 70% ethanol > Methanol > Chloroform> Benzene. The reducing property in 70% ethanol (38.489 ± 0.945 AAE) and methanol (33.792 ± 0.242 AAE) was observed to be comparable. Similarly FRAP results in both these extracts (1.290 ± 0.002 µ mole TE/ g dry mass in 70% ethanol and 1.134 ± 0.002 µ mole TE/ g dry mass in methanol) were also comparable. The reducing property therefore corresponds to the phenolic content of the extracts. Similar results were observed in other medicinal plants (Datta *et al*., 2018a; Datta *et al*., 2018b) [15, 18].

Metal chelating property of *C. trifolia*

Metal ions are known to catalysis of free radical formation and lipid peoxidation that correlates with incidents of cancer and arthritis (Halliwell *et al*., 1995) [19]. The chelating ability of the extracts can be ranked as 70% ethanol > Methanol > Chloroform> Benzene as represented in Table 2. The maximum chelating ability in 70% ethanol (10.647 ± 0.295%/ g dry mass) correlated to the maximum phenolic and flavonoid content in the extract.

Table 2: Reducing activity (mg AAE/ g dm), FRAP (µ mole TE/ g dm) and metal chelating activity (% inhibition/ g dm) of C. trifolia in different solvent systems

Parameters	Extracts of *C. trifolia* in different solvent system			
	70% ethanol	Methanol	Chloroform	Benzene
Reducing activity (mg AAE/ g dm)	38.489 ± 0.945^{a}	33.792 ± 0.242^{b}	22.058 ± 0.201^{c}	10.621 ± 0.237^{d}
FRAP (µ mole TE/ g dm)	1.290 ± 0.002^{a}	$1.134 \pm 0.002^{a,b}$	0.620 ± 0.002^{c}	0.403 ± 0.003^{d}
Metal chelating activity (% inhibition/ g dm)	10.647 ± 0.295^{a}	8.780 ± 0.614^{b}	6.983 ± 0.189^{c}	3.716 ± 0.117^{d}

AAE: Ascorbic acid equivalent; TE: Trolox equivalent. Each value in the table was obtained by calculating the average of three experiments (n=3) and data are presented as Mean ± SEM. Statistical analysis were carried out by Turkeys test at 95% confidence level and statistical significance were accepted at the $p< 0.05$ level. The superscript letter denotes the significance of various parameters.

Radical scavenging property of *C. trifolia*

Scavenging activity of DPPH and ABTS radicals of the different extracts are represented in Table 3. % scavenging activity of ABTS radical was generally observed to higher and ranged from 27.752 ± 3.261%/ g dry mass in 70% ethanol to 6.647 ± 0.528% / g dry mass in benzene. The results correlate to the phenolic acids and flavonoid content in these extracts.

Table 3: Radical scavenging activity (% inhibition/ g dm) of *C. trifolia* in different solvent system using DPPH and ABTS

Parameters	Extracts of *C. trifolia* in different solvent system			
	70% ethanol	Methanol	Chloroform	Benzene
DPPH	5.956 ± 0.328^{a}	$5.714 \pm 0.236^{a,b}$	4.082 ± 0.612^{c}	3.054 ± 0.136^{d}
ABTS	27.752 ± 0.261^{a}	$26.284 \pm 0.502l^{b}$	13.766 ± 0.219^{c}	6.647 ± 0.528^{d}

Each value in the table was obtained by calculating the average of three experiments (n=3) and data are presented as Mean ± SEM. Statistical analysis were carried out by Turkeys test at 95% confidence level and statistical significance were accepted at the $p< 0.05$ level. The superscript letter denotes the significance of various parameters.

Lipid peroxidation of *C. trifolia*

Lipid peroxidation is one of the main factors of food rancidity. Several chemical antioxidants are such as BHA is used as stabilizers to food to increase shelf life. The severe side effects of these synthetic compounds are not unknown. The phenolic compounds are known to suppress lipid peroxidation by inactivating the free alkyl radicals and scavenging the metal ions (Mathew and Abraham, 2006) [20]. In addition, lipid peroxidation of cell membrane is associated with various medical conditions such as atherosclerosis, inflammation and liver injury (Singh *et al*., 2012) [21]. Use of plant based antioxidants has therefore gained demand. The results (Table 4) suggest that 70% ethanol (18.8982 ± 0.104%) and methanol extract (17.509 ± 0.122%) showed maximum inhibition and it correlated to the phenolic and flavonoid content in these extract.

Table 4: Anti-lipid peroxidation assay (% inhibition/ g dm) of *C. trifolia* in different solvent system

Parameters	Extracts of *C. trifolia* in different solvent system			
	70% ethanol	Methanol	Chloroform	Benzene
% inhibition of lipid peroxidation	18.8982 ± 0.104^{a}	17.509 ± 0.122^{b}	8.732 ± 0.612^{c}	3.714 ± 0.534^{d}

Each value in the table was obtained by calculating the average of three experiments (n=3) and data are presented as Mean ± SEM. Statistical analysis were carried out by Turkeys test at 95% confidence level and statistical significance were accepted at the $p< 0.05$ level. The superscript letter denotes the significance of various parameters.

Identification and quantification of different phenolic acids and flavonoids in 70% aq. ethanol extracts of *C. trifolia*

The HPLC chromatogram of the standard phenolic acids and flavonoids is depicted in figure 1 with absorbance at 260nm. Since maximum antioxidant activity was observed in 70% ethanol extract it was chosen for HPLC quantification of phenolic acids and flavonoids (figure 2). The plant contain maximum amount of rutin (Table 5), a flavonol with antidiabetic effect (Srinivasan *et al*., 2005) [22] and anticancer

activity (Lin *et al*., 2012) [23] and can potentially be used as a therapeutic agent. This also justifies the ethnic use of the plant as an anti-diabtic medicine. Ferulic acid is a well-known anti-microbial agent. Presence of ferulic acid validates the ethnic use as a topical application on ulcers. Naringin possess anti-inflammatory and anti-cancerous activity (Chen *et al*. 2016) [24]. Presence of naringin (1.078 ± 0.005 mg/ 100 g dry mass) can contribute to its anti-inflammatory activity.

Table 5: Quantification of phenolic acids and flavonoids in 70% aq. ethanol extract of C. trifolia (mg/ 100g dm) by HPLC

Phenolic acids and flavonoids	Amount mg/100gm dry plant material	Phenolic acids and flavonoids	Amount mg/100gm dry plant material	Phenolic acids and flavonoids	Amount mg/100gm dry plant material
Gallic acid	ND	Caffeic acid	ND	Rutin	6.077 ± 0.001
Protocatechuic acid	ND	Syringic acid	0.322 ± 0.001	Ellagic acid	0.099 ± 0.003
Gentisic acid	ND	p-Coumaric acid	0.208 ± 0.001	Myricetin	ND
p-hydroxybenzoic acid	ND	Ferulic acid	0.261 ± 0.002	Quercetin	ND
Catechin	ND	Sinapic acid	ND	Naringenin	ND
Chlorogenic acid	ND	Salicylic acid	ND	Apigenin	0.469 ± 0.001
Vanillic acid	0.176 ± 0.002	Naringin	1.078 ± 0.005	Kaempferol	0.060 ± 0.001

ND: Not detected. Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM.

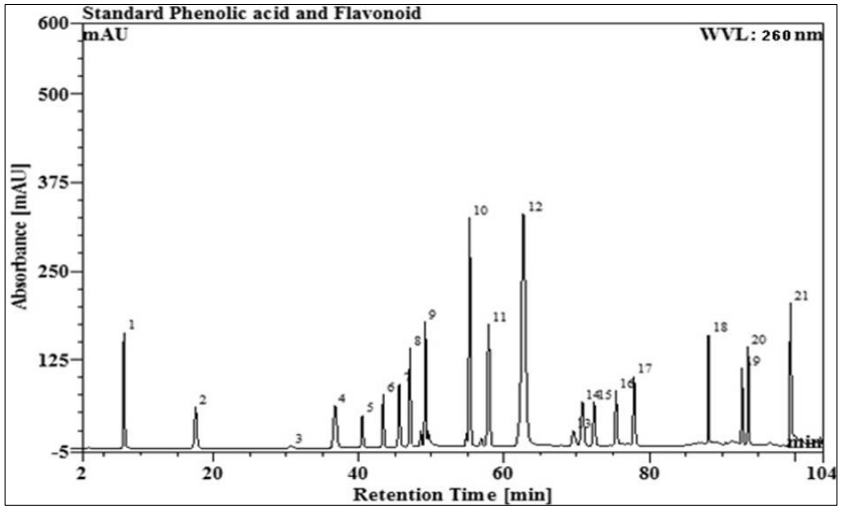

Fig 1: RP-HPLC based separation of standard phenolics and flavonoids

(1. Gallic acid, 2.Protocatechuic acid, 3.Gentisic acid, 4.p-Hydroxy benzoic acid, 5.Catechin, 6.Chlorogenic acid, 7.Vanillic acid, 8.Caffeic acid, 9.Syringic acid, 10.p-Coumaric acid, 11. Ferullic acid, 12.Sinapic acid, 13.Salicylic acid, 14.Naringin, 15.Rutin, 16.Ellagic acid, 17.Myricetin, 18.Quercetin, 19.Naringenin, 20.Apigenin, 21. Kaempferol)

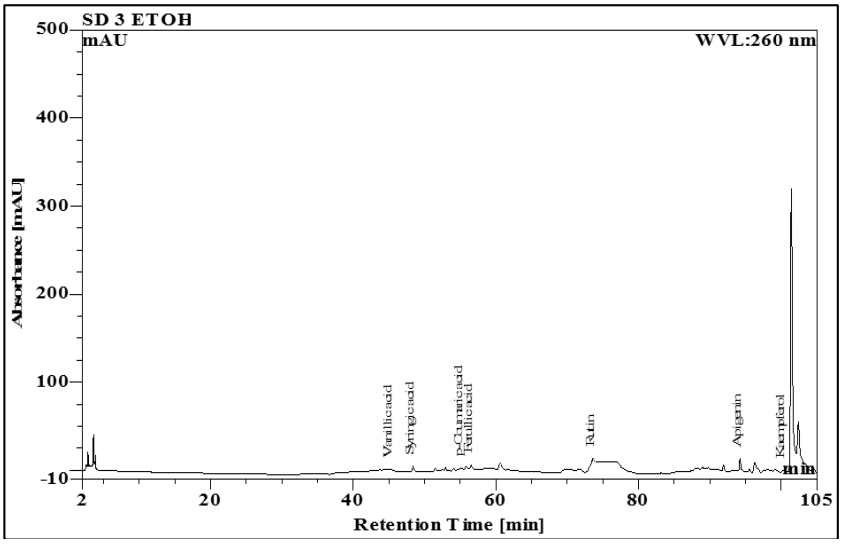

Fig 2: RP-HPLC based separation of phenolics and flavonoids from 70% ethanol extract of aerial parts of *C. trifolia*

Conclusion

The above findings clearly indicate that *C. trifolia* can be used as a natural source of natural antioxidants. Further studies designed to exploit and maximize more practical and effective use of the plant is necessary.

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