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Research Article

Evaluation of Total Phenolic, Flavonoid Contents and Antioxidant Activity of *Acokanthera oppositifolia* and *Leucaena leucocephala*

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ABSTRACT

Objective:The generation of reactive oxygen species and free radicals in human is suggested to contribute to the wide range of pathological disturbances such as, inflammations, cancer, diabetes and arthritis. Therefore the present study was performed to evaluate the antioxidant potential of *Acokanthera oppositifolia* and *Leucaena leucocephala*.

Methods:Three different solvent extractsof both plants were used in thestudy toevaluate their TPC by using Folin Coilteau reaction and flavonoid contents were determined by AlCl3 assay. The antioxidant potential was determined using four different test including metal chelation, reducing power, superoxide anion scavenging and by hydroxyl radical scavenging assay. Results:Total phenol content ranged from 7.51±.02 to 12.06±.03 mg $GAEg^{-1}$ (Gallic acid equivalent) for *A.oppositifolia*and13.40±.08 to 28.49±.02mg $GAEg^{-1}$for *L. leucocephala*while total flavonoid ranged 0.373±0.04 to 1.325±0.08 $mgQEg^{-1}$ (Quercetin equivalent) for *A.oppositifolia*and 2.806±0.05 to 3.587±.003 mg $QEg^{-1}$for *L. leucocephala*. All the extractsunder study exhibited good antioxidant activity in concentration dependent manner. Among all extracts methanolic extract of *A. oppositifolia* andchloroform extract of *L. leucocephala* exhibited better Fe^{2+} chelating activity with IC_{50} 0.065 and 0.112 mg/ml respectively,Fe (III) to Fe (II) reducing capacity with IC_{50} 0.23 and 0.42 mg/ml and superoxide anion scavengingpotential with IC_{50}0.425 and 0.675 mg/ml. In scavenging of hydroxyl radical *A. oppositifolia* acetone and *L. leucocephala* methanolic extract was most effective one with an inhibitory effect of 83.22 % ±.03 and 85.47 % respectively at 1.0 mg/ml (IC_{50} 0.12 & 0.25 mg/ml). Conclusion:All the extracts*A.oppositifolia*and *L. leucocephala*leaves is potentiallygood source of antioxidant, which are found to be quite promising as a multipurpose medicinal agent. Phenolic and flavonoids contents are suggested to be responsible for antioxidant potential.

Keywords: Phytochemicals, Superoxide Anion, Hydroxyl radical, chelation, flavonoid, Phenolic

INTRODUCTION

In living systems, oxidation is a basic part of the normal metabolic process, in which Reactive oxygen species (hydrogen peroxide and hypochlorous acid) and many free radicals (hydroxyl radical (OH) and superoxide anion are, generated[1,2].Rapid production of free radicals and reactive oxygen species (ROS) may cause alteration in the structure and function of cell constituents and membranes and can results in human neurologic and other disorders such as cancer, diabetes, inflammatory disease, asthma, cardiovascular, neurodegenerative diseases, and premature aging and arthritis[3-5]. Exposure of DNA to free radicals causes extensive strand breakage and degradation of deoxyribose[6,7].The mechanistic study of different forms of cancers revealed that its development and progression is linked to multiple mutations related to oxidative DNA damage that affect the integrity of genome and thus leading to malformations.Free radicals are involved in boththe process of aging and the development of cancer[8].Therefore, the prevention of the above conditions requires the presence of antioxidants or the free radical scavenging molecules in the body. The chemical constituents present in herbal drugs are a part of the physiological functions of living flora and hence they are believed to have better compatibility with the human body[9].Studies have shown that natural products derived from food and medicinal plants are the potential sources of antioxidants[10-16].

Acokanthera is a genus of flowering plants in the family Apocynaceae. All parts of *Acokanthera oppositifolia*, except for the pulp of the ripe fruit, contain large amounts of cardiac glycosidesresponsible for the activity as arrow poison, but also act as cardiac stimulant[17,18].This plant has been used for treatment of headache, snake bite & spider bite, in treatment of muscles pain, to treat excessive & irregular menstruation, leaf infusion used intreatment of abdominal pain, Small pieces of stem are chewed to relieve toothache, in intestinal worms treatment and also for aches and colds. This plant is also used in low doses to treat patients suffering from congestive heart failure[19,20].

Leucaena leucocephala belong to family Fabaceaeis a small, fast-growing mimosoid tree native to southern Mexico and northern Central America but is now naturalized throughout the tropics[21]. *L. leucocephala* is used for treatment of intestinal worms, measles, to treat scurf, remedy for diabetes. The roasted seeds help to

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increase menstrual flow[22,23]. In present study *A. oppositifolia*and *L. leucocephala* has been selected to study their phytochemical constituents and antioxidant potential. We selected these plants; because of easy availability and both plants possess various medicinal properties and have been used in traditional system of medicine[19,22].

MATERIALS AND METHOD

Chemical used

Folin-Ciocalteu Reagent,was obtained from Sisco Research laboratories,Pvt. Ltd. Mumbai, India; Gallic acid,aluminium chloride, Quercetin were purchased from HIMEDIA laboratories Pvt. Ltd., Mumbai, India;sodium nitrite, Ferrozine, Ferrous sulphate,TCA, Sodium chloride,phenazine methosulphate PMS,nitrobluetetrazolium NBT and nicotineamide-adenine-dinucleotide NADH, 1, 10-phenanthroline, H_2O_2, $FeCl_2$, EDTA, Mannitol,Methanol, Acetone, Chloroform, Sodium Carbonate, Potassium thiocyanate, potassium ferricyanide were obtained from Central Drug House Pvt. Ltd., New Delhi, India and Hi- Media.

Collection of plants material

Leaves of *A. oppositifolia*and*L. leucocephala*were collected from the Botanical garden of M.M. College, Modinagar and were identified and authenticated by Dr. D.K. Awasthi Professor, Department of Botany, M.M. College, Modinagar, affiliated to CCS University, Meerut, India.

Preparation of crude plant extracts

The collected leaves were washed twice with distilled water and then they were dried in shade. The dried leaves were coarsely crushed using a grinder. 100 gm. of dried leaf powder of different plants leaves were soaked in 250 ml of different organic solvent (Methanol, Acetone, Chloroform) for 10 days. After 10 days it was passed through No.1 Whatman filter paper (Whatman Ltd., England).The extracts obtained were concentrated under vacuum on a rotary evaporator at 40˚C and stored at 4˚C for further use.

Qualitative Phytochemical screening

Test for Phenols

To the extract, few drops of 10 % aqueous ferric chloride were added. Appearance of blue or green color indicates the presence of phenols[24].

Test for Flavonoids

The stock solution (1 mL) was taken in a test tube and added few drop of dilute NaOH solution. An intense yellow colour was appeared in the test tube. It became colourless when on addition of a few drop of dilute acid that indicated the presence of flavonoids[25].

Test for Saponin

The stock solution (1 mL) was taken in a test tube and diluted with 20 mL of distilled water. It was shaken by hand for 15 min. A foam layer was obtained on the top of the test tube. This foam layer indicated the presence of saponins[25].

Test for tannins

The stock solution (3 mL) was taken in a test tube and diluted with chloroform and added acetic anhydride (1 mL). Finally, sulphuric acid (1 mL) was added carefully by the side of test tube to the solution. A green colour was formed which showed the presence of tannin[25].

Test for alkaloids

2ml of Wagner's reagent was added in methanolic extract formation of reddish brown precipitate indicates the presence of alkaloids[24].

Quantification of total phenolic content

The total flavonoid content of the acetone, chloroform and methanolic extracts of leaves *A. oppositifolia* and *L. leucocephala* was determined using colorimetric assay with slight modification[26]. In brief; 0.1ml plant extracts were diluted to 1 ml with distilled water. To this solution 0.5 ml of Folin-Ciocalteu reagent (1:1) and 1.5 ml of 20% sodium carbonate solution was added. The mixture was incubated for 2 hours at room temperature. The volume was raised to 10ml with distilled water and the absorbance of blue colored mixture was measured at 765nm.The standard curve was prepared by using Gallic acid. The total phenolic contents were expressed as mg $GAEg^{-1}$ (Gallic acid equivalent). The regression equation obtained from calibration curve was used to determine total phenol content.

Quantification of total flavonoid content

The total flavonoid content of the acetone, chloroform and methanolic extracts of leaves *A. oppositifolia* and *L. leucocephala*was determined using colorimetric assay given[27]. with slight modification.The mixture consisting of 1ml of extract solution, 0.3ml of sodium nitrite (5%) and 0.3ml of aluminium chloride (10%) was incubated for 5 minutes followed by addition of 1M sodium hydroxide solution (2ml). The volume of the mixture was raised to 10ml and it was then thoroughly vortexed. The absorbance of pink colored solution was determined at 510nm. The flavonoid content was calculated from standard curve of Quercetin and expressed as mg QEg^{-1} (Quercitin equivalent).

Metal Chelation Assay

The potential of plant extracts to chelate Fe^{2+} was determined by colorimetric based ferrozine assay[28]. with slight modifications. Different concentration of extracts (1ml, 20-200µg/ml) was mixed with 3.7ml of methanol and 2mM ferrous sulphate (0.1ml). The reaction was initiated by 5mM of ferrozine (0.2ml). The mixture was kept at room temperature for 10 minutes. The absorbance of the mixture was determined at 562nm.The inhibition percentage was calculated using the formula. Inhibition % = Abs. of Control- Abs. of Sample / Abs. of Control × 100)

Reducing power assay

The reducing power of different fractions was determined by the method of[28]. In briefly, 1 ml of extract of different concentrations was mixed with 2.5 ml of phosphate buffer (200mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50ºC for 20 minutes. A volume of 2.5ml of 10% TCA was then added to the mixture and centrifuged at 3000 rpm for 10 minutes. 2.5 ml of supernatant was mixed with 2.5 ml ofdistilled water and 0.5 ml of FeCl3 (0.1%) and the absorbance was measured spectrophotometrically at 700nm.

Hydroxyl radical scavenging assay

Table 1: Qualitative phytochemical analysis of *A. oppositifolia* and *L. leucocephala*

	A. oppositifolia	*L. leucocephala*
Alkaloid	–	+
Phenol	+	+
Flavonoid	+	+
Saponin	-	-
Tanin	+	+

'+' Represents presence of the phytoconstituent;
'-'represents absence of the phytoconstituent

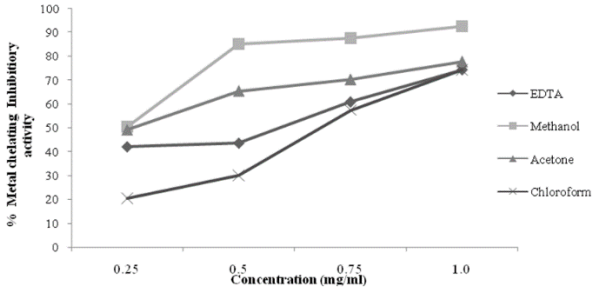

Fig 1: Metal chelating ability of different extract of *A.oppositifolia*leaves as compare to EDTA

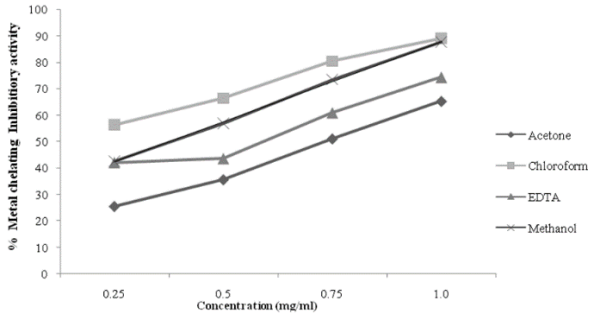

Fig 2: Metal chelating ability of different extract of *L. leucocephala*leaves as compare to EDTA

The hydroxyl radical scavenging activity was assayed according to the method of Jing *et al.*[29]. Reaction mixture contained 60 µl of 1mM $Fecl_2$, 90µl of 1, 10-phenanthroline, 2.4 mL of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17M H_2O_2, and 1.0 mL of extracts at various concentrations. Adding H_2O_2 started the reaction. After incubation at room temperature for 5min. the absorbance of mixture at 560 nm was measured. The inhibition percentage was calculated using the same formula given above.

Superoxide Anion Scavenging Assay

In order to assess the superoxide anion scavenging activity of different plants extracts, 1ml of plant extract of different concentrations (20-200µg/ml) was mixed with 156µM NADH (1ml), 60µM NBT (1ml) and 468µM phenazine methosulphate (1ml) in phosphate buffer (pH = 8.3). The reaction was initiated with the addition of PMS. The reaction mixture was incubated at 25°C for 10 minutes. The absorbance of colored complex was measured at 560nm[30].The inhibition percentage was calculated using the same formula given above. The experimental results were expressed as mean ± standard deviation (SD) of six sample measurements.

RESULTS AND DISCUSSION

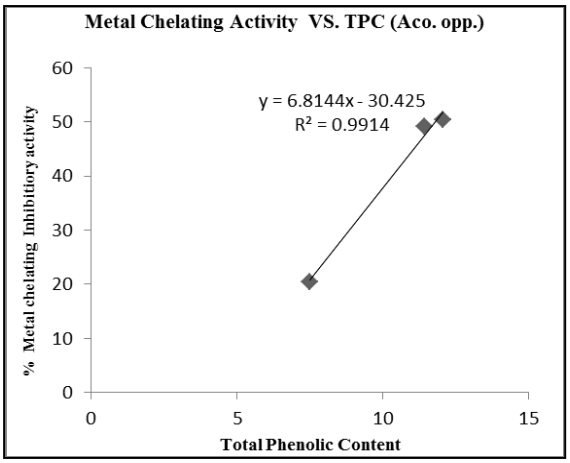

Fig. 3:Correlation between total phenolics and Metal chelating activity, *A. oppositifolia* (r2 = 0.991)

Phytochemical Analysis

Results in (Table 1)indicate the presence of phenolic, flavonoids and tanin in both plants. Alkaloids were present only in *A. oppositifolia* whereas saponins were absent in both the plants.It was observed from (Table 2)leaves extract of both plants contains significant amount of phenolic & flavonoids content. It was found that among the all extract methanol and chloroform extract of *A. oppositifolia* and *L. leucocephala* possess highest phenolic and flavonoids respectively. The total phenolic content was found to be 11.43±.02, 7.51±.02, 12.06±.03 mg/gm. equiv. to Gallic acid for acetone, chloroform and methanolic extracts of *A. oppositifolia* respectively. The total phenolic content was found to be 1.34±.08, 2.849±.02, 1.550±.008 mg/gm. equiv. to Gallic acid for *L. leucocephala* acetone, chloroform and methanolic extracts respectively. The total flavonoid content was found to be 0.823±0.02, 0.373±0.04, 1.325±0.08 mg/gm. equiv. to Quercetin for acetone, chloroform and methanolic extracts of *A. oppositifolia* respectively. The total flavonoid content was found to be 2.806±0.05, 3.587±0.003, 2.933±0.19

Table 2: Quantitative estimation of some phytochemical

Solvent extracts (100 µl of 1mg/ml)	Total phenolic content mg/g equiv. to Gallic acid		Total flavonoid content mg/g equiv. to Quercetin	
	A. opp.	*L. leuco.*	*A. opp.*	*L. leuco.*
Acetone	11.43±.02	1.34±.08	0.823±0.02	2.806±0.05
Chloroform	7.51±.02	2.849±.02	0.373±0.04	3.587±.003
Methanol	12.06±.03	1.550±.008	1.325±0.08	2.933±0.19

Each value is expressed as mean ± standard deviation (n= 6)

Table 3: Comparison of the antioxidant and free radical scavenging capacities of different solvent extracts of *A. oppositifolia* and *L.leucocephala.*

Extracts	Conc. (mg/ml)	Methanol		Acetone		Chloroform	
Plants		*A. opp.*	*L. leuco.*	*A. opp.*	*L. leuco.*	*A. opp.*	*L. leuco.*
Metal Chelation activity % Inhibition	0.25	50.32±.06	42.65±0.3	49.13±.04	25.56±.06	20.52±.07	56.30±.07
	0.50	85.18±.07	56.91±.04	65.36±.05	35.66±.03	30.10±.04	66.37±.02
	0.75	87.60±.03	73.51±.05	70.20±.03	51.10±.06	57.46±.06	80.42±.03
	1.0	92.62±.05	87.66±.02	77.68±.05	65.30±.008	74.40±.03	89.07±.03
IC_{50}		.065 mg/ml	0.37 mg/ml	0.19 mg/ml	0.72 mg/ml	0.68mg/ml	0.11 mg/ml
Reducing power assay Abs.(700nm)	0.25	0.346±.006	0.266±.006	0.293±.003	0.236±.04	0.214±.007	0.372±.010.
	0.50	0.494±.05	0.331±.008	0.347±.007	0.318±.007	0.226±.006	0.392±.007
	0.75	0.502±.003	0.341±.003	0.442±.01	0.334±.004	0.322±.004	0.418±.005
	1.0	0.520±.006	0.364±.006	0.448±.005	0.339±.006	0.353±.01	0.460±.008
IC_{50}		0.23mg/ml	0.40 mg/ml	0.22 mg/ml	0.38 mg/ml	0.24 mg/ml	0.42 mg/ml
Superoxide Scavenging assay % Inhibition	0.25	38.48±.05	20.82±.05	28.22±.05	15.46±.03	18.88±.02	22.08±.02
	0.50	55.78±.06	30.45±.03	37.05±.04	28.80±.008	27.81±.03	39.60±.03
	0.75	70.10±.04	55.12±.02	55.86±.06	42.90±.05	42.57±.04	56.67±.04
	1.0	78.62±.05	62.89±.08	60.11±.05	50.98±.04	66.43±.05	68.91±.05
IC_{50}		0.42 mg/ml	0.75 mg/ml	0.66mg/ml	0.88 mg/ml	0.80 mg/ml	0.67 mg/ml
Hydroxyl assay % Inhibition	0.25	37.18±.05	51.71±.02	56.2±.02	15.66±.02	41.48±.02	24.50±.05
	0.50	44.85±.04	57.85±.04	61.79±.04	36.71±.04	54.33±.04	28.51±.06
	0.75	61.35±.04	75.90±.01	74.55±.03	39.86±.03	70.94±.03	48.20±.04
	1.0	75.51±.02	85.47±.03	83.22±.01	67.11±.06	82.59±.01	72.41±.05
IC_{50}		0.53 mg/ml	0.25mg/ml	0.12 mg/ml	0.78mg/ml	0.40mg/ml	0.72mg/ml

Each value is expressed as mean ± standard deviation (n= 6)

mg/gm. equiv. to Quercetin for *L. leucocephala* acetone, chloroform and methanolic extracts respectively.

Chelating Power assay

It was observed (Table 3)that all organicextracts understudy exhibited Fe^{2+} ions chelating potential in a dose dependent manner. Among different extracts highest metal chelation was observed with methanolextract (IC_{50}0.065 mg/ml) of *A.oppositifolia* Fig. [1] and with chloroform extract (IC_{50} 0.68mg/ml) of *L. leucocephala* Fig. [2].Results was compared with standard metal chelator EDTA (IC_{50} 0.509mg/ml).A significant correlation has been observed between total phenolic contents and metal chelation activity Fig. [3]

Reducing power assay

Results in (Table 3)showed that *A.oppositifolia*methanol extract showed better reducing ability 0.520±.006 absorbance(IC_{50} 0.234 mg/ml) when compared to acetone and chloroform. Both acetone and chloroform extracts also exhibited Fe (III) reducing abilities with 0.448±.005 and 0.353±.01 respectively (IC_{50} 0.22 and 0.242mg/ml).In *L. leucocephala* leaves among the different solvent extracts chloroform extract showed better reducing ability 0.460±.008 (IC_{50} 0.42 mg/ml)in comparison to Methanol 0.364±.006 and acetone 0.339±.006 (IC_{50} 0.40 &0.38 mg/ml respectively). It was found that the tendency to reduce Fe (III) to Fe (II) in a dose dependent manner.Results were compared with standard reducing agent gallic acid (IC_{50} 0.651 mg/ml).

Superoxide anion scavenging assay

All the extracts understudy showed superoxide radical scavenging property (Table 3).At a dose of 1.0 mg/ml 78.62%± 0.05 inhibitions was observed with methanol extract of *A. oppositifolia* followed by chloroform 66.43±.05% and acetone 60.11±.05% under similar experimental condition. In *L .leucocephala* at a dose of 1.0 mg/ml68.91±.05%, 62.89±.08 %, 50.98 ±.04% inhibitions was observed with chloroform, methanol and acetone extracts respectively. It was found that the tendency to scavenge superoxide radical in a dose dependent manner.Results were compared with standard reducing agent quercetin (IC_{50} 0.744 mg/ml).

Hydroxyl radicals scavenging activity

Results in (Table 3)indicate that both the plants understudy showed significant hydroxyl radical scavenging property. At a dose of 1mg/ml acetone and chloroform extracts of *A. oppositifolia* showed around 83% ±.03 inhibition followed by methanol 75.51±.02 % .Under similar experimental condition *L. leucocephala* extracts also showed significant hydroxyl radical scavenging property at a dose of 1mg/ml with 85.47±.03%, 72.41±.05%, 67.11±.06%inhibitions with methanol, chloroform and acetone extracts respectively. It was found that the tendency to scavenge hydroxyl radicals in a dose dependent manner.Results were compared with standard reducing agent mannitol (IC_{50} 0.117mg/ml).

Correlation

Total phenolic content showed good correlation with antioxidant activity in *A. oppositifolia*with r^2value given Fig (3). On the basis of these result we reported, that the phenols are responsible for the antioxidant activity of the plant.Phenolic compounds may contribute directly to the antioxidant action due to the presence of hydroxyl functional groups around the nuclear structure that are potent hydrogen donators. Phenolics content are very

important plant constituents because they can act as reducing agents, hydrogen donors and metal chelator. They also act as radical scavenger due to their hydroxyl groups. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which play an important role as free radical scavengers, reducing agents, quenchers of singlet oxygen and complexes of pro-oxidant metalsFlavonoids found to be strong antioxidants because of their phenolic hydroxyl groups[31,32].

CONCLUSION

Based on the results of this study, it may be concluded that all the extracts*A.oppositifolia*and *L. leucocephala*leaves possess high antioxidant as well as iron chelating properties in vitro. These activities might be due to the presence of phenolics, flavonoids and others phytochemical constituents. Further studies of the plant material in evaluating the antioxidant potential, in vivo may be interesting.

CONFLICT OF INTERESTS

No any conflict of interest.

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