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Research Article

COMPARATIVE *IN VITRO* ANTIOXIDANT ACTIVITY OF ETHYL ACETATE AND ETHANOL EXTRACTS OF *CALLICARPA MACROPHYLLA*

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ABSTRACT

Objective: The objective of the study was to evaluate and compare the antioxidant activity of (EA and E) ethyl acetate and ethanol extracts of (CM) *Callicarpa macrophylla*.

Methods: The physiochemical parameters were assessed according to guidelines given by the world health organization. The total content of phenols and flavonoids was assessed by Folin–Ciocalteu and aluminum chloride methods. *In vitro,* antioxidant activity was screened by (DPPH) 1, 1-diphenyl-2-picrylhydrazyl and(H_2O_2) hydrogen peroxide scavenging and reducing power assay.

Results: The physicochemical parameters fulfilled the standards of WHO guidelines. Total phenol and flavonoid content were more in ethanol extract as compared to ethyl acetate extract of CM. The antioxidant activity of ethanol extract was further high as compared to ethyl acetate extract of *Callicarpa macrophylla*. The IC_{50} of *Callicarpa macrophylla* ethanol extract was less than the ethyl acetate extract. So, more antioxidant activity of ethanol extract compared to ethyl acetate extract of CM.

Conclusion: Overall, both the extracts showed antioxidant activity and can be used further for diseases that can be managed using antioxidants. Ethanol extract possessed significant antioxidant effects than the ethyl acetate extract.

Keywords: *Callicarpa macrophylla*, Antioxidant activity, Total flavonoid content, Phenol content, Physical evaluation.

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INTRODUCTION

Free radicals are those molecules which occupy one or more electron in the outermost orbital. To make themselves stable, they damage other molecules such as DNA, lipids, and proteins. Thus, forming a chain reaction of unstable molecules and resulting in the formation of reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroperoxyl radicals. Reactive oxygen species spawn consistently in living organisms and (FRS) free radical scavengers are the substances that protect cell damage by interrupting the chain reaction of free radicals [1]. Several plants are used traditionally for the treatment of diseases such as Alzheimer's, wound healing, rheumatism, inflammation, heart disease, and esophageal, prostate, and breast cancer and they showed the presence of polyphenolic compounds which are the richest sources of FRS or antioxidants [2]. Thus, FRS (antioxidant activity) is a significant method of authentication of antioxidants in traditionally used medicinal plants. *Callicarpa macrophylla* leaves were selected based on their traditional uses in inflammation and as an analgesic and antipyretic [3,4]. CM is found abundantly in India, Asia, Thailand, and Pakistan. In India, it is widely spread in Kashmir, Odessa, Meghalaya, Tripura, and Punjab up to an altitude of 1800 m [5]. The leaves are used in gout and rheumatic pain [6]. Decoctions of the leaves are used in the treatment of diarrhea, dysentery and to arrest bleeding. The juice made from leaves is used in gastric trouble, headache, and to stop bleeding [7]. Leaves contain calliterpenone, apigenin, docosanoic acid, tricosanoic acid, and tetracosanoic acid. Luteolin, β-sitosterol-β-D-glucoside, ursolic acid, fatty acids, and quercetin are also present [8].

METHODS

Plant materials and chemical reagents

For the current study, the leaves of CM were used. Leaves were purchased from an herbal market in Pune and authenticated by Dr. G.S. Kritikar, Head Pharmacognosist, Samanthak enterprises, Pune (Voucher Specimen Number SE/AC/2019/05). All the chemicals used were of analytical grade. Folin–Ciocalteu reagent, 2, 2-diphenyl-1-picrylhydrazyl Gallic acid, and quercetin were procured from Sigma-Aldrich.

Physiochemical evaluation

The parameters such as foreign matter, ash value, extractive value, and moisture content were assessed according to the WHO guidelines for the standardization of herbal medicines [9].

Soxhlet extraction

The plant materials were defatted with petroleum ether and extraction was done with ethyl acetate and ethanol. The extracts obtained were evaporated to make a concentrated mass and the percentage of yield was calculated based on the air-dried weight of the plant material [10].

Chemical tests for analyzing chemical constituents

The ethyl acetate and ethanol extracts of the leaves of CM (CMEA and CME) were analyzed for the plant constituents according to the standard procedures [10,11].

Estimation of total phenolic content

The total phenol content was determined using the Folin–Ciocalteu method. Briefly, 1 ml of extracts solution was mixed with 2.5 ml of 10% (w/v) Folin–Ciocalteu reagent. After 5 min, 2.0 ml of freshly prepared Na_2CO_3 (7.5%w/v) was subsequently added to the mixtures and incubated at room temperature for 10 min with intermittent agitation. Afterward, the absorbance was measured utilizing a UV Spectrophotometer at 765 nm against a blank without the extract. The outcome data were expressed as mg/g of Gallic acid equivalents in milligrams per gram (mg GAE/g) of the dry extract [12].

Estimation of total flavonoid content

One milliliter of extracts solution was mixed with 0.2 ml of 10% (w/v) $AlCl_3$ solution in methanol, 0.2 ml (1 M) potassium acetate, and

5.6 ml distilled water. The mixture was incubated for 30 min at room temperature followed by the measurement of absorbance at 415 nm against the blank. The outcome data were expressed as mg/g of quercetin equivalents in milligrams per gram (mg Qu/g) of the dry extract [13].

Assessment of FRS activity

This was performed using DPPH, H_2O_2, and reducing power assays.

DPPH assay

The radical scavenging activity of the crude extracts was adapted to measure antioxidant activity using the DPPH assay. Control was prepared by adding 1 ml of methanol in 2 ml of DPPH. The standard used was ascorbic acid at the concentration of 100 μg/ml. Briefly, 2 ml of extract solution (20–100 μg/ml) and methanol were added to 2 ml of DPPH (0.1 mM) solution and control separately. The mixtures were kept aside in a dark area for 30 min and absorbance was measured at λ max 515 nm against an equal amount of DPPH and methanol as a blank. The percentage of DPPH scavenging was estimated using the equation [14,15].

% scavenging of DPPH= [(A0-A1)÷A0] × 100

where A0 = absorbance of the control and A1 = absorbance of the test extracts

H_2O_2 assay

The FRS activity of individual extracts was determined using the H_2O_2 method. Briefly, 2 ml of extract solution (20-100 μg/ml) and methanol were added to 4.0 ml of H_2O_2 (40 mM) solution in phosphate buffer (pH 7.4). After 10 min, the absorbance was measured at λ max 230 nm against the phosphate buffer blank solution. Ascorbic acid was used as standard [14]. The same procedure was used for all extracts.

The percentage scavenging of H_2O_2 was calculated using the equation:

% scavenging of H_2O_2 [(A0−A1)÷ A0] × 100

where A0 = absorbance of the control and A1 = absorbance of the test extracts

Reducing power assay

The reducing powers of the individual extracts, that reflected their antioxidant activity were determined using the modified Fe^{3+} to Fe^{2+} reduction assay. Briefly, 1 ml of extract solution (10-100 μg/ml) and methanol were added to 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% (w/v) potassium ferricyanide [$K_3Fe(CN)_6$] solution. The mixture was vortexed and incubated at 50°C for 20 min assisted with a vortex shaker followed by the addition of 2.5 ml 10% (w/v) trichloroacetic acid and centrifuged at 3000 rpm. Finally, 2.5 ml of the supernatant was mixed with 2.5 ml deionized water and 0.5 ml of 0.1% (w/v) ferric chloride. Perl's Prussian blue color was measured at λ max 700 nm against a blank. Ascorbic acid was used as a positive control [16]. The increased absorbance of the mixture indicates greater reducing power.

RESULTS

At first, an investigation of the physical constants of the leaves of CM was done to check the quality and the purity. The organoleptic characters of CM leaves were mentioned in Table 1.

Table 1: Observations of organoleptic characters

S. No.	Parameters	*Callicarpa macrophylla*
1.	Color	Green
2.	Odor	None
3.	Taste	Bitter
4.	Shape	Ovate-lanceolate
5.	Size	12cm×5.3cm,
6.	Texture	Upper surface- Wrinkled Lower Surface- Glabrous

Physical evaluation

The physical evaluation was done to check the identity, purity, and quality of the crude drug. The foreign organic matter was estimated to check any type of impurity in the drug; it was detected as 1.5% w/w, respectively (Table2). The ash values exposed any type of inorganic constituents such as earthy matter and silica present in the drug, thus helps to disclose the quality of the drug. The total ash value was found to be 7.25% w/w, respectively. Acid insoluble ash was 0.47% w/w and water-soluble ash was 0.8% of CM leaves, respectively (Table2). The extractive value reveals the presence of adulterants due to exhausted or inferior drugs. Alcohol soluble extractive value for CM leaves was 12.2% w/w, while water-soluble extractive value was 11.95% w/w, respectively (Table 2). Loss on drying method helps in exposing the amount of volatile contents and water present in a drug that was noticed as 5.65 % w/w for CM leaves. The percentage yield of CMEA and CME was 8.56 and 7.24 w/w, respectively (Table 2).

Qualitative estimation by chemical tests

Chemical test for ethyl acetate and ethanol extracts of CM leaves showed the presence of different constituents such as alkaloids, carbohydrates, glycosides, phenolic compounds, triterpenoids, and flavonoids (Table 3).

Quantitative estimation

Quantitative estimation of phenolic and flavonoid content was done. The phenolic content of the CMEA extracts was 75.2 mg/g, while the flavonoid content was 62.5 mg/g, respectively. The phenol content

Table 2: Estimation of physical constants

S. No.	Plants name	*Callicarpa macrophylla*
1.	Foreign organic matter (%w/w)	1.5
2.	Total ash (%w/w)	7.25
3.	Water soluble ash (%w/w)	0.80
4.	Acid insoluble ash (%w/w)	0.47
5.	% Alcohol soluble extractable matter (%w/w)	12.2
6.	%Water soluble extractable matter (%w/w)	11.95
7.	Loss on drying (%w/w)	5.65
8.	Percentage yield of CMEA	7.55
9.	Percentage yield of CMEA	8.56

Table 3: Chemical tests for analyzing chemical constituents

S. No.	Chemical tests	CM ethyl acetate extract	CM ethanol extract
1.	Alkaloids	+	+
2.	Carbohydrates	–	–
3.	Proteins	–	–
4.	Amino acids	–	–
5.	Steroids	+	+
6.	Phenolic compounds	+	+
7.	Glycosides	+	+
8.	Flavonoids	+	+
9.	Terpenoids	+	+
10.	Tannins	–	+

Table 4: Total phenol and flavonoid content of ethyl acetate and ethanol extracts of *Callicarpa macrophylla*

S. No.	Methods used	*Callicarpa macrophylla* (mg/g)
1.	Total phenol content ethyl acetate extract	75.2
2	Total flavonoid content ethyl acetate extract	62.5
3.	Total phenol content ethanol extract	88.4
4.	Total flavonoid content ethanol extract	76.4

of CME extract was 88.4mg/g, whereas the flavonoid content was 76.4mg/g, respectively (Table 4).

FRS activity (DPPH, H_2O_2, and reduced power assay)

By the DPPH method, the IC_{50} value of ascorbic acid, CMEA and CME extract was found to be was found to be 23.44, 72.62 and 55.64µg/ml respectively (Table 5).

DPPH assay

The calibration curve of % inhibition versus concentration (µg/ml) of standard ascorbic acid, CMEA, and CME extracts was plotted in Figs. 1-3, respectively.

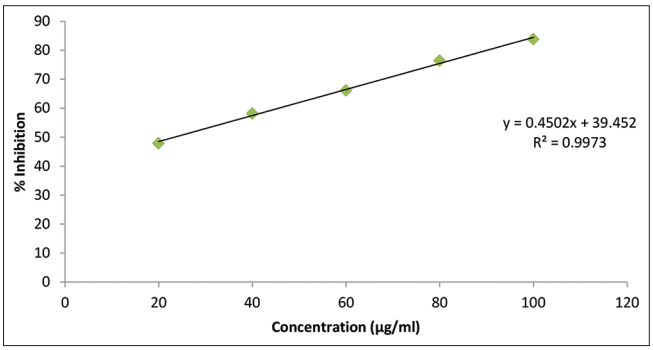

Fig. 1: % Inhibition of ascorbic acid by DPPH assay

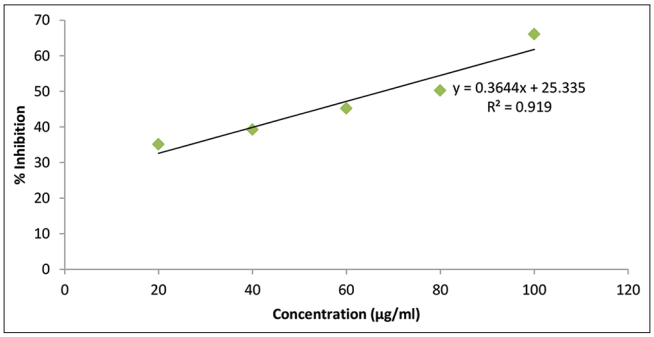

Fig. 2: % Inhibition of CMEA by DPPH assay

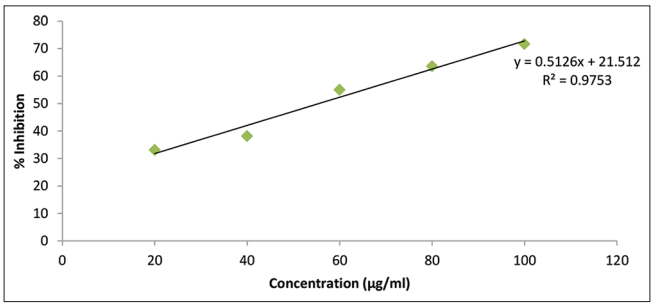

Fig. 3: % Inhibition of CME by DPPH assay

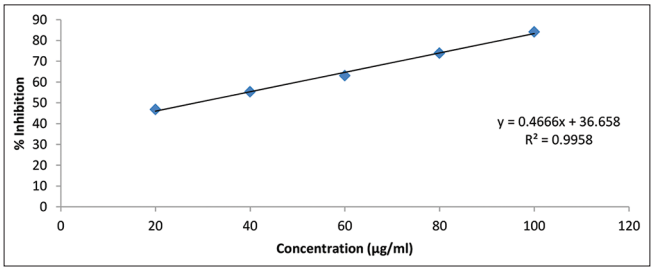

Fig. 4: % Inhibition of standard ascorbic acid by H_2O_2

H_2O_2 assay

By H_2O_2 assay,, the IC_{50} value of ascorbic acid, CMEA and CME extract was found to be was found to be 28.64, 81.77 and 62.79 µg/ml respectively (Table 6).

By H_2O_2 assay, the calibration curve of % inhibition versus concentration (µg/ml) of standard ascorbic acid, CMEA, and CME extracts was plotted in Figs. 4-`6, respectively.

Reducing power assay

The reducing power assay of ethyl acetate extracts showed the absorbance value of 0.182% and 0.128% by ascorbic acid and CMEA extracts, while with the ethanol extracts the absorbance was

Table 5: Observations of percentage inhibition of standard ascorbic acid, ethyl acetate, and ethanol extract by DPPH assay

S. No.	Concentration in µg/ml	% Inhibition ascorbic acid	% Inhibition CMEA	% Inhibition CME
1.	20	47.86±0.41	21.74±0.33	33.08±0.06
2.	40	58.15±0.20	30.52±0.26	38.12±0.25
3.	60	66.15±0.34	44.01±0.22	54.98±0.57
4.	80	76.34±0.33	51.48±0.50	63.53±0.40
5.	100	83.78±0.40	67.06±0.13	71.63±0.48
		IC_{50}=23.44	IC_{50}=72.62	IC_{50}=55.64

All values are Mean± SD of three replicate experiments (n=3)

Table 6: Observations of percentage inhibition of standard ascorbic acid and ethyl acetate and ethanol extract by H_2O_2 assay

S. No.	Concentration in µg/ml	% Inhibition ascorbic acid	% Inhibition *Callicarpa macrophylla*	% Inhibition *Callicarpa macrophylla*
1.	20	46.78±0.12	19.44±0.40	25.62±0.51
2.	40	55.32±0.33	25.52±0.49	32.55±0.48
3.	60	63.12±0.14	31.83±0.24	51.61±0.44
4.	80	73.89±0.15	49.94±0.34	61.63±0.32
5.	100	84.16±0.16	63.67±0.13	70.42±0.13
		IC_{50}=28.64	IC_{50}=81.17	IC_{50}=62.79

Table 7: Observations of reducing power assay of ethyl acetate extracts of all samples

S. No.	Concentration in µg/ml	Ascorbic acid	CMEA
1.	10	0.077±0.001	0.041±0.001
2.	20	0.101±0.002	0.048±0.002
3.	40	0.119±0.002	0.075±0.004
4.	60	0.139±0.001	0.100±0.001
5.	80	0.150±0.002	0.119±0.002
6.	100	0.182±0.001	0.128±0.002

Table 8: Observations of reducing power assay of ethanol extracts of all samples

S. No.	Concentration in µg/ml	Ascorbic acid	CME
1.	10	0.085±0.001	0.045±0.001
2.	20	0.109±0.001	0.054±0.002
3.	40	0.125±0.001	0.081±0.002
4.	60	0.142±0.001	0.101±0.002
5.	80	0.159±0.002	0.122±0.002
6.	100	0.187±0.002	0.139±0.002

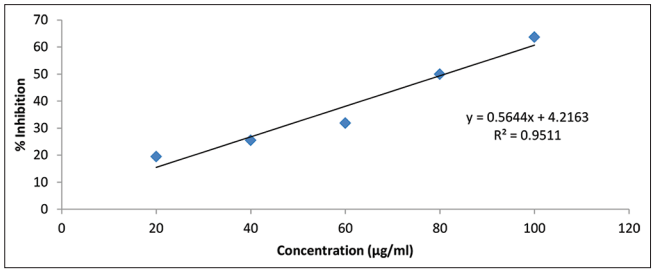

Fig. 5: % Inhibition of CMEA by H_2O_2 assay

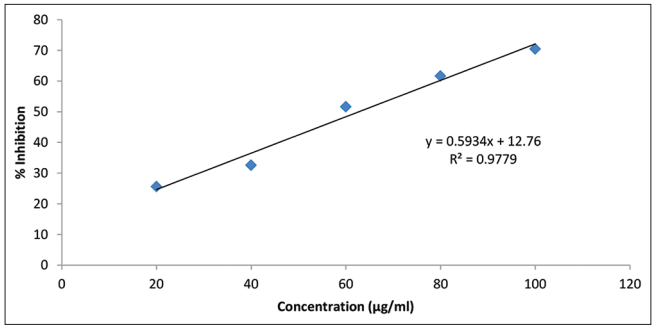

Fig. 6: % Inhibition of CME by H_2O_2 assay

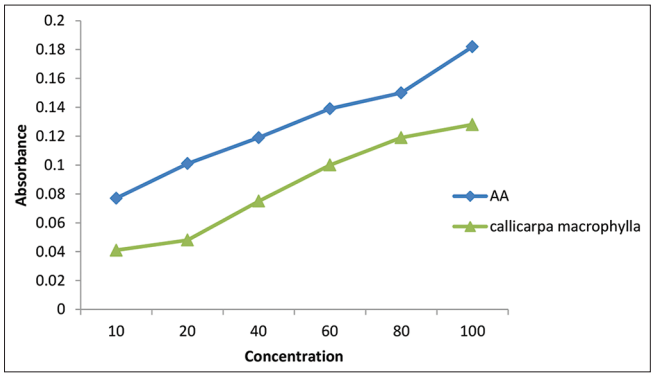

Fig. 7: Reducing power assay of ethyl acetate extracts of all samples

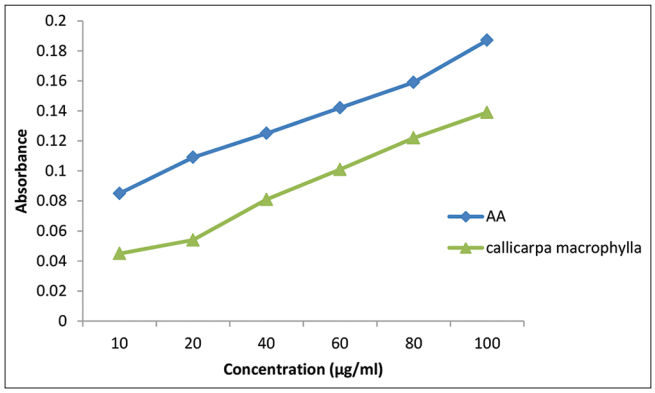

Fig. 8: Reducing power assay of ethanol extracts of all samples

0.187% and 0.139% by ascorbic acid and CME extracts respectively (Tables 7 and 8).

Reducing power assay

By reducing power assay, the calibration curve of % inhibition versus concentration (µg/ml) of standard ascorbic acid and CMEA extracts was plotted in Fig. 7, respectively, and for standard ascorbic acid and CME, it was plotted in Fig. 8, respectively.

DISCUSSION

In our study, a physiochemical evaluation of CM was done which showed that leaves were of the good quality and purity. Further, polyphenolic compounds were evaluated by qualitative and quantitative evaluation. The phenol and flavonoids contents were present in high amount in ethanol extracts which may be due to different chemical nature of the phenolic compounds in different polarity of the solvents. Further, a significant antioxidant activity was observed by both ethyl acetate and ethanol extracts because of the presence of polyphenols, which are responsible for the depletion of 2,2-diphenyl,1-picrylhydrazyl to 2,2-diphenyl,1-picrylhydrazine to form a blue color complex and conversion of hydrogen peroxide to hydroxyl radicals. The results of reducing power assay further supported the results by showing the good absorbance. As, higher the absorbance of the extracts more will be the antioxidant activity. Previous studies also reported that the phenolic and flavonoid contents are responsible for antioxidant activity [17-18]. A study reported in 2016, by Sharma and his coworkers showed that methanol extract of the stem and leaves of *Callicarpa arborea* has an IC_{50} value of 53.65 and 47.20 at the concentrations of 100-500µg/ml, respectively containing phenolic compounds [17]. Another study reported that *Celastrus paniculatus* seeds ethyl acetate extract was IC_{50} value of 558.58 and 601.81 µg/ml respectively, while the ascorbic acid was IC_{50} value of 11.24 and 6.83µg/ml by DPPH and nitric oxide scavenging assay. Many biological activities related to antioxidants are not evaluated yet, so this plant is a better option for those activities [18].

CONCLUSION

The physicochemical evaluation and antioxidant activity of ethyl acetate and ethanol extracts of CM leave results was in the positive direction, so these plants extracts can be investigated further for the lead compounds and for assessment of different therapeutic activities such as anti-inflammatory, anti-rheumatic, anticancer, and anti-aging which could be due to the antioxidant such as phenol, sterols, and flavonoids and can be used as good replacement therapy to the synthetic drugs.

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AUTHORS' CONTRIBUTIONS

All authors are equally contributed in performing the antioxidant activity and preparation of the manuscript.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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Nil.

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