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Carmona retusa (Vahl) Masamune-Potential Antioxidant Natural Medicine

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Abstract : The present study was intended to investigate the phytochemical screening and in vitro free radical scavenging ability of the different solvent extracts of *Carmona retusa* (Vahl) Masamune leaves. Preliminary phytochemical screening of plant extracts showed the presence of alkaloids, phenols, flavonoids, tannins, saponins, terpenoids, steroids, carbohydrates, glycosides, amino acids and proteins. The phytochemical screening of *Carmona retusa* (Vahl) Masamune leaves was carried out by using various solvent system of varying polarity of ethanol, methanol, chloroform, ethyl acetate and aqueous. Radical scavenging assays like 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{+}$), Hydroxyl radical (OH), Nitric oxide (NO) and Ferric reducing ability of Plasma (FRAP) assay were done using renowned protocols. The results obtained in the present study indicated that *Carmona retusa* (Vahl) Masamune leaf as a rich source of natural antioxidants and provides evidence that the solvent extract contains medicinally essential bioactive compounds and the plant species used as traditional medicine for the treatment of some diseases like diarrhea and digestive problems.
Key words : Phytochemicals, *Carmona retusa* (Vahl) Masamune, Metabolites, DPPH, Antioxidant.

Introduction

India has about 8,000 species of known medicinal plants and about 1,000 plants have been used in the traditional system of medicine like Ayurveda, Unani and Sidha[1]. Phytochemical screening is very important in identifying new sources of therapeutically and industrially important compounds like alkaloids, flavonoids, phenolic compounds, saponins, steroids, tannins, terpenoids etc. Diseases are best controlled through pharmacotherapy. The study of the many chemical constituents present in plant is very essential because most drugs used as medicines were later synthesized after a careful study of their constituents and structures[2]. *Carmona retusa* (Vahl) Masamune. Fam.-Boraginaceae (Heliotrope family) previously known as *Ehretia microphylla* Lam [3-5]. *Carmona retusa* (Vahl.) Masamune (*Ehretia microphylla* Lam.) is reported to be medicinally useful in an Indigenous System of Medicine[6].

Carmona retusa found in shrub jungles, river banks and roadsides. They may shrubs or small trees up to 2 meters tall, twigs glabrous to sparsely strigillose. They are evergreen hedge plant to small tree. Leaves were in clustered nearly 3-5, obovate or oblanceolate blade present base onto petiole, coasrsely 3-5 toothed towards apex, apex acute to obtuse or rounded, when leaves are young both surfaces with stiff white hairs, upper surface, petiole 1-5 mm long. Flowers 3-12 flowered scorpioid cymes, unbranched or branched once, sepals 4-

5, lanceolate, 3-4 mm long; corolla white, rotate, 8-10 mm in diam., lobes 4-5, 3-4 mm long. The Fruits are globose, 4-5 mm in diameter. Phytochemical screening is very essential for finding novel sources of therapeutically and pharmaceutically significant compounds like alkaloids, flavonoids, phenolic compounds, saponins, steroids, tannins, terpenoids etc. The present study aimed to find the phytochemical components and antioxidant potential of the various extracts of *Carmona retusa* (Vahl) Masamune.

Materials and Methods

Plant collection and extraction

The leaves of *Carmona retusa* (Vahl) Masamune were collected from Aranoothumalai, Salem District, Tamil Nadu, India. The Botanical recognition and authentication was done by Dr.C.Murugan, Scientist 'D'-In-charge, Botanical Survey of India, Southern Regional Centre, Coimbatore, Tamilnadu, India and the authentication number was BSI/SRC/5/23/2016/Tech/770.The voucher specimen has been deposited in the Department of Botany, Periyar University, Salem, Tamil Nadu, India.

Qualitative Phytochemical analysis of the leaf extract

Preparation of plant extract

The plant leaves were thoroughly washed in the running tap water for the removal of dust particles which are adhering on the leaf surfaces. Then the leaves were shade dried and powdered using mixer grinder and stored in an air tight bottle to avoid the effect of humidity. The powdered material (10 g) was extracted with 100 ml of selected organic solvents (Water, Methanol, Ethanol, Chloroform and Ethyl acetate) using soxhlet apparatus and filtered through Whatmann No 1 filter paper. The filtrate was concentrated and dried under reduced pressure and controlled temperature. The concentrated extracts of the leaves were stored in small vials at -20º C and used for further analysis.

The phytochemical screening aqueous, ethanol, methanol, chloroform and ethyl acetate extracts of sample was subjected to different chemical tests for the detection of different phytoconstituents using standard procedures [7-8]. The ultimate aim of the present study is to identify the presence of alkaloids, flavonoids, tannins, saponins, steroids, terpenoids, glycosides, phenols, carbohydrates and proteins and to study the in vitro free radical scavenging ability of the different solvent extracts of *Carmona retusa* (Vahl) Masamune leaves.

Test for Alkaloids-Meyar's reagent (potassium iodide): 1.3 g of mercuric chloride was dissolved in 60 ml distilled water and 5.0 g of potassium iodide in 10 ml of water. The two solutions were mixed and diluted to 100 ml with distilled water. To 1.0 ml of leaf extract, few drops of reagent were added. Formation of white or pale yellow precipitate showed the presence of alkaloids.

Phenols-Ferric chloride test: To 1.0 ml of leaf extract 2.0 ml of distilled water, followed by a few drops of 10 % aqueous $FeCl_3$ solution was added. Formation of blue or green colour indicates the presence of phenols.

Flavanoids: The test tubes containing 0.5 ml of leaf extract, 5-10 drops of dilute HCl and small piece of zinc or magnesium were added and the solution was boiled for a few minutes. In the presence of flavonoids, reddish pink or dirty brown colour was produced.

Tannins- Ferric chloride test: To 1- 2 ml of leaf extract, few drops of 5 % aqueous $FeCl_3$ solution was added. A bluish black colour, which disappears on the addition of a few ml of dilute H_2SO_4 was followed by the formation of a yellowish brown precipitate.

Saponins: In a test tube containing about 5.0 ml of leaf extract, a drop of sodium bicarbonate solution was added. The mixture was shaken vigorously and kept for 3 minutes. A honey comb like froth was formed and it showed the presence of saponins.

Terpenoids - Salkwski reaction: About5.0 ml of leaf extract was mixed in 2.0 ml of chloroform and 3.0 ml of concentrated $H_2SO_4$was carefully added to form a layer. A reddish brown coloration in the inter phase formed to show positive results for the presence of terpenoids.

Steroids- Libermann-Burchard's test: To 1.0 ml of leaf extract, 1.0 ml of concentrated H_2SO_4 was added, followed by the addition of 2.0 ml of acetic anhydride solution. Greenish colour developed and turned blue indicates the presence of steroids.

Carbohydrates-Benedict's test: 173 g of sodium citrate and 100 mg of sodium carbonate was dissolved in 500 ml of water. To this solution 17.3 g of copper sulphate dissolved in 100 ml of water was added. To 0.5 ml of the leaf extract, 5.0 ml of Benedict's reagent was added and boiled for 5 minutes. Formation of a bluish green colour showed the presence of carbohydrates.

Glycosides: About 1.0ml of leaf extract was dissolved in 1.0 ml of water and then an aqueous sodium hydroxide solution was added. Formation of a yellow colour indicates the presence of glycosides.

Aminoacids and Proteins-Biuret's test: To 1.0 ml of leaf extract, 5-8 drops of 5 % sodium hydroxide solution were added, followed by one or two drops of 1 % copper sulphate. Formation of pink or purple colour confirmed the presence of amino acids and proteins.

Free radical scavenging activity of plant leaves

The free radical scavenging activity such as DPPH, $ABTS^+$ and hydroxyl radical scavenging, FRAP assay, and nitric oxide scavenging assays were determined.

DPPH radical scavenging activity [9]

DPPH radical is scavenged by antioxidants through the donation of a proton forming the reduced DPPH. The colour change from purple to yellow after reduction can be quantified by its decrease in absorbance at wavelength 517 nm.Various concentrations (100 - 500 µg / ml) of a leaf extract (4.0 ml) were mixed with 1.0 ml of different solvents like ethanol, methanol, chloroform, ethyl acetate and aqueous solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.2 mM. The mixture was shaken vigorously and left for30 minutes at room temperature and the absorbance was measured at 517 nm. Vitamin C was used as a control. The percentage of DPPH decolorization of the sample was calculated as follows;

% decolorization = [1- (ABS sample/ ABS control) X 100]

$I = A_o – A_1 / A_o \times 100$,

Where, A_o is absorbance of control reaction, A_1 is absorbance of test compound.

$ABTS^+$ radical scavenging activity [10]

$ABTS^+$ decolourisation assay involves the generation of the $ABTS^+$ chromophore by the oxidation of $ABTS^+$ with potassium persulphate. It is applicable for both hydrophilic and lipophilic compounds. The scavenging activity of the leaf extracts on $ABTS^+$ radical cation was measured at 734 nm. The reaction was initiated by the addition of 1.0 ml of diluted $ABTS^+$ to 10 µl of different concentrations (50 - 250 µg / ml) of leaf extract and also to 10 µl of distilled water as a control. Ascorbic acid was used as positive control. The absorbance was read at 734 nm after 6 minutes and the percentage inhibitions were calculated. The inhibition was calculated according to the equation,

$I = A_o – A_1/A_o \times 100$,

Where, A_o is absorbance of control reaction, A_1 is absorbance of test compound.

Hydroxyl radical scavenging assay [11]

Hydroxyl radicals were generated from $FeSO_4$ and hydrogen peroxide and detected by their ability to hydroxylate salicylate and the hydroxylated salicylate complex is measured at 562 nm.A reaction mixture of 3.0 ml volume contained, 1.0 ml of 1.5 mM $FeSO_4$, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate and 1.0 ml of different concentrations (100 - 500 mg / ml) of leaf extract. After incubation for an hour at 37^{o}C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. Vitamin E was used as positive control. The percentage scavenging effect was calculated as,

Scavenging activity= $[1-(A_1-A_2)/A_0] \times 100$

Where, A_o is absorbance of the control, A_1 is absorbance in the presence of the extract; A2 is absorbance without sodium salicylate.

FRAP assay [12]

The total antioxidant potential of sample was determined using ferric reducing ability of plasma FRAP assay as a measure of antioxidant power. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue coloured Fe II-tripyridyl triazine compound from colourless oxidized Fe III form by the action of electron donating antioxidants.The stock solution of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ and 0.3 M acetate buffer (pH 3.6) was prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml acetate buffer. It was freshly prepared and warmed to 37°C. Then, 900 µl FRAP reagent was mixed with 90 µl water and 30 µl test sample/methanol/distilled water/standard antioxidant solution. The reaction mixture was then incubated at 37°C for 30 minutes and the absorbance was recorded at 593 nm. An intense blue coloured complex were formed when ferric tripyridyl triazine (Fe^{3+}-TPTZ) complex were reduced to ferrous (Fe^{2+}) form. The absorption at 540 nm was recorded. The calibration was plotted with absorbance at 593 nm Vs concentration of ferrous sulphate in the range 0.1 mM both aqueous and ethanol solutions. The concentrations of $FeSO_4$ were in turn plotted against concentration of standard antioxidants L-ascorbic acid.

Nitric oxide scavenging assay [13]

The interaction of plant extract with nitric oxide was assessed by the nitride detection method. Nitric oxide was generated from sodium nitroprusside and measured by the Griess illosvoy reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generated nitric oxide, which interacts with oxygen to produce nitrite, which can be estimated by the use of Griess illosvoy reagent, in the present experiment, nitrite ion was measured by using Griess illosvoy reagent, which is modified by using naphthyl ethylene diamine dihydrochloride instead of 1-naphthylamine.Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which were measured at 540 nm. The reaction mixture (6.0 ml) containing sodium nitroprusside (4.0 ml), phosphate buffer saline (PBS, 1.0 ml) and different concentrations (100 - 500 µg / ml) of a leaf extract (1.0 ml) in DMSO was incubated at 25°C for 15 minutes after incubation,0.5 ml of the reaction mixture containing nitrite was removed, 1.0 ml of sulphanilic acid reagent was added, mixed well and allowed to stand for 5 minutes for completion of diazotization and 1.0 ml of naphthyl ethylene diamine dihydrochloride was added, mixed well and allowed to stand for 30 minutes in diffused light. A pink coloured chromophore was formed. The absorbance of these solutions was measured at 540 nm against corresponding blank solutions. Rutin was used as a standard.

The inhibition was calculated according to the equation,

$I = A_o - A_1/A_o \times 100$,

Where, A_o is absorbance of control reaction, A_1 is absorbance of test compound.

Statistical analysis

All the data acquired were analyzed statistically and recoded in triplicates (n=3) values of the Mean ± Standard deviation of the results. Data were taken as one way analysis of the variance (ANOVA). The difference between the different concentrations were determined by DMRT test and using SPSS software. The P values of < 0.05 were considered significant.

Results and Discussion

Phytochemical screening of *Carmona retusa* (Vahl) Masamune leaves extracts

The phytochemicals such as a flavonoids, alkaloids and terpenoids were responsible for prevention of the oxidative damage to lipids and helps to protect liver and other tissues [14-16]. In this present study the *C. retusa* plant extracts showed presence of various secondary metabolites (Table 1).

Free Radical Scavenging Assays

Several free radical scavenging assays were followed because the antioxidant capacity of the plant extracts were could not be estimated by a single method. Hence the radical scavenging activity performs a vital role in the protection of free radical damages it is essential to study the functions of antioxidant capacity of the plant extracts. The free radicals are responsible for any kind of biochemical process and symbolize an indispensable element of aerobic life and metabolism. Most of the diseases were associated with oxidative stress because of free radicals [17]. In nature the human body is present with endogenous antioxidants and this property has the potential to stop formation of free radicals [18]. The presence of flavonoids seen in *Tilia* species shows antioxidant activity and performs as reactive oxygen species (ROS) scavengers, especially with hydrogen peroxide and superoxide anion which were responsible for various diseases like cancer [19].

Table 1: Phytoconstituents present in different solvent extracts of leaves

Phytochemicals	Aqueous	Ethanol	Methanol	Chloroform	Ethyl acetate
Alkaloids	+	+	+	-	-
Phenols	++	+++	+++	++	++
Flavonoids	++	+++	+++	++	++
Tannins	+	++	++	+	+
Saponins	+	++	++	++	+
Terpenoids	++	+++	+++	++	++
Steroids	-	+	+	-	-
Carbohydrates	-	-	-	-	-
Glycosides	+	+	+	+	+
Amino acids	-	+	+	-	-
Proteins	+	-	-	-	-

+ → present in small concentration; ++ → present in moderately high concentration;
+++ → present in very high concentration; -- → absent.

Table 2: DPPH Scavenging Assay

Sample	% inhibition				
	100 (µg/ml)	**200(µg/ml)**	**300 (µg/ml)**	**400 (µg/ml)**	**500 (µg/ml)**
Aqueous extract	10.20 ± 0.22	23.44 ± 0.34	36.10 ± 0.20	48.40 ± 0.30	62.10 ± 0.30
Standard (Vitamin C)	16.20 ± 0.40	32.10 ± 0.20	43.20± 0.50	57.40 ± 0.55	68.30 ± 0.20

The experiment was conducted in triplicates (n=3)

DPPH scavenging assay

DPPH free radical scavenging activity the conservative method which is used for screening the antioxidant activity of the selected plant extracts. Table 2 shows the DPPH radical scavenging activity of the aqueous extract of *C. retusa.* The free radical activity of plant extracts on DPPH was examined at the different concentrations (100, 200, 300, 400, 500µg/ml).

The DPPH radical scavenging activity of the *C. retusa* leaves extract at 500 (µg/ml) showed 62.10 % the radical scavenging activity also increased in dose dependent manner. The standard Vitamin C showed 68.30 % at the concentration range from 100 to 500µg/ml and the scavenging activity increased in a dose dependent manner. The sample concentration required to decrease the initial concentration of DPPH by 50% (IC_{50}). The lower IC_{50} value shows the higher antioxidant activity. IC_{50} values of aqueous extracts of *C. retusa* leaves and Vitamin C were 412.25 µg/ml, 348.67 µg/ml respectively (Fig. 1). The ability of antioxidants for DPPH radical scavenging is supposed to be due to their hydrogen donating property

[20]. When DPPH radicals react with suitable reducing agents, the electrons become paired off and the solution loses colour stoichiometrically depending on the number of electrons taken up [21].

ABTS$^+$ scavenging assay

ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants and of chain-breaking antioxidants. The extract efficiently scavenged ABTS radicals generated by the reaction between 2, 2'-azinobis (3- ethylbenzothiazolin-6-sulphonic acid) (ABTS) and potassium persulfate [22]. In this present study, the ABTS scavenging activity was increased in a dose dependent manner from 14.10% to 61.45% at the concentration of 50-250 µg/ml (Table 3). The aqueous extract and standard vitamin C showed an IC_{50} value of 198.21µg/ml and 173.06 µg/ml respectively. The aqueous leaves extract of *Carmona retusa* (Vahl) Masamune shows the ability for free radical scavenging (Fig. 2).

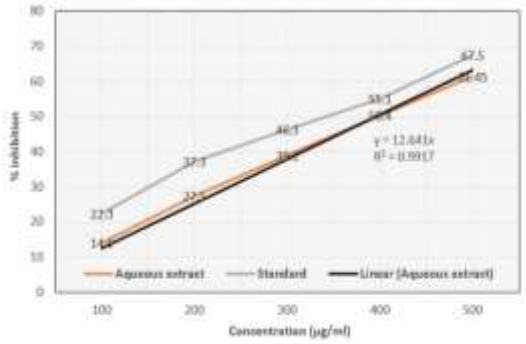

Figure 1: DPPH radical scavenging activity of *C. retusa* (IC_{50}-412.25 µg/ml)

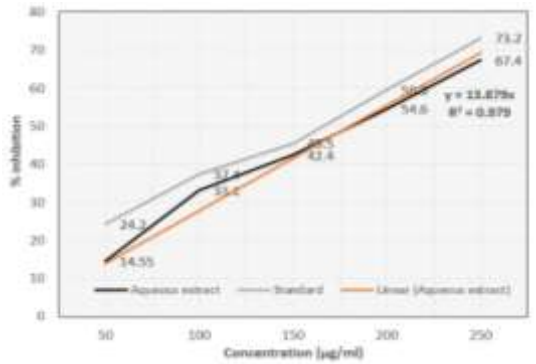

Figure 2: ABTS radical scavenging activity of *C. retusa* (IC_{50}-198.21µg/ml)

Table 3: ABTS scavenging activity of aqueous extract of *C. retusa*

Sample	Absorbance at 593 nm				
	100 (µg/ml)	200 (µg/ml)	300 (µg/ml)	400 (µg/ml)	500 (µg/ml)
Aqueous extract	0.35 ± 0.05	0.45 ± 0.30	0.52 ± 0.34	0.60 ± 0.20	0.74 ± 0.25
Standard (Vitamin C)	0.40 ± 0.10	0.54 ± 0.30	0.65 ± 0.35	0.70 ± 0.40	0.85 ± 0.20

The experiment was conducted in triplicates (n=3).

Table 4: Hydroxyl radical scavenging assay of aqueous extract of *C. retusa*

Sample	% inhibition				
	100 (µg/ml)	**200 (µg/ml)**	**300 (µg/ml)**	**400 (µg/ml)**	**500 (µg/ml)**
Aqueous extract	14.55 ± 0.20	33.10 ± 0.50	42.40 ± 0.55	54.60 ± 0.30	67.40 ± 0.80
Standard (Vitamin C)	24.20 ± 0.50	37.40 ± 0.30	45.50 ± 0.20	59.60 ± 0.50	73.20 ± 0.55

The experiment was conducted in triplicates (n=3)

Hydroxyl radical scavenging assay

The scavenging activity of the leaves extract of *Carmona retusa* (Vahl) Masamune was found 67.40%. The standard Vitamin C showed the inhibition value of 73.20% at 500 (µg/ml) concentrations. The inhibition value of standard vitamin C was higher than leaves extract of *C. retusa* (Table 4). The hydroxyl radical is the most reactive of the reactive oxygen species and it induces severe damage in adjacent biomolecules. The hydroxyl radical can cause oxidative damage to DNA, lipids and proteins [23-24].

The mutagenic capability of free radicals is due to the straight relations of hydroxyl radicals with DNA, resulting in DNA breakdown and therefore playing a significant work in a cancer development [25]. The aqueous extract and standard vitamin C showed an IC_{50} value of 365.26 µg/mland 336.12 µg/ml respectively (Fig. 3). Hydroxyl radical can be formed by the Fenton reaction in the presence of reduced transition metals such as Fe^{2+} and H_2O_2, which is known to be the most reactive of all the reduced forms of dioxygen and is thought to initiate the cell damage *in vivo* [26-27].

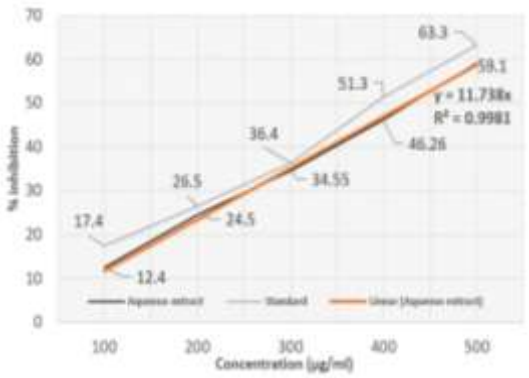

Figure 3: Hydroxyl radical scavenging assay*of C. retusa* (IC_{50}-365.26µg/ml)

Nitric oxide scavenging assay

Nitric oxide is a very unstable species under aerobic condition. It reacts with O_2 to produce stable products, nitrate and nitrite through intermediates. It also plays an important role in the pathogenesis of pain, inflammation, neural signal transmission, immune response, control vasodialation and blood pressuren [28].The scavenging of nitric oxide by leaf extract increased in a dose dependent manner as illustrated in (Table 5).

Table 5: Nitric oxide scavenging assay of *C. retusa*

Sample	% inhibition				
	100 (µg/ml)	**200 (µg/ml)**	**300 (µg/ml)**	**400 (µg/ml)**	**500 (µg/ml)**
Aqueous extract	12.40 ± 0.30	24.50 ± 0.40	34.55 ± 0.50	46.26 ± 0.30	59.10 ± 0.45
Standard (Vitamin C)	17.40 ± 0.20	26.50 ± 0.30	36.40 ± 0.45	51.30 ± 0.40	63.30 ± 0.20

The experiment was conducted in triplicates (n=3)

The scavenging activity of the leaves extract of *Carmona retusa* (Vahl) Masamune was found 59.10%. The standard Vitamin C showed the inhibition value of 63.30% at 500 (µg/ml) concentrations. The aqueous extract and standard vitamin C showed an IC_{50} value of 429.53 µg/ml and 391.04 µg/ml respectively (Fig. 4). Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases. β cells are particularly sensitive to damage by nitric oxide and free radicals because of their low levels of free radical scavenging enzymes [29].

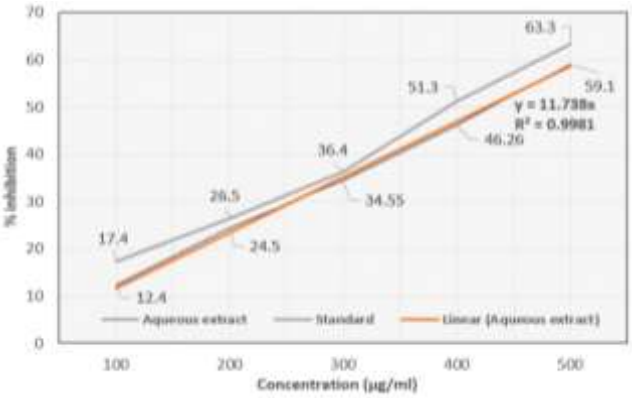

Figure 4: Nitric oxide scavenging assay *of C. retusa* (IC_{50}-429.53µg/ml)

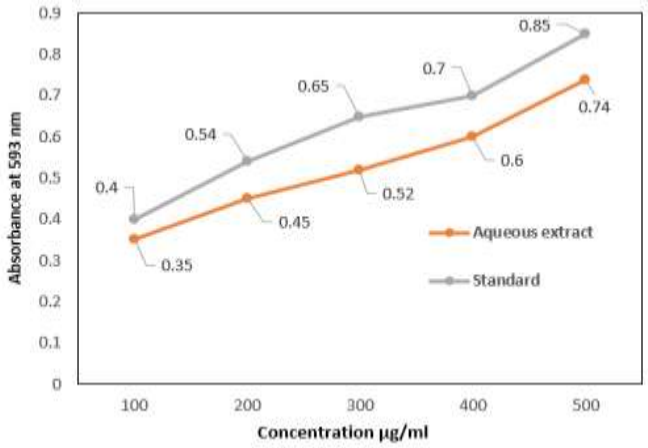

Figure 5: FRAP assay aqueous extract of *C. retusa*

FRAP assay

Present investigation showed the higher ferric reducing potency. The aqueous leaves extract of *C. retusa* and Vitamin C were showed in the Figure 5. Different concentrations of the plant extracts were used (100-500µg/ml) and the aqueous extract of the leaves showed the strong reducing power at the concentration of 500 µg/ml the leaves and its activity increases in a dose dependent manner when compared to the standard Vitamin C at the same concentration. Ferric Reducing Antioxidant Power (FRAP) assay is clearly reproducible and linearly interconnected to the molar concentration of the antioxidant present and it was described that its work as a free radical scavenger, proficient of transforming reactive free radical species into conventional non radical products [30].

Conclusion

The phytochemicals screening of the extracts showed the presence of alkaloids, phenols, flavonoids, tannins, saponins, terpenoids, steroids, carbohydrates, glycosides, amino acids and proteins. The phytochemical investigation and screening of antioxidant potential of the aqueous leaves extract of *Carmona retusa* (Vahl) Masamune shows the higher antioxidant activity.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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