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Research Article

EVALUATION OF *IN VITRO* ANTIOXIDANT ACTIVITY OF A TRITEREPNE ISOLATED FROM *MADHUCA LONGIFOLIA L* LEAVES

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ABSTRACT

Objective: To evaluate methanolic extract and dMA of *Madhuca longifolia L* leaves for possible antioxidant potential.

Methods: *In vitro* antioxidant activity of the methanolic extract and derivative of Madhucic Acid was evaluated by using Reducing power assay, Super oxide radical scavenging activity, Hydroxyl radical scavenging activity.

Result: MLME and dMA effectively scavenged free radicals at all different concentrations showed its potent antioxidant activity. Further, these effects were in a dose dependent manner. Results were compared to standard antioxidant such as butylated hydroxyl anisole.

Conclusion: The result indicated that *Madhuca longifolia L* leaves has significant natural radical scavengers.

Keywords: *Madhuca longifolia L*, Antioxidant, Triterpene, Reducing power assay, Super oxide radical scavenging activity, Hydroxyl radical scavenging activity.

INTRODUCTION

Oxygen is survival of all on this earth. During the process of oxygen utilization in normal physiological and metabolic processes approximately 5 % of oxygen gets univalently reduced to oxygen derived free radicals [1,2] like reducing power, super oxide radical, hydroxyl scavenging, nitric oxide. All these radicals are known as reactive oxygen species (ROS) exert oxidative stress towards the cells of human body rendering each cell to face about 10000 oxidative hits per second [3]. When generation of ROS overtakes the antioxidants defence of the cells. The free radicals start attacking the cell proteins, lipids and carbohydrates [4-6] and this leads to a number of physiological disorder. Free radicals are involved in the development of degenerative diseases. Many plants often contain substantial amounts of antioxidants including vitamin C and E, caretenoids, flavonids and tannins etc., and thus can be utilized to scavenge the excess free radicals from human body. *Madhuca longifolia L* is a folklore medicinal plant; it is commonly used for the treatment of snakebite as antidote in Southern part of India [7]. It is found in sub-tropical region. The plant also possesses antidiabetic and immunomodulators activity. The seed oil is used for cooking purpose. Flowers and leaves of the plant is widely used for making local liquor and also used in headache [8, 9]. Thus the present study was to investigate the antioxidant property of *Madhuca longifolia L* leaves in different *in vitro* models.

MATERIALS AND METHOD

Plant material

The leaves of *M.longifolia* were collected in Nov 2009 from Konchavaram forest Gulbarga, Karnataka, India and authentication was done by Prof Y.N. Seetharam, Dept of Botany, Gulbarga University Gulbarga, Karnataka India where a voucher specimen has been deposited in the herbarium (HGUG no: 723).

Extraction and isolation

Air dried leaves (500 g) of *M. longifolia* were reduced to a fine powder, which was subjected to hot continuous extraction in a soxhlet extractor, successively with petroleum ether (40-60˚C). Each time before extracting with the next solvent, the powder material was dried in hot air oven below 50˚C . Each extract was concentrated by distilling off the solvent followed by evaporation to dryness on a water bath. All extracts were kept in a desiccator and stored in a refrigerator for phytochemical and pharmacological studies. The methanolic extract was subjected for column chromatography on silica gel (60-120 mesh) and eluted with following solvent systems. The chloroform: methanol (1:9) fraction was repeatedly chromatograph on column and the collected fraction was checked on TLC until it gave a single spot of bright red color (100 mg) (R_f value : 0.89). The isolated compound was subjected for spectral analysis and the compound was identified as 10-(Carboxyoxy) 1,2,2,6a,9,9,hexamethyldocosahydropicene-4a-Carboxylic acid which showed m.p at 310˚C, λ_{max} 254nm, the IR (KBr) V_{max} cm^{-1} 3468.32 (OH) stretching, 2922-2808(C-H) stretching, 1709(C=O),1612(COOH), 1213 C-O-C); ^{1}H-NMR (DMSO) suggesting the structural similarities with Madhucic acid [10] which was identified and confirmed by LCMS,IR, ^{1}H-NMR (Fig 1).

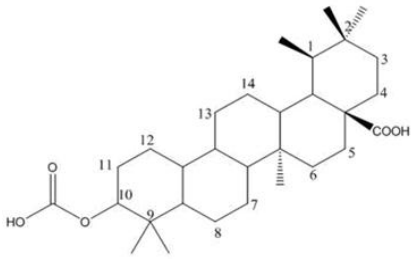

Fig. 1: Chemical structure of compound isolated from *Madhuca longifolia L* leaves 10-(Carboxyoxy)-1, 2, 2, 6a, 9, 9-hexamethyldocosahydropicene-4a-carboxylic acid

Drugs and chemicals

Nitro blue tetrazolium, and all the solvents used in the study were of analytical grade and were procured from SD Fine Chemicals Limited, Mumbai, India. Thiobarbituric acid, malondialdehyde and other chemicals were obtained from Sigma Chemical Company.

In vitro antioxidant activity

Reducing power activity

Various concentrations of methanolic extract and derivative of Madhucic Acid (50 µg, 75 µg and 100µg) were mixed with 2.5 ml of 200 m mole/L sodium phosphate buffer (P^H 6.6) and 2.5 ml of 1 % potassium ferricyanide. The mixture was incubated at 50^0 C for 20 min. 2.5 ml of 1% trichloroacetic acid (TCA w/v) was added. This was followed by the addition of 5 ml of distilled water and 1 ml of 0.1% of ferric chloride was added. The absorbance was measured spectrophotometrically at 700 nm. Butalylated hydroxy anisole (BHA) was used as standard antioxidant. [11].

Superoxide radical scavenging activity

The superoxide radical scavenging activity of MLME and dMA was measured according to the method of [12]. Super oxide radical

radicals were generated in 3.0 ml of tris–HCl buffer (16 mM, pH 8.0), containing 0.5 ml of nitroblue tetrazolium (NBT) (0.3 mM), 0.5 ml NADH (0.936 mM) solution, 1.0 ml of test samples and 0.5 ml Tris–HCl buffer (16 mM, pH 8.0). The reaction was initiated by adding 0.5 ml phenazine methosulfate (PMS) solution (0.12 mM) to the mixture, incubated at 25o C for 5 m and then the absorbance of various concentrations of MLME and dMA were measured at 560 nm against a blank sample. Percentage inhibition in absorbance was calculated as super oxide radical scavenging activity % inhibition = (absorbance of blank- absorbance of test) / absorbance of blank × 100.

Hydroxyl radical scavenging activity

various concentration of extracts were taken in different test tubes and made up to 250 µL with 0.1 M phosphate buffer 1 ml of iron-EDTA solution (0.13 % ferrous ammonium sulphate and 0.26 % EDTA) 0.5 ml of EDTA (0.018) and 1 ml of di-methyl sulphoxides (0.85 %) in 0.1 M phosphate buffer pH 7.4 were added to these tubes and the reaction was initiated by adding 0.5 ml of 0.22 % ascorbic acid. These reaction mixtures were incubated at room temperature for 15 m. The reaction was terminated by adding 1 ml of ice cold TCA (17.5 % w/v). 3ml of Nash reagent (150 gm of ammonium acetate, 3ml of glacial acetic acid and 2 ml of acetyl acetone were mixed and the volume was made to 1 liter with distilled water) was added to all the tubes and kept at room temperature for 15 m. The intensity of color formed was measured spectrophotometrically at 412 nm against blank. Percentage inhibition in absorbance was calculated as % inhibition = (absorbance of blank- absorbance of test) / absorbance of blank × 100. [13]

Statistical analysis

The data of the current experiment are presented as mean ± SEM (standard error mean).The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnet's t-test and the results were regarded as significant at P < 0.05 *.

RESULT

Table 1 represent the reductive capabilities of *Madhuca longifolia L* leaves compared to BHA. The reducing power of compound serves as a basis of an indicator for its potential antioxidant activity. In our studies the reducing power was found to be increased with increased concentration of extracts. Both methanolic extract and dMA showed significant (P<0.01** and P<0.05** respectively) ferric reducing activity. However, the result seemed to be more significant with methanolic extract and dMA, at 100 µg there was 0.962 nm and 0.462 nm reduction in reducing equivalent which corresponds to P<0.01**.

Table 1: Reducing power of *M.longifolia L* leaves.

Group	Treatment µg	Absorbance at 700 nm (Mean ± SEM)
I	Standard BHA	
	50	0.687 ± 0.00
	75	1.62 ± 0.00
	100	2.14 ± 0.00
II	MLME	
	50	0.36 ± 0.00
	75	0.51 ±0.00
	100	0.96 ±0.00
III	dMA	
	50	0.21 ±0.00
	75	0.24 ± 0.00
	100	0.46 ± 0.00

Values are mean **±** SEM; number of each group = 3, MLME – *Madhuca longifolia* methanolic extract, dMA - derivative of Madhucic Acid.

Table 2 represent the reductive capabilities of Madhuca longifolia L leaves compared to BHA. Methanolic extract showed significant (P<0.01**) results at all the concentrations. The percentage inhibition of super oxide radical scavenging activity is being highest 74.10 % at 100 µg.The dMA also exhibited super oxide scavenging activity; it also showed maximum reduction i.e. 70 % at 100 µg concentration.

Table 2: Super oxide radical scavenging of *M.longifolia L* leaves

Group	Treatment µg	Percentage inhibition (Mean ± SEM)
I	Standard BHA	65.42 ± 0.00
	50	
	75	72.01 ± 0.00
	100	86.26 ± 0.04
II	MLME	48.82 ± 0.00
	50	
	75	52.68 ± 0.00
	100	75.56 ± 0.00
III	dMA	40.82 ± 0.00
	50	
	75	50.01 ±0.00
	100	60.56 ±0.00

Values are mean **±** SEM; number of each group = 3, MLME – *Madhuca longifolia* methanolic extract, dMA - derivative of madhucic acid

Table 3 represent the percentage of hydroxyl radical scavenging activity of *Madhuca longifolia* leaf extract. Hydroxyl radical is the most reactive among ROS and it bears the shortest half-life compared with other ROS. In this study administration of leaf extract to the reaction mixture significantly inhibited the hydroxyl radical activity. Both methanolic extract and dMA exhibited significant (P<0.001**) activity at all three concentrations. This was quite more when compared with the standard: it means that the test extract is definitely better hydroxyl scavenger than that of standard. These results were found statistically significant (P<0.01**).

Table 3: Hydroxy radical scavenging activity of *M.longifolia L* leaves

Group	Treatment µg	Percentage inhibition (Mean ± SEM)
I	Standard BHA	
	50	4.86 ± 0.00
	75	8.68 ± 0.00
	100	16.86 ± 0.00
	MLME	
II	50	68.03 ± 0.00
	75	72.65 ± 0.00
	100	79.32 ± 0.00
	dMA	
III	50	41.13 ± 0.00
	75	43.15 ± 0.00
	100	46.77 ± 0.00

Values are mean **±** SEM; number of each group = 3, MLME – *Madhuca longifolia* methanolic extract, dMA - derivative of madhucic acid

DISCUSSION

The antioxidant activities of natural components may have a reciprocal correlation with there reducing powers [14].The reducing power of methanolic extract and dMA of *Madhuca longifolia* was determined. The reducing power increased as the extract concentration increased, indicating some compounds in *Madhuca* are both electron donors and could react with free radicals to convert them into more stable products and to terminate radical chain reactions. It has been shown that the antioxidant effect exponentially increased as a function of the development of reducing power, suggesting that the antioxidant properties be associated with the development of reducing power [15]. Super oxide is produced from molecular oxygen due to oxidative enzymes [16] of body as well as via non enzymatic reaction such as auto oxidation by catecholamine. In the present study super oxide radical reduces NBT to a blue colored formazan is measured at 560 nm. The effect of MLME and dMA in this regard is shown in table 1. The probable mechanism of scavenging the super oxide anions may be due to the inhibitory effect of MLME and dMA towards the generation of super oxides in the reaction mixture.

As observed from the data the components presents in the extract of *Madhuca longifolia* have high antioxidant activities and its various antioxidant mechanisms may be attributed to the strong reducing power and its effectiveness as a good scavenger of free radicals. According to many reports, highly positive relationship between triterpene and antioxidant activity has been found in extract of many plants species. Numerous *in vitro* studies have shown that some of the phytochemical are potent antioxidants, metal chelators or free radical scavengers which may account for their health promoting properties [17]. Naturally occurring triterpene compounds are reported to possess free radical scavenging properties, due to their hydroxyl groups. It is apparent from the present study that the components present in leaves not only scavengers off the free radicals but also inhibits the generation of free radicals.

CONCLUSION

It may be thus concluded that the fraction obtained from methanolic extract of *Madhuca longifolia L* leaves possess significant antioxidant activity. The antioxidant potential may be attributed due to the presence of bioactive isolated.

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