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RESEARCH ARTICLE

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TYROSINASE INHIBITION AND ANTI-OXIDANT PROPERTIES OF *MUNTINGIA CALABURA* EXTRACTS: IN *VITRO* STUDIES

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ABSTRACT

The present investigation deals with identifying new potential skin-lightening and antioxidant agents from natural origin for treating the hyperpigmentation disorders. The medicinal plant, *Muntingia calabura* was selected as the candidate plant for the present study. 10 % (w/v) extract of various parts (leaf, flower and fruit) of *M.calabura* in different solvents (aqueous, ethanol, hydroethanol and petroleum ether) was prepared by decoction method. The antityrosinase activity of the extracts from various parts of the plant was determined by using mushroom tyrosinase as an apt model system and it was found that the hydroethanolic extract of leaves from *M. calabura* possessed the maximum tyrosinase inhibiting potential among the various parts examined. The antioxidant activity of leaf extract of the plant was also ascertained by using 2, 2-diphenyl 1-picryl hydrazyl (DPPH) scavenging assay and ferric thiocyanate assay. The results showed that DPPH scavenging activity of hydroethanolic extract of leaves was in a dose dependant manner with the IC_{50} value of 8.5 μg. The inhibition of lipid peroxidation was almost similar in aqueous and hydroethanolic leaf extracts of *M.calabura*. The phenolic content of various solvent extracts of *M.calabura* leaves was also determined. The hydroethanolic extract of *M.calabura* leaves exhibited the phenolic content to a greater extent. Our findings revealed that leaves of *M.calabura* exerted the potent antityrosinase and antioxidant activities.

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KEY WORDS

Antityrosinase, DPPH, hyperpigmentation, melanin, *Muntingia calabura*, skin lightening.

INTRODUCTION

The skin is an important barrier that protects our body from damage due to its direct contact with the outside environment. Melanin is the important pigment in the skin. It protects our skin from UV damage by absorbing sunlight and removing reactive oxygen species. About 10 % of skin cells in the innermost layer of epidermis produce melanin. Upon exposure to UV radiation, melanogenesis is initiated by the enzyme tyrosinase resulting in skin darkening (Vamos, 1981).

Tyrosinase is a multifunctional, glycosylated, copper containing oxidase and found exclusively in melanocytes (Strothkemp *et al.*, 1976). The biosynthetic pathway is initiated with the hydroxylation of L-tyrosine to 3-4-dihydroxyphenylalanine (L-DOPA) and the oxidation of L-DOPA to o-dopaquinone. This o-quinone is a highly reactive compound and can polymerize spontaneously to form the pigment melanin; this presents a serious aesthetic problem in human beings (Briganti et al., 2003).

The hyper-pigmented skin is observed in various dermatological disorders namely melasoma, solar lentigines and ephelides (Maeda and Fukuda, 1991). The above mentioned clinical conditions are due to frequent exposure to UV rays, certain drugs and chemicals resulting in skin darkening. Depigmenting agents commonly are prescribed to treat disorders of hyper pigmentation (Kubo, 1986; Jang et al., 1997).

The most common skin lightening and depigmentation agents available commercially are kojic acid, arbutin, catechins, hydroquinone (HQ) and azelaic acid (Maeda and Fukuda, 1996). Some adverse effects of these synthetic compounds are irreversible cutaneous damage, ochronosis etc. These adverse effects have lead to the search for safer plant-based skin lightening ingredients.

Muntingia calbura. L. (also known as Jamaica cherry) selected as the target plant for the present study belonging to the family Elaeocarpaceae (Morton, 1987). It is native to the American continent and is widely cultivated in warm areas of Asian region (Chin, 1989). Its leaves, barks and flowers are believed to possess medicinal value as reported in Peru folklore medicinal uses. This plant is rich in flavonoids, flavones and flavanones, rendering to its potent antitumor activities (Nshimo et al., 1993). The roots have been employed as an emmenogogue in Vietnam and as an abortifacient in Malaysia. In the Philippines, the flowers of this species have been used to treat headaches, incipient colds, as an antidyspeptic, antispasmodic and diaphoretic. Infusions of the flowers of this plant used as a tranquillizer and tonic in Colombia (Perez-Arbelaez, 1975).
Scientifically, this plant has been proven to possess anti-tumor (Kaneda et al., 1991; Su et al., 2003), anti-inflammatory, anti-pyretic properties (Zakaria et al., 2007 b, 2008), antibacterial activity (Zakaria et al., 2006) and antistaphyloccocal activity (Zakaria et al., 2007 a). Plant extracts having an inhibitory effect on melanogenesis may be a good choice for cosmetic applications because of their natural

origin and with very low side effects. Thus, the present investigation was carried-out to analyze the skin whitening and antioxidant potential of *M. calabura*.

MATERIALS AND METHODS

Medicinal Plant

Leaves, flowers and fruits of *M.calabura* were obtained from the gardens of ITC R&D, Bangalore during the month of August 2010. The plant material was authenticated by the Botanist, Mount Carmel College, Bangalore.

Preparation of plant extract

Various parts of *M.calabura* was thoroughly washed with distilled water and dried under shade. The dried materials were ground separately into powder and used for experimentation. One gram of herbal powder was taken and dissolved in 10ml of water/ethanol/hydroethanol(75:25)/petroleum ether. The solution was heated in the boiling water bath at 60 degree centigrade for 60 minutes. The mixture was cooled to room temperature and centrifuged at 6000 rpm for 10 minutes. The supernatant was filtered and the filtrate was collected and used for the analysis.

Skin whitening assay

Antityrosinase assay (Lee et al., 2003)

Tyrosinase (Phenoloxidase activity) which catalyses the transformation of L-tyrosine into L-DOPA by hydroxylation and further into O-dopaquinone by oxidation. Then, through a series of non-enzymatic reactions, O-dopaquinone is rapidly transformed into melanins, which is measured at 492 nm in a spectrophotometer. Each plant extract was assayed for tyrosinase inhibition by measuring its effect on tyrosinase activity using a 96-well reader. The reaction was carried out in a 50 mM potassium phosphate buffer (pH 6.8) containing 20 mM L-tyrosine and 125 U/mL mushroom tyrosinase at 30°C. The reaction mixture was pre-incubated for 10 min before adding the enzyme. The reaction mixture without the enzyme serves as blank. The reaction mixture with the corresponding solvents (without plant material) serves as control. The change of the absorbance at 492 nm was measured. The percent inhibition of tyrosinase was calculated as follows:

$$\% \text{ Inhibition of tyrosinase} = \frac{\text{OD of Control- OD of Test}}{\text{OD of Control}} \text{X100}$$

Antioxidant assays

The antioxidant activity of plant material was evaluated by employing the following methods.

DPPH radical scavenging assay (Mensor et al., 2001)

DPPH (2, 2-diphenyl picryl hydrazyl) is a commercially available, commonly used, stable free radical, which is purple in colour. Antioxidant molecules when incubated, reacts with DPPH and converts it into di-phenyl hydrazine, which is yellow in colour. The degree of discoloration of purple to yellow was measured at 520 nm, which is a measure of scavenging potential of plant extracts. 5 µl of plant extract was added to 195 µl of DPPH solution (0.1mM DPPH in methanol) in a microtitre plate. The reaction mixture was incubated at 25 ^{0} C for 10 minutes, after that the absorbance was measured at 520 nm. The DPPH with corresponding solvents (without plant material) serves as control. The methanol with respective plant extracts serves

as blank. The DPPH radical scavenging activity of the plant extract was calculated as the percentage inhibition.

$$\% \text{ Inhibition of DPPH radical} = \frac{\text{OD of Control- OD of Test}}{\text{OD of Control}} \times 100$$

Lipid peroxidation by Ferric thiocyanate method (Mistuda et al., 1996)

Cell membrane is more susceptible to free radicals that reacts rapidly with the unsaturated fatty acids (like linoleic acid and arachidonic acid) embedded in the membrane resulting in lipid peroxidation. In this assay, linoleic acid is used as the model system for measuring the levels of lipid peroxidation. This was used to determine the amount of peroxide formed during the lipid peroxidation, in which peroxide will react with ferrous chloride and form ferric ions. Ferric ions will then unite with ammonium thiocyanate and produce ferric thiocyanate whose colour is measured at 500nm. A mixture containing 10 ml of 0.05 M-phosphate buffer (pH 7.0), 5.9ml of water, 0.1 ml of plant extract and 4 ml of 2.5% linoleic acid in absolute ethanol was placed in a vial with a screw cap and then placed in a dark oven at 40 degree centigrade for overnight. To 0.1ml of this incubation mixture, added 9.7ml of 75% ethanol and 0.1 ml of 0.02M ferrous chloride in 3.5% HCl. Add 0.1ml of 30% ammonium thiocyanate, precisely 3 minutes after the addition of ferrous chloride. The absorbance of the red colour was measured at 500nm. A mixture without the plant sample was used as the negative control. (Note: Instead of plant extract, use 0.1 ml of water/ethanol/ petroleum ether as control).

Determination of total phenolics (Mallick and Singh, 1980)

Phenols react with phosphomolybdic acid in Folin-ciocalteau reagent in alkaline medium and produce a blue colored complex (molybdenum blue) that can be estimated colorimetrically at 650 nm. Pipetted out different aliquots (0.1 to 0.5 ml) of plant extracts into the test tubes. Made up the volume in each tube to 3.0 ml with water. Added 0.5 ml of Folin-Ciocalteau reagent. After 3 minutes, added 2.0 ml of 20% sodium carbonate solution to each tube. Mixed thoroughly, placed the tubes in a boiling water bath for exactly 1 minute, cooled and measured the absorbance at 650nm against reagent blank.

Statistical analysis:

Samples were analyzed in triplicate and the results were given as Mean ± S.D.

RESULTS AND DISCUSSION

The reactive oxygen species produced in the human body enhances the DNA damage, melanin biosynthesis and induces the proliferation of melanocytes (Yasui and Sakurai, 2003) which results in hyperpigmentation disorders. The production of melanin in the body can be reduced by several mechanisms including direct inhibition of tyrosinase enzyme, inhibiting the transport of melanosome to the stratum corneum, the supplementation of antioxidants (Pawelek and kormer, 1982) and so on.

Skin whitening agents bring the attention of researchers in recent days in order to find the solution for hyperpigmentation disorders. Many tyrosinase inhibitors and antioxidant agents have been tested as a way of preventing overproduction of melanin in epidermal layers (Cabanes et al., 1994). The current study

employs the antityrosinase and antioxidant efficacy of various parts of *M. calabura.*

Antityrosinase activity of various parts of *M.calabura* in different solvents.

The antityrosinase activity of various parts of *M. calabura* in different solvents was summarized in Table 1. The hydroethanolic extract of *M.calabura* leaf exhibited the potent inhibition of tyrosinase enzyme when compared to the aqueous and petroleum ether extract of leaf. The tyrosinase inhibition values of the ethanolic extract of *M.calabura* leaves were discarded from the results because this extract formed a white precipitate when mixed with other reagents during the assay in the reaction mixture. In case of *M.calabura* flowers, the ethanolic extract exerted high tyrosinase inhibiting potential when compared to aqueous extract of flowers. No activity was observed in petroleum ether extract of flowers. The tyrosinase inhibiting potency of fruit of *M.calabura* was maximum in ethanolic extract as compared to the aqueous extract. As in the case of flowers, the petroleum ether extract of *M.calabura* fruits also showed no tyrosinase inhibition.

Table 1
Antityrosinase activity of various parts of M.calabura in different solvents.

Part used	Type of extract	% inhibition of tyrosinase
Leaves	Aqueous	81.59±2.3
	Hydroethanolic	94.00±1.97
	Petroleum ether	01.02±0.084
Flowers	Aqueous	43.98±2.32
	Ethanol	52.52±3.12
	Petroleum ether	-
Fruits	Aqueous	1.32±0.098
	Ethanol	10.20±0.87
	Petroleum ether	-

Among the different parts of the *M.calabura* screened, the ethanolic extract of the various parts namely, leaves, flowers and fruits showed better tyrosinase inhibition when compared to the aqueous and petroleum ether extracts. In order to compare the tyrosinase inhibiting potential of various parts, leaves show very good skin whitening efficacy when compared to flowers and fruits irrespective of the solvents used for the extraction. The aqueous extract of the *M.calabura* leaves shows 81.59% inhibition of tyrosinase which is superior to all other values of the other parts in the same solvent. The hydroethanolic extract of *M.calabura* shows very good skin whitening potential (94.00%) which is better than the aqueous extract of the leaves.

Lipid peroxidation by ferric thiocyanate method

Biological membranes are rich in unsaturated fatty acids that are most susceptible to

oxidative damage. More particularly, linoleic acid and arachidonic acid are targets of lipid peroxidation (Yu, 2001). The various solvent extracts of *M.calabura* leaves have been tested for their antioxidant potential through the inhibition of lipid peroxidation on linoleic acid oxidation (Figure 1). The results showed that aqueous and hydroethanolic extract of *M.calabura* leaves exhibited almost the similar inhibition of linoleic acid oxidation followed by ethanolic extract and petroleum ether extract of leaves. The antioxidant activity of plant extracts may be due to the hydrogen donating ability of phenolic compounds that stabilize the free radicals.

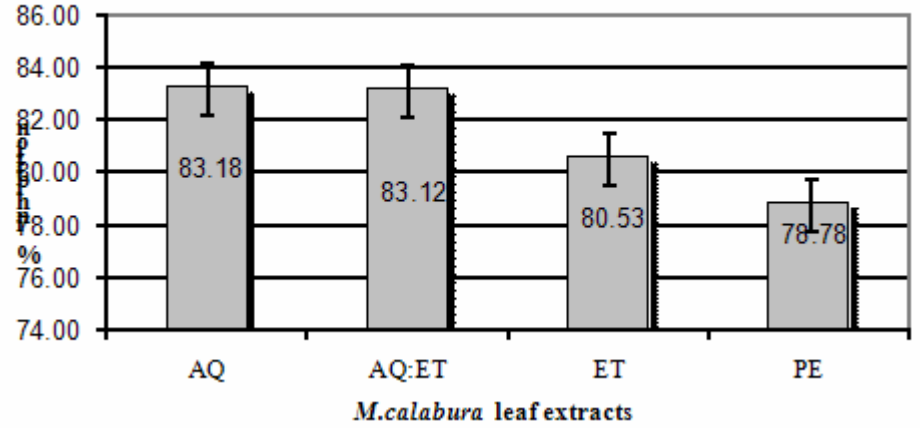

Figure 1
Inhibition of lipid peroxidation by various solvent extracts of M.calabura leaves.

DPPH radical scavenging ability of various solvent extracts of *M.calabura* leaves.

DPPH is generally used as a substrate to evaluate the antioxidant activity of plant extracts. The capacity of different extracts of *M.calabura* to scavenge the DPPH radical was measured as shown in Figure 2. The marginal difference was observed between ethanolic and hydroethanolic extract of *M.calabura* leaves for scavenging the DPPH radical. This is followed by aqueous and petroleum ether extract of leaves. Since the hydroethanolic extract of plant leaves exhibited the maximum scavenging activity, it was further examined for determining the IC_{50} value.

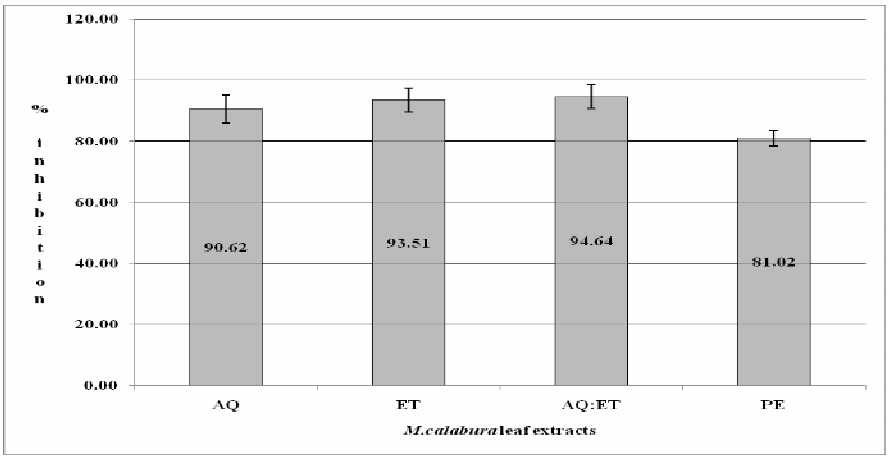

Figure 2
DPPH-scavenging activity of M. calabura leaves in various solvents.

Concentration dependant DPPH radical scavenging ability of hydroethanolic extract of *M.calabura* leaves.

Figure 3 demonstrates the DPPH scavenging ability of hydroethanolic extract of *M.calabura* leaves in a dose dependant manner. The scavenging of the DPPH radical was in a concentration dependant manner with the IC_{50} value of 8.5 μg. The strong inhibition of DPPH radical was recorded as 94.03 % at 40 μg concentration. The scavenging ability of the plant extract attained saturation at the dose of 40 μg onwards. It appears that hydroethanolic extract of *M.calabura* possesses hydrogen-donating capability that confers antioxidant efficacy.

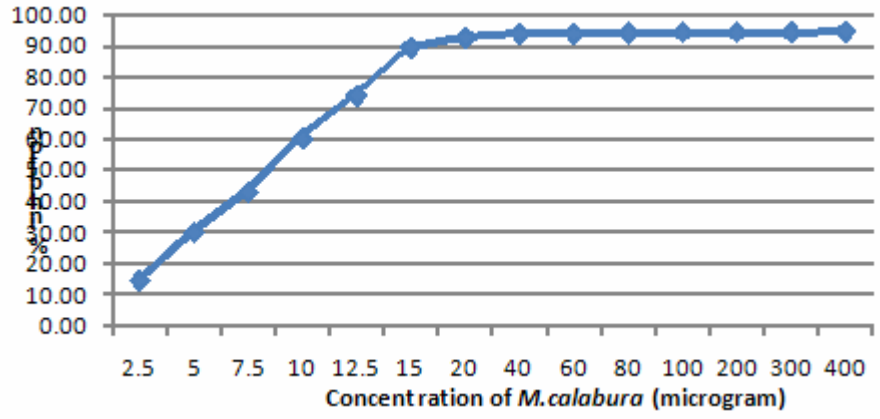

Figure 3
DPPH-scavenging activity of M. calabura leaves at various concentration.

Determination of total phenolics in various solvent extracts of *M.calabura* leaves.

The phenolics are a class of antioxidant compounds which serve as free radical terminators by scavenging or chelating process. The free radical induced lipid peroxidation is inhibited by the proton donating ability of hydroxyl groups in phenolic compounds. Figure 4 indicates the total phenolic contents of different solvent extracts of *M.calabura* leaves. The order of phenolic contents in various extracts of leaves was as follows.AQ: ET>ET>AQ>PE. There was a good correlation between the phenolic content and antioxidant potential of *M.calabura* leaves studied in various solvents.

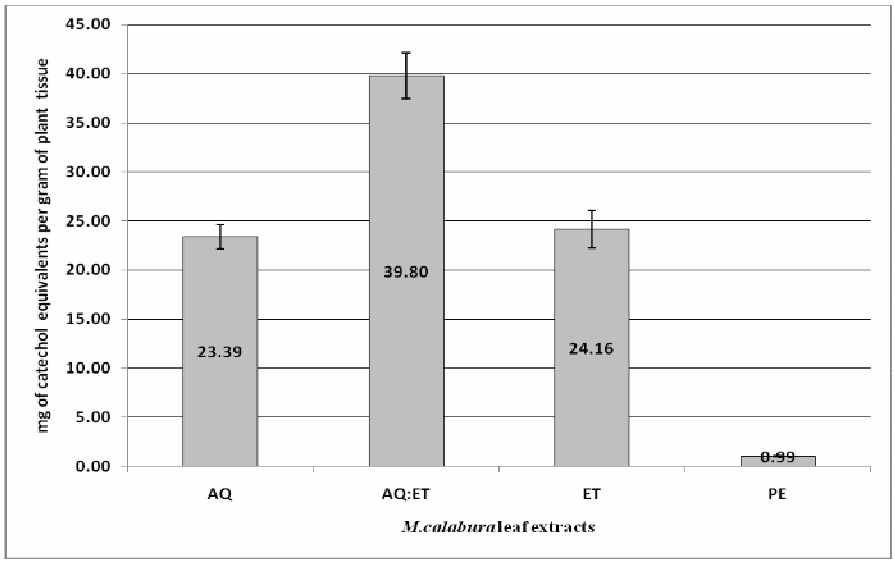

Figure 4
Total phenolics of M.calabura leaves in various solvents.

CONCLUSION

Our findings indicate that antityrosinase and antioxidant activity of hydroethanolic extract of leaves from *M.calabura* was significantly higher when compared to the flowers and/or fruit. The preliminary chemical examination of the leaf extracts has demonstrated the presence of phenolic compounds, which may be responsible for its strong antioxidant and lipid peroxidation inhibition activities. The potent skin whitening and antioxidant ability of *M.calabura* leaves make it a suitable candidate for the remedy of hyperpigmentation disorders. This needs to be done after establishing the dermal safety of the plant.

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