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PHYTOCHEMICAL SCREENING AND BIOACTIVITY STUDIES OF IMMATURE AND MATURE LEAVES OF CALOPHYLLUM INOPHYLLUM.L

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ABSTRACT

Plants have been widely used as a source of inspiration for new drug compounds, since plant derived medicines have made many gifts to human health and its wellbeing. The role of medicinal plants is double in the development of new drugs. *Calophyllum Inophyllum.*L has got vast medicinal values and almost all parts of this plant can be used as antiseptic, expectorant, astringent, purgative and diuretics. The present study was carried out to study the presence of various phytochemical and also to have an insight on the bioactivity of Ethanol extracts of immature (IE) and mature (ME) leaves of *calophyllum inophyllum*. Ethanol extract showed the presence of phytochemicals such as alkaloids, carbohydrates, proteins, flavonoids, phenols, glycosides, terpenoids and volatile oils. The total phenolics content were found to be 77mg GAE/g and 60 mg GAE/g for immatured and matured leaves and flavonoid was 157.5 mg/g and 180 mg/g of for immatured and matured leaves. Immature leaf extract exhibited comparatively more free radical scavenging activity than mature leaves extract. Brine shrimp lethality assay indicates that the bioactive components present in this plant can be accounted for its pharmacological effects. Thereby this results support the uses of these plant species in traditional medicine.

Key words: *Calophyllum inophyllum*, phytochemicals, Brine shrimp assay, Antioxidant activity, DPPH.

INTRODUCTION

Global plant biodiversity serves as the main source of herbal medicine and almost ¾ th of the world population depends on plant related medicines for basic health care. Medicinal plants have great value to the health of humans (Farnsworth N R, 1991) (Sofowora A, 1993). In traditional medicine, for the healing of many ailments medicinal plants are usually used (Kokate, 2001).Bioactive compounds in medicinal plants can be improved by different technologies (Abdin, 2007). Phytochemicals plays a very important role in defence mechanisms against many diseases and various researches on this can lead to the discovery of many anti-infective compounds (Ogukwe, 2004). The most imperative bioactive compounds include tannins, alkaloids, flavonoids, and phenolic compounds.

Calophyllum inophyllum Linn is an evergreen shrub commonly distributed in tropical areas and it belongs to the family Guttiferae. Traditional Chinese folk medicine employs this for the treatment of wounds,eye diseases, inflammations and rheumatism (Chen, 1965), (Dai, 2007). The chemical literature shows the presence of diverse biomolecules such as flavonoids (Li, 2007), triterpenes (Govindachari, 1967) which have assorted bioactives such as anti-microbial (Yimdjo, 2004), cytotoxic activities (Xiao, 2008), and anti-HIV-1 (Patil, 1993).Almost all the parts of the plant are useful. It is supposed to have diuretic characteristics. The gum extracted from the plant can be used as a purgative and it can be used for the healing of wounds and ulcers. The leaves saturated in water are useful for inflamed eyes. The leaf cocktail can be used internally for heatstroke (Edeoga, 2005). The plant has anti-inflammatory and pain relieving properties. All the above mentioned traditional uses of this plant indicates that there are many properties lying within this plant. In the present investigation, phytochemical analysis and bioactivity studies of immature and mature leaves in terms of free radical scavenging using DPPH have been done along with cytotoxicity analysis using brine shrimp.

MATERIALS AND METHODS

Sample collection: The Plant material, *Calophyllum inophyllum* immature and mature leaves were collected from the campus of Sathyabama University, Chennai. The leaves were shade dried for about 2 weeks and milled using an electric blender.

Preparation of extracts: 10 gms of each powdered immature and mature leaves of *Calophyllum inophyllum* were extracted with 100 ml of petroleum at room temperature for 24 hours with continuous stirring for the elimination of fats. After 24 hours, it was filtrate was collected and again extracted with 100 ml of ethanol by cold extraction method. The crude extract was obtained by reducing the supernatant under pressure. % yield of the extract was found out using the following equation:

% yield = wt. of crude extract/wt. of dried plant x 100.

Phytochemical screening: Phytochemical screening were performed for all the extracts as per the typical methods (Sofowara, 1993; Harborne, 1973). Detection of Carbohydrate (Fehlings test), Proteins (Bradford's test), Phenols (Ferric chloride test), Glycoside (Keller-Killani test), Alkaloids (Mayer's test), Flavonoids (Aluminium chloride test), Volatile oils and Terpenoids.

Quantification of phytochemicals:

Estimation of total phenol content (TPC): The total phenol content was calculated using modified Folin-Ciocalteau's reagent method (Gulcin, 2004). A standard curve was plotted using gallic acid with a concentration range from 0.5 - 25 µg/mL. Total phenolic content was demonstrated as mg gallic acid equivalents (GAE)/g of samples. 100µl of 80% ethanolic extract is mixed with Folin-Ciocalteau's reagent (500 µl) and distilled water (3 ml), and incubated at room temperature for 3 minutes. Sodium carbonate, 20% (2 ml) was added to the mix and the test tubes were kept at boiling water bath for 1min. After 2 hrs, the optical density was measured at 650 nm using Spectrophotometer.

Estimation of Total Flavonoid Content (TFC): The total flavonoid content was estimated by modified Aluminium chloride colorimetric method (Park, 2008). 2.5 ml of distilled water was added to 1 ml of 80% methanolic extract (1mg/ml) of sample and other test tubes containing standard solution (Quercetin) with different concentrations. 75µl of 5% Sodium nitrite was added and incubated at room temperature for 5 mins and 150 µl of 10% Aluminium chloride were added to the sample. 0.5 ml of Sodium hydroxide (1M) was added after 6 min. The absorbance of the reaction mixture was recorded at 510 nm with Spectrophotometer. The total flavonoid content of the extract was calculated from the Quercetin standard plot.

Thin layer chromatography: The ethanol extract of the immature and mature leaves were chromatographed on an Aluminum foil backed normal particle silica gel 60 F254 plates (TLC, #5554 from Merck, Darmstadt, Germany) TLC layer; and was developed with different solvent systems. Developed plates were dried, visualized and detected with iodine vapour exposure. Rf value for the developed bands were calculated.

$$R_f = \frac{Distance\ traveled\ by\ the\ compound}{Distance\ traveled\ by\ the\ Solvent\ front}$$

Antioxidant assays:

DPPH Radical Scavenging Assay: The antioxidant action of the extracts can be calculated by using 1, 1- diphenyl 2-picrylhyorazyl (DPPH) method of Brand-Williams E *et al.*, 1995 with trivial modifications. 1ml of plant extract solution was diluted successively to attain subsequent concentrations (35.25,62.5 125, 250,500, and1000 µg/ml) which was then added with 1 ml of 0.1mM DPPH solution and made up to 1ml with methanol . L-Ascorbic acid was used as reference standard and corresponding blank sample were made 1ml of each methanol and DPPH solution without any extract was used as control. The reaction was done in triplicates and the absorbance was read using UV Visible spectrophotometer at 517nm after 30 minutes of incubation in dark. The inhibition % was calculated using the following formula.

Inhibition % = Ac-As/Ac×100

Ac - absorbance of the control, As - absorbance of the sample

***Artemia salina* lethality assay:** After extraction, the crude extract was tested for the *Artemia salina* toxicity assay, which is a simple, rapid and inexpensive bench top assay and considered as a useful tool for preliminary assessment of general toxicity. A separation funnel is filled with 1L of filtered seawater and 200 mg of *A. salina* eggs and kept under permanent light and at constant temperature until the larva hatch. In a 96 well micro titer plate. 100µL of 1mg/mL solutions of each extracts in DMSO or water is added to first well and serial diluted to obtain desired concentration and to each well 10 larvae are added carefully. The tests are carried out in duplicate. For Positive control, 100 µL of DMSO and for negative control, 100 µL of sea water was used. After incubation of 24h at permanent light and constant temperature, dead larvae are counted. The total number of dead larva is calculated for each well.

$$\%\ of\ mortality = \frac{No.\ of\ dead\ naupli}{Total\ number} \times 100$$

RESULTS AND DISCUSSION

In the present study undergone, it was found that all major phytochemicals like alkaloids, carbohydrates, proteins, flavonoids, phenols, glycosides, terpenoids and volatile oils were present in both the immature and mature

extracts. The Phytochemical compounds identified in this study were earlier been proved as bioactive. Broad chemical investigation of this genus has resulted in the isolation of a wide variety of natural products**.** The presence of some of these compounds has been confirmed to have medicinal and physiological activity. **(**Isaias, 2004, Da Silva, 2001; Ito, 2002, Oger, 2003**).**

Table.1.Phytochemical screening of the plant extracts

Phytochemicals	IE plant extract	ME plant extract
Alkaloids	+	+
Carbohydrates	+	+
Flavonoids	+	+
Glycosides	+	+
Phenols	+	+
Proteins	+	+
Terpenoids	+	+
Volatile oils	+	+

The total phenolics content was calculated from the standard graph plotted, and it was found to be 77 GAEmg/g in Immatuee leave extract and in Mature leaf extract contains 60 GAEmg/g .The total flavonoid content was also calculated and IE plant extract contains 180 GAE mg/g and ME plant extract contains 157.5 GAE mg/g. The IE and ME extracts of *C.inophyllum* displayed the presence of high amount of phenolic and flavonoid contents. Both of these secondary metabolites have efficient antioxidant activity and their effects are significant on the human health and disease prevention. Phenolics comprises of simple phenols, phenolic acids, lignans, lignins coumarins, flavonoids,, hydrolyzable and condensed tannins. These includes the most commonly seen secondary metabolites in the plant biodiversity and it also acts as antioxidants, and defending agents against UV light (Gottlieb, 2000). Phenolics and flavonoids hold a wide spectrum of chemical and biological activities which includes radical scavenging properties. It is said that phenolic compounds are powerful chain breaking antioxidants (Shahidi *et al*., 1992). The scavenging activity of phenolic group is because of its hydroxyl group. (Hateno, 1987).

TLC analysis of the fractions: Both immature and mature ethanolic extract of *C.inophyllum* was separated using different solvent system in TLC and found that solvent system of Hexane (5): Ethyl Acetate (2): Chloroform (3) was showing good separation. IE separated into 5 and ME into 4 bands. The Rf (Retention factors) for the immature and mature extracts in this solvent systems was tabulated in Table 2.

Table.2.Rf values of the plant extracts (Hexane (5): Ethyl Acetate (2): Chloroform (3)

Band	**Rf value of IE**	**Rf value of ME**
1	0.591	0.836
2	0.836	0.938
3	0.938	0.969
4	0.969	0.979
5	0.979	-

Antioxidant assays:

DPPH assay: *In vitro* antioxidant potentials of immatured ethanolic (IE) and matured ethanolic (ME) extracts of *C. inophyllum* were studied and found that the activity was concentration dependent increase in concentration leads to increase activity (Fig 1, 2). IE is having more antioxidant activity when compared to ME which may be because of more amount of total phenol and flavonoid contents. A number of diseases are related to oxidative stress and free radical-induced damage. Antioxidants are compounds which check the oxidation of organic molecules and thus protect the body from various chronic diseases. Several studies have documented the antioxidant activities of the phenolic components. Free radical scavenging activity is the most important feature of poly phenols and flavonoids. Flavonoids stabilize the free radicals by reacting with the radicals (Ebrahimzadeh, 2008). Here IE seems more promising for antioxidant potentials than ME.

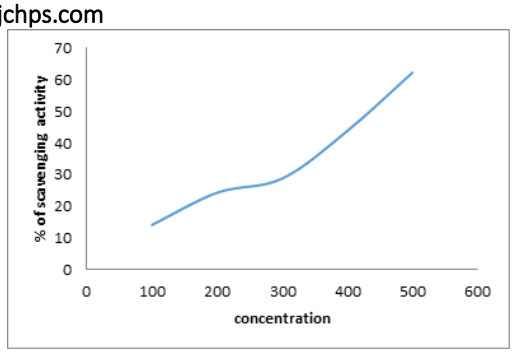

Figure.1.Graph showing the DPPH assay of immature ethanolic plant extract

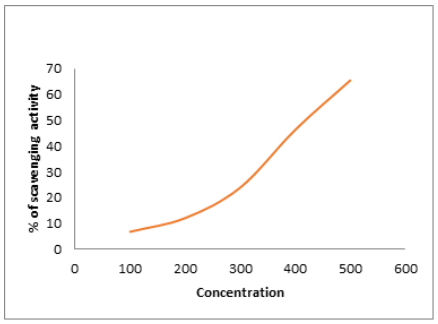

Figure.2.Graph showing the Dpph scavenging activity of mature ethanolic plant extract

***Artemia salina* lethality assay:** The brine shrimp lethality assay (Michael, 1983) was carried out to determine the lethal concentration of the immature and mature plant extracts. Percentage lethality/mortality of Brine shrime by different concentration of ethanolic extract of ME and IM was represented in the Fig 4 The brine shrimp lethality bioassay represents a rapid, low-cost simple bioassay for testing extract's bioactivity which in most cases correlates reasonably well with cytotoxic and antitumor properties of extracts (McLaughlin, 1993). The cytotoxicity bioassay of crude extracts using brine shrimps is regarded as an important tool useful for the preliminary assessment of toxicity and it has been used for the detection of fungal toxins, cyanobacterial toxins, plant extract toxicity, heavy metals, pesticides and cytotoxicity testing of dental materials (Jaki, 1999; Harwig and Scott, 1971; McLaughlin, 1991; Martinez, 1999; Barahona and Sanchez-Fortun, 1999; Pelka, 2000).

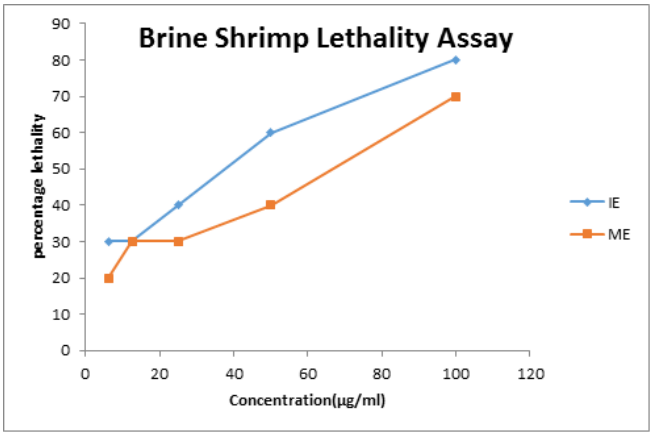

Figure.3.Brine shrimp cytotoxicity assay for plant extracts (IE, ME)

SUMMARY AND CONCLUSION

The ethanolic extract of both matured and immature leaves of *Calophyllum Inophyllum*.L showed the presence of important phytochemicals. TLC profiling shows fair results that can direct the presence of number of phytochemicals. Antioxidant properties of *Calophyllum Inophyllum*.L immature and mature ethanolic leaf extracts were carried out using DPPH method. It was found that immature leaf extract has more antioxidant activity. The leaf extracts of *Calophyllum inophyllum* exhibited cytotoxic activity against the brine shrimp and considered as containing active or potent components. Although, BSLA is inadequate in determining the mechanism of action of the bioactive substances in the plant, it is very useful by providing a preliminary screen that can be supported by a more specific bioassay, once the active compound has been isolated. The selected plant leaves are the potential source to produce drugs. The traditional medicine practice is recommended strongly for this plants.

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