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Phytochemical Composition, Antioxidant Studies and HPTLC Analysis of Methanolic Extract of *Curcuma amada* Linn.

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ABSTRACT

The present study was undertaken to evaluate phytochemical composition, antioxidant and HPTLC (High Performance Thin Layer Liquid Chromatography) of methanolic extract of *Curcuma amada* leaves. Phytochemical analysis of the plant has shown the presence of proteins, oil and fats, phenolic compounds, flavonoids, saponins, tannins and carbohydrates as major phytochemical groups. Antioxidant activity was performed using DPPH radical scavenging and Total antioxidant assays. The extract has shown highest DPPH radical scavenging activity (IC₅₀= 25.16µg/ml) and total antioxidant capacity. Extract also showed the presence of high amount of phenols (16.99 mg GAE/gm of extract) and flavanoids (266.99 mg QE/gm of extract). Antioxidant potential might be due to the presence of polyphenols in the extract and thus HPTLC finger printing and FT-IR (Fourier Transform Infra Red) analysis has been done. As a result, HPTLC analysis identified one polyphenol and FT-IR analysis revealed the presence of functional groups in the extract that might be responsible for the antioxidant activities. Therefore the present study this article throws a light to the major active components as well as the biological activities of the *C. amada* that may be considered as important source from the pharmacological point of view.

Keywords: *Curcuma amada* leaves, Agar well diffusion method, DPPH radical scavenging activity, HPTLC finger printing.

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INTRODUCTION

Biodiversity of the natural resources has served not only for the human needs but also for the health care from the ancient times. The major traditional health care system harbors one of the oldest histories of Indian civilization. The medicinal plants play a vital role and constitute the backbone of all the traditional systems of medicinal practices [1, 2].

A free radical is a highly unstable atom, molecule or compound because of its atomic or molecular structure. It is responsible for accelerated aging, destruction of DNA, clogging of arteries, tissue damage etc. However, many free radicals are produced due to environmental factors like pollution, radiation, smoking or inhaling smoke, pesticides, herbicides, and stress. Therefore Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Thus, it can cause disruptions in normal mechanisms of cellular signaling [3-5].

Antioxidants act like scavengers for free radicals, giving them one of their electrons. The antioxidant have a stabilizing effect that can repair the damage caused by free radicals [6] and also improves body's immune system, to overcome diseases and their complications [7-9].

C. amada is a herb which is rhizomatous aromatic leafy tuft with a height of 60-90 cm and is commonly known as mango ginger. Leaves are long, petiolate, oblong-lanceolate, tapering at both ends, glabrous and green on both sides. In terms of medicinal uses this has been used for healing of wounds, cuts, itching, sprains, skin diseases, carminative properties as well as rhizome was being used as a decoction for colds and coughs [10]. The biological activities of *C. amada* include antioxidant activity, antifungal activity, antibacterial activity, platelet aggregation inhibitory activity, cytotoxicity, antiallergic activity, hypotriglyceridemic activity, brine-shrimp lethal activity, enterokinase inhibitory activity, CNS depressant and analgesic activity. The major chemical components include phenolic acids, volatile oils, starch, curcuminoids and terpenoids like difurocumenonol, amadannulen and amadaldehyde. More than 130 bioactive chemical constituents have been reported in *C. amada* rhizomes [10, 13].

The presence of unique chemicals in *C. amada* rhizome also imparts diverse therapeutic efficacies such as insecticidal, aphrodisiac, antipyretic, anti-inflammatory, anti-mycobacterial and anti-hyper-cholesterolemic [10, 14- 17]. T has been also reported that the extract of *C. amada* can able to inhibit trypsin enzyme activity in the case of pancreatitis [18]. Hyper triglyceridemic activity of *C. amada* in triton-induced hyperlipidemic rats has been reported [19]. inspite of these reports, the studies on HPTLC analysis of *C. amada* was not available. Hence, the present study was focused on phytochemical composition, antioxidant and HPTLC analysis of methanolic extract of *C. amada* leaves.



MATERIALS AND METHODS

Chemicals

Quercetin and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA). Sodium carbonate (Na_2CO_3), Sodium phosphate (NaH_2PO_4) was purchased from HI media Laboratories Pvt. Ltd. (Mumbai, India). Methanol, Ferrozine, Ferrous chloride (FeCl_2), Ferric chloride (FeCl_3), Potassium Ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), Trichloroacetic acid, Ascorbic acid, Folin- Ciocalteu reagent, Ethanol, Ascorbic acid, Gallic acid were purchased from SRL Pvt. Ltd. (Mumbai, India). Ammonium molybdate ($(\text{NH}_4)_2\text{MoO}_4$) and Aluminium chloride (AlCl_3) were purchased from SD Fine-Chem Chem. Ltd. (Mumbai, India). All other chemicals used were of analytical grade.

Collection and processing of the Plant material

The Sample *C. amada* was collected from agricultural field of mango ginger, from Alwaye, Kerala, India. The leaves of *C. amada* were taken and washed in distilled water and then dried in shade at room temperature. The leaves which are dried were made into fine powder by using mechanical grinder. The powder is then extracted in methanol at room temperature for 48 hrs, filtered and concentrated with Rotary Evaporator. Thus obtained extract was collected in air tight container and stored at 4°C for further use.

Phytochemical Screening

Preliminary phytochemical screening was carried out to assess the qualitative chemical composition of the methanol extracts of *C. amada* leaves were tested for tannins, flavonoids, saponins, carbohydrates, proteins, oils and fats using the standard protocols [21].

Antioxidant studies

DPPH based free radical scavenging activity

The DPPH radical scavenging activity was performed according to the method of Gunjan et al., 2010 with few modifications [22]. The plant extracts were diluted to make 10, 20, 40, 60, 80 and 100 µg/ml dilutions. Two milliliters of each dilution was mixed with 1 ml of DPPH solution (0.2 mM/ml in methanol) and mixed thoroughly. The mixture was incubated in dark at 20°C for 40 min. Absorbance was measured at 517 nm using a Cary 50 UV-vis spectrophotometer (Varian, Inc., CA, USA) with methanol as blank. Gallic acid was used as positive control. The percentage scavenging of DPPH by the extract was calculated according to the following formula:

$$\% \text{ DPPH Radical scavenging} = [(A_c - A_t) / A_c] \times 100$$

Here:

A_c is the absorbance of the control (DPPH), A_t is the absorbance of test sample.

Estimation of total antioxidant assay

A concentration of 125, 250, 500 and 1000 $\mu\text{g/ml}$ of methanol extract of *C. amada* was prepared. These dilutions were made in triplicates [23]. A reaction mixture comprising of 3.3ml of concentrated sulphuric acid and 0.335grams of sodium phosphate monobasic with 0.495grams of ammonium molybdate in 96.67ml was prepared. To 1ml of each dilution, 3ml of the reaction mixture was added and incubated for 1 hour at 95°C . 6ml of reaction mixture in 2ml distilled water was used as the blank. The absorbance was measured using a spectrophotometer at 695nm.

Estimation of Total Phenolic content

Total phenolic content of the methanol extract of *C. amada* was determined using the Folin-Ciocalteu reagent method [24]. The crude methanol extract were diluted to obtain different concentrations (250, 500, 750 and 1000 $\mu\text{g/ml}$). 50 μl of extract was mixed with 2.5 ml of Folin-Ciocalteu reagent (1/10 dilution in purified water) and 2 ml of 7.5% Na_2CO_3 (w/v in purified water). The mixture was incubated at 45°C for 15 min. The absorbance was measured at 765 nm. Na_2CO_3 solution (2 ml of 7.5% Na_2CO_3 in 2.55 ml of distilled water) was used as blank. The results were expressed as Gallic acid equivalence in μg .

Estimation of Total Flavanoids content

The determination of total flavonoids of the methanol extract of *C. amada* leaves was carried out using the modified procedure [25]. A volume of 1 ml (containing 125, 250, 500 and 1000 $\mu\text{g/ml}$ extract) was mixed with 1 ml of AlCl_3 (2% in ethanol). The mixture was incubated at room temperature for 60 minutes. AlCl_3 solution (1 ml of 2% AlCl_3 in 1 ml of water) was used as blank. The absorbance was measured at 420 nm using UV-Vis spectrophotometer. Total flavonoid content was expressed as Quercetin equivalence (QE) in μg . Experiment was performed in triplicates at each concentration.

FT-IR analysis of the plant sample

The dried leaves *C. amada* were ground into fine powder using mechanical grinder. Two milligrams of the sample were mixed with 200 mg KBr (FT-IR grade) and pressed into a pellet. The sample was placed into the sample holder and FT-IR spectra were recorded in the range $4000\text{-}450\text{ cm}^{-1}$ in FT-IR spectroscopy (AVATAR 300 FT-IR, Thermo Nicolet, USA) [26].

HPTLC finger printing for the identification of Polyphenols

HPTLC analysis was carried out by following a standard protocol to identify polyphenols present in the extract [27]. About 10 mg of the extract was dissolved in 1 ml of distilled water which was then centrifuged at 3000 rpm for 5 mins and used for HPTLC analysis. A volume of 2 μl of test solution was loaded as 5mm band length in the 2 x 10 Silica gel 60F₂₅₄ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plate was held in TLC

twin trough developing chamber (after saturated with Solvent vapor) with corresponding mobile phase Toluene-Acetone-Formic acid (4.5 : 4.5 : 1) and the plate was developed in the respective mobile phase up to 90mm. The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at Day light, UV 254nm and UV366nm. At last, before derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at UV 254nm. The Peak table, Peak display and Peak densitogram were recorded. The software used was win CATS 1.3.4 version.

RESULT AND DISCUSSION

The identification and investigation of pharmacological active compounds from the medicinal plants is widely expanding field of research. In the view of upsurging interest in the health benefits of medicinal plants, we examined the phytochemical composition and antioxidant activities of the crude methanolic extract of leaves of *C. amada* [27].

Phytochemical screening showed the presence of proteins, oil, fats, phenolic compounds, flavonoids, saponins, tannins and carbohydrates in the methanolic extract of *C. amada* leaves. The results of the phytochemical analysis are shown in Table 1. Since preliminary phytochemical screening confirmed the presence of polyphenols, extract was further quantified for phenolics and flavonoids. Extract had shown the presence of high amount of phenols (16.99 mg GAE/gm of extract) and flavanoids (266.99 mg QE/gm. of extract) which was expressed as gallic acid and quercitin equivalence and was shown in the figures 3 and 4 respectively.

Table 1: Phytochemical screening of the leaves of *Carcuma amada*. L

Phytochemical Test	<i>C. amada</i> Leaves
Flavonoids	+
Saponins	+
Tannins	+
Carbohydrates	+
Oils and fats	+
Proteins	+

Here, +: positive, -: negative

As per literature survey, polyphenols are found to possess good antioxidant activities [28] and other biological activities [29]. Hence, extract was evaluated for antioxidant capability by performing the standard DPPH radical scavenging assay and total antioxidant assays. DPPH is a steady free radical, due to the delocalization of spare electrons over the molecules gives deep violet color which is been absorbed at 517 nm, when DPPH reacts with the substance which can donate H⁺ atom. Reduced form is generated which is accompanied by loose of violet color. Methanol extract of *C. amada* leaves has shown 93% of DPPH radical scavenging activity with an IC₅₀. value of 25.16 µg/ml.

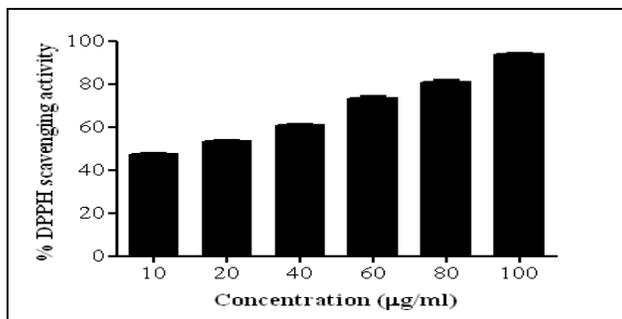


Figure 1: Percentage DPPH radical scavenging activity of methanolic extract of *C. amada* leaves; All the values are represented as Mean \pm SD; n=3.

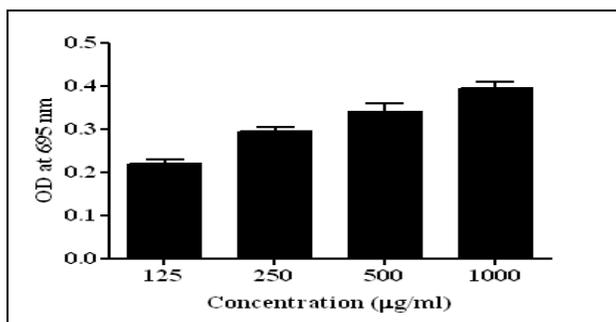


Figure2: Total antioxidant assay of methanolic extract of *C. amada* leaves; All the values are represented as Mean \pm SD; n=3.

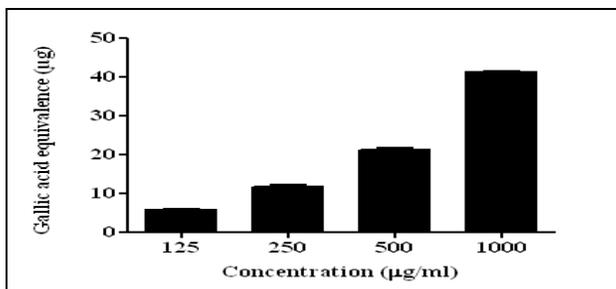


Figure 3: Quantitative analysis of Total Phenolic content of *C. amada* leaves; All the values are represented as Mean \pm SD; n=3.

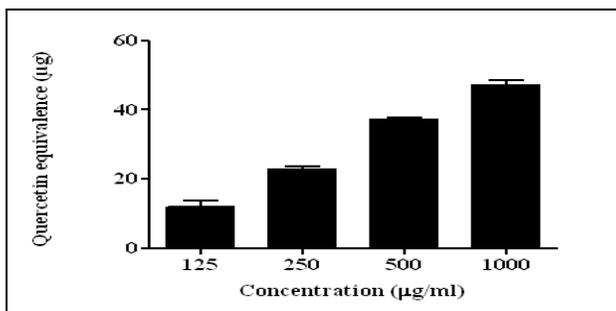


Figure 4: Quantitative estimation of Total flavanoids content of *C. amada* leaves; All the values are represented as Mean \pm SD; n=3.

Total antioxidant assay is based on reduction of molybdate VI to V which is accompanied by green phosphate complex at acidic pH that can be observed at 695 nm. It is a function to estimate the total radicals scavenging activity of the extracts. Extract exhibited good antioxidant potential in a dose dependent manner.

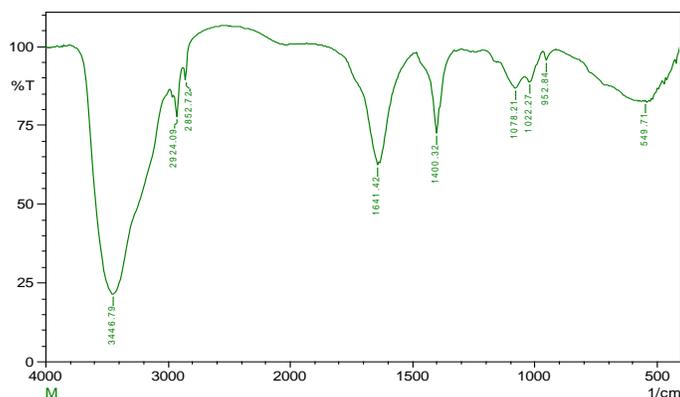


Figure 5: FTIR analysis of methanolic extract of *C. amada* leaves

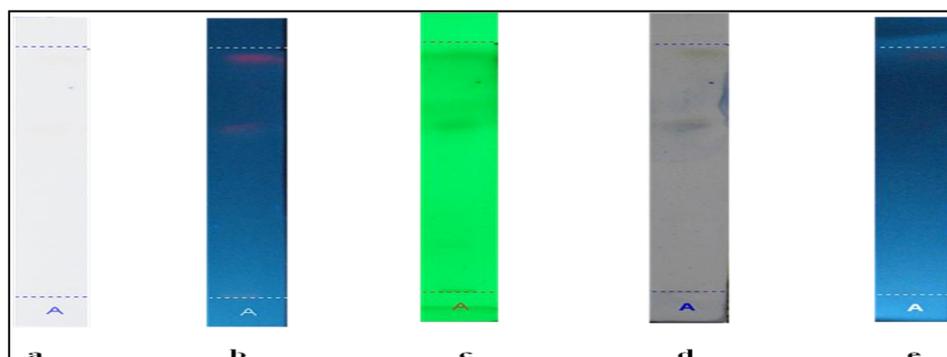


Figure 6. Chromatograms of the extracts in HPTLC analysis (a) Before derivatization under day light, (b) Under UV 366nm & (c) Under UV 254 nm; (d) After derivatization under day light and (e) Under UV.

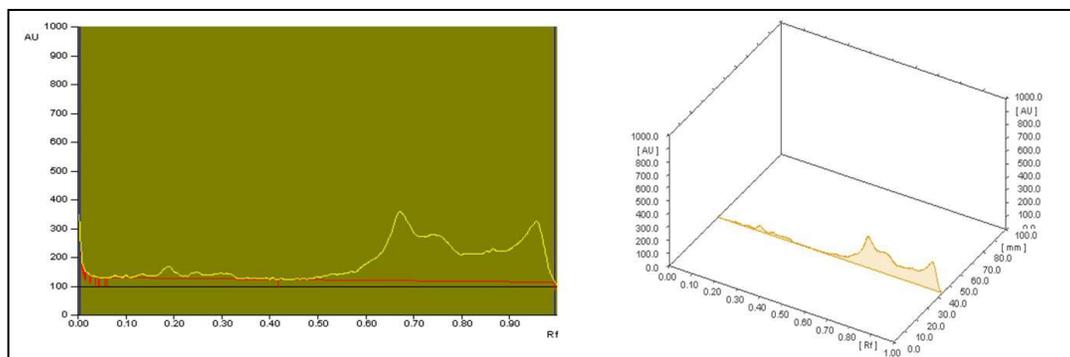


Figure 7. Sample baseline put on view (Scanned at 254nm); Figure 8: 3D display (Scanned at 254nm).

FT-IR Spectroscopy analysis was performed to identify the –OH functional groups in the extract. Extract has shown the presence of –OH functional groups which has been shown in the below figure 5. Therefore to identify the number of polyphenols in the extract, HPTLC analysis was carried out. HPTLC finger printing profile of the methanolic extract of *C. amada* leaves was shown in the table 4. Among the peaks noted before derivatization and after derivatization, peak 6 has shown the presence of one polyphenol, hence this polyphenol might be responsible for the antioxidant activities of the plant.

Table 4: HPTLC analysis showing the peaks, Rf value, height, area of polyphenol in *C. amada* leaves

Peak	Rf	Height	Area	Assigned substance
1	0.13	14.8	361.4	Unknown
2	0.19	43.0	1136.1	Unknown
3	0.25	24.5	503.3	Unknown
4	0.53	22.4	518.0	Unknown
5	0.57	30.9	587.0	Unknown
6	0.67	239.8	14209.8	Polyphenol 1
7	0.74	161.7	8133.8	Unknown
8	0.86	113.4	3930.4	Unknown
9	0.95	209.8	11043.9	Unknown

CONCLUSION

In recent years, medicinal plants have been significantly studied for their phytomedicinal properties. Phytochemical screening, and antioxidant activities of *C. amada* reveals that it is a valuable medicinal plant with numerous medicinal properties. As it is a best source of antioxidant, a typical research and developmental work should be carried out for the conservation, for the better therapeutic and commercial utilization of *C. amada*. With the results obtained in this study, it has been concluded that the leaves of *C. amada* can be used as a good source for the isolation of safe and natural antioxidant compounds.

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