



ANTIFUNGAL AND ANTIOXIDATIVE POTENTIAL OF OIL AND EXTRACTS DERIVED FROM LEAVES OF INDIAN SPICE PLANT *Cinnamomum tamala*

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Abstract

Plant-based antimicrobials and antioxidants represent a vast untapped source for medicines and food supplements and hence have enormous therapeutic potential. Present work reports the fungicidal potential of *Cinnamomum tamala* Nees & Eberm (Lauraceae) leaf oil against five food spoilage and pathogenic fungi. In addition antioxidant efficacy of seven different solvent extracts derived from leaf was also evaluated using *in vitro* models. The oil demonstrated potent antifungal activity against *Aspergillus niger*, *A. fumigatus*, *Candida albicans*, *Rhizopus stolonifer* and *Penicillium* spp. in agar diffusion assay. Zone of inhibition ranged from 17-25 mm. The MFC values of oil against all the test fungi were found to be 230µg/ml. Phytochemicals present in *C. tamala* leaf were extracted in several solvents for assessing their effect in oxidative defense. The extracts exhibited appreciable antioxidant activity in β-carotene bleaching assay and reducing power assay. The antioxidative activities of extracts were compared with the activities of standard antioxidant compounds BHA and ascorbic acid. Petroleum ether, ethanol, acetone and chloroform extracts exhibited about 30-67% antioxidant activity in β-carotene bleaching assay. Aqueous and ethanol extracts exhibited better reducing power which increased gradually with increasing amount of the extract concentration showing dose dependent response. Results indicated that natural phytochemicals present in *C. tamala* leaf extracts have potential to prevent growth of food spoilage/pathogenic fungi. In addition they also have capability to mitigate the oxidative stress by antioxidant response.

Key words: *Cinnamomum tamala*, extract, oil, antifungal, antioxidant, phytochemicals.

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INTRODUCTION

Antimicrobial properties of herbs and spices have been recognized and used since time immemorial for food preservation and medicine (29). In light of the evidence of rapid global spread of resistant clinical isolates, researchers are increasingly turning their attention to herbal products, looking for new leads to develop better drugs against multi drug resistant (MDR) microbial strains (22, 24). Mycosis constitutes a common health problem, especially in tropical and subtropical developing countries. Dermatophytes, *Malassezia* spp. and *Candida* spp. are the most frequent pathogens in humans and animals (6). Many of the available antifungal drugs have undesirable side effects or are very toxic (amphotericin B). They have been reported to produce recurrence, show drug-drug interactions (azoles) or lead to the development of resistance (fluconazole, 5-flucytosine). Some of the drugs even show ineffectiveness (19, 28) and have become therefore less successful as therapeutic agents. To overcome these problems continuing search for more effective and less toxic novel antifungal agents is required. Interestingly, plant extracts and oil are widely employed in folk medicine, mainly in communities with inadequate conditions of public health and sanitation. Several medicinal plants have been extensively studied in order to find more effective and less toxic compounds (20).

Antioxidants are substances that neutralize free radical or their actions. Recent developments in biomedics have pointed to the involvement of free radicals in many diseases such as cancer, atherosclerosis, diabetes, neurodegenerative disorders and ageing (27). Although living organisms possess enzymatic and non-enzymatic defense systems against excessive production of free radicals, different external factors (smoke, diet, alcohol, some drugs

and ageing decrease the efficiency of such protecting system, resulting in disturbance of redox equilibrium established under healthy conditions. It is believed that higher intake of antioxidant rich food is associated with decreased risk of degenerative diseases particularly cardiovascular diseases and cancer. Plant derived secondary metabolites such as polyphenols, alkaloids, tannins, carotenoids etc. have been found to act as antioxidant, free radical scavengers, quenchers of singlet and triplet oxygen and inhibitors of peroxidation (10, 15).

Indian Cassia lignea, *Cinnamomum tamala* Nees & Eberm (Hindi- Tejpat) is an evergreen tropical tree, belonging to the family Lauraceae. It is mainly used for flavouring food and widely used in pharmaceutical preparation because of its hypoglycemic, stimulant and carminative properties (2). The leaves of this tree are used as spice having clove like taste and pepper like odour. Essential oil and extracts of *Cinnamomum* spp. have been shown to possess antibacterial (16, 18), antifungal (17) and antioxidant activities (23) by our research group. As a part of our ongoing research programme to explore the medicinal virtues of the *Cinnamomum* spp., the present paper reports the antifungal and antioxidant efficacy of oil and extracts, respectively, derived from *C. tamala* leaves.

MATERIALS AND METHODS

Plant Material

C. tamala (Lauraceae) leaves were collected during October/November 2008 from Ranchi (Jharkhand, India) and dried in a shaded and airy place at room temperature for 10–15 days. Identification was confirmed by Prof. D. K. Chauhan, Department of Botany, Faculty of Science, University of Allahabad, Allahabad, India. The plant material was reduced to a fine powder with mortar and pestle.

Preparation of extracts

Powdered sample was sequentially extracted with different solvents in a Soxhlet apparatus for 8h (17). The solvents used for extraction included petroleum ether (b.p. 40-60°C) (PE), benzene (BZ), chloroform (CH), ethyl acetate (EA), acetone (AC), ethanol (ET) and water (AQ). The respective extracts were filtered and dried under reduced pressure using rotary evaporator to yield solid/semisolid residues. The residues were stored in amber coloured vials and kept in refrigerator until used.

Extraction of essential oil

Air dried leaves of *C. tamala* were chopped and ground into small pieces. The known amounts of processed samples were placed in a round bottom flask (1L) together with distilled water and hydrodistillation was performed using Clevenger apparatus. Essential oil was collected, dehydrated using sodium sulphate and dispensed into dark bottles. Oil was stored at 4°C until used. Tween 20 (0.1%) was used as dispersing agent.

Fungal strains and growth conditions

Five fungal isolates were tested for their susceptibility to oil. These fungi included *Candida albicans*, *Aspergillus niger*, *A. fumigatus*, *Rhizopus stolonifer* and *Penicillium* spp. The isolates were obtained from the Mycopathology laboratory, Department of Botany, University of Allahabad, Allahabad, India. These fungi were grown on PDA slants at 28 ± 1°C and then stored at 4 ± 1°C for further use. The organisms were sub-cultured once in every fifteen days and the purity of the cultures was checked regularly under microscope.

Testing of antifungal efficacy of oil

Agar diffusion method was used to study the antifungal efficacy of oil against test fungi. Briefly, 0.1 ml of fungal broth culture was spread on the surface of PDA plates. About 50 µl oil was poured in separate wells with the help of micropipette. Tween 20 (0.1%) was used as control. All the operations were carried out aseptically in the laminar chamber. Plates were incubated at 28 ± 1°C for 5 days. Nystatin (30 µg/disc) was used as positive control. Antifungal activity was determined by measuring diameter of the zone of inhibition (ZOI) surrounding wells.

Determination of minimum fungicidal concentration (MFC) of oil

MFC of oil was determined following the method of Pandey *et al.* (23). Malt extract tubes containing various concentrations of oil (115, 230, 460 and 690 µg/ml) dispersed in 0.1% Tween 20 were prepared. Different fungal spore suspensions (0.1 ml) were inoculated separately in 10 ml of malt extract solution aseptically and tubes were incubated at 28 ± 1°C for 5-7 days. The lowest concentration of the essential oil that resulted in a complete inhibition of visible growth of the test fungi in broth medium was regarded as the minimum inhibitory concentration. The contents of the tubes showing no visible fungal growth or turbidity were further subcultured on freshly prepared PDA plates. The plates were incubated at 28 ± 1°C for 5-7 days. The minimum fungicidal concentrations (MFCs) were determined as the lowest concentration of the test oil which did not allow any visible growth of the microorga-

nisms after subculture.

Determination of Antioxidant activity of extracts

Antioxidant assay using β-Carotene linoleate model system

Antioxidant activity of *C. tamala* leaf extracts was evaluated by the β-carotene linoleate model system (11) with slight modification. β-carotene 0.2 mg, linoleic acid 20 mg, and 200 mg of Tween-40 (polyoxyethylene sorbitan monopalmitate) were mixed in 0.5ml of chloroform. Chloroform was evaporated at 40°C under vacuum using rotary evaporator. The resulting mixture was immediately diluted with 10ml of triple distilled water and was further made up to 50 ml with oxygenated water. Aliquots (4ml) of this emulsion were transferred into different test tubes containing 0.2ml of test sample in DMSO (100 µg/ml) and 0.2 ml ethanol. BHA was used as standard antioxidant for comparative purpose. A control containing 0.2ml of ethanol, 0.2 ml pure DMSO and 4 ml of the above emulsion was prepared. The tubes were placed at 50°C in water bath. Absorbance of all the sample at 470nm were taken at zero time (t = 0). Measurement of absorbance was continued until the colour of β-carotene disappeared in the control reaction (t = 180min) at 15 min intervals. Percent antioxidant (% AO) activity was calculated using the formula (8):

$$\% \text{ AO Activity} = 100[1 - (A_0 - A_t)/(A_0^c - A_t^c)]$$

where A_0 and A_0^c are the absorbance values measured at zero time of the incubation for test sample and control, respectively, A_t and A_t^c are the absorbance measured in the test sample and control, respectively after incubation for 180 min.

Reducing Power assay

The reducing power was determined by the method of Gordon (7) with minor modification. DMSO was used as dissolving medium for all test extracts instead of methyl alcohol. Two milliliter DMSO containing different amounts of *C. tamala* leaf extracts (200, 400, 600, 800 and 1000 µg) was mixed with 2.5 ml potassium phosphate buffer (0.2 M, pH 6.6) followed by addition of 2.5 ml potassium ferricyanide (10 g/l). The tubes were incubated at 50°C for 20 min. After incubation 2.5 ml trichloroacetic acid (10%) was added to the mixture. Tubes were then centrifuged at 5000g for 10 min. One milliliter of the upper layer of the solution was mixed with 1.0 ml of distilled water followed by addition of 1.0 ml of 0.1% FeCl₃ and the absorbance was measured at 700 nm. Ascorbic acid was used as reference material. Increase in absorbance of the reaction mixture indicated the increased reducing power of the samples.

Statistical analysis

All experiments were carried out in triplicate. The results were averaged for evaluating antifungal efficacy of oil. However, for determinations of antioxidant activity the data were expressed as mean ± standard error of mean (SEM) and the plots were prepared using Graphpad Prism software.

RESULTS

Antifungal susceptibility and determination of minimum fungicidal concentration (MFC) of oil

C. tamala essential oil exhibited appreciable inhibitory efficacy against test fungi in agar diffusion tests (Table 1).

Table 1. Inhibitory activity and minimum fungicidal concentration (MFC) of *C. tamala* leaf oil against fungi.

Fungi	ZOI with oil	ZOI with Nystatin	Minimum fungicidal concentration (MFC) in $\mu\text{g/ml}$
<i>Aspergillus niger</i>	20	20	230
<i>A. fumigatus</i>	17	18	230
<i>Rhizopus stolnifer</i>	19	16	230
<i>Penicillium spp.</i>	21	18	230
<i>Candida albicans</i>	25	23	230

Numbers represent ZOI (zone of inhibition) which are shown in mm. Concentration of nystatin was $30\mu\text{g/disc}$. Experiments were performed in triplicate and values represent average of three replicates.

Most of the fungi (*C. albicans*, *A. fumigatus*, *A. niger*; *R. stolnifer* and *Penicillium spp.*) showed susceptibility to test oil. Highest growth retardation effect of oil was observed on *C. albicans* showing ZOI of 25 mm. In comparison the inhibitory efficacy of oil against rest of the fungal isolates was a little low as evident from inhibition zone sizes (ZOI 17-21 mm). All the test fungi were susceptible to the standard antifungal agent nystatin with ZOI in the range of 16-23mm. However, fungal growth inhibition effect of nystatin was a little lower as compared with the effect of oil.

The MFC of oil extracted from *C. tamala* leaves was evaluated against test fungi at four different concentrations of oil (115, 230, 460 and $690\mu\text{g/ml}$). Oil at higher concentrations demonstrated complete fungicidal activity in the bioassay. The lowest concentration of the oil that did not produce any fungal growth on the solid medium was regarded as MFC. The values are depicted in Table 1. The MFC values of oil against all the fungal isolates tested were found to be $230\mu\text{g/ml}$.

Antioxidant activity of *C. tamala* leaf extracts

In β -carotene linoleate model system (Fig. 1 and Table 2), PE extract demonstrated substantial free radical scavenging ability as indicated by retention of colour of β -carotene after 180 minutes showing percentage antioxidant activity (%AO) of about 66%. The EA and AQ fractions accounted for the lowest activity. The %AO recorded for BHA, the standard antioxidant compound, under similar test conditions was 84.3%. ET, AC, CH and BZ extracts also exhibited appreciable bleaching reduction potential up to 135 min and there after the antioxidant activity dropped to 30-35% in next 45 minutes. So the decreasing order of antioxidant activity in different solvent extracts by β -carotene linoleate model system may be summarized as PE, ET, AC, CH, BZ, EA and AQ.

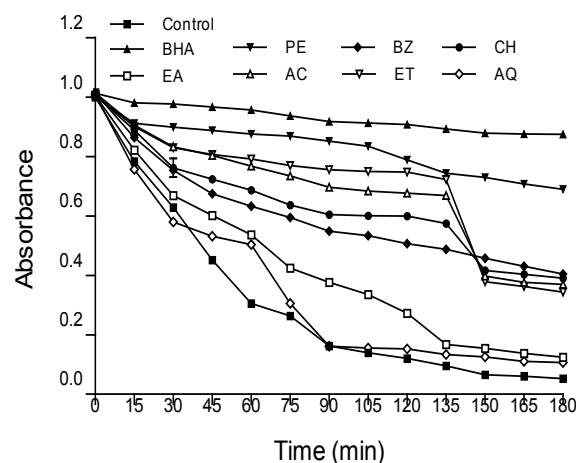
The reducing power of various *C. tamala* leaf extracts was evaluated using the potassium ferricyanide reduction method. Five different test concentrations of the extracts (200, 400, 600, 800 and $1000\mu\text{g/ml}$) were used. Reducing power of extracts was directly proportional to the concentration of extracts exhibiting dose dependent response. Results are depicted in Fig 2. Higher absorbance values indicated higher reducing power. Among all the test extracts, AQ extract demonstrated comparably better reducing activity (absorbance ≈ 1.1) followed by ET (≈ 0.5) and EA (≈ 0.3) extracts. Rest of the extracts (PE, BZ, AC and CH extracts) accounted for lower reducing power. In comparison to test extracts, the standard antioxidant com-

pound exhibited better reducing power.

Table 2. Percent antioxidant activity (% AO) of *C. tamala* leaf extracts after three hours using β -carotene linoleate model system.

Extracts	%AO
BHA	84.3
PE	66.3
BZ	34.8
CH	38.9
EA	5.2
AC	31.1
ET	28.5
AQ	4.0

Abbreviations: BHA: butylated hydroxyanisole; PE: petroleum ether; BZ: benzene; CH: chloroform; EA: ethyl acetate; AC: acetone; ET: ethyl alcohol; AQ: water.

**Figure 1.** Antioxidant activity of *C. tamala* leaf extracts by β -carotene linoleate model system (The leaf extracts were prepared in petroleum ether (PE), benzene (BZ), chloroform (CH), ethyl acetate (EA), acetone (AC), ethanol (ET), and water (AQ) as described in Methods section. Antioxidant activity of extracts ($100\mu\text{g/ml}$) and standard antioxidant BHA was evaluated for 3h under experimental conditions and absor-

bance was measured at 470nm. Data represent mean \pm SEM of three replicates.

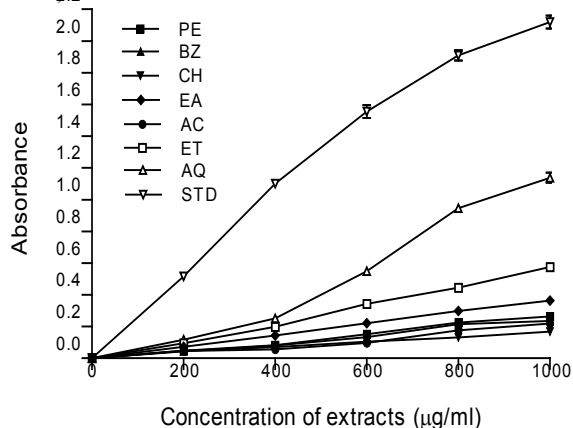


Figure 2. Reducing power assay of *C. tamala* leaf extracts (The extracts were prepared in PE, BZ, CH, EA, AC, ET and AQ as described in Methods section. Reducing power of extracts and standard antioxidant ascorbic acid (STD) was measured at different concentrations and absorbance was recorded at 700nm. Data represent mean \pm SEM of three replicates).

DISCUSSION

Antifungal susceptibility testing remains an area of intense interest. Susceptibility testing can be used for drug discovery and epidemiology. Most of the fungal isolates in our experiments exhibited susceptibility to *C. tamala* leaf oil. The fungicidal effect was most pronounced against *C. albicans* and *Penicillium spp.* MFC of oil (230 µg/ml) against test fungi demonstrated appreciable antifungal potential (Table 1). Biological activity of oil has been attributed to the presence of terpenes and terpenoids which have been shown to possess activity against bacteria, fungi and viruses (3, 26). The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds. It has been shown that increasing the hydrophilicity of kaurene diterpenoids by addition of a methyl group drastically reduced their antimicrobial activity (3).

Phenylpropanoids have been reported to be major component (about 66%) in *C. tamala* leaf oil. The marker phenylpropanoids include eugenol, cinnamaldehyde, cinnamyl alcohol, cinnamyl acetate and cinnamic acid. In addition several other components such as B-caryophyllene, spathulinal, sesquiterpenoids and viridiflorene etc. are also present in appreciable quantity in the oil (2, 12). Eugenol and cinnamaldehyde have been reported to be major fungitoxic component present in *C. zeylanicum* bark and leaf oil (25). Presence of these chemical moieties in *C. tamala* leaf oil may also be accounted for the observed antifungal properties. Reports indicate that the concentration of many oils required for total inhibition of growth of microorganisms is generally greater than 500µg/ml. However, cinnamon oil at lesser concentration has been shown to possess similar inhibitory potential (9). So the lower MFC values (230µg/ml) of *C. tamala* oil against test fungi in our experiments are in conformity with earlier reports.

C. tamala leaf extracts prepared in different solvents exhibited various degrees of antioxidant activities in β -carotene bleaching assay (Fig. 1). Potential extract frac-

tions exhibited about 30 to 67% antioxidant activities at a concentration of 100 µg/ml (Table 2). The mechanism of bleaching of β -carotene is a free radical mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. β -carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β -carotene molecules. As β -carotene molecules lose their double bonds by oxidation, the compound loses its chromophore and characteristic orange color, which is monitored spectrophotometrically (11). The presence of *C. tamala* extracts may hinder the extent of β -carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system. Higher β -carotene bleaching reduction potential observed in some of the extracts could be accounted for by the presence of specific antioxidant phytochemicals in those extracts (15).

The reducing properties are generally associated with the presence of reductones (4). Gordon (7) reported that the antioxidant action of reductones is based on the breaking of the free radical chain by the donation of a hydrogen atom. Reductones also react with certain precursors of peroxide, thus preventing peroxide formation. Natural phytochemicals such as polyphenols may act in a similar fashion as reductones by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction (23). Results indicate that AQ and ET extracts possess noticeable reducing power (Fig. 2).

Our group has earlier reported the presence of many phytochemical moieties such as phenolics, flavonoids, tannins, terpenoids, alkaloids and saponins in *C. tamala* leaf extracts (18). So the observed free radical scavenging potential and the reducing power in *C. tamala* extracts could be attributed to the presence of these phytochemicals. In addition variations observed in antioxidant activity pattern of extracts could be attributed to the presence of differential amount of phytochemicals in respective extracts (5). The results indicate that leaves of *C. tamala* are rich sources of the antioxidant compounds. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (14). Presence of significant antioxidant activity in some of the extracts indicated that the selective extraction of antioxidant from natural sources by appropriate solvent is very important in obtaining fractions with high antioxidant activity.

The antioxidant activity of polyphenols is mainly due to their redox properties, which can play an important role in adsorbing neutralizing free radicals, quenching oxygen, or decomposing peroxidase. Most living species have potential system against oxidative stress and toxic effects of ROS. Several studies have demonstrated that the antioxidant properties of plant compounds could be correlated with oxidative stress defense. Okuda *et al.* (21) reported that the reducing power of tannins prevents liver injury by inhibiting the formation of lipid peroxides. Flavonoids have been reported to exhibit the antioxidative, antiviral, antimicrobial and anti-platelet activities (13). Polyphenols, particularly flavonoids, which are present in considerable amounts in fruits, vegetables, spices, medicinal herbs and beverages, have been used to treat many human diseases, such as diabetes, cancer and coronary heart disease (1, 5).

Based on the above discussion it may be concluded that nature is the best combinatorial chemist and possibly has answers to all diseases. Till now, natural products and compounds discovered from medicinal plants (and their analogues thereof) have provided numerous clinically useful drugs. In spite of the various challenges encountered in the medicinal plant-based drug discovery, natural products isolated from plants will still remain an essential component in the search for new medicines.

The results have established the intense antifungal and antioxidative potential of *C. tamala* oil and extracts, respectively. This indicates that the phytoconstituents present in test plant have considerable potential to prevent spoilage of food and diseases caused by fungi as well as the diseases resulting from overproduction of radicals. So the natural phytochemicals derived from *C. tamala* could be regarded as promising alternative to synthetic antifungal and antioxidant preparations for future use.

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Other articles in this theme issue include references (30-57).

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