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Full Proceeding Paper

IN VITRO ANTIMICROBIAL AND ANTICANCER ACTIVITY OF *CINNAMOMUM ZEYLANICUM* LINN BARK EXTRACTS

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ABSTRACT

One of the most common threats is the spread of multidrug resistant pathogens. Cancer is another major problem leads to death. Hence a search for new, plant based, risk-free, superior compounds with novel antimicrobial and anticancer activities is the need of the day. The aim of this study was to evaluate the antimicrobial and anticancer effects of bark of *Cinnamomum zeylanicum.* The antimicrobial activity of extracts of *Cinnamomum zeylanicum* bark (aqueous, methanol and chloroform) against bacterial and fungal clinical isolates like *Bacillus subtilis, Staphylococcus aureus*, *Escherichia coli, Aspergillus niger* and *Candida albicans* was determined. The anticancer activity was studied by MTT and AO/EB staining on hepato carcinoma cell lines ((Hep G2 cell line). The result of antimicrobial study showed that methanolic extract had better antibacterial and antifungal activity. The most susceptible bacterial and fungal strains were *Bacillus subtilis* and *Aspergillus niger*, respectively. The methanolic extract showed MIC value of 2.5mg/ml for *Bacillus subtilis* and 5mg/ml for *Aspergillus niger.* The results of *in vitro* anticancer studies by MTT assay on Hep G2 cell line in the presence of methanolic extract of *Cinnamomum zeylanicum* bark showed an IC_{50} value of 150µg/ ml. The AO/EB staining also showed that the methanolic extract was able to induce apoptotic activity in HepG2 cells after 24 hours of incubation at a concentration 150µg/ ml. This study proved that *Cinnamomum zeylanicum* bark is a reliable and safer herbal drug that can be used in pharmaceutical preparations for infectious and malignant diseases.

Keywords: *Cinnamomum zeylanicum*, Antimicrobial, MIC, Anticancer, MTT, AO/EB staining, Apoptosis.

INTRODUCTION

There has been a progress in the prevention, control and eradication of microbial diseases by the development of antimicrobials. Due to the evolution and adaptation of microbes, there is threats of new diseases and re-emergence of old diseases [1]. Cancer is a life-threatening disease and leads to high rates of mortality worldwide. Investigations for finding new plant based antimicrobial and anticancer compounds are imperative and interesting. There are many studies on anticancer herb/plant extracts in cell line models [2-4].

Cinnamomum zeylanicum tree belongs to the family, Lauraceae most noted for its bark, which provides the world with the commonly known culinary spice, cinnamon. Cinnamon has medicinal property and has been used to treat gastrointestinal complaints and other ailments [5]. Cinnamon possesses antiallergenic, anti-inflammatory, anti-ulcerogenic, anti-pyretic, antioxidant, anaesthetic activities [6]. Antioxidant studies with *Cinnamomum zeylanicum* bark showed better free radical scavenging capacity against a battery of free radicals [7]. In the present study, *Cinnamomum zeylanicum* bark was analyzed for its antimicrobial activity against clinical bacterial and fungal pathogens. Apoptosis is the mode of cell death induced by stimuli such as drugs, stress, radiation etc., The study was also aimed to evaluate the anticancer ability of *Cinnamomum zeylanicum* bark extract by inducing apoptosis in human hepato carcinoma cells (Hep G2 cell lines).

MATERIALS AND METHODS

The cinnamon bark was purchased from local market, Tiruchirappalli. The bark was dried, powdered and stored in a sterile container until use. A voucher specimen was deposited at the Rapinath Herbarium (BV001) and identified by Dr. John Britto of St. Joseph's College, Tiruchirappalli. The solvents and chemicals used were of analytical grade.

Test Microrganisms

The bacterial and fungal strains used were clinical isolates obtained from Eumic Analytical Lab and Research Institute, Tiruchirappalli. The bacterial strains used were *Bacillus subtilis, Escherichia coli, and Staphylococcus aureus.* The fungal strains used were *Aspergillus niger* and *Candida albicans.*

Preparation of Plant Extracts and Inoculation Medium

10g of powered bark of *Cinnamomum zeylanicum* was homogenized in 100ml of the solvents of varying polarity such as water, methanol and chloroform. The organic extracts were dried at 60°C kept away from sunlight. The residue was weighed and dissolved in Dimethyl Sulfoxide (DMSO; 20mg/50µl) to obtain the desired concentration. Aqueous extracts were prepared fresh.

The nutrient broth was prepared and sterilized by autoclaving at 121°C and at 15lbs for 15 minutes. Bacterial and fungal cultures were sub cultured in liquid medium at 37°C for 8h and further used for the test (10^4-10^5CFU /ml).

Antibacterial Assay

Kirby bauer Agar Well Diffusion method was used to study the effect of various bark extracts on the selected bacterial strains [8]. The sterilized nutrient agar medium was aseptically poured (20ml) into the sterile petri-plates and allowed to solidify. The bacterial broth cultures were separately swabbed on petri-plate using a sterile bud. Then wells were made by well cutter. The organic and aqueous extracts of bark (20 µl) were added to each well aseptically and were incubated at 37°C for 24 hours. The zone of inhibition was measured. Amphicillin disc was used as a positive control [9]. The experiment was repeated and the mean of triplicates was calculated.

Antifungal Assay

The antifungal activity of bark extracts against *Aspergillus niger* and *Candida albicans* were assayed by agar well diffusion method similar to antibacterial assay, except that Rose Bengal Agar medium [10] was used. Amphotericin B disc was used as standard. The petriplates were incubated at 37°C for 48 hrs. The antifungal activity was assayed by measuring the zone inhibition [9]. The experiment was repeated in triplicates and mean value was calculated.

Assay of Minimum Inhibition Concentration (MIC)

The MIC was determined with the methanolic extract which showed the maximum zone of inhibition, according to the NCCLS protocol [11]. Sterile nutrient broth was used for bacteria and Rose Bengal broth was used for fungal strains. Serial dilutions of the methanolic extract of *Cinnamomum zeylanicum* bark in DMSO was done to obtain the concentrations ranging from 10, 5, 2.5, 1.25, 0.63, 0.31,

0.16, 0.08 and 0.04 mg/ml. 100 µl from each tube was then transferred into 96-well microtitre plates. Each well was then filled with 100 µl microorganism suspension. Well containing microorganism suspension without methanolic extract served as positive control and the well without microorganism suspension served as negative control. The microtitre plates were incubated at 37ºC for 24 hours for bacterial strains and 37ºC for 48 hours for fungal strains with intermittent shaking and observed for visible growth.

Anticancer Drug Screening by MTT Assay

The anticancer assay was performed on Human hepatocellular carcinoma cells (HepG-2 cell line) supplied by National Centre for Cell Science (NCCS), Pune, India, with non-toxic dose of the bark extract and its dilutions. The cells were cultured in standard RPMI-1640 medium containing 2 mM glutamine supplemented with 12 % (v/v) heat inactivated fetal bovine serum and antibiotics (100 U/ml penicillin and 100µg/ml streptomycin) in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C. Cells were maintained in 25 cm^2 flasks and were sub cultured every 3 – 4 days.

The cell viability was determined by MTT (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) Assay. MTT is cleaved by mitochondrial enzyme dehydrogenase of viable cells, yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity [12].

HepG-2 cells were seeded onto a 96-well plate at a density of 5000 cells/well in 200 µL of medium for 24 hours. Various concentrations of methanolic extract of *Cinnamomum zeylanicum* bark (50, 100, 150, 200 µg/ml), was added to the wells and were incubated for 24 and 48 h. A control well without bark extract was maintained to compare the hundreds percent cell viability. After the incubation period 100 µl MTT (5 mg/ml in phosphate buffered saline) was added to each well and incubated for 3 hours in dark. Then MTT was discarded and 150 µl of DMSO was added to each well. The purple colour developed was measured at 570 nm with microplate reader (Bio-Rad). The IC_{50} value was calculated from the graph.

Percent cell viability was calculated as follows:
% of cell death = 100 - (OD of Test/OD of Control X 100)

Acridine Orange-Ethidium Bromide (AO/EB) Staining

Acridine orange is a vital dye and will stain both live and dead cells. Ethidium bromide will stain only cells that have lost membrane integrity [13].

HepG-2 cells were grown in 6-well plates treated with methanolic extract of *Cinnamomum zeylanicum* bark at 150 µg/ml (IC_{50}) for 24 and 48 h. The cells grown without methanolic extract of *Cinnamomum zeylanicum* bark served as control. The culture medium was removed and the cells were washed thrice with PBS, stained with 10µL of fluorescent dyes i.e. AO/EB (1:1 ratio at 100µg/mL) (Sigma Aldrich, USA) for 5 min. After washing thrice with PBS, morphological changes were observed under an epifluorescence microscope (Carl zeiss, Germany) for apoptotic changes at Ex/Em=510/595 nm for ethidium bromide and at Ex/Em=500/530 nm for acridine orange.

RESULTS

Antibacterial Activity of *Cinnamomum zeylanicum* Bark

The bark extracts were evaluated for their antibacterial activity. Figure 1 and Plate 1 shows the antibacterial function of aqueous, methanol and chloroform extracts of the bark of *Cinnamomum zeylanicum* on the selected bacterial strains which were *Bacillus subtilis Escherichia coli* and *Staphylococcus aureus.*

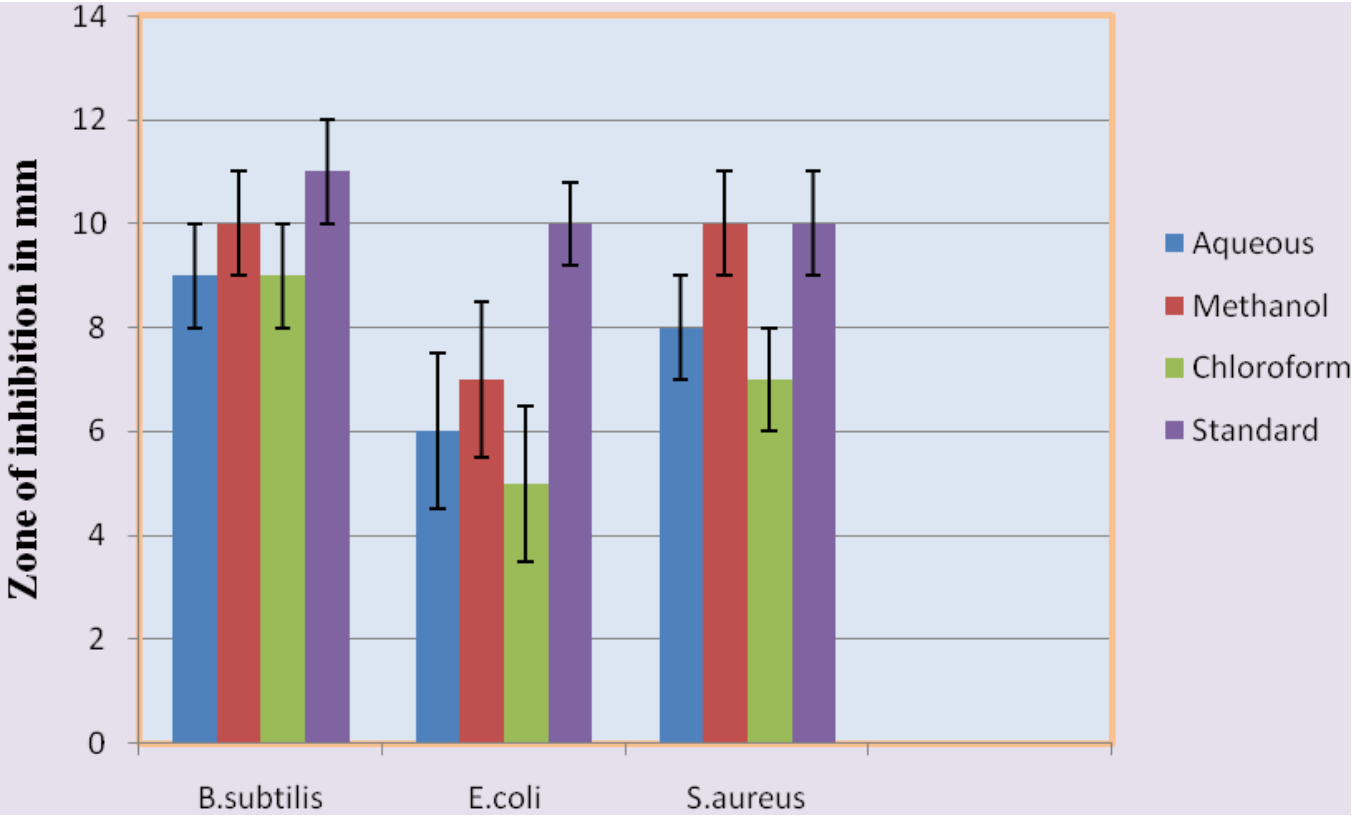
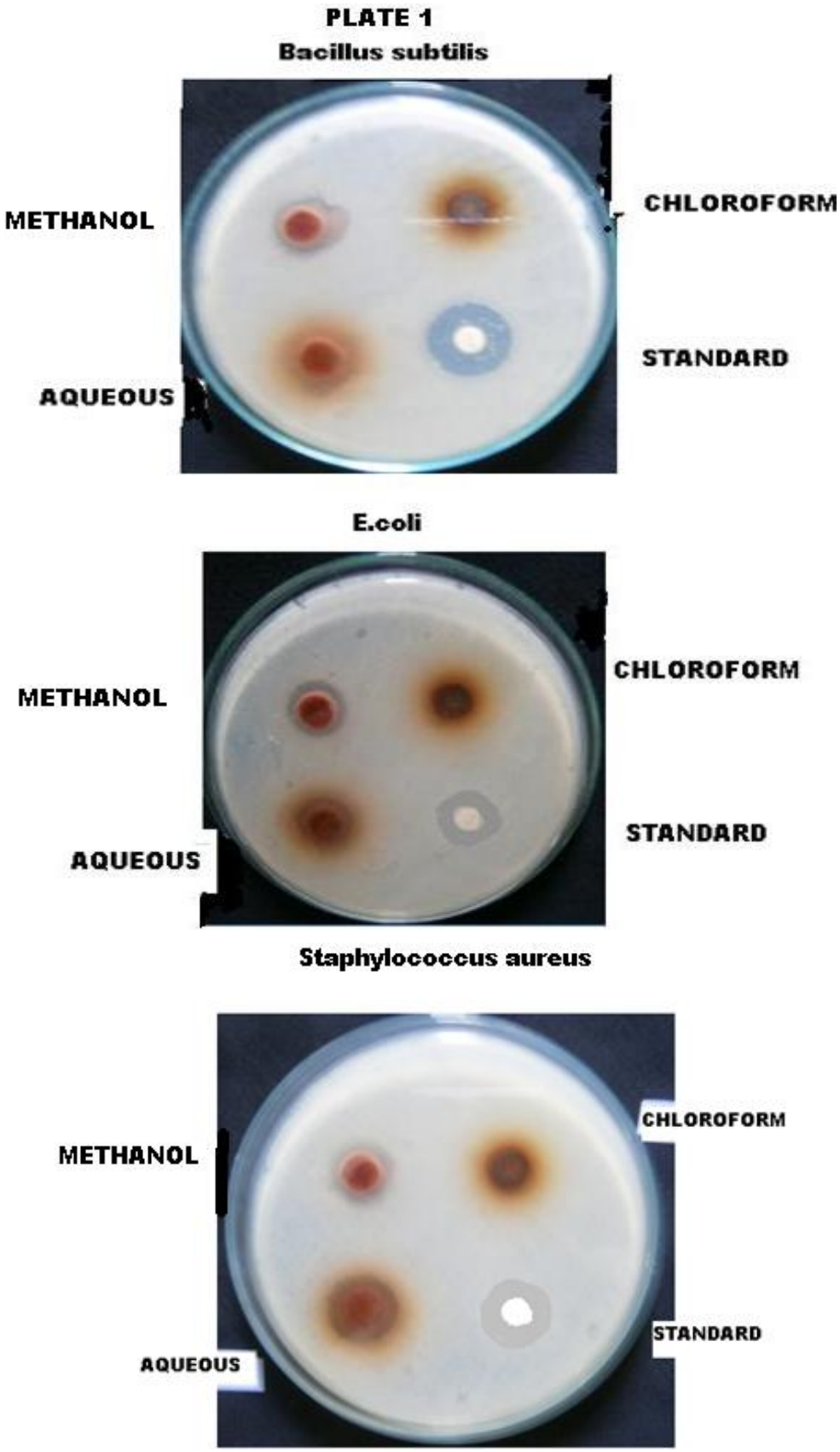


Fig. 1: Antibacterial effect of *Cinnamomum zeylanicum* bark extracts

The zone of inhibition for *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* were found to be the more in the methanolic extract which is comparable with the standard drug ampicillin and followed by aqueous and chloroform extracts.



Antifungal Activity of *Cinnamomum zeylanicum* Bark

The antifungal function of the bark extracts were determined against the fungal clinical isolates like *Candida albicans* and *Aspergillus niger*. The methanolic extract of the bark was found to cause more inhibition of the growth of *Aspergillus niger* than aqueous and chloroform extracts (Figure II and Plate II) and compared with that of standard drug amphotericin B. All the three extracts showed moderate inhibition on *Candida albicans*.

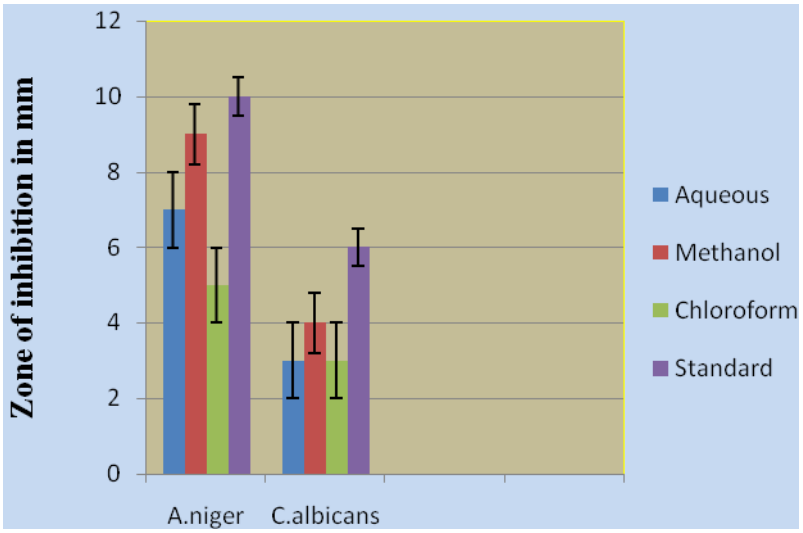


Fig. 2: Antifungal effect of *Cinnamomum zeylanicum* bark extracts

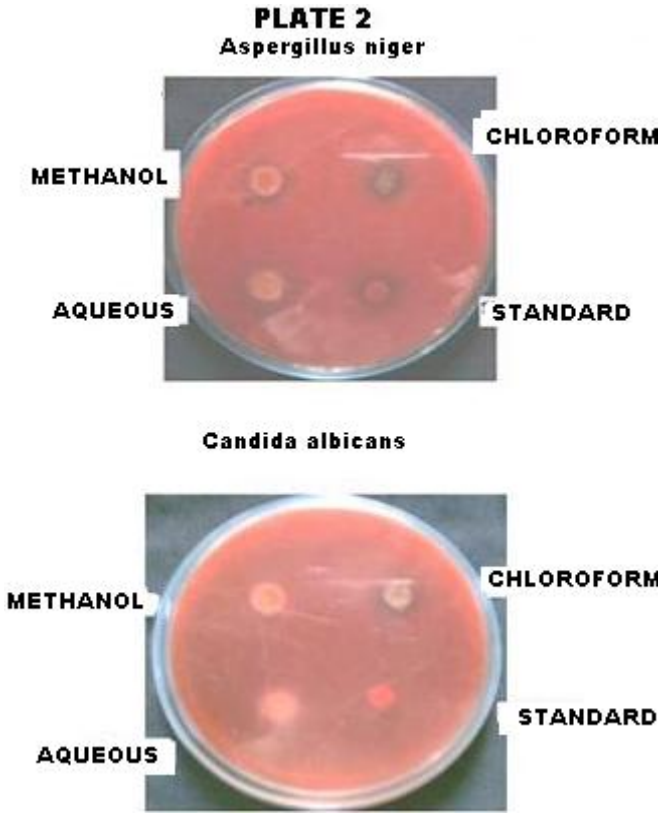


Table 1: Minimum Inhibitory Concentration

S. No.	Microorganims	Methanolic extract concentration (mg/ml)
1	*Bacillus subtilis*	2.5
2	*Eschericia coli*	>5
3	*Staphylococcs aureus*	2.5
4	*Asperillus niger*	5
5	*Candida albicans*	>5

By collective observation it was revealed that methanolic extract showed a good antibacterial activity and moderate antifungal activity. The aqueous and chloroform extracts of the bark showed the moderate antibacterial and lesser antifungal activity.

Minimum Inhibition Concentration

Table 1 depicted the MIC values for methanolic extract against the selected microorganisms. The methanolic extract of bark showed a low MIC of 2.5mg/ml against *Bacillus subtilis* and *Staphylococcus*

aureus but *E.coli* showed MIC of more than 5mg/ml and hence the least susceptible bacteria among the three. The methanolic extract of bark showed the lowest MIC of 5mg/ml against *Aspergillus niger* but *Candida albicans* showed MIC of more than 5mg/ml and hence the lesser susceptible fungi.

Anticancer Activity by MTT Assay

Significant dose and time-dependent cytotoxicity was observed by MTT assay. The IC_{50} values of methanolic extract of *Cinnamomum zeylanicum* bark were found to be 200 and 150µg/ml after 24 and 48 hr respectively. At this concentration the extract was toxic to more than 50% of HepG2 cells. The control tube (without bark extract) showed 100% viability of cancer cells (Figure III).

Acridine Orange and Ethidium Bromide Staining

Treatment of HepG2 cells with methanolic extract of *Cinnamomum zeylanicum* bark for 24 and 48 hours and staining with AO/EB fluorescent stain exhibited characteristic apoptotic morphology, i.e., cell shrinkage, nuclear condensation and fragmentation and the results were represented in Plate 3.

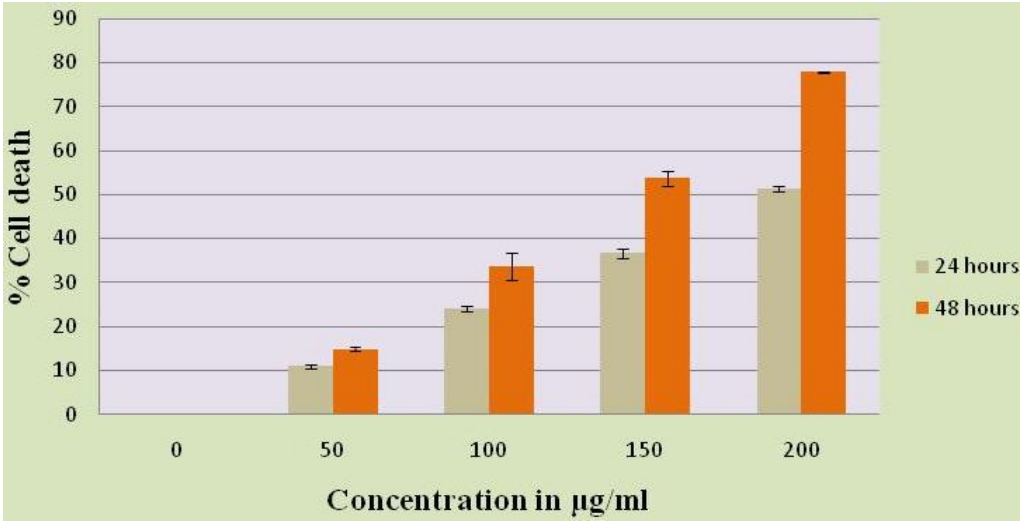
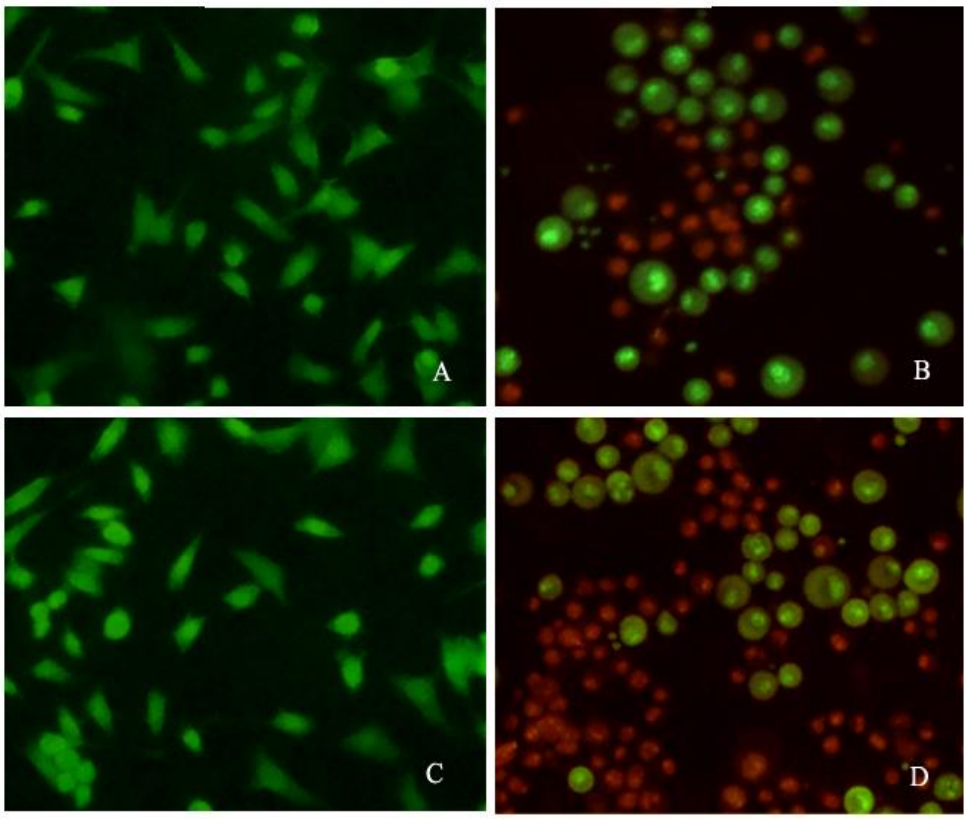


Fig. 3: MTT Assay

Plate - 3 AO and EtBr Staining of HepG2 cells



A- Control cell at 24 hrs
C- Control cell at 48 hrs

B-Cells +extract at 24 hrs
D-Cells +extract at 48 hrs

Under fluorescence microscope the control cells treated for 24 hours and 48 hours had no morphological changes; nucleus was smoother and was uniformly bright green together with the cytoplasm (Plate 3A & 3C). The cells treated with 150 µg/ml of methanolic extract of *Cinnamomum zeylanicum* bark for 24 h stained green and contained bright green dots in the nuclei showed the presence of early apoptotic cells (Plate 3B). HepG2 cells treated with 150 µg/ ml of methanolic extract of *Cinnamomum zeylanicum* bark for 48 hours integrated ethidium bromide and stained orange indicated the presence of condensed and fragmented nuclei which showed the presence of late apoptotic cells (Plate 3D). Dead cells were stained orange (Plate 3B and 3D).

DISCUSSION

Antimicrobial activity

The bacterial strains used were of different taxonomy. *Bacillus subtilis* is a Gram positive spore forming rods*; Staphylococcus aureus* is a Gram-positive cocci and *Escherichia coli* is a Gram-negative enterobacteria. The findings of this study showed that *Cinnamomum zeylanicum* bark extracts had inhibited both Gram-positive bacteria and Gram-negative bacteria indicating broad spectrum inhibitory effect. Gram positive bacteria were more susceptible than Gram-negative bacteria by the action of *Cinnamomum zeylanicum* bark extracts, demonstrating antibacterial effect which was comparable with that of the standard drug amphicillin.

There are several reports in the literature indicating the antibacterial and antifungal activity of the medicinal plants. Many studies reported the incapability of herbal antimicrobial agents to inhibit growth of Gram-negative bacteria [14] due to the presence of complex cell wall structure which decreases the penetration of bacterial cells by herbal extracts. But in the present study, *Cinnamomum zeylanicum* bark extracts moderately inhibited the growth of *E.coli*, proving penetrating ability of extracts in to bacterial cells.

The methanolic extract of *Cinnamomum zeylanicum* bark was found to cause significant inhibition of the growth of *Aspergillus niger* and *Candida albicans* compared to aqueous and chloroform extracts and was comparable with that of standard drug amphotericin B.

Studies by Kil *et al.,* (2009)proved that n-hexane, ethyl acetate, n-butanol, methanol and water fractions of sorghum (*Sorghum bicolor* Moench), the methanol extract elicited the maximum antimicrobial activity against *Escherichia coli, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Bacillus subtilis* and *Candida albicans* [15].

Studies by Liwei *et al.,*(2004)[16] showed that dimeric, trimeric, and higher oligomeric proanthocyandins with doubly linked bis-flavan-3-ol units were present in *Cinnamomum zeylanicum* bark. In this study the antimicrobial activity of *Cinnamomum zeylanicum* may be due to the presence of these secondary metabolites. The methanolic extract of the bark of *Cinnamomum zeylanicum* had strong antimicrobial activity showed that the active components have been extracted in methanol.

In the current investigation, the methanolic extract of *Cinnamomum zeylanicum* bark exerted low MIC of 2.5mg/ml against *Bacillus subtilis* and *Staphylococcus aureus* but *Escherichia coli* showed MIC of more than 5mg/ml and hence the least susceptible bacteria among all. The methanolic extract of bark showed the lowest MIC of 5mg/ml against *Aspergillus niger* but *Candida albicans* showed MIC of more than 5mg/ml and hence the lesser susceptible fungi.

The Chinese medicinal plants extracted with hot water, methanol and acetone were evaluated for their antifungal activity, among which the acetone extracts had the lowest MIC values indicating their effective antifungal property [17].

Manvi Malwal and Renu Sarin (2010)studied the *invitro* antimicrobial efficacy and MIC of root extracts of Murraya koenigii (Linn) Spreng against four bacterial strains (*Escherichia coli, Staphylococcus aureus, Bacillus subtilis,* and *Salmonella typhi)* and three fungal strains *(Candida albicans, Aspergillus niger* and *Trichophyton rubrum*) [18]. The most susceptible bacterial and fungal strains were *Staphylococcus aureus and Trichophyton rubrum*, for methanolic of roots showed MIC value of 0.078mg/ml and 0.156 mg/ml respectively.

Our results were congruent with the results of others.

Anticancer activity

MTT is considered to be a reliable assay to determine the extent of cell viability. In the present study the IC_{50} values of methanolic extract of *Cinnamomum zeylanicum* bark were found to be 200 and 150µg/ml after 24 and 48 hr respectively. The results of the study also showed that the induction of apoptosis by methanolic extract of *Cinnamomum zeylanicum* bark in human hepatoma cancer cells indicates its anticancer activity.The study by Venkatakrishnan *et al*., (2010) reported that the ethanolic extract of *Pleurotus ostreatus* repressed the cell proliferation in a dose-dependent manner in leukemia cells (HL-60 cell line) [19]. The methanolic extract of *Piper sarmentosum* possessed anticarcinogenic properties in HepG2 cells [20]. Similarly, Zhang and Poporich (2008) estimated, using the MTT assay, the inhibition of cell proliferation in Hep G2 (liver carcinoma) cells by soya saponins, which was found to be dose-dependent [21].

Shahrul Hisham *et al*., (2009), reported on the anticarcinogenic activity of an ethanolic extract from *Piper sarmentosum* in HepG2 by MTT assay [22]. The IC_{50} value for HepG2 cells was 12.5 µg mL^{-1}.Treatment with 10, 12 and 14 µg mL-1 of ethanolic extracts caused typical apoptotic morphological changes in HepG2 cells on staining with acridine orange and ethidium bromide.

Lingadurai *et al.,* (2011) studied the cytotoxic effect of the leaves of *Bichofia javanica* extract on human leukemic cell lines (U937, K562, and HL60). 10 µg/ml methanolic extract of *Bichofia javanica* showed significant cytotoxicity [23].

Our result was congruent with result of others.

CONCLUSION

The present study strongly iterates the antimicrobial and anticancer capacity of the bark of *Cinnamomum zeylanicum* and scientifically validates it for use as a component in medicinal preparations, especially against pathogenic attack and proliferative diseases.

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