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Identification of Bioactive Compounds and Toxicity Study of *Araucaria columnaris* Bark Extract on Human Embryonic Kidney Cell Line

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ABSTRACT

Plants produce a diverse range of bioactive compounds making them a rich source of different types of medicines. Ornamental plants are cultivated for adornment and to enhance the appearance of houses and also for commercial purposes. However, only very few of these ornamental plant species have found to be used in medicine and only little literature exit on their chemical and biological actions. In the present study, the evaluation of antimicrobial activities and identification of bioactive compounds using TLC and GC-MS of the *A. columnaris* bark extract were performed. In GC-MS bioactive compounds with medicinal value were identified, such as Benzoic acid, 1H-N-Hydroxynaphth (2,3) imidazole-6,7-dicarboximide, 2-Propenoic acid, 3-(4-methoxyphenyl), 1H-N-Hydroxynaphth (2,3-d) imidazole-6,7-dicarboximi. To prove the nontoxic nature of the plant, its crude bark extract was subjected to toxicity study using human embryonic kidney cell line. It reveal that the plant is minimal toxic to the human kidney cell line so usage of appropriate level will found to be safe and also carrying out some structural modification will help in the extraction of new drugs for pharmaceutical purpose.

Key words: Benzoic acid, imidazole, propenoic acid, MTT assay, ornamental plant

INTRODUCTION

Plants are the gift of the nature which has high medicinal value. Diseases that remain most challenging for today's health care system tend to be more complex than could be treated by current combination therapies. However, plant based drugs contain a mixture of multiple components which saves the effective control of disease (Karnath, 2002). The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body (Rajesh *et al.,* 2009). Recently, however, plant derived compounds offer a potential source of new antimicrobial, anticancer and anti-HIV agents among various pharmaceuticals. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic components (Hill, 1952). The substances that can inhibit pathogens and have little toxicity to host cells are considered in developing new antimicrobial drugs. In comparison to modern medicine, the incidence of side-effects are lesser with herbal products but they cannot be considered completely free from side-effects. The growing number of herbal drug users around the globe and scarcity of scientific reports regarding the safety aspects of herbal products makes it imperative to conduct a toxicity study of herbal drugs (Saad *et al*., 2006). Plant preparations which were medically useful should be nontoxic or of low toxicity towards human cells. Therefore, it is necessary to carry out toxicity studies on medicinal plants, even though they have been used for decades (Rajeh *et al.,* 2012).

Araucaria columnaris is large evergreen, ornamental tree, commonly known as Christmas tree. The genus *Araucaria* belongs to Araucariaceae comprises about 38 species. Various reports say that different parts of *Araucaria* species were widely used in traditional medicine for the treatment of antiulcer and antipyretic (*Araucaria bidwillii)* (Anderson, 1986), gastroprotective and wound healing action (*Araucaria araucana*) (Schmeda-Hirschmanna *et al*., 2005), antibacterial activity (*Araucaria angustifolia*) and antimicrobial activity (*Araucaria cunninghamii*) (Chen *et al*., 2011). The aim of the present study was to determine the bioactive compound of *Araucaria columnaris* bark using GC-MS and study its cytotoxicity ability in human embryonic kidney cell line (HEK 293). In future the plant extract can be further formulated and used as drug for various diseases.

MATERIALS AND METHODS

Identification and collection of plant materials: The taxonomic identity of the plant was confirmed by the Botanist of the Institute of Forest Genetics and Tree Breeding Center (IFGTB), R.S. Puram, Coimbatore, Tamilnadu, India. The bark of the plant *Araucaria columnaris* was collected from the garden of Nehru Arts and Science College, T.M. Palayam, Coimbatore, Tamil Nadu, India. The bark samples were shade dried, powdered and stored in polypropylene air-tight containers under proper conditions for further uses.

Extracts preparation: The crude powdered samples were weighed and subjected to solvent extraction for 8-10 h repeatedly in methanol, ethyl acetate, benzene and water using soxhelt apparatus. The extracts were then concentrated in 40-45°C and air dried. The dried samples were then stored in airtight bottles at 4°C for further analysis.

Antibacterial screening of TLC fractions of bark extract: The TLC was performed in glass plates with silica gel G (Hi Media) of 60-120 meshes, was used as stationary phase and the mobile phase used was chloroform: Methonal (29:0.3). The plates were spotted with 10 μL of different extracts. For visualization of phenolic compound, Folin-cioclaute reagent was used as the spraying agent. Different bands appeared on TLC plate (Stahl, 1969; Chatwal, 1998). The bands were scraped individually and were dispensed in appropriate solvent and filtered to get the pure sample. Further, these samples were tested for antibacterial activity against human pathogens (*E. coli, Staphylococcus aureus* and *Bacillus subtilis*) by agar well diffusion method.

Bioactive compound identification using GC-MS: Based on the antimicrobial assay, the most effective fraction of the methanolic extract of the Bark (F3) was further used for the identification of bioactive constituents by GC-MS analysis. Analysis by GC/MS was performed using a Thermo Gc-Trace Ultra Ver: 5.0, Pyrolysis auto sampler interfaced to a Perkin Elmer Turbomass Gold equipped with a fused silica capillary column (J and W; DBI; 30m length×0.25 mm id. Film thickness 0.25 mm). The fraction was pyrolysed at 610°C and then introduced to the GC column. The transfer line was held at 280°C and the source temperature was maintained at 180°C and ionization energy was set at 70 eV. Helium was employed as carrier gas (1 mL m^{-1}). The GC oven temperature was programmed: The column held initially at 70°C m^{-1} (isothermal) and then increased by at 8-260°C m^{-1} min^{-1} (isothermal).

Interpretation of mass spectra of GC-MS was done using the database of the National Institute of Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the

unknown component was compared with the spectrum of the known components stored in the NIST08 and Wiley08 library. The name and molecular weight of the components of the test materials were ascertained.

Study on the toxicity of bark extract: Based on identification of bioactive compounds the crude bark extract was subjected to toxicity study. The presence of non toxic nature of plant material was essential in the case of herbal medicines. The *in vitro* toxicity of the aqueous extract of bark was performed using human kidney cell line (Mosmann, 1983; Monks *et al*., 1991).

Human embryonic kidney cell line study: The human embryonic kidney cell line (HEK 293) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium (EMEM) containing 10% Fetal Bovine Serum (FBS). All cells were maintained at 37°C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly and the culture medium was changed twice a week.

The monolayer cells were detached with trypsin-EDTA to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give a final density of 1×10^5 cells mL^{-1}. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 15,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO_2, 95% air and 100% relative humidity. After 24 h, the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and diluted to twice the desired final maximum test concentration with serum free medium. Additional four, 2 fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 μL of these different sample dilutions were added to the appropriate wells already containing 100 μL of medium, resulted the required final sample concentrations. Following drug addition the plates were incubated for an additional 48 h at 37°C, 5% CO_2, 95% air and 100% relative humidity. The medium without samples were served as control and triplicate was maintained for all concentrations.

MTT assay: The 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 h of incubation, 15 μL of MTT (5 mg mL^{-1}) in Phosphate Buffered Saline (PBS) was added to each well and incubated at 37°C for 4 h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 μL of DMSO and then measured the absorbance at 570 nm using a micro plate reader. The percentage cell inhibition was determined using the following formula.

$$\text{Cell Inhibition (\%)} = \frac{\text{100-Abs (sample)}}{\text{Abs (control)}}\times 100$$

Nonlinear regression graph was plotted between percentage cell inhibition and Log_{10} concentration and IC_{50} was determined using graph pad prism software.

RESULTS AND DISCUSSION

Antibacterial activity of TLC fractions of bark extracts: On performing TLC, 7 spots were observed from different extracts of bark. The methanolic extract (F3) fraction has shown maximum zone of inhibition against pathogenic bacterial strains shown in Fig. 1. The maximum zone of

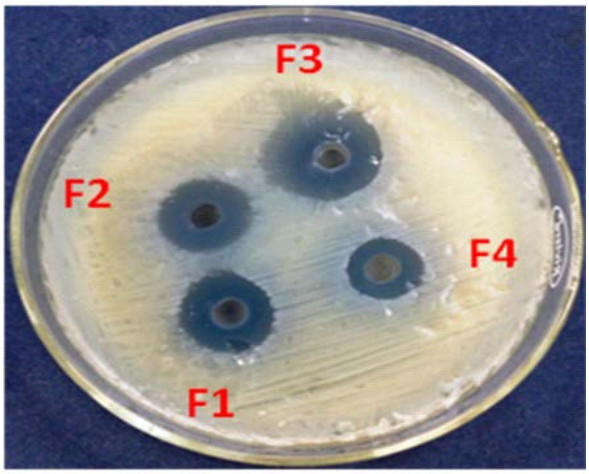

Fig. 1: Antibacterial property of the bark extract scraped from TLC (Method adopted from Stahl, 1969; Chatwal, 1998), Antibacterial activity of different bark extract of TLC fractions, F3 fraction shows the maximum zone of inhibition against *Staphylococcus aureus*

inhibition was noticed against *Staphylococcus aureus* followed by *E. coli* and *Bacillus subtillis* (20, 18 and 15 mm, respectively), when compared to other fractions. The active fraction was selected for GC-MS analysis.

The variation in RF values of the phytochemicals provides a very important clue in understanding of their polarity and also helps in selection of appropriate solvent system for separation of pure compounds by different chromatographic techniques. Compound showing high RF value in less polar, solvent system have low polarity and with less RF value have high polarity. Mixture of solvents can be used for separation of different polarity compounds from plant extracts. The selection of appropriate solvent system for a particular plant extracts can only be achieved by analyzing the RF values of compounds in different solvent system (Talukdar *et al*., 2010).

GC-MS analysis of TLC fractions: The GC-MS study of *Araucaria columnaris* revealed the qualitative identification of different constituent. The chemical identities of terpenoids, flavonoids and phenolic derivatives and other compounds from bark extracts were determined by matching their recorded mass spectra with the data bank mass spectra (NIST and WILLEY libraries) provided by the instrument software and by comparing their calculated retention indices with literature values measured on columns with identical polarity (Fig. 2 and Table1). The GC-MS of bark results showed that the compounds found mostly the derivatives of terpenoids and flavonoids. This is the first report on identification of bioactive compounds in *Araucaria columnaris* bark.

Ponnamma and Manjunath (2012) reported about 2-propenoic acid,3-(2-hydroxyphenyl) or its synonym cinnamic acid dihydro having antibacterial, anesthetic, anti-inflammatory, antimutagenic, antispasmodic, cancer preventive, dermatitigenic, fungicide, herbicide, laxative, pesticide activities. In the current study similar compound 2-propenoic acid, 3-(4-methoxyphenyl) with retention time 28.57 had been detected in the bark of *Araucaria columnaris*. Benzoic acid was also detected in the bark, used as a food preservatives and inhibits the growth of mold, yeast and some bacteria, antifungal (tinea, ringworm and athlete's foot), analgesic, antiseptic properties, decongestants and also as expectorant (Warth, 1991; Wilson *et al*., 2004; Lillard, 1919).

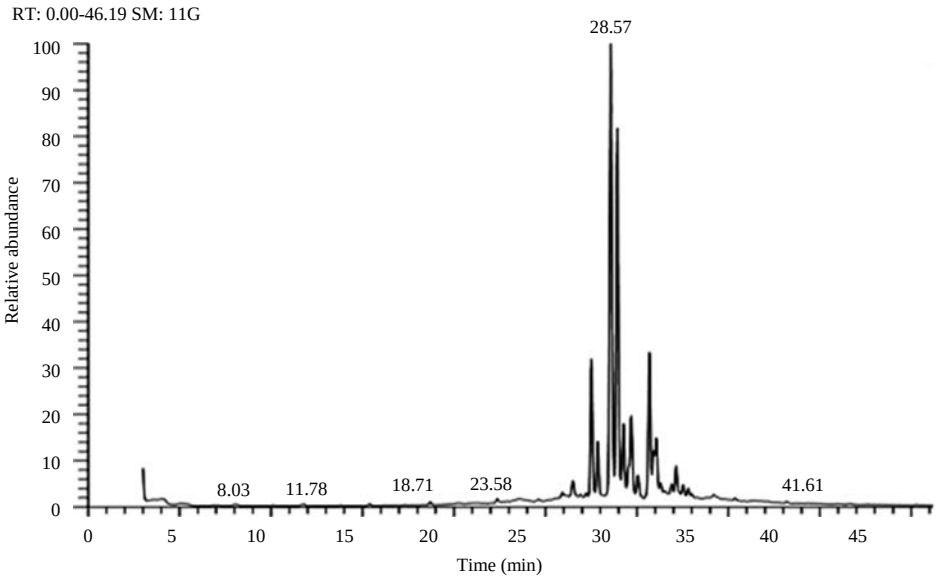

Fig. 2: GC-MS analysis of *Araucaria columnaris* bark extract (Method adopted from Adams, 1995)

Table 1: GC- MS bioactive compounds identified from bark extract

RT	Name of the compounds	Molecular formula	Molecular weight	Peak area (%)
28.57	Benzoic acid	$C_{16}H_{16}N_4O_5$	344	30.76
18.71	1H-N-Hydrxynaphth(2,3)imidazole-6,7 -dicarboximide	$C_{13}H_7N_3O_3$	253	0.20
28.57	2-Propenoic acid, 3-(4-methoxyphenyl)	$C_{10}H_{10}O_3$	178	30.76
28.57	Benzoic acid, 4-[[[[(1,2-dichloroethylidene) amino]oxy]carbonyl]amin o]-, methyl ester	$C_{19}H_{22}N_4O_2$	338	30.76
11.78	Tert Butoxy 2 ethoxyethane	$C_8H_{18}O_2$	146	0.23
15.41	1H-N-Hydroxynaphth(2,3-d)imidazole-6,7-dicarboximide	$C_{13}H_7N_3O_3$	253	0.20
18.71	6-Methoxy-2-methyl-2-phenyl-2H-1-benzopyran	$C_{17}H_{16}O_2$	252	0.20
11.78	2,3-Diamino-2-methylpropanoic acid	$C_4H_{10}N_2O_2$	118	0.23
23.56	2,4-Dimethylfuran	C_6H_8O	96	0.16
23.56	Cyclopentane, ethylidene (CAS)	C_7H_{12}	96	0.16

The another compound named 1H-N Hydrxynaphth(2,3)imidazole6,7dicarboximide have various antifungal, antiprotozoal, antihypertensive and anticancer activity, medications in inflammation, neurodegenerative diseases and also have antioxidant property (Khalid *et al.,* 2005; Bogle *et al*., 1994; Almasirad *et al*., 2014). Thus it could be concluded that *Araucaria columnaris* plant is of pharmaceutical importance.

Study on cytotoxicity of bark extracts of *Araucaria columnaris*: The cytotoxicity was checked using different concentrations of the bark extract on human kidney cell line (HEK) and it was found to be minimal toxic to the human kidney cell line, its IC_{50} is 95.0 μg mL^{-1} and R^2 is 0.999 (Table 2 and Fig. 3). It was concluded that the usage of this plant is safe up to 75 μg but over dose will be found to be toxic. Similar work was reported by Hirschmann *et al*. (2005) in the *Araucaria cunninghamii,* diterpenes, where the compounds 15-Acetoxy-8, 9-epoxylabdane-19-oic acid and 15, 19-Dihydroxy-8, 17-epoxylabdane presented a strong gastroprotective effect (68 and 54%, respectively) with cytotoxicities higher than 1000 μm. Considering that simple structural modifications led to lower cytotoxicity maintaining the gastroprotective effect, selective modifications can afford labdane diterpenes with a better potential as anti-ulcer drugs.

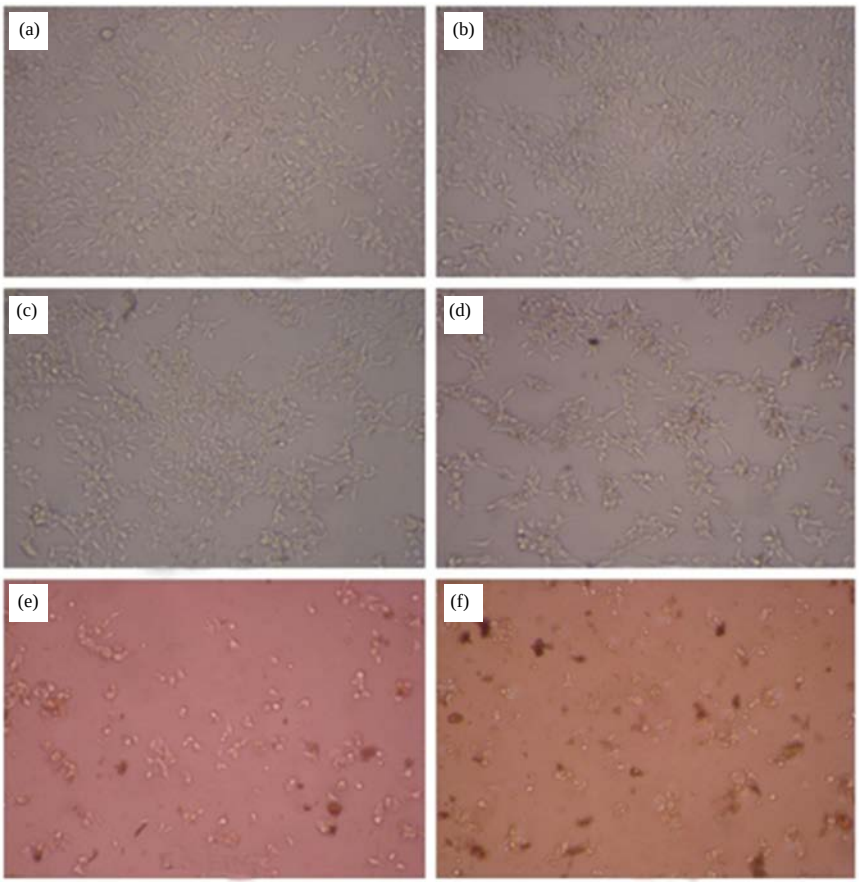

Fig. 3(a-f): Growth inhibition of HKCL by the addition of different concentration of bark extract. (a) Control (without bark extract) no growth inhibition, (b) 18.75 (c) 37.5 µg no growth inhibition was observed, (d) 75 µg mild cell growth inhibition was observed, (e) 150 and (f) 300 µg complete growth inhibition was found, Toxicity study using human kidney cell line (Method adopted from Mosmann, 1983; Monks *et al*., 1991)

Table 2: Percentage cell inhibition of bark extract of *Araucaria columnaris*

Concentration (µg)	Cell inhibition (%)	IC_{50}	R^2
18.75	0.357995	95.0 µg mL^{-1}	0.999
37.5	2.386635		
75	30.548930		
150	83.651550		
300	95.823390		

CONCLUSION

Therefore, it is recommended that more work to be conducted to help optimally extract all the bioactive compounds in the plant and thus formulated into an appropriate dosage for the treatment of infectious diseases. The plant *Araucaria columnaris* holds promise for the production of novel pharmaceuticals as well as a nutraceutical. It would be worthwhile to further isolate the compounds and determine their specific activity and also to understand the synergistic effect of compounds for therapeutic roles. Further animal study will be conducted for proper use of this plant extract as a pharmaceutical product.

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