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INHIBITORY POTENTIALS OF *TINOSPORA CORDIFOLIA* LEAF EXTRACTS AGAINST PATHOGENIC BACTERIA AND COLON CANCER CELL LINE

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Herbal alternates are being preferred over synthetic medicines nowadays, owing to the side effects exhibited by them. Preliminary investigations of the inhibitory potentials of *Tinosporacordifolia* leaf extracts against pathogenic bacteria and colon cancer cells were analysed in the current study. Phytochemical analysis showed the presence of various secondary metabolites in the crude leaf extract. Upon studying the antibacterial efficiency of the extracts, the ethyl acetate and methanolic extract showed maximum inhibition against *Staphylococcus aureus* and *Aeromonashydrophila*. 68% of HT29 cells treated with the ethyl acetate extract displayed cytotoxicity. A laddering pattern was observed after DNA fragmentation assay was performed, hinting at the apoptosis induced by the ethyl acetate extract.

Key words: Antibacterial, Anti-cancer, DNA fragmentation, Cytotoxicity

INTRODUCTION

Plants are invaluable sources of pharmaceutical products (Sadqui *et al.*, 2006) and are recognized for their ability to produce a wealth of secondary metabolites. Mankind has used many species for centuries to treat a variety of disorders (Olalde 2005). The complex secondary metabolites produced by plants have found various therapeutic uses in medicine. The early history of modern medicine contains descriptions of plant-derived phytochemicals, many of which are still in use for treatment (Rishton 2008). Traditional medicine refers to the knowledge, application, approach and belief in incorporating plant or animal based properties in remedies, singularly or in combination, for the purpose of treating or preventing disease as well as to maintain the well-being of an individual (Mouli *et al.*, 2009). *Tinosporacordifolia* (Guduchi) is a widely used plant in folk and ayurvedic systems of medicine (Kavitha *et al.*, 2009). It is distributed throughout tropical Indian subcontinent ascending from Himalayas down to the southern part of peninsular India at an altitude of 300m asl. It is also reported in neighboring countries like Bangladesh, Pakistan and Sri Lanka (Preeti 2011). The leaves are membranous, cordate and heart in shape.

The plant is well known, Indian, bitter and prescribed for fevers, diabetes, dyspepsia, jaundice, urinary problems, skin diseases, and chronic diarrhea and dysentery. It has been also indicated useful in the treatment of heart diseases, leprosy, helmenthiasis and rheumatoid arthritis. The starch obtained from "guduchisatva" is highly nutritive, digestive and used in many diseases (Kirtikar *et al.*, 1933). The bitter principle present shows antiperiodic, antispasmodic, anti-inflammatory, anti-fertility and antipyretic properties (Dahanukar *et al.*, 1988). *T.cordifolia* has been reported to treat throat cancer in humans (Nisha *et al.*, 2005). This plant also possesses antimicrobial activity against many pathogenic organisms (Verma *et al.*, 2011). Cancer is the second leading cause of

death (Hoyer *et al.*, 2005), where one in four deaths is due to cancer. Cancer is a major public health burden in both developed and developing countries. It was estimated that there were 10.9 million new cases, 6.7 million deaths, and 24.6 million persons living with cancer (Parkinet *al.*, 2002). The use of plant products in the treatment of cancer has been of recent interest (Bauer 2000). In the current study, the leaf extracts of the plant was subjected to phytochemical studies and its antibacterial and anticancer properties were recorded.

MATERIALS AND METHODS

Plant Material Collection

The fresh leaves of *Tinosporacordifolia* were collected from Irular Tribal Women Welfare Institute, Thandrai, Chengalpet, Chennai, Tamil Nadu, India. The plant specimen was identified by Dr. Narasimhan, Department of Botany, Madras Christian College, Chennai. The collected plant leaves were allowed for Shade drying for 4 days completely. The dried samples were powdered with electrical blender and made into coarse powder and used for further extraction.

Cell Line

The colon cancer cell line HT 29 was procured from National Centre for Cell Line Sciences (NCCS) Pune.

Sequential extraction

About 50 gm of dry sample was weighed and macerated with 200 ml of hexane separately and kept overnight in shaker. The extract was collected after filtration using Whatman No:1 filter paper and was stored. Another 200 ml of ethyl acetate was added to the residual mixture and incubated in shaker for 24 hours and the extract was collected again using a Whatman No:1 filter paper. This procedure was repeated once again with methanol and the extract was evaporated (Manjamalai 2011), which was used for further phytochemical analysis, antimicrobial activity and anticancer activity.

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Preliminary Phytochemical Screening

The extracts were subjected to preliminary phytochemical screening (Harborne 1998) for their presence or absence of active phytochemical constituents such as alkaloids, carbohydrate, quinones, flavonoids, phenols, coumarins, tannins, glycosides, cardiac glycosides, anthraquinones, proteins, saponins and terpenes.

Estimation of total flavonoids

Aluminium chloride colorimetric method was used for flavonoids determination. Each plant extracts (10mg/ml) was prepared and 0.5ml of each sample was separately mixed with 1.5ml of methanol, 0.1ml of 10% aluminium chloride and 0.1ml of 1M potassium acetate. 2.8ml of methanol was added and kept at room temperature for 30min, the absorbance of the reaction mixture was measured at 415nm. The content of flavonoid was expressed in mg/g (Chang *et al.*, 2002).

Estimation of Total Tannin

Tannin concentration in the extract was measured by Folin Denis method (Schanderi 1970) with minor modification. The crude extract (0.1ml) was diluted with 0.4ml of distilled water and then mixed with 0.5ml of Folin Denis reagent. The reaction mixture was alkalized by the addition of 1ml of 15% (w/v) sodium carbonate solution and kept in dark at room temperature for 30min. The absorbance of the solution was read at 700nm using spectrometer and the concentration. Tannins in the extract was determined using pure tannic acid as standard (10mg/ml).

Agar Diffusion Method

Effects of Hexane, ethyl acetate and methanolic extracts of *Tinosporacordifolia* leaves were analyzed against three pathogenic bacterial strains by agar diffusion method (Jehanbakht *et al.*, 2011). The bacterial strains were *Aeromonashydrophila*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Ciprofloxacin was used as standard and the zone of inhibition was measured.

Minimum Inhibitory Concentration (MIC)

The Nutrient broth was prepared for 15ml with distilled water. Hexane, Ethyl acetate and Methanolic extracts of *Tinosporacordifolia* leaves were analyzed against three pathogenic bacterial strains and the MIC was determined in a 96-well microplate²². Ethyl acetate and methanolic extracts at 2mg and 5mg DMSO were added in the wells with different pathogens. This was incubated at 37°C overnight. After one day incubation, the OD values were read in a micro plate reader at 600 nm.

$$\frac{\text{Absorbance of the control} - \text{Absorbance of the sample}}{\text{Absorbance of control}} \times 100$$

MTT Assay

MTT assay is based on the ability of live but not dead cells to reduce a yellow tetrazolium dye to a purple formazan product (Mossman 1983). In brief, approximately 5×10^3 cells/well

(cell line) were seeded into 96 well plate, 100µl of RPMI 1640 medium was added and incubated at 37°C. After 24 hours, the medium was discarded and fresh medium was added with different concentration of plant extract (Hexane, ethyl acetate, methanol). The plates were incubated for 2 days at 37°C in a CO₂ incubator. After respective incubation period, medium was discarded and 100µl fresh medium was added with 10µl of MTT (5mg/ml). After incubation at 37°C in for 4h, the medium was discarded and 200µl of DMSO was added to dissolve the formazan crystals. Then the absorbance was read in a microplate reader at 570nm and cell survival was calculated by the following formula.

$$\text{Viability \%} = \frac{\text{Test OD}}{\text{Control OD}} \times 100$$

$$\text{Cytotoxicity \%} = 100 - \text{viability \%}$$

Cyclophosphamide (90µg) was used as a positive control.

DNA Fragmentation

This method is used to analyze the genotoxicity of the plant extract in the cell lines. Grown cells were seeded in 24 well plate and kept in CO₂ incubator. Cells were treated with ethyl acetate extract in three different concentrations (50,100,150µg) for 24 hours. Cells were centrifuged for 1000rpm for 3mins at 14°C. The pellet was incubated with 100µl of cell lysis buffer at 37°C for 1hour. This was centrifuged at 3000rpm for 15mins. To the supernatant equal volume of phenol: chloroform: isoamyl alcohol mixture was added and mixed well. This was centrifuged at 10000rpm for 15mins. The supernatant was transferred to a new tube. To the final aqueous phase 40µl of 3.5M ammonium acetate was added. To this ice cold isopropanol was added to precipitate the DNA. This was incubated at -20°C for 1hour followed by the centrifugation at 10000rpm for 15mins. The pellet was retained and washed with 70% ethanol and stored in 20-50µl of TE buffer. The samples were analyzed in 2% agarose gel stained with ethidium bromide (Maniatis, 1982).

RESULTS AND DISCUSSION

Fresh and healthy leaves of the plant were collected and shade dried. The leaves were then powdered and sequential extraction was performed using hexane, ethyl acetate and methanol solvents. Plant-derived compounds and their semi-synthetic, as well as synthetic analogs, serve as major source of pharmaceuticals for human diseases. It is estimated that approximately 25% of prescriptions handled in United States contain a plant-derived natural product and 74% of the 119 most important drugs currently contain ingredients from plants used in traditional medicine. Hence, for the treatment of disease states where in drug therapy is a rational approach, plant materials represent legitimate starting materials for the discovery of new agents (Grifo *et al.*, 1997). The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavanoids, tannins and phenolic compounds (Edeoga *et al.*, 2005). Preliminary phytochemical investigations showed the presence of various secondary metabolites (Table 1). Table 2 represents the quantitative analysis of secondary metabolites of *Tinosporacordifolia*. This observation suggests that *Tinosporacordifolia* leaves contain 87µg/mg of flavonoids in methanolic extract, which is

significantly higher than the other extracts. The tannin content in methanol extract was about 32µg/mg. In earlier studies, *Tinosporacordifolia* has been reported to significant amount of tannic acid (5,852.10 µg/g). Out of the six different Phenolic acids, Ferulic acid and Cinnamic acid showed amount content of 45.70 µg/g and 10.46 µg/g which are detected only in *Tinosporacordifolia* (Singh *et al.*, 2010). Table 3 represents the antibacterial activity of *Tinosporacordifolia* in different solvent extracts tested by agar diffusion assay. The ethyl acetate and methanolic extract of *Tinosporacordifolia* showed the maximum inhibition against *Staphylococcus aureus* and *Aeromonashydrophila*. (10 and 30mm). Previously, *Tinosporacordifolia* leaf extracts had showed almost similar zone of inhibition against all the tested bacteria such as *E. coli*, *S. aureus* and *B. subtilis* (Mahesh *et al.*, 2008).

Table 4 represents the antibacterial activity of ethyl acetate and methanolic extract of *Tinosporacordifolia* against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Aeromonashydrophila* by Minimum Inhibitory Concentration (MIC) method. The MIC values of ethyl acetate extract against *A. hydrophila*, *P. aeruginosa* and *S. aureus* were 72 mg/ml, 51 mg/ml and 64.5 mg/ml respectively. The MIC values of methanolic extract against *A. hydrophila*, *P. aeruginosa* and *S. aureus* were 62mg/ml, 67 mg/ml and 64 mg/ml respectively. Verma *et al.*, (2009) had previously reported that the minimum inhibitory concentration of *Tinosporacordifolia* stem methanolic extract against *Escherichia coli* was 2.5 mg/ml, *Staphylococcus aureus* 5.0 mg/ml and *Staphylococcus albus* 7.5 mg/ml. Further studies, are needed to isolate and characterize the active principles to elucidate their different antimicrobial mechanism. Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Geissman 1963). These compounds protect the plant from microbial infection and deterioration (Cowan 1999).

Some of these phytochemicals can significantly reduce the risk of cancer due to polyphenol antioxidant and anti-inflammatory effects. Some preclinical studies suggest that phytochemicals can prevent colorectal cancer and other cancers (Michaud *et al.*, 2000; Greenberg *et al.*, 1994; Birt *et al.*, 2001). Fig. 1 records the cytotoxic effect of the leaf extracts against HT 29 cell line. The cytotoxic activity of leaves extract of ethyl acetate against the cell line HT 29 was found to be 68.25%. MTT assay revealed the potent cytotoxic activity of the *T. cordifolia* leaf extracts on colon cancer cells. Apoptosis has been defined as programmed cell death and is a normal physiologic process. Under diseased circumstances like cancer, natural cell death does not occur in proportion to their proliferation. This leads to formation of tumors, which may later metastasise. Hence, for a compound to be a potential anticancer drug, it should be able to induce apoptosis in the cancerous cells. Apoptosis has been characterized biochemically by the activation of a nuclear endonuclease that cleaves the DNA into multimers of 180-200 basepairs and can be visualized as an 'oligosomal ladder' by standard agarose gel electrophoresis (Alexei G. Basnakian and S. Jill James, 1994). Fig. 2 represents the results of the DNA fragmentation assay reflecting the genotoxic potentials of *Tinosporacordifolia* leaf extracts against HT29 Colorectal carcinoma cell line. The DNA from HT 29 cells treated with ethyl acetate extract

displayed genotoxicity. The highest damage of DNA was noticed in cells treated with 150µg ethyl acetate extract, reflected by the laddering observed. This is concurrent with the earlier studies where the ethyl acetate extract of *Tinosporacordifolia* showed high antiproliferative activities against lung carcinoma cells, murine lewis lung carcinoma (LLC) cells, A549 human lung adenocarcinoma cells in the DNA fragmentation assay (Rudeewan Tungpradit *et al.*, 2010).

Table 1. Qualitative analysis of Hexane, Ethyl Acetate, Methanol Extract of *Tinosporacordifolia* leaves

PHYTOCHEMICAL TEST	HEXANE	ETHYL ACETATE	METHANOL
Carbohydrates	+	+	+
Tannins	-	-	+
Flavonoids	-	+	+
Quinones	+	+	+
Terpenoids	+	-	+
Coumarins	-	-	+
Steroids & phytosteroids	P+	S+	-

+ indicates Present, - indicates absent, S indicates Steroids, P indicates Phytosteroids

Table 2. Quantitative analysis of total Flavonoids and Tannins in ethyl acetate and methanol extracts of *Tinosporacordifolia* leaves

S.NO	LEAVES EXTRACTS	FLAVONOIDS (µg/mg)	TANNINS (µg/mg)
1	Ethyl acetate	60	NIL
2	Methanol	87	32

Table 3. Antibacterial activity of *Tinosporacordifolia* leaf extracts by agar diffusion assay

Pathogens	Zone of inhibition (mm)			
	Hexane	Ethyl acetate	Methanol	Control (Ciproflaxcin)
<i>Aeromonas hydrophila</i>	14±0.5	16±1.1	8±1.0	30±0.01
<i>Pseudomonas aeruginosa</i>	Nil	18±0.3	24±0.8	20±0.01
<i>Staphylococcus aureus</i>	Nil	20±0.5	30±0.5	50±0.01

Values are mean of inhibition zone (mm) ± S.D of triplicates.

Table 4. Determination of Minimal Inhibitory Concentration (MIC) of ethyl acetate and methanol extracts of *Tinosporacordifolia* leaves against pathogenic bacteria

Organisms	Leaf Extracts (mg/ml)	
	Ethyl acetate	Methanol
<i>Aeromonashydrophila</i>	79.67	62
<i>Pseudomonas aeruginosa</i>	51.03	67.38
<i>Staphylococcus aureus</i>	64.53	64.55

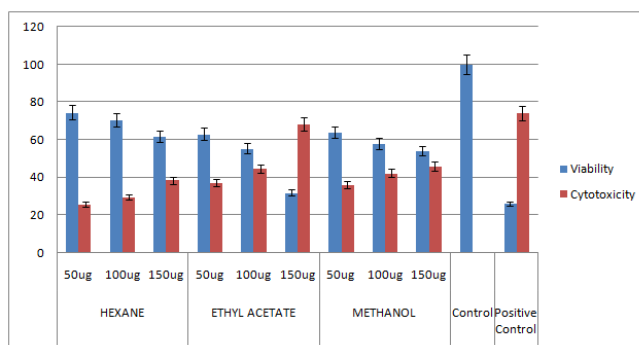


Fig. 1. Cytotoxicity of *T. cordifolia* leaf extracts against HT29 cells

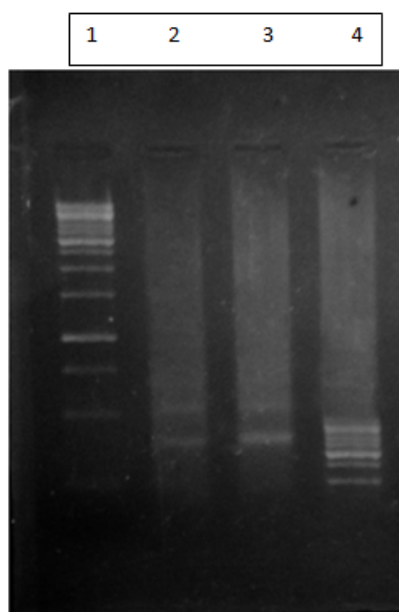


Fig. 2. DNA Fragmentation assay. Lane 1: 1kb Ladder, Lane 2: DNA from HT29 cells treated with 50µg Ethyl acetate extract, Lane 3: DNA from HT29 cells treated with 100µg Ethyl acetate extract, Lane 4: DNA from HT29 cells treated with 150µg Ethyl acetate extract

Conclusion

Throughout history plants have been used by human beings for medicinal purposes and even in modern times have formed the basis of many pharmaceuticals in use. The results of the current study provide rationale to develop *Tinosporacordifolia* leaves extract enriched with secondary metabolites into value added nutraceutical product for cancer prevention. There is a need for further investigation of this plant in order to identify and isolate its active anti cancer principles.

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