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Research Article

In-vitro Anti-inflammatory and Anti-cancer activity of extracts of stem bark of *Zanthoxylum tetraspermum* Wight & Arn.

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Abstract

This study was subjected to investigate *in-vitro* anti-inflammatory and cytotoxic activity of ethanol and aqueous extract of stem bark of *Zanthoxylum tetraspermum* Wight & Arn. The anti-inflammatory activity was studied by Human Red Blood Cell membrane stabilization method (HRBC). The prevention of hypotonicity-induced HRBC membrane lysis was taken as a measure of the anti-inflammatory activity. Ethanolic extract showed maximum inhibition than aqueous extract. Their activities are comparable to that of the standard drug indomethacin. Cytotoxicity activity was carried out ethanol and aqueous extract of stem bark of *Zanthoxylum tetraspermum* Wight & Arn against Dalton's Lymphoma Ascites cells (DLA tumor cells). Percentage cell viability of cell lines were carried out by using Trypan blue dye exclusion technique was used to evaluate the reduction of viability of cell cultures in the presence and absence of the extract. Cell viability was inhibited to different extents by different concentrations of the extract. The present study states that the methanolic extract of *Zanthoxylum tetraspermum* Wight & Arn showed a significant *in-vitro* anti- inflammatory and Cytotoxicity activity.

Keywords: *Zanthoxylum tetraspermum* Wight & Arn, anti-inflammatory, cytotoxicity, tumor cell line

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Introduction

Inflammation was described as "the succession of changes which occurs in a living tissue when it is injured provided that the injury is not of such a degree as to at once destroy its structure and vitality" (Sanderson, JB 1871), or "the reaction to injury of the living microcirculation and related tissues (Spector, WG and Willoughby, DA 1963). Inflammatory response to tissue injury involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair (Vane, JR and Botting, RM. 1995) which are aimed at host defense and usually activated in most disease conditions. HRBC or erythrocyte membrane is analogous to the lysosomal membrane 11 and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of human red blood cell membrane (HRBC) by hypo tonicity induced membrane lysis can be taken as an in vitro measure of anti -inflammatory activity of the drugs or plant extracts.

Cancer is a dreadful disease and combating this disease is of great importance to public health (Jayaprakashan, B et al., 2003). There is a necessity for search of new compounds with cytotoxic activity as the treatment of cancer with the available anticancer

drugs is often unsatisfactory due to the problem cytotoxicity to the normal cells. Phytochemical examination has been making rapid progress and herbal products are becoming popular as sources of plausible anticancer compounds (Kleinsmith, LJ 2007).

Zanthoxylum tetraspermum Wight & Arn (Rutaceae) is a thorny, stout, aromatic climbing shrub, with brown bark having short recurved prickles, found in the Western Ghats in the Nilgiri and Annaimalai hills and in Kolli hills (Namakal District TN) at altitudes of 1,200 - 1,800 m and in Kerala and Karnataka. The plant is credited in Srilanka with stimulant, astringent and digestive properties and prescribed in dyspepsia and diarrhoes (Ambasata, SP 1998, Chandra, YR 1994, Hajra, PK et al., 1997). It contains 8-acetonyldihydronitidine, 8-acetonyldihydroavicine, Liriodenine, seaamin, Lichexanthone, (+),(-) pipertiol-gamma dimethylallyl ether have been reported (Nissanka, APK et al., 2001). However, to the best of our understanding there is no information in the literature about the in vitro anti-inflammatory and cytotoxicity activities of an aqueous and ethanolic extracts of this plant. Therefore, this study was undertaken in order to investigate the in vitro anti-inflammatory and cytotoxicity activity.

Materials and Methods

Plant material

The stem bark of *Zanthoxylum tetraspermum* Wight & Arn were collected from Cholakkadu, a village of Kolli hills, 60km away from Namakkal (TN) in the month of August (2007). The plant was identified by local people of that village and authenticated by G.V.S Murthy, Join Director, botanical survey of India, Southern circle, Coimbatore, (No. Bsi /sc/5/23/07-08/ Tech 715). A herbarium specimen of the plant was preserved in the department of pharmacognosy of our institute for further references.

Chemicals

All the reagents used were of analytical grade obtained from S.D. fine Chemicals Ltd, Mumbai.

Preparation of stem Bark Extracts

The stem bark of *Zanthoxylum tetraspermum* were washed with water, air dried at room temperature and then reduced to coarse powder. The dried powder (250 gm) was subjected to soxhelt extraction with ethanol and water. The extraction was carried out for 72 hr room temperature Horbone (1973). The extract was filtered, concentrated and the weight of the residue was recorded and percentage yield was calculated.

Methods

In vitro Anti-Inflammatory Activity (Heat Induced Hemolysis)

The human red blood cell membrane stabilization method (HRBC) has been used as a method to study the *in-vitro* anti-inflammatory activity. (Seema Chaitanaya Chippada, et al., 2011). Human blood was purchased and mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride in water). The blood was centrifuged at 3000 rpm for 10 min and the packed cells were washed three times with isosaline (0.85%, pH 7.2). The volume of the blood was measured and reconstituted as 10% v/v suspension was made with isosaline. The drug sample was prepared by suspending the residues in hot water. The assay mixture contains 1 ml phosphate buffer (pH 7.4, 0.15M), 2 ml hyposaline (0.36%), 0.5 ml HRBC suspension (10% v/v) with 0.5 ml plant extract and standard drug Indomethacin of various concentrations.(100,200,300,400,500 µg/ml). Instead of hyposaline 2 ml of distilled water was used in the control to produce 100% hemolysis. All the assay mixture were incubated at 37 ˚ C for 30 min and centrifuged respectively. The hemoglobin content in the supernated solution was estimated using spectrophotometer at 560 nm. The percentage hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water as 100%. The percentage of HRBC membrane stabilization was calculated using the formula, Percentage production= 100- [(Optical density of Test Sample / Optical density of Control) × 100].

In vitro Cytotoxicity Studies

The cytotoxicity study was determined by Trypan blue dye exclusion method (Babu, et al., 1995). Tryphan blue is a blue acid dye that has two azo chromophores group. Tryphan blue is an essential dye, use in estimating the number of viable cells present in a population. It based on the principle that live cells possess intact cell membrane that excludes the dye

while the dead cells do not have blue colored cytoplasm under light microscope. The test compounds were studied for short term *in-vitro* cytotoxicity using Dalton's lymphoma ascites cells (DLA). The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with PBS (phosphate buffered saline) or normal saline. Cell viability was determined by trypan blue exclusion method. Viable cell suspension (1×106 cells in 0.1 ml) was added to tubes containing various concentrations was the of the test compounds and volume was made up to 1 ml using phosphate buffered saline (PBS). Control tube contained only cell suspension. The sample dissolved in DMSO. This assay mixture was incubated for three hours at 37°C. Further cell suspension was mixed with 0.1ml of 1% trypan blue and kept for 2-3 minutes and loaded on a haemocytometer. Dead cells take up the blue colour of trypan blue while living cells did not take up the dye. The number of stained and unstained cells was counted separately.

% Cytotoxicity = No. of dead cells / No of live cells +No of dead cells × 100

In-vitro Anti-Inflammatory Activity by HRBC Membrane Stabilization

The lysosomal enzymes released during inflammation produce a variety of disorders. The extracellular activity of these enzymes is said to be related to acute or chronic inflammation. The main action of anti-inflammatory agents is the inhibition of cyclooxygenase enzyme which is responsible for conversion of arachidonic acid to prostaglandins (PG). The non-steroidal drugs (NSAIDs) act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membranes by means of inhibiting the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes (cyclooxygenase) and proteases, which cause further tissue inflammation and damage upon extra cellular release or by stabilizing the lysosomal membrane (Arun Shirwaikar, et al., 2011). Since HRBC membrane are similar to lysosomal membrane components, the prevention of hypotonicity induced HRBC membrane lysis is taken as a measure of anti-inflammatory activity of drugs. The results were shown in table-1 and figure-1.

Results and Discussion

Table 1 *In-vitro* Anti-Inflammatory Activity of the Ethanolic and Aqueous Extract of Stem Bark of *Zanthoxylum tetraspermum* Wight & Arn by HRBC Membrane Stabilization Model.

Treatment	Concentration (µg/ml)	Absorbance	% protection
Ethanol extract	Control	1.226	-
	100	0.536	56.28
	200	0.502	59.05
	300	0.471	61.58
	400	0.442	63.94
	500	0.378	69.16
Aqueous extract	Control	1.226	-
	100	0.645	47.38
	200	0.604	50.73
	300	0.584	52.36
	400	0.542	55.79
	500	0.472	61.09
Indomethacin	Control	1.226	-
	100	0.420	65.74
	200	0.396	67.80
	300	0.340	72.26
	400	0.287	76.59
	500	0.273	77.73

From the results it was observed that the standard drug Indomethacin has the percentage protection of 65.74, 67.8, 72.26, 76.59, and 77.73. The ethanolic extract has 56.28, 59.05, 61.58, 63.94, 69.16 and the aqueous extract has 47.38, 50.73, 52.36, 55.79, and 61.09 for 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml and 500 µg/ml respectively. It indicates that ethanolic and aqueous extract of stem bark of *Zanthoxylum tetraspermum* Wight & Arn (500 µg/ml) shows maximum anti-inflammatory activity and was comparable to standard drug Indomethacin.

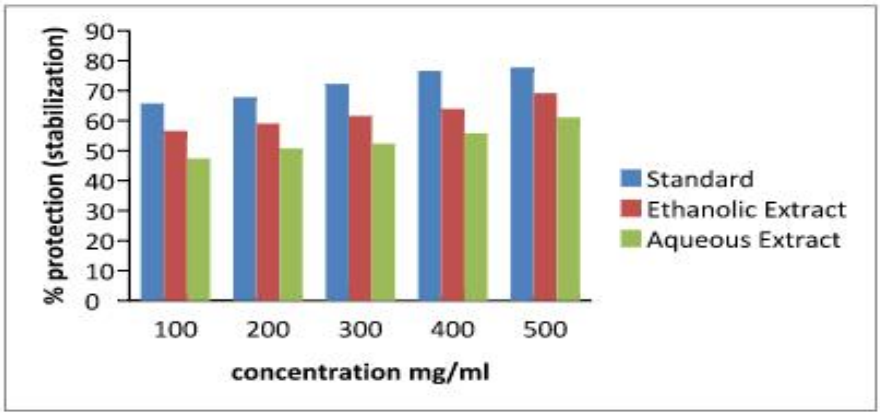

Fig. 1 *In-vitro* anti-inflammatory activity of the ethanolic and aqueous extract of stem bark of *Zanthoxylum tetraspermum* Wight & Arn by HRBC membrane stabilization model.

Ethanolic extracts showed the maximum inhibition (69.16 %) at 500 µg/ml than aqueous extract (61.09 %) stem bark of *Zanthoxylum tetraspermum* Wight & Arn. The anti-inflammatory activity of extracts was concentration dependent. Stabilization of the HRBC's membrane by hypo tonicity induced membrane lysis was studied to establish the mechanism of anti-inflammatory action of stem Bark of *Zanthoxylum tetraspermum* Wight & Arn. Due to the presence of active principles such as alkaloids, flavonoids, glycosides and Polyphenols may responsible for this activity. Hence, *Zanthoxylum tetraspermum* Wight & Arn can be used as potent anti-inflammatory and dental analgesic agent.

Cytotoxicity Activity by Tryphan blue exclusion method

Short term in vitro cytotoxic effect (Trypan Blue method) of ethanolic extract of *Zanthoxylum tetraspermum* Wight & Arn" against Dalton Lymphoma Ascites cell lines result was tabulated in Table 2 and figure 2. The cytotoixcity increased with increase in concentration of both the extract, 50 µg/ml concentration showed 6 and 4% cell death respectively where as in high concentration (200 µg/ml) 28% and 18 cell death respectively was noticed. Both ethanolic and aqueous extracts of "*Zanthoxylum tetraspermum* Wight & Arn" showed significant anticancer activity at the concentration (200, 100 µg/ml). When compared to the control.

Table 2 Data showing *in-vitro* Cytotoxicity activity of ethanolic and aqueous extracts of "*Zanthoxylum tertraspermum* Wight & Arn".

S.No	Drug Concentration (µg/ml)	(%) percent cell death	
		Ethanol Extracts	Aqueous Extracts
1	200	28	18
2	100	12	10
3	50	6	4
4	20	2	2

Control tube contains only two dead cells.

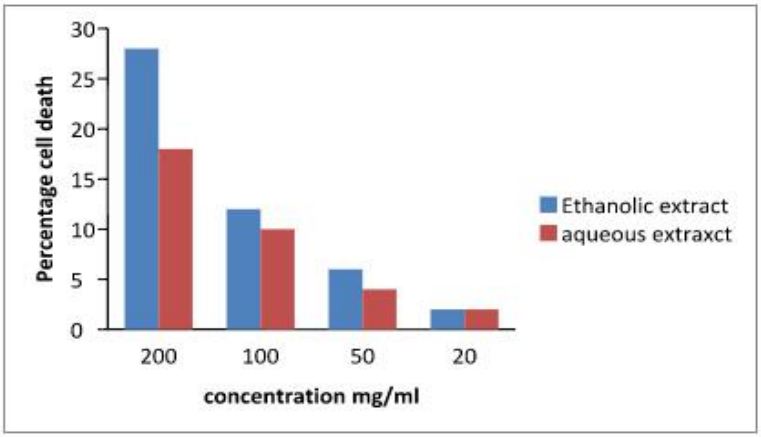

Fig. 2 *In-vitro* cyto toxicity activity of the ethanolic and aqueous extract of stem bark of *Zanthoxylum tetraspermum* Wight & Arn by tryphan blue exclusion method

The ethanolic extracts showed more significant cytotoxicity activity than aqueous extract of "*Zanthoxylum tetraspermum* Wight & Arn" at different dose level. Further, in depth cytotoxic activity of the plant extract under study were evaluated against Dalton Lymphoma Ascites cell line. 24hrs treatment with plant extracts showed growth inhibition of Dalton Lymphoma Ascites cell line. The death of the cells caused by the extract under study was due to the loss of mitochondria which is one of the hallmarks of the apoptosis pathway. From the results it was clearly evident that at minimum concentration of the extract activities and resulted in death of Dalton Lymphoma Ascites cell line.

Conclusion

In conclusion, it was observed from the present study that had a significant *in-vitro* inflammatory and cytotoxicity activity when the percentage mortality increased with increase in concentration. The result of this study (anti-inflammatory) shown that the ethanolic extract of *Zanthoxylum tetraspermum* Wight & Arn possess anti-inflammatory and analgesic properties mediated by prostaglandin synthesis inhibition. The data of the results of the in-vitro cytotoxicity studies clearly depicted that even minimum concentration of the ethanolic extract of *Zanthoxylum tetraspermum* Wight & Arn showed potent anticancer activity against Dalton Lymphoma Ascites cell lines.

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