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In vitro studies of the anticancer action of *Tectaria cicutaria* in human cancer cell lines: G_0/G_1 p53-associated cell cycle arrest-Part I

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ABSTRACT

Objective: The rhizome of Tectaria cicutaria has been used in Indian traditional medicine for the treatment of various disorders. The objective of present investigation is to screen various extracts of the rhizomes of *Tectaria cicutaria* for anti-cancer activity and to investigate the mechanism involved.
Materials and methods: The rhizomes of *Tectaria cicutaria* were extracted with different solvents. In vitro anti-cancer activity of different rhizome extracts were studied in Human cancer Cell Lines using Sulphorodamine B (SRB) colorimetric cytotoxicity assay. The effect of ethanolic extract (TCe) on cell growth inhibition, modulation in gene expression, and induction of apoptosis using the K562 human leukemia cell line were studied. The extract was analyzed by GC-MS to identify their major chemical compounds.
Results: TCe shows antioxidant potential in both DPPH scavenging assay and reducing capacity. Flow cytometric analysis showed that 11 μg/ml of TCe arrested cell cycle progression at the G_0/G_1 phase. In the TCe treated K562 cells, the mRNA and protein expression level of p53 was strongly up-regulated in reverse transcription polymerase chain reaction. Furthermore, its downstream target p21 level was also increased. The GC-MS study has depicted results with the presence of twelve different compounds which will require significant further efforts for structure and putative identification.
Conclusion: The present work has for the first time, tried to elucidate the anti leukemic potential of *Tectaria cicutaria*. TCe was more potent in K562 cells, altering the cell cycle progression and inducing apoptosis.

1. Introduction

For prevention and treatment of several diseases plant and plant extracts have been widely used all over the world. According to recent studies conducted by the World Health Organization (WHO), about 80% of the world's population relies on traditional medicine.[1] Traditional medicine involves the use of plant extracts as well as their active principles. Indeed, several anti-cancer agents derived from plants have been discovered in the last decades.[2,3] Besides, many anticancer agents obtained from plant source have achieved pre-clinical or clinical development.[4,5]

Tectaria cicutaria (L.) Copel is a species of fern in the Tectariaceae family. Its rhizomes are short (Fig. 1), erect and dark brown or black in color with distinctive hard thick leaf bases. This fern is found all over in India as well as in the tropics throughout the world. It is known as *Kombadnakhi* in the local language. In Ayurveda rhizomes of *Tectaria cicutaria* has been used for the treatment of wide variety of disorders and conditions such as chest complaints, sprain, rheumatic pain, burns, poisonous bites,[6] toothache, gum complaints, and diarrhea.[7] In folklore medicine, it is also used in clinical conditions like tonsillitis, mental disorders, and obesity. Decoction as well as an infusion is used for the treatment of different types of gynecological disorders and inflammatory conditions in Ayurvedic medicine. So far there is no studies available pertaining to anti-cancer activity of *Tectaria cicutaria*.

In view of this fact, the present study was aimed to investigate the phytochemicals present in the *Tectaria cicutaria,* in vitro anti cancer activity of rhizome extracts of *Tectaria cicutaria* on different

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List of abbreviations

RT-PCR	Reverse transcription polymerase chain reaction
TCe	Ethanolic extract of *Tectaria cicutaria* rhizome
TGI	Total growth inhibition
GI_{50}	Growth inhibition of 50%
PBS	Phosphate-buffered saline
PS	Phosphatidylserine
FACS	Fluorescence activated cell sorting
TE buffer	Tris/EDTA buffer
Annexin FITC	fluorescein isothiocyanate

human cancer cell lines and further to understand the underlying molecular mechanism.

2. Materials and methods

2.1. Collection of plant material and preparation of extracts

Tectaria cicutaria was collected from Koyana Wildlife Sanctuary, Western Ghats of Sahyadri hills, Satara, India. The species of fern were identified with the help of experts using the standard floras.[8–10] Identification of the fern was confirmed by Dr. S. S. Sathe, Head/Dept of Botany, Padmabhushan Dr. Vasantraodada Patil Mahavidyalaya, Tasgoan, Maharashtra, India. Washed rhizomes were dried under shade, ground into powder and used for successive extraction. Coarse powder was subjected to hot successive continuous extraction in Soxhlet apparatus. 50 gm of powder was extracted with petroleum ether (400 mL). It was sequentially extracted with solvents of increasing polarity (ethyl acetate, ethanol and water). Each time before extracting with the next solvent, the material was dried at room temperature. The extracts were filtered and evaporated to dryness. Yield of the extract was recorded and extracts were stored at 4°, until analysis. These extracts were tested for the presence of active phytochemicals viz: tannins, alkaloids, phytosterols, triterpenoids, flavonoids, glycosides, saponins, carbohydrates, proteins, amino acids and fixed oils & fats using standard tests.[11]

Fig. 1. Rhizome of *Tectaria cicutaria.*

2.2. DPPH radicals scavenging and reducing power assay

DPPH radical is a widely used method to evaluate the free radical scavenging ability of natural compounds. This assay is based on the measurement of the scavenging ability of antioxidant substances toward the stable radical. 1.0 mL of various concentrations of extracts (10–50μg/mL) was mixed with 1.0 mL of 0.8 mmol/L DPPH solution. Each mixture was kept in the dark for 30 min and the absorbance was measured at 517 nm against a blank.[12] Ascorbic acid was used as standard. The inhibition percentage for scavenging DPPH radical was calculated according to the equation:

$$\%\text{decolorization} = \left[1 - \left(\frac{\text{AB sample}}{\text{AB control}}\right) * 100\right] \quad (1)$$

The reducing power ability of the extracts was evaluated by the method described by Oyaizu. The reaction mixture contained 1.0 mL of TCe (20–100 μg/mL), 2.5 mL of 1% potassium ferricyanide and 2.5 mL of 0.2 mol/L sodium phosphate buffer. The mixture was incubated at 50 °C for 30 min and the reaction was terminated by the addition of 2.5 mL of 10% trichloroacetic acid, followed by centrifugation at 3000 rpm for 10 min. 2.5 mL of the upper layer was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride and absorbance was measured at 700 nm against blank that contained distilled water and phosphate buffer. Increase in absorbance indicates increased reducing power of the sample. BHT was used as standard.[13]

2.3. Determination of cell proliferation in vitro (sulphorodamine B assay)

K562 (Human Leukemia Cell Line), KB (Human Nasopharyngeal Cancer Cell Line), HT29 (Human Colon Cancer Cell Line) and Colo205 (Human Colon Cancer Cell Line) were used for screening the anticancer activity of the different extracts of *Tectaria cicutaria* using the Sulphorodamine B (SRB) colorimetric cytotoxicity assay. The extracts were dissolved in DMSO and tested at concentrations of 10, 20, 40, 80 μg/ml. Adriamycin (Doxorubicin) was used as a positive control at the same concentration range. Cytotoxicity was expressed as the percent viable cells relative to cells incubated in the presence of 0.1% DMSO vehicle control. Growth inhibition of 50% (GI_{50}) was calculated from drug concentration resulting in a 50% reduction in the net protein increase. Each measurement was performed in triplicate.[14]

2.4. Apoptosis detection

The cells (1×10^6) were harvested and washed twice with ice-cold PBS. Subsequently, the cells were labeled with Annexin V and Dead Cell assay kit according to the manufacturer's instructions. This assay is based on the phosphatidylserine (PS) detection on the apoptotic cells surface, using fluorescently labeled Annexin V. The samples were determined by the Muse Cell Analyzer (Millipore, USA) and analyzed by software provided by Merck Millipore.

2.5. Cell cycle analysis

Analysis of DNA content and cell cycle distribution was done using muse cell cycle kit by flow cytometry. For fluorescence activated cell sorting (FACS) analysis, the treated K562 cells were incubated for 24 h. After overnight incubation cells were detached and pipetted out and spun at 300 × g for 5 min and washed once with 1 × Phosphate-buffered saline (PBS). The cells were fixed with 1 ml of ice cold ethanol and incubated at -20 °C for overnight. The

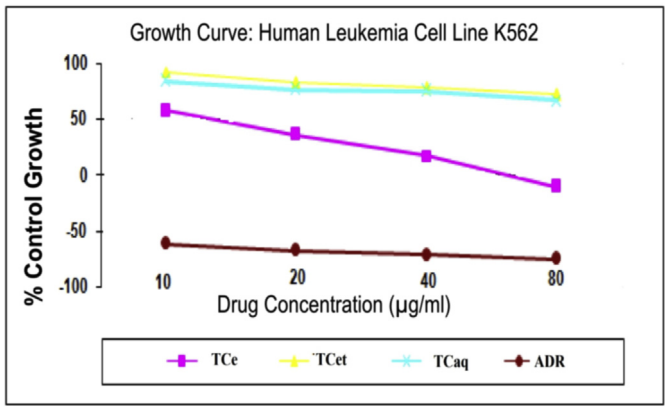

Fig. 2. Growth curve: The plot of percentage control growth vs. Molar drug concentration shows the effective drug concentration on the *Human Leukemia Cell Line K562.*

ethanol fixed cells were washed once with PBS followed by 200 µL of Muse cell cycle reagent. The tubes were incubated for 30 min at dark and analyzed on Muse flow cytometer (Millipore, USA)

2.6. Gene expression

2.6.1. Isolation of total RNA (trizol method)

Total RNA was isolated using the total RNA isolation kit according to the manufactures instruction (Invitrogen). Addition of Trizol solution causes the disruption of cells and the release of RNA. Chloroform extraction following centrifugation, exclusively in the aqueous phase whereas proteins are in the inter phase and organic phase. 80% confluent cells were treated with sample and incubated overnight. On mixing with isopropanol RNA gets precipitated as a white pellet on the side and the bottom of the tube. 1 ml of trizol reagent was added to the cultured plate and homogenized until it formed a fine paste and transferred it into an eppendorf tube. 200 µl of chloroform was added and shaking was done vigorously for 15 s and incubated for 2–3 min at room temperature. The sample was then centrifuged at 14000 rpm for 15 min at 4 °C. The aqueous layer was collected and 100% 500 µl of isopropanol was added. It was incubated for 10 min at room temperature. Supernatant was discarded and pellet was collected, washed with 1 ml of 75% of ethanol (Merck, Germany). It was then centrifuged at 10000 rpm for 5 min at 4 °C. The RNA pellet was dried and dissolved in Tris/EDTA (TE) buffer.

2.6.2. Reverse transcriptase PCR (RT-PCR)

cDNA synthesis was performed as per thermo scientific verso cDNA synthesis kit manual and amplification was performed using Invitrogen Thermo Script RT-PCR System manual. After the amplification, the PCR product was separated by agarose gel electrophoresis. Sequences of primers used in real-time PCR analysis are as below.

ERK2 FORWARD-GAGCACCAGACCTACTGCCAG
ERK2 REVERSE- AATTTCTGGAGCCCTGTACCA
P53 F FORWARD - CCACCATGAGCGCTGCTCA
P53 F REVERSE - GCAGGGAGGGAGAGATG
P21 FORWARD- GCAGATCCACAGCGATATCC
P21 REVERSE – CAACTGCTCACTGTCCACGG

Agarose gel electrophoresis method was used for separating and visualizing DNA fragments.

2.7. GC-MS analysis

GC-MS analysis of the active ethanol extract of *Tectaria cicutaria* was carried out by using the GCMS instrument equipped with a capillary column DB-%MS (30 m × 0.25mm × 0.25 mm length). The detector temperature was 300 °C. The carrier gas used was helium at a flow rate of 1.5 ml/min. The oven temperature was initially programmed at 50 °C (isothermal for 2 min) and then increased to 300 °C at 7 °C/min. The identification of compounds from the spectral data was based on the available mass spectral records (NIST05 libraries).

3. Result

3.1. Phytochemical investigation

Phytochemical screening of rhizomes of *Tectaria cicutaria* confirmed the presence of triterpenoid, flavanoids, saponins, tannins, phenols, and carbohydrate.

3.2. DPPH radicals scavenging and reducing power assay

The DPPH method is fast and reliable method. Standards and extracts showed a dose dependent inhibition of the DPPH radicals. TCe exhibited strongest DPPH radical scavenging activity compared to other extracts. Radical scavenging activity of different extracts were in the order TCe > TCet > $TC_{aq.}$ Concentration of the extract necessary to decrease the initial concentration of DPPH by 50% (IC_{50}) was calculated. A lower IC_{50} value indicates a higher antioxidant activity. Ascorbic acid was used as standard at the concentration 10–50 µg/mL. IC_{50} value was found to be 26.50 and 27.02 µg/mL for standard and TCe respectively.

Presence of reductones causes breaking of free radical chain by donating a hydrogen atom and shows reducing power.[15] Like antioxidant activity, reducing power of TCe increases with the increase in concentration. TCe showed good reducing power ability in a dose dependent manner. This activity was comparable to that of standards. The antioxidant principles present in the extracts caused the reduction of Fe3+/ferricyanide complex to the ferrous form and thus proved the reducing power ability.

Table 1
Effect of extracts of *Tectaria cicutaria* on Human Leukemia Cell Line K562.

Drug Concentrations (µg/ml)	% Growth Control Average Values				Drug concentrations (µg/ml) calculated		
	10	20	40	80	LC_{50}	TGI	GI_{50}
TCe	57.6	36.0	17.4	−10.2	53.2	32.6	**11.9**
TCet	92.1	82.5	77.9	72.0	>80	>80	>80
TCaq	84.2	76.8	74.7	67.0	>80	>80	>80
ADR	−61.5	−67.1	−71.1	−74.3	21.5	<10	<10

TCe- Ethanolic Extract of *Tectaria cicutaria*, TCet –ethyl acetate extract of *Tectaria cicutaria*, TCaq-aqueous Extract of *Tectaria cicutaria*, ADR- Adriamycin (Doxorubicin). Average Values are average of three sets of experiments.

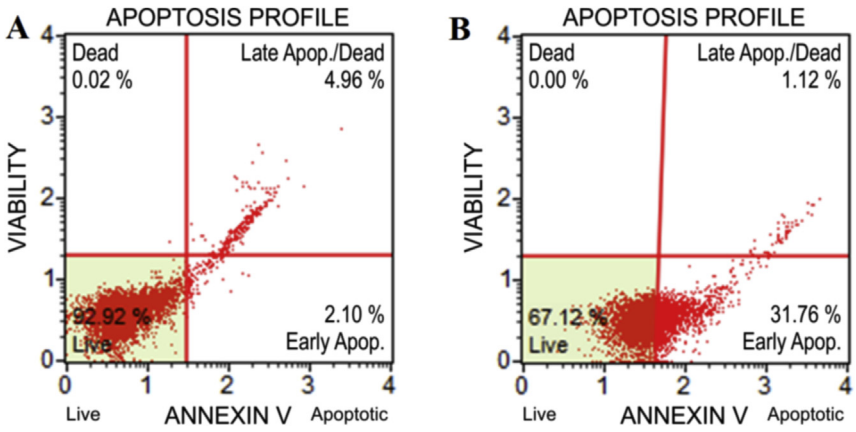

Fig. 3. Flow cytometry with FITC-Annexin V. analysis of the effect of TCe on cell cycle of Cell Line K562 A- **Control and B- Test**.

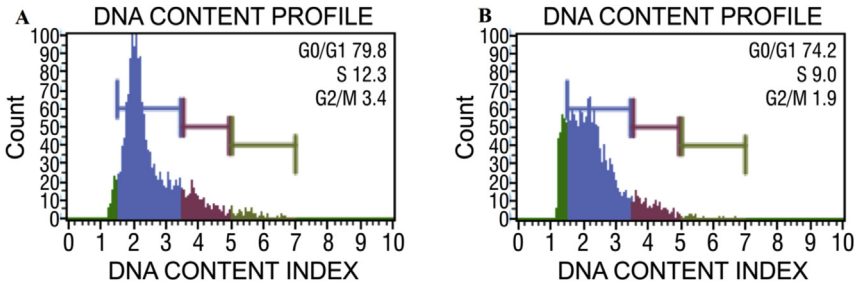

Fig. 4. Population and DNA content profile of A- **Control and B- Test**.

3.3. Cell proliferation in vitro

The anticancer activity of different extracts of *Tectaria cicutaria* was conducted against four different Human Cancer Cell Line using sulforhodamine B (SRB) assay. Adriamycin was used as the positive control. Fig. 2 shows activity of different extracts against K562 Cell Line (Data for other cell lines not shown). The results showed that (Table 1) TCe extract was the most potent extract in the K562 (GI_{50} value **11.9 μg/mL** and LC_{50} value 53.2 μg/mL). This is probably due to the different chemical composition of the extract hence; further analyses were performed with the TCe.

3.4. Mode of cancer cell death (apoptosis)

Cytotoxic anticancer drugs have potential to elicit cancer cell death by apoptosis or by necrosis.[16] After staining the treated cells with Annexin V-FITC/PI, the mode of cell death was determined from the transition paths of the cells in the dot plot diagrams. The plots are divided into four quadrants, so that viable cells fall into the lower left quadrant (Annexin V-negative, PI-negative), early apoptosis is at lower right (Annexin V-positive, PI-negative), late apoptosis is at upper right (Annexin V-positive, PI-positive), and the nonviable cells are at upper left (Annexin V-negative, PI-positive). After treatment the highest early apoptotic populations was 31.76 % with the populations of viable cells 67.12% [Fig. 3 B] as compared to control [Fig. 3A].

3.5. Cell cycle analysis

Importance of phytochemicals that function as cell-cycle modulators is increased due to the recent confirmation of concomitant involvement of apoptosis and cell cycle inhibition.[17] Flow cytometry analysis was performed to analyze the various cell cycle checkpoints. We performed an experiment to evaluate the effect of TCe on the DNA content of K-562 cells by cell cycle phase distribution (G_0, G_1, S, G_2 and M). This data suggested that cells exposed

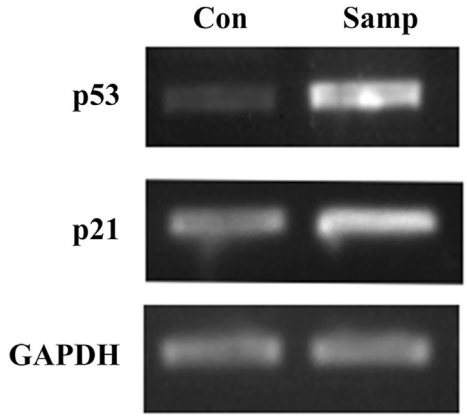

Fig. 5. Effect of TCe on the regulation of p53 and p21 mRNA expression.

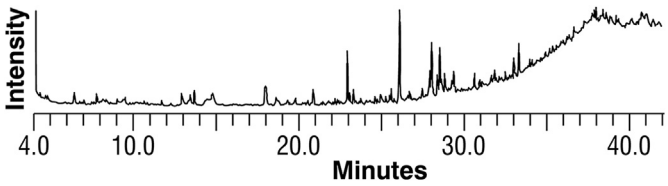

Fig. 6. GCMS chromatogram of TCe.

Table 2
Probable compounds in TCe identified by GCMS.

Sr. No.	Retention time	Name of the compound	Area %	Molecular formula	Molecular weight
1	4.092	2-propanone	3.37	$C_3H_6O_2$	74
2	4.133	Silanediol	3.90	$C_2H_8O_2Si$	92
3	4.169	Dihydroxydimethylsilane	10.71	$C_2H_8O_2Si$	92
4	20.899	Hexasiloxane	2.90	$C_{14}H_{42}O_5Si_6$	458
5	22.974	Phenol, 2-methyl-5-(1,2,2- trimethyl cyclopentyl)-, (S)	11.40	$C_{15}H_{22}O$	218
6	26.042	Phthalic acid, dibutyl ester	2.95	$C_{16}H_{22}O_4$	278
7	26.116	n-Hexadecanoic acid	17.13	$C_{16}H_{32}O_2$	256
8	28.054	Oleic acid, Methyl ester	9.21	$C_{19}H_{36}O_2$	296
9	28.401	Steric acid. Methyl ester	3.57	$C_{19}H_{38}O2$	298
10	28.536	9-Octadecenoic acid	10.89	$C_{16}H_{30}O_2$	254
11	28.608	l3-Octadecenal	4.13	$C_{18}H_{34}O$	266
12	29.385	Heptadecane	2.95	$C_{17}H_{36}$	240

to 11 μg/ml of TCe significantly increased the accumulation of the DNA contents up to 74.2% in the G_0/G_1 phase. Both S and G_2/M phase cells decreased in the treatment [Fig. 4 B]. It has been observed that different plant extracts with known antitumor activity such as ginseng extract, the extract of Tinospora cordifolia, or the Marsdenia tenacissima extract have similar effects; arresting cells in the G_0/G_1 phase of the cell cycle.[18]

3.6. Gene expression

Tumor suppressor action of p53 is by preventing the uncontrolled proliferation of cells. The p53 is found to mediates cell cycle arrest and apoptosis in response to DNA damage.[19] As mentioned in earlier studies p53 mediate cell arrest at late G_1phase, G_0/G_1 phase as well as G_2/M phase. P53 functions as a transcription factor and up-regulates a number of genes involved in these processes.[20] Analysis of p53 protein expression and of its downstream transcription targets, p21 revealed a p53 associated growth arrest after extract treatment and apoptosis within 24 h. Our results demonstrate for the first time that TCe induces cell cycle arrest and apoptosis via p53-dependent mechanisms [Fig. 5].

3.7. GC-MS analysis

The mass of the compounds and fragments recorded were matched with NIST database for identification of probable compounds present in the sample. The present communication describes preliminary analysis of the TCe as it showed good antileukemic activity. It is for the first time that the composition of extract has been investigated through GC-MS analysis [Fig. 6]. It revealed 12 probable compounds which are mentioned in Table 2 which indicates the active principles with their retention time, name of compound, concentration (% peak area), molecular formula and molecular weight. The most prevailing compounds are 9-Octadecenoic acid, oleic acid, n-hexadecanoic acid (palmitic acid), octadecanoic acid (stearic acid) and phthalic acid dibutyl ester. Hexadecanoic acid, octadecanoic acid and oleic acids are among the fatty acids known to have potential antibacterial and antifungal activity.[21] Hexadecanoic acid is responsible for analgesic, anesthetic, antibacterial, anti inflammatory, antioxidant and antifungal activity activity.[22] Oleic acid has been found to be fungistatic against a wide spectrum of moulds and yeasts. The compound 9-Octadecenoic acid is unsaturated fatty acid present in several plants. It lowers the level of cholesterol and the risk of heart problem. It is also responsible for hypotensive, antherosclerosis and aids in cancer prevention.[23] Further studies are in process for the isolation and identification of individual compounds from the plant crude extracts.

4. Discussion

Herbs, herbal extracts and herbal formulations are known to possess curative potential.[24–26] *Tectaria cicutaria* was reported to be rich in polyphenols.[27] Advanced Centre for Treatment, Research & Education in Cancer (*ACTREC* Mumbai) recommended that GI_{50} value $\leq$ 20 μg/mL is considered to demonstrate activity.

Present study implicit the observation that *Tecatria cicutaria* (GI_{50} 11 μg/mL) has a promising anti-cancer activity against the selected cell line. Flow cytometric analysis of apoptosis showed that the TCe induced apoptosis in K562 cell line though early apoptosis. The increased proportions of cells in the G_0/G_1 phase confirmed that TCe induced apoptosis in K-562 cells, resulting in DNA degradation. The percentage of cells at the G_2/M phase decreased, which corresponded to an increase in the percentage of cells at the G_0/G_1 phase, indicating an arrest of the cell cycle at the G_0/G_1 phase by extracts after treatment for 24 h. The above results reveal that TCe showed considerable inhibitory activity on cellular growth, more by apoptosis than by necrosis in the K562 cells. Inactivation of p53 is commonly seen in tumors. P53 has become the focus on most intensive cancer based research. Interestingly, we observed an increase in the levels of p53 upon treatment with TCe. The increased p53 level also coincided with increase in its downstream target p21. Based on the preliminary phytochemical tests and antileukemic results of different extracts, only ethanol fraction was selected for GC-MS studies.

The presence of various bioactive compounds justifies the use of the plant rhizomes for various ailments by traditional practitioners. However, isolation of individual phytochemical constituents and subjecting it to pharmacological activity will definitely give fruitful results.

Conflict of interest statement

The authors declare no conflicts of interest.

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