GC-MS ANALYSIS OF METHANOL EXTRACT OF CASSIA FISTULA AND ITS IN VITRO ANTICANCER ACTIVITY ON HUMAN PROSTATE CANCER CELL LINE

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ABSTRACT

Methanol extracts of Cassia fistula was used for phytochemical analysis and totally 10 different phytoconstituents were identified from GC-MS. Two compounds (citronellol and linoleic acid) out of ten have already shown as anticancer agents. In MTT assay, in 30 µg treated human cancer cells showed less viability (5.06%). If the concentration of extract decreases the viability was less reason is that drug dose dependent. The anticancer activity was observed in extract treated cancer cells by Acridine orange assay, observed the necrosis and arrested the cell cycle at different stages. The extract induced the activities of caspase -3, 7, 9 and 10, these enzymes are crucial for apoptosis and they are quantified and compared with untreated control. The activity of these enzymes was increased from 2 fold to 5 fold. The genomic DNA fragmentation was observed in extract treated cancer cells. We conclude that the methanol extract of C.fistula having anticancer agents and they showed anticancer property in MTT assay confirmed by acridine orange test. The extract inhibited the cell growth and induced the cell death by modulating caspase enzymes and cleaving genomic DNA. Further work is needed to identify the exact anticancer agent in methanol extract of C. fistula.

Keywords
Cassia Fistula,
Phytochemicals,
GC-MS,
Cytotoxicity,
Caspases,
DNA Fragmentation.

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INTRODUCTION

*Cassia fistula* (Fabaceae) using as ayurvedic formulations for treatments of heart disease, hematemesis, pruritus, leucoderma, abdominal lump, metabolic disorder and used as laxatives and purgative [1,2]. The *Cassia fistula* extracts have been used for various pharmacological activities like anti-inflammatory [3], antioxidant [4], antimicrobial [5], wound healing properties [6] and anticaner activity on MCF-7 and SiHa cell lines [2]. Rhein as isolated from ethyl acetate extracts and showed anticancer activity against colon cancer cell line [7] and ethyl acetate and n-butanol extracts have shown anti-proliferative activity against human cervical cancer (SiHa) and breast cancer (MCF-7) cell lines [2]. The present research investigation was aimed to identify phytochemicals in methanol extract and evaluated against prostate cancer cell line. Our work is different from earlier work in analyze phytochemical from GC-MS and evaluated against prostate cancer cell line.

MATERIALS AND METHODS

Collection plant and extract preparation

The plant *Cassia fistula* was collected during August 2013 from Western Ghat region (Udupi) of Karnataka. After shade dried, the whole plant was used for extraction using methanol. Coarse powdered crude plant were placed in stopper container with the solvent and allowed to stand at room temperature (26±2°C) for a period of 3 days with frequent agitation [8].

Thin Layer Chromatography (TLC)

TLC was carried out using methanol extract and the mobile phase was toluene, ethyl acetate and methanol at the ratio of 1:1:1. The methanol extract was mounted on TLC sheets, which was previously made for the activity by marking spots with equal distance spaces. The separated metabolites was observed and analysed under UV light [9].

GC-MS Analysis

The bioactive crude extract was separated into various fractions by column chromatography. The column was packed with silica gel (mesh 60-120) and run with n-hexane: EtOH (8:2). The earlier stated 6th fraction showed a clear band in TLC and is selected for GC-MS analyses. GC-MS analyses were performed at Central Instrumentation Department, Indian Institute of Sciences (IISc), Bangalore, India. GC-MS measurements were performed with a Shimadzu instrument equipped with GC: Agilent 7890 A, MS: MS detector 5975C, Ionization for MS: Electron Impact Ionization, Mass Analyzer: Quadrupole, Software: Data Analysis, Library: Nist 2008, column: HP 5 ms, Dimensions: 30m L X 0.25mm ID x 0.25μm film thickness, initial temperature is 0 to 40°C 2 min hold time, ram temperature is 100°C to 3100°C 10 min is the hold time, total time is 34 min, carrier gas is helium, flow (ml/min) is 1.0, split flow: 1ml/min, injection volume: 1μl, Scan mass range: 30m/z-600m/z and polarity +ve. GC-MS performed based on the database having more than many patterns. The spectrum of the unknown compound was compared with the spectrum of the known compounds in library [10].

*In vitro* anti-prostate cancer activity

*In vitro* anti-prostate activity was done in Raghanvedra Biotechnologies, Bengaluru and they maintaining all kind of human cancer lines. The cell line was grown in T-25 flasks and sub-cultured twice a week in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 mg/l streptomycin) in a humidified atmosphere of 5%CO₂ at 37°C [2].

Cytotoxicity assays

MTT assay

The stock culture was trypsinized and all the cells were collected in a 15 ml centrifuge tube. The mixture was centrifuged at 1500rpm for 10 min. Then the media (supernatant) was removed and the cells were re-suspended to 1 ml with complete media. Amount of cells per ml was counted and recorded. Care was taken to remove cells aseptically, while counting. Then the cells were diluted to 75,000 cells per ml, where complete media was used to dilute cells. Then 100 μl of cells (7500 total cells) were added to each well and were incubated overnight. On the second day, the cells were treated with the purified plant extract. Final volume was maintained to be 100 μl per well, by removing media. On third day, 20 μl of 5 mg/ml MTT was added to each well, aseptically. It was incubated for 3.5 h at 37°C. Carefully media was removed and 100 μl MTT solvent was added. It was covered with tinfoil and the cells were agitated on orbital shaker for 15 min. Later, the absorbance was read at 545 nm [11].

Acridine Orange Staining

The acridine orange is a vital dye and will stain both live and dead cells. This method is helping to know their effectiveness by visibly. Incubate 25 μl of cell suspension (0.5 x 106 to 2.0 x 106 cells/ml) with 1 μl of AO/EB solution. Mix gently. Each sample should be mixed just prior to microscopy and quantification. Samples must be evaluated immediately. Place 10 μl of cell suspension onto a microscopic slide, cover with a glass cover-slip, and examine at least 300 cells in a fluorescence microscope using a fluorescent filter and a 60X objective (higher or lower magnification may be desired depending on cell type and nuclear morphology should be discernible). Pictures of the cells were captured along with the conduction of test [12].

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Quantification of Caspase Enzymes

Prostate cancer cell lines were treated with methanol extract for 48h. At the end of treatment duration, cells were harvested and washed in PBS at 4°C. The caspase -3, 7, 9 and 10 enzymes activities were quantified by the caspase apoptosis assay kit (G-Biosciences, USA) [2].

DNA ladder Assay

*Cassia fistula* methanol extract incubated with prostate cancer cell line for 48 h. At the end of treatment duration, cells were harvested and washed in PBS at 4°C. The cells pellets were used for genomic DNA fragmentation assay using DNA ladder assay kit (G-Biosciences, USA). The fragmented DNA was analysed on 1.8% (w/v) agarose gel and documented using the gel-documented system Biotron Healthcare gel-documented system, model-gel vision DC [2].

RESULTS

In TLC, the methanol extract of *C. fistula* have showed much cleared bands under UV light but compounds were not prominent without UV light (Fig 1). The GC-MS analysis yielded totally 10 prominent peaks with different retention times of 5.0 to 17.5 min (Fig 2). The different compounds were compared with library search (main library) and identified the major compounds as viz., Cyclopentasiloxane, Decamethyl-, Cyclohexasiloxane, dodecamethyl-, Mome- Inositol, Citronellol, Isophytol, 1,3-Cyclopentadiene, 5-(1-methylethylidene)-, Phytol, Pyridine, Trimethylsilanol and Linolenic acid. The spectrum of unknown compounds was identified with library based on retention time and mass spectra. The chemical structure, biological activity and other sources of the identified compounds are depicted in Table 1. Based on available literature regarding identified compounds in GC-MS, only two compounds (citronellol and linolenic acid) have reported as anticancer agents. May be these compounds in combination or individually acted as anticancer agent by inhibiting prostate cancer cells.

Fig 1. Methanol extract of *Cassia fistula* showing different phytochemical presence, A. absence of UV, B. Under UV light.

Fig 2. GC-MS analysis of methanolic extract of *Cassia fistula*. 
Table 1. Identification of phytochemicals in methanol extract of *Cassia fistula* using GC-MS analysis and their biological significances.

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>CAS</th>
<th>Compounds name</th>
<th>RT</th>
<th>Molecular formula</th>
<th>Biological significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>541026</td>
<td>Cyclopentasiloxane, Decamethyl-</td>
<td>9.4002</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;30&lt;/sub&gt;O&lt;sub&gt;5&lt;/sub&gt;Si&lt;sub&gt;5&lt;/sub&gt;</td>
<td>Skincare, Cosmetics, Induction of Hepatic xenobiotic metabolizing enzyme</td>
</tr>
<tr>
<td>2.</td>
<td>540976</td>
<td>Cyclohexasiloxane, dodecamethyl-</td>
<td>11.0808</td>
<td>C&lt;sub&gt;12&lt;/sub&gt;H&lt;sub&gt;36&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;Si&lt;sub&gt;6&lt;/sub&gt;</td>
<td>Cosmetics and personal care products</td>
</tr>
<tr>
<td>3.</td>
<td>0</td>
<td>Mome- Inositol</td>
<td>15.4199</td>
<td>C&lt;sub&gt;7&lt;/sub&gt;H&lt;sub&gt;14&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
<td>No significance reported</td>
</tr>
<tr>
<td>4.</td>
<td>106229</td>
<td>Citronellol</td>
<td>17.2461</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;20&lt;/sub&gt;O</td>
<td>Has anticancer, anti-inflammatory and wound healing properties</td>
</tr>
<tr>
<td>5.</td>
<td>505328</td>
<td>Isophytol</td>
<td>17.2461</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;40&lt;/sub&gt;O</td>
<td>Synthesis of Vitamin A and K, Used as a flavoring substance</td>
</tr>
<tr>
<td>6.</td>
<td>2175919</td>
<td>1,3-Cyclopentadiene, 5-</td>
<td>6.4564</td>
<td>C&lt;sub&gt;4&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;</td>
<td>Addressing the issues of arthritis and rheumatism, increasing excretory potency of urinary bladder, treatment of viral infections and cancer, modulating uric acid levels, agonist for treatment of dyslipidemia, hypercholesterolemia and diabetes, treatment of cardiovascular diseases, biological assays.</td>
</tr>
<tr>
<td>7.</td>
<td>150867</td>
<td>Phytol</td>
<td>17.2461</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;40&lt;/sub&gt;O</td>
<td>Antinociceptive (reducing sensitivity to painful stimuli) and antioxidant properties, Antimicrobial, anticancer, anti-inflammatory, anti-diuretic, immune-stimulatory and anti-diabetic CNS depressant</td>
</tr>
<tr>
<td>8.</td>
<td>110861</td>
<td>Pyridine</td>
<td>6.0902</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;N</td>
<td>CNS depressant</td>
</tr>
<tr>
<td>9.</td>
<td>1066406</td>
<td>Trimethylsilanol</td>
<td>5.3845</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;OSi</td>
<td>Antimicrobial properties</td>
</tr>
<tr>
<td>10.</td>
<td>463401</td>
<td>Linolenic acid</td>
<td>17.1704</td>
<td>C&lt;sub&gt;18&lt;/sub&gt;H&lt;sub&gt;28&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Anticancer properties and reduces proliferative activity</td>
</tr>
</tbody>
</table>

*Cassia fistula* methanol extract reduced the prostate human cancer cell lines viability by dose dependent manner in MTT assay (Fig 3 & 4). We have tried, 7 different doses (0.625, 1.25, 2.5, 5, 10, 20 and 30 µg) of methanol extract on cancer cell line by increasing dose. Depending dose increased the viability of cancer cells were decreased after 48 h treatment. The lowest viability of cancer cells was observed with 30 µg showed 5.06%, whereas 20 µg showed 5.87%. This result clearly indicates that viabilities of cells was depending on dose of the extract. The vehicle control showed 97.77% viability of cells. Acridine staining taken by apoptosis cells by orange colour and orange coloured cells are necrosis cells (Fig 5).
Fig 3. Viability of prostate cancer cell line treated with *C. fistula* extract in MTT assay. A. vehicle control shows 97.77% viability, B. 0.625 μg shows 92.71% viability, C. 1.25 μg extract shows 79.35% viability, D. 2.5 μg shows 67.61% viability, E. 5 μg shows 48.58% viability, F. 10 μg shows 12.55%, F. 20 μg shows 5.87%, G. 30 μg shows 5.06% of viability.

Fig 4. *In vitro* anti-prostate cancer activity analysis using MTT assay on methanol extract of *Cassia fistula* (24 h observation).
Significantly enhanced the different caspase enzymes (3, 7, 9 &10) activities in prostate cancer cells due to *C. fistula* extract treatment (Fig 6). The caspase 3 was enhanced 2 fold (0.18), 7 enhanced 4 fold (0.21), 9 enhanced 5 fold (0.33) and 10 enhanced 3 fold (0.18).

**DISCUSSION**

The present investigation was aimed to identify phytochemicals present in methanol extract of *Cassia fistula* and was evaluated against prostate cancer cell line to know their activity. The GC- MS yielded 10 different compounds in methanol extract, whereas citronellol and linoleic acid have already reported as anticancer agents from different sources [13,14,15] and they induced cell cycle arrest at different stages. At different concentration the viability of cancer cell line was observed and viability is decreased when high dose provided. The results are confirmation with the findings of Irshad *et al.* (2014) [2] but they used different cancer cell lines and also different parts and solvents. Acridine orange staining assay is a widely used test in cytotoxicity studies and it is more sensitive indicators of cellular damage as compared to MTT assay [16,17,18]. This assay concluded that the methanol extract inhibited the cell division and induced the cell arrest of prostate cell line [2, 19]. The extract may cause effects on cell membrane integrity and inhibited the cell at various stages. The *C. fistula* methanol extract treated cancer cells were highly condensed nuclei and fragmented chromatin, it is due to formation of apoptosis or fragmentation of genomic DNA [2, 20].

The extract increased the activities of caspase enzymes (3, 7, 9 and 10) as compared with untreated control. These enzymes activities were increased from 2 fold to 5 fold. Caspases are crucial mediators of programmed cell death (apoptosis). Among them caspase-3 is frequently associated protease, catalyzing the target specific cleavage of many key cellular proteins [21]. Caspase -7 is executioner caspases, activates the intrinsic apoptotic pathways through cleavage of BID to induce efficient cell death, apoptotic cell development. Caspase-9 initiates apoptosis by cleaving and thereby activating executioner caspases, mitochondrial morphological changes, ROS production by cleaving and activating BID into tBID [22]. Caspase-10 is a initiator caspase in death receptor signaling [23].
The methanol extract of *C. fistula* treated prostate cancer cell line showed fragmentation of genomic DNA, which indicates the presence of anticancer agents in extract might be induced apoptosis regulatory activity and induced caspase enzymes activities. The above results strongly demonstrated that the *C. fistula* methanol extract inhibited the proliferation of prostate cancer cells by inducing the apoptosis.

**CONCLUSION**

As per earlier report, GC-MS yielded 10 different compounds, out of which two (citronellol and lenoleic acid) have already possessed anticancer property. Our experimental results, MTT and acridine orange tests confirmed the cytotoxicity activity of the extract. Further, extract induced the caspase enzymes activity and fragmentation of genomic DNA. The anticancer activity may be due to combination of all the compounds or individual compound. The extract can be successfully exploited in the herbal formulation of cancer prevention in Soliga Tribal, BR Hill region of Western Ghat, Karnataka.

**Conflict of interest**

We declared that we have no any conflict of interest.

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