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Anticancer Activity of Friedelin Isolated from Ethanolic Leaf Extract of *Cassia tora* on HeLa and HSC-1 Cell Lines

KEYWORDS: *Cassia tora*; ethanolic extract; cytotoxic activity; bioactive principle; terpene fraction; friedelin

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ABSTRACT *Cassia tora, an annual shrub growing in the wild is traditionally well-known for its therapeutic properties against certain human diseases. Considering some reports on the anticancer activity of this plant, a study was carried out to identify its constituent(s) with potential cytotoxic effect on cancer cells. IC(50) values of ethanolic leaf extract of C. tora on two human cancer cell lines, HeLa and HSC-1 were determined using Trypan blue exclusion and Methylthiazolyl tetrazolium assays. The crude extract showed a substantial cytotoxicity against both the cell lines. Fractionation of the crude extract by Acid-base fractionation yielded an alkaloid, phenolic and terpene fractions which were confirmed by respective qualitative tests. These three fractions were screened for their cytotoxicity, and only terpene was found to possess the substantial activity. Fractionation of terpene by TLC yielded 4 subfractions. the subfraction that showed significant anticancer activity (P<0.001) was characterized by spectroscopic techniques. Infrared spectroscopic data indicated that absorption peaks corresponding to terpenes. Mass spectrum revealed the presence of parent ion peak (m/z) at 427.3. 1H-NMR spectrum of the subfraction was indicative of the prominent chemical shifts at δ, cohering to the properties of friedelin. Thus, the present study confirms that C. tora leaf contains friedelin, which has potential anticancer activity on HeLa and HSC-1 cells. In comparison, HeLa cell line was more sensitive than HSC-1 for cytotoxic effect of friedelin.*

1. Introduction

Plants used in folk and traditional medicine have been accepted as one of the main sources of chemopreventive drug discovery and development. There is a long standing association between purified phytochemicals of medicinal plants and cancer chemoprevention (Abdullaev, 2001). *Cassia tora* L. (Family: Caesalpiniaceae) is an annual shrub, common to Southeast Asia, Fiji, Northern Australia, Africa and Latin America (Parsons and Cuthbertson, 1992). In Asian countries, variety of traditional medicines prepared from parts of *C. tora* for several ailments are well-known, and its leaf is composed of many constituents that are of pharmacological importance (Jain and Patil, 2010; Meena *et al*., 2010). *C. tora* leaf extracts possess potential synergistic cytotoxic effect with anticancer drugs, such as, cisplatin (Rejiya *et al*., 2009) and doxorubicin (Yang *et al*., 2011). However, the literature review reveals that leaf extract of *C. tora* as a single agent has not been studied for its anticancer activity either *in vitro* or *in vivo*. In this context, we carried out a study to assess the *C. tora* ethanolic leaf extract for the identification of potential anticancer constituent against human cancer cell lines, viz., HeLa (cervical carcinoma) and HSC-1 (squamous carcinoma).

2. Materials and methods

2.1. Reagents

Thiazolyl blue tetrazolium bromide (CAS No. 298-93-1; S. D. Fine Chem. Ltd. Mumbai, India), Cyclophosphamide (CP: CAS No. 6055-19-2; Endoxan, Astra-Pharma Ag, Germany, marketed by German Remedies Ltd., Ponda, India), Trypan blue 0.5% solution in Dulbecco's phosphate buffered saline (CAS No. 72-57-1; Himedia Lab Pvt. Ltd., Mumbai, India), Trypsin (CAS No. 9002-07-7; S. D. Fine Chemicals, Mumbai), Dulbecco's Modified Eagle Medium (Product Code: AL007; Himedia Lab Pvt. Ltd., Mumbai, India), DMSO (CAS No. 67-68-5; S. D. Fine Chem. Ltd. Mumbai, India). All other chemicals/reagents were procured from Merck India and Sisco Research Laboratories Pvt. Ltd (SRL) India.

Cell lines: HeLa (Batch No. P80) and HSC-I (Batch No. PN12) were procured from National Center for Cell Sciences (NCCS), Pune, India.

2.2. Preparation of the extract and phytochemical characterization

C. tora leaves were collected from the site of Mangalore University. Identification of the plant was authenticated at the Department of Applied Botany, Mangalore University. The leaves were dried up in a shade, cut and powdered. The crude ethanolic extract of powdered *C. tora* leaves was prepared by employing Soxhlet extraction process. The extract was filtered through Whatman Grade No.1 paper. The filtrate remove comma, was concentrated using Rotary evaporator at 40°C, lyophilized and stored at 4°C . The yield of the crude extract was 7.9 % on a dry weight basis. The ethanolic leaf extract of *C. tora* was subjected to preliminary phytochemical screening (Ramaan, 2006).

Extract was fractionated into three fractions, namely, alkaloid (CTF1), phenolic (CTF2) and terpene (CTF3) using an acid and alkali extraction process. CTF refers to *Cassia tora* fraction.

CTF3 fraction was subjected to Thin Layer chromatography (TLC) on silica gel using Chloroform: methanol (9:1) as a mobile phase, which resulted in four subfractions, subsequently designated the CTF3-I, CTF3-II, CTF3-III and CTF3-IV.

2.3. Cytotoxic activity

A stock solution of the extract was prepared in ethanol at a concentration of 1000 µg/mL. Initially, a wide range of concentration of the crude extract in a log increment (1 to1000 µg/mL) was used for cytotoxicity tests. Based on the results

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of these tests, subsequent tests were performed with concentrations in a narrower range, viz., 10 to 200 µg/mL (linear order) of the extract. The fractions and subfractions of the extract with the concentrations ranging from 10 to 200 µg/mL were taken for cytotoxicity assays. The selected concentrations of the crude extract, fractions and sub-fractions were dissolved in ethanol. The conventional anticancer drug, CP (0.5 to 10.0 µg/mL) was used as the positive control.

Trypan blue exclusion (TBE) test

HeLa and HSC-I cells in the exponential growth phase were washed with phosphate buffered saline solution, trypsinized and resuspended in Dulbecco's Modified Eagle Medium. Cells were plated at a concentration of 30,000 cells/well in 6 well plates and incubated for an entire day during which a partial monolayer was formed. After the incubation, the cells were exposed to the selected concentrations of *C. tora* extract, its fractions and sub-fractions. CP was used as the standard. Control well contained only cell suspension. The plates were incubated at 37^0 C in a humidified incubator with 5 % CO_2 for a period of 24 hours. At the end of it, 50 µL of the cell suspension was mixed with 50µL of 0.4% trypan blue dye and left for 1-2 min at room temperature. The non-viable (stained) and viable (unstained) cells were counted using the hemocytometer, and the percentage of dead cells was determined (Freshney, 2006).

2.3.2. Methyl thiazolyl tetrazolium (MTT) assay

MTT assay was done as per the modified method of Mosmann (1983). HeLa and HSC-I cells were seeded in 96 well flat bottom tissue culture plates at a density of approximately 1x10^4 cells per well and allowed to attach to the plate for 24 hrs at 37^{0}C. The growth medium was aspirated off and substituted with the fresh medium containing the selected concentrations of the extract of *C. tora* leaves, its fractions, sub-fractions and CP. Control wells contained 200 µL of the medium. After exposing the cells to the test samples (crude extract, fractions and sub-fractions) for 24 hours, the cells were grown for another 24 hrs in the fresh medium. At the end, the cells in the microtiter wells were treated with 50 µL of MTT solution and incubated for 4 hrs in a humidified atmosphere at 37^0 C. MTT was aspirated from the wells and replaced with 200 µL of DMSO to all of the wells to dissolve MTT-formazan crystals. In the spectroscopic studies, the absorbance was recorded at wavelength of 570 nm with the aid of Double beam UV-VIS spectrophotometer. For each test sample, the IC(50) value was determined from the dose response curves. The assay was performed in triplicate for each of the test samples and mean IC(50) values were calculated.

2.4. Spectroscopic analyses

CTF3-IV was characterized using Infrared (IR) spectroscopy, Nuclear Magnetic Resonance (NMR) and Mass spectroscopy (MS). IR spectrum was recorded on KBr pellets using Jasco FT/IR-4100 model. The Mass spectrum was recorded by Turbo-Spray method using Applied Biosystems API 4000 model. ^{1}H- NMR spectra was recorded on Bruker Advance-II 400 NMR.

2.5. Statistical analysis

The results are presented as IC(50) (± SD). The experimental data obtained were analyzed for multiple comparisons using one-way ANOVA then by Dunnett's test with the aid of Graph Pad Prism version 5.0. P values less than 0.5 were considered as significant.

3. Results

3.1. Phytochemical screening

Preliminary phytochemical screening of the *C. tora* ethanolic leaf extract indicated the presence of alkaloids, glycosides, phytosterols, triterpenoids and phenolic compounds. The acid-alkali fractionation yielded three fractions viz.; alkaloid (CTF1), phenolic (CTF2) and terpene (CTF3) from the rudimentary extract. The CTF1 fraction on evaporation yielded a green amorphous powder and answered affirmatively for Mayer's, Wagner's, Hayer's and Dragendorff's tests, thereby confirmed the presence of alkaloids in the fraction. The CTF2 fraction on evaporation resulted a yellow amorphous powder, containing phenolic compounds and glycosides. The CTF3 fraction was obtained as a yellow amorphous powder, and gave positive results for triterpenoids and phytosterols.

The CTF3 fraction on Thin Layer Chromatographic separation yielded four subfractions, namely, CTF3-I, CTF3-II, CTF3-III and CTF3-IV. Out of these four, CTF3-I, CTF3-II and CTF3-III were isolated from preparative TLC plate and evaporated, which yielded yellow amorphous powder. These subfractions gave positive results for phytosterols. CTF3-IV subfraction was isolated as greenish white amorphous powder, gave an affirmative result for triterpenoids.

3.2. Cytotoxic activity:

The results obtained for cytotoxic activity are expressed as IC(50) values and presented in Table 1. The crude leaf extract of *C. tora* exhibited cytotoxic activity against HeLa and HSC-1 cells with IC(50) values ranging between 129.3 µg/mL and 169.3 µg/mL, as evident from TBE and MTT assays. It is observed that HeLa cell line was more responsive in comparison to HSC-1 cell line ($p<0.05$). Among the three fractions of the extract, CTF3 was most effective in cytotoxicity IC(50) values ranging from 82.0 µg/mL to 141.7 µg/mL), while CTF1 and CTF2, whose values were >200 µg/mL. Among the all subfractions of CTF3, CTF3-IV was highly cytotoxic with IC(50) values ranging from 19.3 µg/mL to 31.0 µg/mL against HeLa and HSC-1 cells. The anticancer activity of CTF3-IV was highly significant compared to that of crude extract on both the cell lines ($p<0.001$). The positive control CP exhibited cytotoxicity with IC(50) values ranging from 3.9 µg/mL to 5.4 µg/mL against HeLa and HSC-1 cells, which was roughly five times more potent than CTF3-IV (Fig. 1).

3.3. Spectroscopic analyses

The IR spectroscopic analysis of CTF3-IV showed the absorption bands at 2956 and 2851 cm^{-1} due to asymmetric and symmetric stretching of methyl and methylene groups, the absorption band appeared at 1746 cm^{-1} was due to the presence of carbonyl group, 1463 cm^{-1} was due to C-H bending vibration of CH_3 group. (Fig.2). The Mass spectrum showed a base peak (m/e) at 427.3 along with other significant peaks of fragments at 381, 355, 283, 208, 166, 136 and 121 (Fig. 3). ^{1}H- NMR spectrum had the prominent chemical shift values at δ = 0.92, 0.94, 0.98, 1.01, 1.05, 1.07, 1.09, 1.16, 1.18, 1.23, 1.25, 1.28, 1.29, 1.33, 1.36, 1.38, 1.50, 1.54, 1.62, 2.00, 2.50, 3.30, 4.01, 5.31 and 5.32 ppm (Fig. 4). All these spectral data and their analyses, confirmed that CTF3-IV was friedelin (Fig. 5).

Table 1: Cytotoxicity of (#IC50 values - µg/mL) of ethanolic leaf extract of C. tora, its fractions and sub-fractions against HeLa and HSC-1 cell lines.

Test samples	HeLa cell line		HSC-1 cell line	
	TBE test	MTT assay	TBE test	MTT assay
Crude extract	141.6 (±3.89)*	129.3(±4.26)*	169.3(±5.21)	161.7(±3.65)
CTF1	>200	>200	>200	>200
CTF2	>200	>200	>200	>200
CTF3	90.3(±2.86)*	82.0(±3.42)*	141.7(±4.22)	131.0(±3.41)
CTF3-I	>200	>200	>200	>200
CTF3-II	>200	>200	>200	>200
CTF3-III	>200	>200	>200	>200
CTF3-1V	22.7(±1.24)**	19.3(±1.27)**	31.0(±2.13)**	28.7(±1.98)**
CP	4.7(±0.85)	3.9(±0.73)	5.4(±0.96)	4.2(±0.87)

***p<0.05 HeLa** *vs.* **HSC-1 cell line; ****p<0.001 CTF3-IV** *vs.* **crude extract.**

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#IC_{50} - concentration that induced 50% growth inhibition, obtained from dose-response curves, Mean of triplicate wells.
CTF: *Cassia tora* **fraction; CP-Cyclophosphamide**

4. Discussion

The current work has been carried out with the goal of exploring the anticancer property and identifying the dynamic principle in *C. tora* ethanolic leaf extract. The preliminary phytochemical screening of the extract indicated the presence of alkaloids, glycosides, phytosterols, triterpenoids and phenolic compounds. All these plant-based compounds are renowned for their anticancer property, with several reports published elsewhere. In the present study, we observed the cytotoxic effect of the plant extract against the cell lines, HeLa and HSC-1 (Table-1 and Fig.1). The results obtained from TBE and MTT assays are comparable, which confirmed the antiproliferative activity of the extract. The observed cytotoxic effect is probably due to the synergistic effect of various non-polar active principles present in the extract. Active constituents present in *C. tora* leaves that have been identified for their anticancer property include emodin, gallic acid and friedelin (Jain and Patil, 2010; Rejiya *et al.*, 2009; Shibata *et al.*, 1969).

Based on the results obtained from the crude extract of the plant, we further proceeded with the identification of the probable constituent, which imparted the anticancer activity. The acid-alkali fractionation a commonly employed method was used to obtain various fractions from plant extracts (Harwood and Moody, 1989), thus we obtained three fractions from the crude extract, viz.; alkaloid (CTF1), phenolic (CTF2) and terpene (CTF3). All the three fractions have been evaluated for their anticancer activity using the same endpoints. Among three fractions, the terpene fraction of the extract showed the positive response, whose IC(50) values were within the maximum dose (<200 µg/mL) selected (Table-1). Anticancer/antitumor activities of terpene fraction of certain therapeutic plants have been established using *in vitro* and *in vivo* experimental models (Fan *et al.*, 2010; Mullauer *et al.*, 2010; Zhang *et al.*, 2010). Thousands of secondary terpenes are found in the plant kingdom, but each species is capable of synthesizing only a small fraction of this total (Chen *et al.*, 2011). In order to identify the specific terpene of *C. tora* leaves which is responsible for anticancer activity, CTF3 fraction was further subjected to fractionation by TLC. The subfractions so obtained were designated as CTF3-I, CTF3-II and CTF3-III and CTF3-IV. All the four subfractions were screened for their anticancer efficacy, of which only CTF3-IV was found to be potent on both the cell lines (Table-1). CTF3-IV subfraction was isolated as greenish white amorphous powder, which gave affirmative result for triterpenoids. Consequently, CTF3-IV was subjected to spectroscopic analyses and the three spectroscopic (IR, NMR and Mass) data proved CTF3-IV as friedelin (Fig. 5).

Friedelin is a friedelane triterpene, whose presence has been identified in many plant species and its anticancer activity was studied in a wide range of cancer cell lines (Chang *et al.*, 2003; Ee *et al.*, 2005; Li *et al.*, 2010; Lu *et al.*, 2010; Olmedo *et al.*, 2008). In the present study, both HeLa and HSC-1 cells have responded for antiproliferative effect of friedelin with high efficiency. Therefore, the study can be extended further to explore the anticancer activity of the friedelin in *in vivo* tumor models (Human cervical carcinoma and Human squamous carcinoma) to evaluate the pharmacological behavior of the compound. Cyclophosphamide, an anticancer agent was used as the reference drug whose IC(50) value was 5.0 µg/mL, which was roughly five times more potent than friedelin. However, any two agents cannot directly be compared for their efficacy, since it is the pharmacotoxicological profile that determines the therapeutic dose of a drug.

Many reports are available with respect to friedelin-induced anticancer property, but its mode of action is yet to be clarified. Friedelin is derived from the parent compound friedelane. Moiteiro *et al.* (2001) and Olmedo *et al.* (2008) verified the anticancer activity of a few derivatives of friedelane. Various triterpenoids-induced anticancer activity in *in vitro* and *in vivo* models have been reported (Chang *et al.*, 2003; Ee *et al.*, 2005; Li *et al.*, 2010; Li *et al.*, 2006; Mullauer *et al.*, 2010; Mujoo *et al.*, 2001; Shanmugam *et al.*, 2012; Zhang *et al.*, 2010). Triterpenoids-induced anticancer/antitumor activities and their various modes of action have been reviewed (Patlolla and Rao, 2012; Petronelli *et al.*, 2010; Shanmugam *et al.*, 2012). According to Li *et al.* (2006), apoptosis and arresting the cells at G0/G1 phase are the two mechanisms, by which triterpenes induce anticancer activity against B16 melanoma and sarcoma S180 cell lines. Triterpenoids-induced antitumor activity was by a mechanism of apoptosis (Mujoo *et al.*, 2001; Petronelli *et al.*, 2009). Many triterpenes have been elucidated for their roles in upregulation and downregulation of several important genes that potentiate the apoptotic effects (Prasad *et al.*, 2008; Prasad *et al.*, 2001; Sung *et al.*, 2010]. Hence, friedelin as a tritepernoid, whose anticancer result as observed in the present study and elsewhere, may be due to its proapoptotic action. However, additional studies are required to substantiate this hypothesis. Investigation on the possible gene-specific action of friedelin, may give insight into the molecular mechanism of its apoptotic/antiproliferative effect.

The crude ethanolic leaf extract of *C. tora* contains various cytotoxic constituents imparting the synergistic anticancer effect. However, when the subfraction composed of friedelin was tested, it was found to possess approximately 5-fold higher efficiency than the extract ($P<0.001$). It indicates that friedelin in its pure form has higher anticancer activity than the crude extract, where it is in combination with other constituents. The presence of other phytochemicals in the crude extract may interfere with the antiproliferative action and/or dilute anticancer constituent(s). This may be the reason for lower efficiency of the crude extract compared to friedelin. The similar kinds of a result wherein the pure compounds/sub-fractions have been shown to possess higher anticancer potentiality than crude extact of the plants. (Afify *et al.*, 2011; Hamzah *et al.*, 2010). When compared the IC(50) values, the crude extract and the active principle were more effective on HeLa cells than the HSC-1 cells ($P<0.05$) as indicated from both the assays (Table 1 and Fig. 1). Such a kind of responses by different cancer cells for cytotoxic agents was also demonstrated by Chang *et al.* (Chang et al., 2012). Nevertheless, the IC(50) values in the range from 19.3 to 31.0 µg/mL indicate that both the cell lines, HeLa and HSC-1 are highly sensitive to the antiproliferative effect of friedelin. There are some reports on friedelin-induced cytotoxic activity against other kinds of cancer cell lines as well (Li *et al.*, 2010; Lu *et al.*, 2010; Olmedo *et al.*, 2008). Therefore, friedelin can be a drug candidate for a wide range of cancers. Thus, it can be concluded that ethanolic leaf extract of *C. tora* is a good source of friedelin, which exhibits potentially broad spectrum anticancer activity. Moreover, *C. tora*, which is generally neglected as a weed indeed a very resourceful plant for bioactive principles with special reference to friedelin.

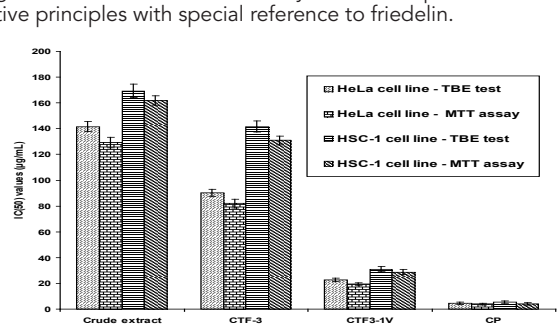

Figure 1

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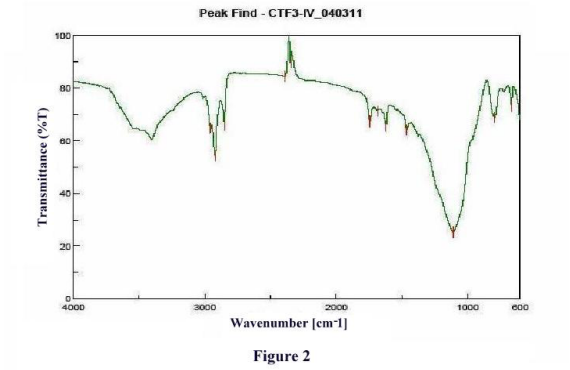

Figure 2

Figure-2 IR Spectroscopic Pattern of CTF3-IV

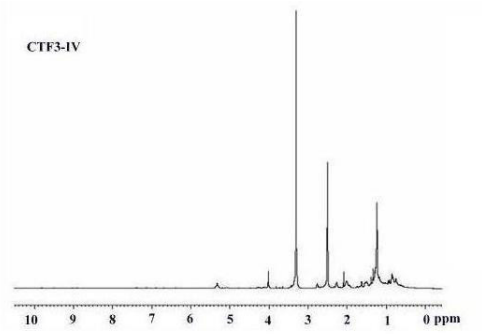

Figure 4

Figure-4 ^{1}H NMR spectrum for CTF3-IV

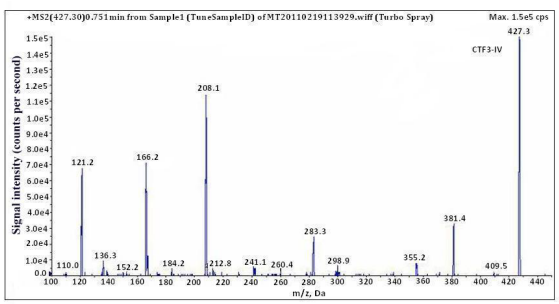

Figure 3

Figure-3 Mass spectroscopic and fragmentation pattern of CTF3-IV

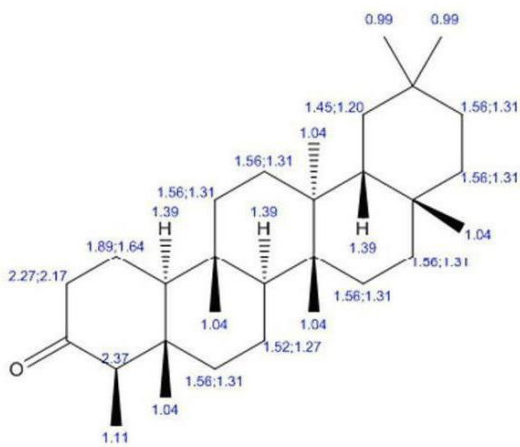

Figure 5

Figure-5 Friedelin - $C_{30}H_{50}O$

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