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ORIGINAL ARTICLE

Screening of *Chonemorpha fragrans* Bioactive Extracts for Cytotoxicity Potential and Inhibition Studies of Key Enzymes Involved in Replication

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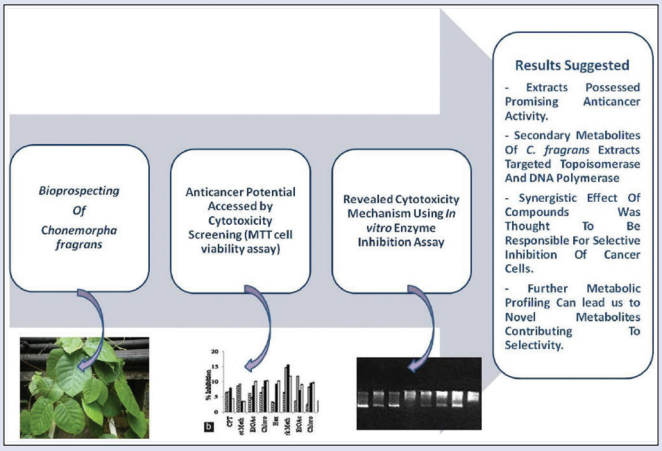
ABSTRACT

Background: *Chonemorpha fragrans* (Moon) Alston, a liana belonging to family *Apocynaceae*, is used in traditional medicinal systems for the treatment of various ailments. It is an unexplored medicinal plant with respect to its anticancer potential. **Objective:** Cytotoxicity of sequential as well as crude extracts of *in vivo* plant parts (leaves, bark, and roots), *in vitro* cultures, and callus were compared. **Materials and Methods:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay was used to compare the extracts of various *in vivo* plant parts (leaves, bark, and roots) along with *in vitro* culture systems (*in vitro* plantlets, callus). Furthermore, the extracts were used to evaluate inhibition of key enzymes involved in replication, i.e. topoisomerase (Topo) I and II, DNA polymerase, to check the probable mechanism of action for this cytotoxicity. **Results:** MTT assay showed that the chloroform extract of callus has potent anticancer potential. The plant has a promising anticancer activity against human colon epithelium, lung carcinoma, and epidermoidal carcinoma cell lines. It was found to possess Topo as well as DNA polymerase inhibitory activity. **Conclusion:** The results have pointed toward pharmaceutical importance of this plant. This study is the first report of exploring the antiproliferative potential as well as inhibition studies of key enzymes involved in replication, which was useful to point out probable mechanism of action for extracts of *C. fragrans*.

Key words: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, *Chonemorpha fragrans,* DNA polymerase, inhibitory activity, topoisomerase I and II

SUMMARY

- It's a first report of cytotoxicity studies and inhibition of enzyme involved in the replication process by *Chonemorpha fragrans* plant extracts. The results reveal the pharmaceutical importance of this plant. From various assays performed here, a potent anticancer potential of chloroform extract of callus was revealed showing Topo I (*E. coli* and human) inhibitory activity, DNA pol inhibitory activity. Considering the importance of these activities, plant further needs to be explored in detail for *in vivo* cancer studies as well as for its metabolite content.



Abbreviations used: CPT: Camptothecin, EDTA: Ethylenediaminetetraacetic acid, MTT: 3-4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide, Pol: Polymerase, Topo - Topoisomerase.

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INTRODUCTION

Chonemorpha fragrans (Moon) Alston is a liana belonging to family *Apocynaceae*. It is known to possess diverse biological activities including muscle relaxant, antiparasitic properties,[1] and antihyperglycemic effect.[2] *C. fragrans* is also used in traditional systems of medicines for the treatment of different ailments such as gynecological disorders,[3] skin diseases and inflammations,[4] and fever and stomach disorder.[1] In spite of traditional use in the treatment of variety of ailments, metabolic content of plant has not been explored. Phytochemical analysis of *C. fragrans* has revealed the presence of alkaloids, such as camptothecin (CPT), chonemorphine, and funtumafrine.[1] CPT is a plant-derived monoterpene indole alkaloid, currently is in clinical use for the treatment of various types of cancer. This compound exhibits a broad spectrum of antitumor activity in the treatment of lung cancer, uterine cervical cancer, and ovarian cancer.[5] According to Vijayan *et al.*,[6] steroidal alkaloids are also responsible for anticancer potential of plant extract. Such steroidal alkaloids of plant origin have potential as cytotoxic drugs for treating multidrug resistant cancer.[7]

We know from literature that severe side effects of chemotherapeutic agents and increasing recurrence of tumors reduce the clinical

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effectiveness of a large variety of currently used anticancer agents. Also, acquired resistance of tumor cells to multiple cytotoxic drugs is a major cause of failure of cancer chemotherapy. Hence, there is a constant need to develop alternative anticancer drugs with minimal side effects. Anticancer agents derived from natural sources are preferred over chemical agents due to their effectiveness for cancer prevention and therapeutics.[8]

Considering these facts, we have attempted to unveil the cytotoxicity potential of *C. fragrans* using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Instead of using a conventional approach for evaluating the cytotoxicity of crude plant extract, we have followed different approach wherein the sequential and crude extracts of various *in vivo* plant parts (leaves, bark and roots) along with *in vitro* culture and callus were compared. In cancer, cell begins to divide uncontrollably.[9] Inhibiting the key enzymes in this division process can slowdown or hinder this uncontrolled cell division. Therefore, the work was further extended to disclose inhibitory activity of such enzymes such as topoisomerase (Topo) I and II, DNA polymerase (DNA pol) which play a key role in replication. This has also helped in evaluation of probable mechanism for this cytotoxicity.

MATERIALS AND METHODS

Preparation of plant extracts of *Chonemorpha fragrans*

Plant was identified and authenticated by Botanical Survey of India, Pune (Voucher Specimen No. BSI/WRC/Cert./2015/PK01). *In vivo* plant parts (leaves, bark, and roots) of *C. fragrans in vitro* shoots grown on B5 medium supplemented with 2.2 mg/l 6-Benzylaminopurine and callus grown on B5 medium supplemented with 2.2 mg/l BAP + 0.6 mg/l 1-Naphthaleneacetic acid were shade-dried and were coarsely powdered using grinder. The extracts were prepared according to Kedari and Malpathak.[10] All the obtained fractions were dissolved in 100 mg/ml of 0.1% dimethyl sulfoxide (DMSO) and diluted to yield various final working concentrations. These extracts were filtered using a 0.45 µm cellulose nitrate membrane and stored at −20°C till further analysis was carried out.

Anticancer activity

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay

MTT assay standard method was used to assess cell viability.[11] L929 (Murine fibroblast cell line), HT29 (human colon epithelium), A549 (human lung carcinoma), and A431 (human skin epithelium) were chosen to evaluate cytotoxicity by means of MTT assay. All the cell lines were procured from National Centre for Cell Science, Pune, India.

1.0×10^5 cells/well were seeded in 96-well microtiter plates. After incubation with various extracts (2 µg, 4 µg, 6 µg, 8 µg of each extract) for 24 h, 50 µl MTT was added to each well and the plates were incubated for additional 4 h at 37°C. To achieve solubilization of the formazan crystal formed in viable cells, 200 µl DMSO was added to each well-followed by gentle shaking. Absorbance was read at 550 nm and surviving cell fraction was calculated. 0.1% DMSO was used as negative control and CPT was used as positive control. Data are presented as mean ± standard deviation (SD) of 3 experiments. Different letters within a column for a particular treatment represent significance at $P < 0.05$. The inhibition of cell was calculated by following formula:

$$\% \text{ Inhibition} = ([A_C - A_S]/A_C) \times 100$$

where, A_C = Absorbance of control

A_S = Absorbance of standard/extracts

Determination of topoisomerase inhibitory activity

Topoisomerase I (Escherichia coli) inhibitory activity

Topo I inhibitory activity was determined using *Escherichia coli* Topo I (New England Biolabs). Reactions were carried out as described by Diwan and Malpathak[12] method.

Topoisomerase I (human) inhibitory activity

Topo I inhibitory activity was determined using Human Topo I (TopoGEN). Reactions were carried out as described by Diwan and Malpathak[12] method.

Topoisomerase II (human) inhibitory activity

Inhibitory activity of test compounds on Topo II (Topo GEN) activity was evaluated by detecting the conversion of supercoiled pUC19 DNA to monomer as described by Snapka *et al* (1998).[13]

DNA polymerase inhibitory activity

The procedure for assaying DNA pol inhibitory activity involves use of fluorescence dye PicoGreen with double-stranded DNA as described by Tveit and Kristensen.[14] PicoGreen dsDNA quantitation reagent was purchased from Invitrogen (USA), *E. coli* DNA pol I large fragment (Klenow fragment) was purchased from fermentas. The method described here allows quantitation of polymerase activity exemplified by the Klenow fragment of DNA pol I from *E. coli* at reaction temperatures between 37°C and 72°C.

Primer template annealing

The annealed primer template mixture 1.0 pmole and 1.5 pmole (forward and reverse primers) was mixed with 2 mM each dNTP's, ×10 reaction buffer, and the final volume was made to 20 µl by adding sterile distil water. The reaction was initiated by addition of 0.05 U of DNA pol (Klenow fragment) to the above mixture. The reaction mixture was incubated for 30 min at 37°C. The reaction was stopped by addition of 0.25 M ethylenediaminetetraacetic acid (EDTA) followed by 100 µl of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and 100 µl of Picogreen solution in TE. This mixture was incubated for 5 min and fluorescence was estimated using Pro5 (Molecular probes) spectrometer at 538 nm using an excitation wavelength of 485 nm.

Statistical analysis

The data are expressed as mean ± SD for at least three independent determinations in triplicate for each experimental point. SPSS (Version 18.0, Chicago, IL, USA) was used for all statistical analyses.

RESULTS

3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide cell proliferation assay

In the current study, we initially investigated the effects of several extracts on the viability of human cancer cell lines of various origins, including HT29 (human colon epithelium), A549 (human lung carcinoma), and A431 (human skin epithelium) cell lines. To check the adverse effect of these extracts on normal cells, these results were compared with the effect of extracts on normal cell line L929 (murine fibroblast cell line).

In vivo (root, bark, and leaves) extracts showed high inhibition of cells as compared to *in vitro* (*in vitro* shoots and callus) extracts on L929 cell line. Highest cytotoxicity was seen in chloroform extract of root (25.37%) at 6 µg, methanol extract of bark (24.505%) at 2 µg, ethyl acetate extract of bark (24.089%) at 6 µg, and equivalent to CPT (20.86%) at 8 µg [Figure 1a]. The lowest activity was observed in

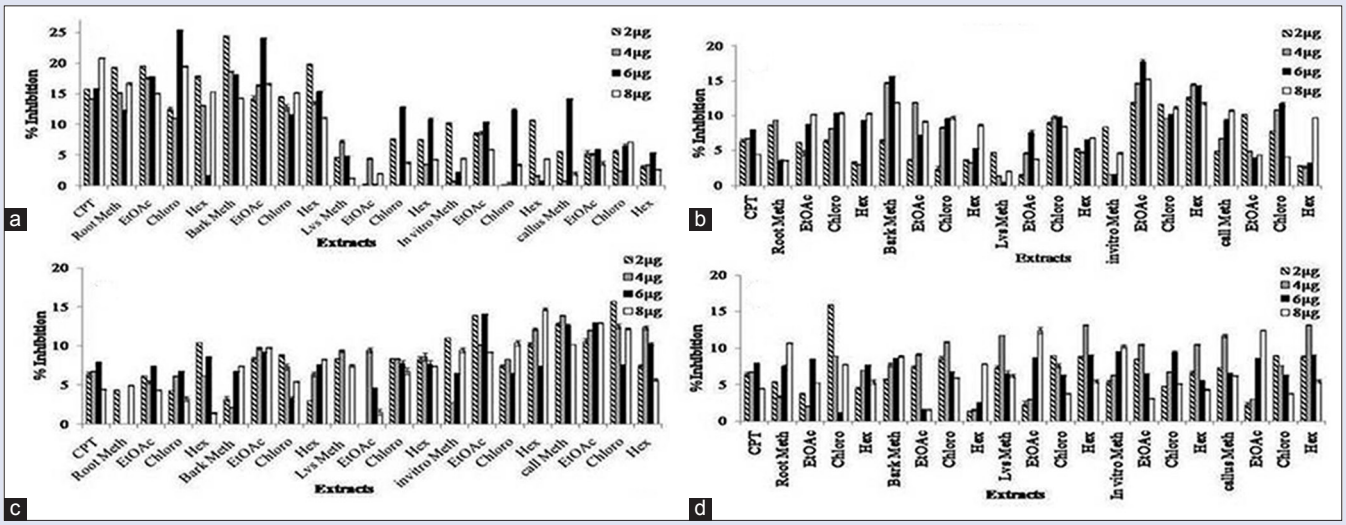

Figure 1: (a) Cytotoxic effect of *in vivo* and *in vitro* extracts of *Chonemorpha fragrans* on cell line L929; (b) cell line HT29; (c) cell line A549; and (d) cell line A431

chloroform extract of *in vitro* shoot (0.08%) at 2 µg, methanol extract of callus (0.83%) at 4 µg.

In HT29, maximum inhibition of cells were observed by *in vitro* cultures, i.e., chloroform extract of callus (15.77%) at 2 µg, hexane extract of *in vitro* shoot (14.70%) at 8 µg, ethyl acetate extract of *in vitro* shoot (14.06%) at 6 µg, methanol extract of callus (13.89%) at 4 µg, ethyl acetate extract of callus (12.96%) at 8 µg, and hexane extract of callus (12.36%) at 4 µg [Figure 1b]. These extracts showed higher inhibition of HT29 cell line than standard CPT (7.96%) at 6 µg. Lowest activity was shown by *in vivo* extracts such as hexane extract of root (1.42%) at 8 µg and ethyl acetate extract of leaves (1.568%) at 8 µg.

In case of cell line A549% inhibition ranged from 1 to 18% [Figure 1c]. Highest activity was seen by ethyl acetate extract of *in vitro* shoots (17.68%) at 6 µg concentration, chloroform extract of *in vitro* shoot (11.665%) at 2 µg, chloroform extract of callus (11.67%) at 8 µg, and hexane extract of *in vitro* shoots (14.48%) at 4 µg. Similar to HT29 cell line, *in vitro* shoot and callus extracts were effective as compared to *in vivo* plant part extracts (root, bark, and leaves) on A549 cell line.

The present inhibition of A431 ranged from 5 to 15% of inhibition [Figure 1d]. For all the analyzed extracts, percent inhibition was higher than standard CPT (7.90%) at 6 µg. The maximum activity was shown by chloroform extract of roots (15.94%) at 2 µg, hexane extract of callus (13.185%) at 4 µg, ethyl acetate extracts of leaves (12.45%) at 8 µg, and methanol extract of callus (11.75%) at 4 µg. Lowest cytotoxicity was shown by chloroform extract of roots (1.148%) at 6 µg, hexane extract of bark (1.58%) at 4 µg.

Percent inhibition of these extracts was at least twice to that of the standard CPT (4.468%) at 8 µg. Lowest activity was seen by methanol extract of leaves (0.33%) at 8 µg extract. In this case, *in vitro* shoots and callus extracts were effective than *in vivo* plant extracts (root, bark, leaves).

Escherichia coli topoisomerase I inhibitory activity

As seen from Figure 2a, extracts showed retention of supercoiled form of DNA suggesting the inhibitory activity of *C. fragrans* extracts against Topo I. In ethyl acetate (Lane V), chloroform (Lane W), and hexane (Lane X) extract of callus, it is clearly seen that there is very low Topo I activity or no relaxed form.

In vitro shoot extracts (Lane Q–T) showed weak inhibition of Topo I activity while the crude methanol extracts of roots (Lane E), bark (Lane I) leaves (Lane M), and callus (Lane U) did not inhibit the activity of Topo I and caused complete relaxation of plasmid DNA indicating that all *in vitro* shoots extracts were weak inhibitors of Topo I and allowed Topo I to relax the plasmid DNA. We can observe that root (Lane F–H) and bark (Lane J–L) sequential extracts are also inhibitors of Topo I. Leaves (Lane N–P) sequential extracts have shown Topo I inhibition as well.

Human topoisomerase I inhibitory activity

Although the extracts vary in inhibiting *E. coli* Topo I, in case of human Topo I, all the extracts showed inhibitory activity. All the root, leaf, and callus extracts showed inhibitory activity comparable to CPT (2 µg/ml) (Lane C) [Figure 2b]. Bark (Lane I–L) and *in vitro* shoot (Lane Q–T) possessed weak activity. Ethyl acetate (Lane V) and hexane (Lane X) extract of callus showed strong activity. Ethyl acetate extract of bark (Lane J) and leaves (Lane N) showed strong inhibition of human Topo I.

Human topoisomerase II inhibitory activity

The assay results showed that all the extracts formed monomers of relaxed plasmid [Figure 2c].

DNA polymerase inhibitory activity

Evaluation of *C. fragrans* extracts for DNA pol inhibitory assay revealed that the highest activity was seen in methanol extract of roots (82.54%) at 6 µg, chloroform extract of roots (72.22%) at 100 µg, methanol extract of bark (87.85%) at 8 µg, hexane extract of bark (87.10%) at 8 µg, methanol extract of leaves (85.087%) at 4 µg, and chloroform extract of *in vitro* shoots (86.79%) at 4 µg concentration [Figure 3].

DISCUSSION

The objective of this study was to investigate and compare cytotoxic activity of *in vivo*, *in vitro* shoot and callus sequential extracts of *C. fragrans*. The National Cancer Institute, USA, has listed most common types of cancer. Cell lines were chosen for most common types of cancer and were used for studying the effect of our extracts. L929, murine fibroblast cell line is a normal cell line in comparison to which the other cancerous cell lines A431, A549, and HT29 were analyzed.

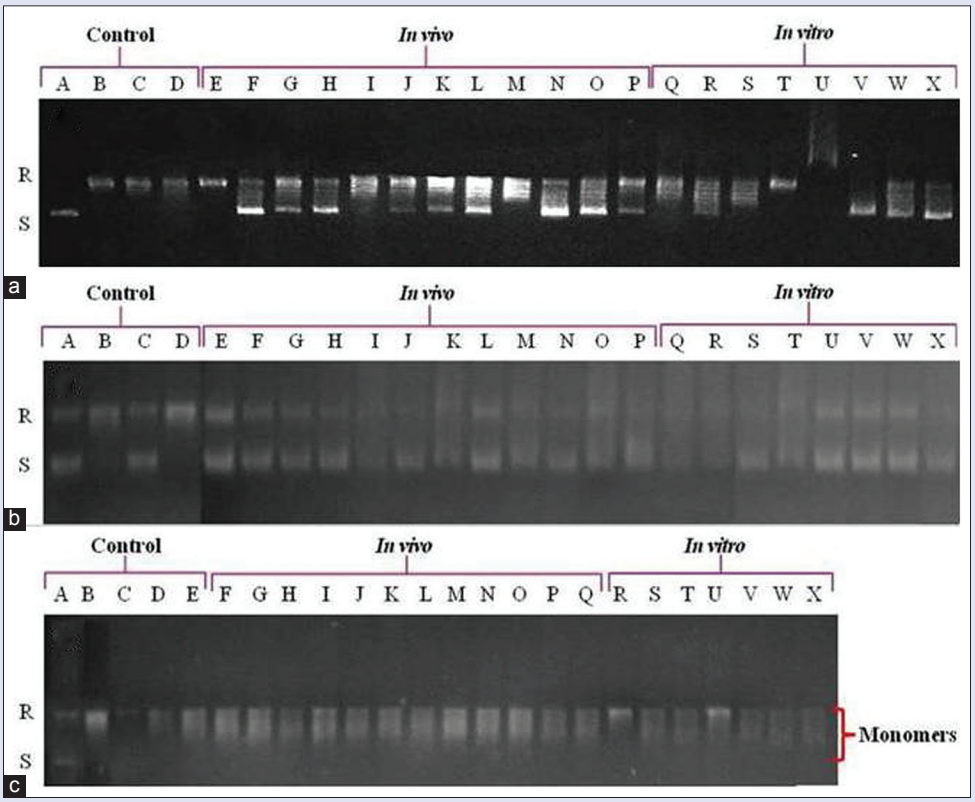

Figure 2: Lane A–E (a) Coli topoisomerase I inhibition by extracts of *Chonemorpha fragrans*; (b) human topoisomerase I inhibition; (c) human topoisomerase II inhibition, (reproduction size 11.9 cm × 15.4 cm) Lane A: Supercolied pUC 19 DNA, Lane B: DNA + topoisomerase enzyme, Lane C: 1 µg camptothecin, Lane D: 0.1% dimethyl sulfoxide, Lane E–X: DNA + topoisomerase + 2 µg/test compound, E–H: Root MeOH, EtOAc, CH_3Cl, C_6H_{12} extracts resp., Lane I–L: Bark MeOH, EtOAc, CH_3Cl, C_6H_{12} extracts, respectively, Lane M–P: Lvs MeOH, EtOAc, CH_3Cl, C_6H_{12} extracts, respectively, Lane Q–T: *In vitro* MeOH, EtOAc, CH_3Cl, C_6H_{12} extracts, respectively, Lane U–X: Callus MeOH, EtOAc, CH_3Cl, C_6H_{12} extracts, respectively. (R) Relaxed and (S) supercoiled plasmids

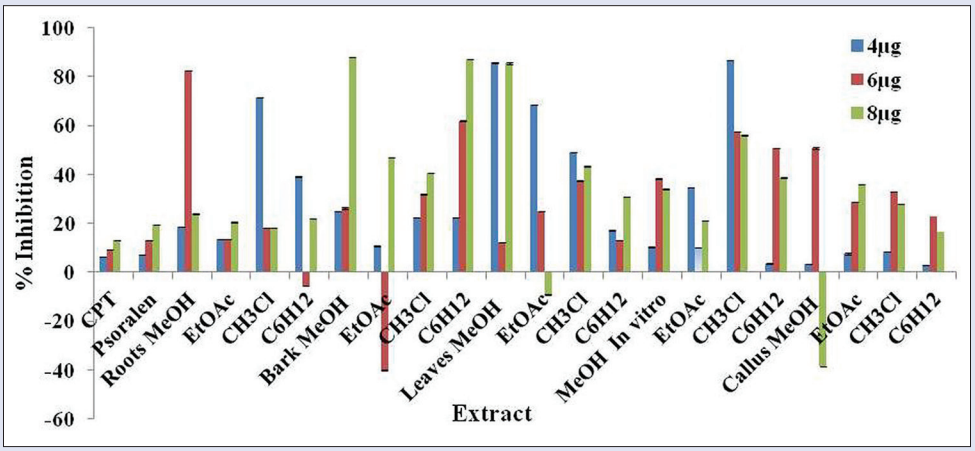

Figure 3: DNA polymerase inhibitory activity of *Chonemorpha fragrans*

Different extracts of the plant exhibited varying activity on different cell lines. Lack of morphological differentiation of plants leads to change in the regular metabolic pathways.[15] This can contribute to change in the metabolic content of an extract causing varying degree of anticancer activity.

Unlike cell line L929, in all the other cell lines, proliferation was strongly inhibited by *in vitro* shoots and callus extracts which was significantly higher than standard CPT (≥90% pure, Sigma-Aldrich). Standard CPT, which is clinically used in the treatment of various cancers, showed 15% inhibition for cell line L929 whereas it showed 5–10% inhibition level for other cancer cell lines suggesting a harmful effect of CPT on normal cells. Same is the case for *in vivo* roots and bark extracts. Leaves showed moderate activity on every cell lines but *in vitro* shoot and callus cultures showed the best activity on cancer cells with comparatively low activity on normal cells. This observation suggested that these extracts probably act selectively on cancer cells.

Maximum CPT is known to present in the root, bark, and leaves extracts as compared to *in vitro* plantlets and callus extracts[10] whereas the antiproliferative effect was seen to be maximum in the extracts in *in vitro* plantlets and callus extracts which has lower amount of CPT or in hexane extracts where CPT is absent.[10] The presence of higher antiproliferating activity by extracts with low or no presence of CPT content as compared to standard CPT indicates the presence of other antiproliferative compounds in the extracts. Anticancer molecules such as CPT show severe side effects on normal cells, on the other hand, in this experiment extracts with low CPT or no CPT with high activity on cancer cell line showed very low toxicity toward normal cell line suggesting reduced side effects. When a combination of compounds exhibits a more potent therapeutic effect than that of individual compounds, the effect is described to be a synergistic.[16] This type of mode of action offers an opportunity for more precise control of biological systems.[17]

In vitro shoot and callus of *C. fragrans* strongly inhibited all the cancer cell lines (HT29, A549, and A431) with percent inhibition ranging from 1 to 18% whereas it showed 0.5–4% inhibition of murine fibroblast cell line (L929). Standard CPT showed 5–10% inhibition of cancer cell lines (HT29, A549, and A431) and 15% inhibition for murine fibroblast cell line (L929). These results are indicating that the *in vitro* shoots and callus extracts exhibit comparatively higher inhibition of growth of cancer cell lines compared to CPT.

In-vitro cytotoxicity analysis using MTT assays indicated that this plant is very effective and could inhibit the proliferation of different cancerous cell lines such as A431 (human epidermoidal carcinoma cell line), A549 (human lung carcinoma cell line), and HT29 (human colon epithelium cell line) suggesting the potential of *C. fragrans* as an anticancer agent. The most potent extracts were *in vitro* shoots and callus extracts which also showed selectivity toward cancer cells whereas roots, bark, and leaves extracts were less toxic to cancer cells. Considering the need of exploring the possible mechanism of action, further assays were carried out to understand inhibition of various key enzymes playing role in replication.

Topo is one of the crucial enzymes for cellular genetic processes, such as DNA replication, transcription. Topo I is known to relax the supercoilled DNA in the absence of an energy cofactor, by nicking the DNA and allowing rotation of the broken strand around the Topo I-bound DNA strand. The inhibition of DNA Topo I has proven to be a successful approach in designing of anticancer drugs.[18]

In the MTT cell proliferation assay, callus extracts have shown highest activity against HT29 (human colon epithelium cell line), A549 (human lung carcinoma cell line), and A431 (human epidermoidal carcinoma cell line) as compared to *in vivo* roots, bark, and leaves extracts as well as standard anticancer drug CPT. Whereas it did not show antiproliferative effect against L929 (normal murine fibroblast cell line) which supports the observation that the callus extracts are selectively killing cancerous cell line. In human Topo I inhibition assay, callus extract of chloroform have shown superior inhibition of human Topo I activity as compared to in vivo extracts. This suggested that Topo I inhibition by this extract might be a possible mechanism for its strong anticancer activity where it can inhibit cancer cell lines.

In vitro shoot extracts proved to be weak inhibitors of *E. coli* Topo I activity while crude methanol extracts of roots, bark, leaves, and callus extracts did not inhibit the activity of Topo I and caused complete relaxation of plasmid DNA indicating that all *in vitro* shoots extracts were weak inhibitors of *E. coli* Topo I and allowed *E. coli* Topo I to relax the plasmid DNA. We can observe that root and bark sequential extracts are also inhibitors of *E. coli* Topo I, but these extracts also showed inhibitory effect on L929, therefore cannot consider having selective antiproliferation potential.

In our previous finding,[10] quantification of the extracts by HPLC and HPTLC has showed that the content of CPT was lowest in hexane extracts of *in vitro* shoots and callus. Our results have showed that these extracts were promising Topo I inhibitors as compared to extracts which showed highest CPT content (methanol extracts). This was thought to be due to the synergistic effect of compounds in extracts with high activity or antagonistic effect of various compounds in the extracts with low activity in spite of presence of high CPT.

Strong activity of ethyl acetate and hexane extract of callus confirmed mechanism of action is through Topo I inhibition. Here, we can clearly observe that ethyl acetate extracts of bark and ethyl acetate extracts of leaves were also strongly inhibiting the activity as no relaxed form is seen in a gel [Figure 2b].

Ethyl acetate extract of leaves and bark showed strong inhibition of human Topo I. According to previous reports, both the extracts contain high CPT than *in vitro* extracts (callus and shoots). As ethyl acetate extract of bark showed greater inhibition of normal cells too, bark was not considered as having anticancer potential but was considered to be cytotoxic in nature.

Topo II makes transient breaks in both strands of one DNA molecule allowing the passage of another DNA duplex through the gap and changing the linking number by steps of two. Topo II inhibitory activity was also analyzed to find out the probable mechanism for anticancer activity of these extracts as cancer cells rely on these enzyme more than healthy cells, since they divide more rapidly,[19] and therefore this enzyme is one of the targets in developing anticancer assay.

The results suggested that all the extracts are weak inhibitors of Human Topo II enzymes. From previous results, the extracts were thought to have inhibitory effect on Human Topo I activity.

All the extracts were proved to have weak human Topo II inhibitory activity. According to Pavillard *et al*.,[20] who explored the cytotoxicity of combinations of a Topo I inhibitor (CPT) and a Topo II inhibitor (doxorubicin or etoposide) at several molar ratios, the simultaneous combination of Topo I and Topo II inhibitor was antagonistic in C6 cells. The cytotoxicity of etoposide (Topo inhibitor) was diminished in the presence of CPT. Likewise, treatment with CPT diminished the cytotoxicity of Topo II inhibitors, namely, aminoacridine [4-(9-acridinylamlno)-N~(methanesulfonyl)-m-anisidine] and anthracycline dau norubicin. CPT also antagonized the cytotoxicity of 4'-(9-acridinylamino) methanesulfon-M-anisidide and daunorubicin, two structurally unrelated Topo II-directed agents. Topotecan, a CPT analog currently undergoing Phase I clinical trials, had a similar effect.[17] This suggests that CPT reduces the cytotoxicity of different, structurally distinct Topo II directed chemotherapeutic agents. An inhibitory effect of CPT on etoposide induced cytotoxicity was observable at concentrations of CPT as low as 0.01 μM. These results have proved that CPT shows inhibitory effect in presence of Topo II inhibitors.[17] In this case, also, presence of CPT could be related to the weak activity of Topo II inhibitory activity of *C. fragrans* extracts.

In case of hexane extracts, although CPT is absent, the extracts have shown presence of Topo I inhibitors. Literature survey has revealed that presence of Topo I inhibitor (CPT) gives antagonistic effect to Topo II inhibitor and vice versa. Same could be the reason for weak Topo II inhibitory activity of hexane extracts.

DNA pol is actively being targeted for the development of novel anticancer agents. The most common method used to measure DNA pol activity *in vitro* depends upon the incorporation of radiolabel nucleotides.[21] However, routine use of such assays is detrimental due to risks and restrictions associated with radioisotopes.[22] We have used a rapid, highly sensitive, and quantitative assay capable of measuring DNA pol extension activity. This fluorescence-based assay measures the

inhibition of DNA pol activity on the basis of specific reaction of the dye PicoGreen with double-stranded DNA.[12]

The extracts showed highest inhibition of DNA pol activity as compared to activity of pure CPT (13.09%) at 8 µg suggesting that mere presence of CPT is not responsible for this DNA pol inhibition. It should be noted that chloroform extract of *in vitro* shoots and methanol extract of leaves showed highest activity at lowest concentration (4 µg). Lowest activity was seen in CPT (13.90%) at 8 µg, hexane extract of callus (16.48%) at 8 µg whereas ethyl acetate extract of bark and methanol extract of callus showed negative activity.

The study also indicates good DNA pol inhibitory activity of *C. fragrans* suggesting that the extracts have a potential of inhibiting DNA polymerase at low concentration. The inhibitors of DNA pol has been reported to impair the growth of cancer cells.[23]

CONCLUSION

We can arrive at the conclusion that this plant further needs to be explored in detail for *in vivo* cancer studies. From MTT assay, we have concluded that chloroform extract of callus is a potent anticancer agent showing Topo I (*E. coli* and human) inhibitory activity and also is an inhibitor of DNA pol. The plant has promising anticancer activity against human colon epithelium, lung carcinoma, and epidermoidal carcinoma. This study is the first report of exploring the antiproliferative potential and probable mechanism of action using crude and sequential extracts of *C. fragrans*.

From different assays, a potent anticancer potential of chloroform extract of callus was revealed showing Topo I (*E. coli* and human) inhibitory activity, DNA pol inhibitory activity suggesting that different compounds are contributing different activities. Also, synergistic effect of compounds was thought to be responsible for inhibitory activity of Topo I as well as DNA pol from *C. fragrans* extracts as seen from the results of hexane extracts. Considering the importance of these activities, the extracts should be further explored for its metabolite content.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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