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Original Article

Cytotoxicity Assay, Antioxidant Activity and Investigation of Bioactive Compounds Using GC-MS in the Leaves Extracts of *Cinnamomum tamala* (Buch. -Ham.)

Sushmita Semwal[1], Neeraj Kumar[1,*], Bhuwan Chandra Joshi[2], Pankaj Budhlakoti[3]

[1]Department of Pharmacology, School of Pharmaceutical Sciences, Shri Guru Ram Rai University, Dehradun, Uttarakhand, INDIA.
[2]Department of Pharmaceutical Sciences, Faculty of Technology, Sir J.C. Bose Technical Campus, Bhimtal, Kumaun University, Nainital, Uttarakhand, INDIA.
[3]Central Laboratory, Patanjali Food and Herbal Park Pvt. Ltd., Haridwar, Uttarakhand, INDIA.

ABSTRACT

Background: *Cinnamomum tamala* (Lauraceae), commonly known as Tejpaat or Bay leaves, find application in foods, along with traditional uses for curing a number of ailments. The objective of the current study was to investigate the cytotoxic efficacies of different solvent extracts of *Cinnamomum tamala* (Buch. -Ham.) leaves on some cancer cell lines. **Materials and Methods:** The powdered leaves were extracted using petroleum ether, chloroform, ethyl acetate and ethanol and tested for the presence of phytoconstituents. The extracts were evaluated for cytotoxic activity using four cell lines i.e. MDA-MB-231, A549, HepG2 and HT-29 by MTT assay. The most effective extract was then evaluated for antioxidant activity using the DPPH assay. The extract exhibiting notable cytotoxic activity was further subjected to GC-MS profiling to determine its bioactive constituents. The cytotoxicity shown by all the extracts was time and dose-dependent at low concentrations. **Results:** The Cytotoxic activity shown by Chloroform Extract (CTCE) was effective against human colon cancer cell line (HT-29) having IC_{50} 26.15 µg/mL. CTCE also revealed good DPPH antioxidant activity with IC_{50} 46.31±0.15 µg/mL. The GC-MS profiling of CTCE showed 50 constituents, among which the component phytosterols like γ-Sitosterol and Campesterol were present. **Conclusion:** This study highlights the greater cytotoxicity of CTCE observed on HT-29 cell lines. The cytotoxicity can be due to presence of detected compounds, particularly phytosterols in the CTCE. So, it can be concluded that *Cinnamomum tamala* leaves have significant cytotoxic activity and suggest further isolation of active components and evaluating their mechanism(s) of action.

Keywords: Antioxidant, Cancer cell lines, *Cinnamomum tamala*, Cytotoxic Activity.

Correspondence:
Dr. Neeraj Kumar
Professor, Department of Pharmacology, School of Pharmaceutical Sciences, Shri Guru Ram Rai University, Dehradun-248001, Uttarakhand, INDIA.
Email: neerajkumar@sgrru.ac.in
ORCID: 0000-0002-5584-8522

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INTRODUCTION

Cancer is the second most prevalent cause of mortality and is quickly overtaking other causes as individual's age. The term "cancer" refers to a broad range of malignant tumors that can affect almost all human organs and tissues. The result of genetic defects within a cell is essentially the proliferation of aberrant cells.[1] A fast-paced, unhealthy lifestyle, urbanization, industrialization, population expansion and an increase in life expectancy might all be contributing causes to the rise in cancer occurrences.[2] In this regard, higher plants provide a wealth of possible phytochemicals that might be effective in the fight against cancer.[3] Over the past few years, the researches on the clinical applications of plant secondary metabolites have paved the way towards the battle against cancer and showcased the significant impact that discovering new drugs from medicinal plants has had in combating this disease.[4] Cancer develops and progresses primarily as a result of the suppression of apoptosis in carcinogenesis. To prevent apoptosis, tumour cells employ a range of molecular strategies. In order to treat cancer chemotherapy, one particular therapeutic technique is to induce apoptosis in tumour cells.[3,5,6] Apart from their ability to induce apoptosis, studies have shown that the majority of phytochemicals cause cell cycle arrest, differentiation induction and/or interference with multiple cell-signalling pathways.[3,7] Numerous research has documented the medicinal potential of phytoconstituents in the prevention and treatment of cancer. The mechanisms include immune function enhancement as well as antioxidant and antiproliferative actions.[8]

Cinnamomum tamala (Buch.-Ham.) T.Nees & C.H. Eberm. *(C. tamala)*, also known as Tejpata or Indian bay leaf, is an aromatic evergreen tree native to the Indian subcontinent. Beyond its

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culinary applications, this plant has gained interest in recent years due to its remarkable pharmacological properties, with a specific focus on its cytotoxic potential against cancer cells. The plant leaves contain essential oils, polyphenols and alkaloids, which contribute to its pharmacological activities. Researchers have demonstrated that the extracts of the plant have cytotoxic effects on a variety of cancer cell lines, such as those from the breast, lung, prostate, glioblastoma, colon and ovarian cancers.[9-11] The cytotoxicity is attributed to bioactive compounds like cinnamaldehyde, eugenol and polyphenols, which induce apoptosis (programmed cell death) in cancer cells while inhibiting their proliferation and metastasis.[12] Additionally, the cytotoxic potential of a plant is greatly influenced by its anti-inflammatory and antioxidant effects.[13,14] Pharmacological activities reported for *C. tamala* include antioxidant,[13] anti-inflammatory,[14] antidiabetic,[15] immunosuppressive,[16] antimicrobial,[17] and cytotoxicity against cancer cells.[12] Considering the significance of *C. tamala* and its reported constituents along with several pharmacological activities, this study was done to further decipher its active constituents particularly related to cytotoxicity in some cancer cell lines. Furthermore, the study is going to provide an experimental base for the synthesis of plant-based medicines and the isolation of active ingredients from CTCE that have cytotoxic and antioxidant properties.

MATERIALS AND METHODS

Plant material

The leaves of *C. tamala* were collected from a local garden of Dehradun district of Uttarakhand, India and authentication by Dr. S.K. Singh (Scientist-E) from Botanical Survey of India (BSI), Northern regional centre, Dehradun, Uttarakhand, India with voucher specimen number Ref. BSI/NRC/Tech./Herb (Ident.)/2020-21/613/435 March 2021.

Extract preparation

The shade dried leaves (250 g) were coarsely powdered and by continuous hot extraction procedure utilising Soxhlet's equipment the leaves were extracted using petroleum ether at 40-60°C. After defatting, the resulting marc was dried in the open air before being extracted again using chloroform, ethylacetate and ethanol. The yield was calculated for the extracted materials and then stored in a desiccator until further analysis.

Phytochemical screening

The various extracts of *C. tamala* in petroleum ether (CTPE), chloroform (CTCE), ethylacetate (CTEE) and ethanol (CTAE) were tested for identification of saponins. flavonoids, steroids, terpenoids, phenolic compounds and tannins by phytochemical screening as per standard methods.[18-20]

Cytotoxicity Assay

Cell lines

Human cancer cell lines for the breast (MDA-MB-231), lung (A549), colon (HT-29) and liver (HepG2) were obtained from NCCS, Pune, India. Using DMEM media supplemented with 10% FBS and 1% antibiotic solution, the cells (10000 cells/well) were grown in 96-well plates for 24 hr at 37°C with 5% CO_2.[21,22]

Cell viability

The cytotoxicity of the various solvent extracts (CTPE, CTCE, CTEE and CTAE) was determined against the four human cancer cell lines: MDA-MB-231, A549, HT-29 and HepG2 using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This test assesses the quantity of MTT that is converted to a blue formazan product by mitochondrial dehydrogenase. The intensity of colour also gives a measure of viable cells.[23] The cultured cells were treated with different concentrations (prepared in incomplete medium) of the formulations ranging from 1-1000 µg/mL. A final concentration of 250 µg/mL of MTT solution was added to the cell culture after a 24-hr incubation period and the culture was then incubated for an additional 2 hr. After collecting the culture supernatant at the end of the experiment, the cell layer matrix was dissolved in 100 µL of Dimethyl Sulfoxide (DMSO) and read using an Elisa plate reader (iMark, Biorad, USA) at reference wavelengths of 540 and 660 nm. The data was represented by the IC_{50} value. IC_{50} (Half maximum inhibitory concentration) is the drug concentration that results in a 50% reduction in cell viability or growth.

Antioxidant Activity

The potent extract (CTCE) was evaluated for its antioxidant capacity based on its potential to scavenge free radicals.

1,1-Diphenyl-2-Picrylhydrazyl (DPPH) radical scavenging assay

The assay was performed by taking 0.2 mL of CTCE in varying concentrations (20-100 µg) and mixed with 0.2 mL DPPH ethanolic solution. After the resulting solutions were retained in the dark for 30 min at 37°C, the absorbance was measured at 517 nm wavelength. As a reference, ascorbic acid was used. The inhibition of DPPH and IC_{50} values were calculated using following equation:

Inhibition (%)=(Acontrol-A sample)/Acontrolx100

Where, $A_{control}$ and A_{sample} indicate the absorbance of DPPH solution alone and with the extracts, respectively. All tests were carried out in triplicate. To determine the half maximum Inhibitory Concentration (IC_{50}) value, the percentage of inhibition at each sample concentration was plotted.[24,25]

GC-MS Analysis of potent fraction of *C. tamala*

GC-MS Protocol

The Gas Chromatography (GC-MS) profiling of chloroform extract (CTCE) of *C. tamala*, was performed on Shimadzu GCMS system, model-TQ8040 equipped with AOC-20i robotic arm auto sampler, Split/Splitless (S/SL) Injector. The samples were prepared in suitable solvents and diluted in the same solvent. 1µL of the sample was injected into the S/SL injector through a programmed autosampler using 10µL syringe in splitless mode. Capillary column (SH-Rxi-5Sil MS, specification- 30 mmx0.25 mm IDx0.25 µm, stationary phase-5% biphenyl 95% dimethyl polysiloxone) was used for separating different components present in the sample. As the carrier gas, helium (99.999%) was utilised at a steady flow rate of 1 mL/min. Gas chromatograph was programmed with the inlet temperature set at 250°C. The initial oven temperature was kept at 50°C (hold for 2.0 min), temperature was increased to 310°C @ 10°C per minute (hold for 25.0 min). Total run time calculated by the system was 53.0 min. The sample was ionized by using an EI source maintained at a temperature of 230°C with ionization energy set to 70 eV. MS Interface temperature was set to 250°C to avoid the condensation of sample components while entering the MS system.[26]

Determination of constituents

For calculating the relative percentage of constituents present, the peak area of each peak was compared with the total area for all the peaks integrated from the chromatogram. By comparing the recorded mass spectra of the extract components with the database of National Institute Standard and Technology (NIST), India, the components were identified.

Statistical Analysis

All the experiments were conducted in triplicate and the results are represented as Mean±SEM (n=3). Analysis was performed using the SPSS software package. The regression analysis was done using the best fit method.

RESULTS

Extraction Yield

Table 1 lists the percentage yield, color and consistency of each of the four extracts. The percentage yield is different with different solvent systems used. The alcoholic extract (CTAE) showed the highest yield (3.85% w/w) while Ethylacetate Extract (CTEE) had the lowest yield (1.15% w/w). The percentage yield is an indicator of solubility of particular constituents in particular solvent.

Phytochemical Analysis

Phytochemical analysis of various extracts showed the presence of flavonoids, alkaloids, phenolic chemicals, proteins, carbohydrates and steroids/terpenoids shown in Table 2.

Cytotoxicity Assay

The leaf extracts CTPE, CTCE, CTEE and CTAE of *C. tamala* were taken to evaluate the effectiveness on cytotoxicity against four human cancer cell lines (MDA-MB-231, A549, HT-29 and HepG2) of different histological origins by MTT assay. The results indicated that all the four extracts exhibited cytotoxicity on all cell lines in time- and dose- dependent manner at low dose. The lowest IC_{50} values, corresponding to the most cytotoxic substances, were found for the Chloroform Extract (CTCE) of *C. tamala* in all four cell lines but more prominently to colon cancer cell line (HT-29) with IC_{50} value 26.15 µg/mL (Figure 1) (Table 3). This may be due to the presence of phytoconstituents in this plant. CTCE exhibited significant cytotoxic activity relative to other extracts.

Morphological observation

The effect of CTPE, CTCE, CTEE and CTAE treatment was examined for cell morphology on all four cell lines. The extracts treatment was done at different concentrations (0-1000 µg/mL). Cells treated with CTCE exhibited morphological characteristics associated with the apoptotic process (Lane b-c) in a dose-dependent manner, in contrast to the control (Lane a). Morphological alteration, cell shrinkage and an increase in number of floating and dead cells was evident in the treated cells. HT-29 cell lines showed a substantial decrease in proliferation rate when exposed to high concentrations of CTCE (500 and 1000 µg/mL) (Figure 2).

Antioxidant Activity

The potent extract (CTCE) was evaluated for the antioxidant activity using DPPH assay. The DPPH radical scavenging activity of CTCE and STD (ascorbic acid), with IC_{50} values of 46.31±0.15 µg/mL and 27.38±0.16 µg/mL, respectively was noted. The antioxidant capacity of the samples is estimated using free radical tests. The ethanolic solution of DPPH is a purple-coloured stable free radical that becomes yellow on interaction with

Table 1: Characterization of different extracts of *C. tamala* leaves.

Extracts	Color	Odor	Consistency	Yield (%w/w)
CTPE	Dark Green	Characteristic	Semi-solid	3.26
CTCE	Greenish brown	Characteristic	Semi-solid	1.31
CTEA	Greenish brown	Characteristic	Semi-Solid	1.15
CTAE	Brown	Characteristic	Semi-solid	3.85

Table 2: Phytochemical screening of different extracts of *C. tamala* leaves.

Phytochemical	Test	CTPE	CTCE	CTEE	CTAE
Alkaloids	Mayer's test	-	+	-	-
	Dragendorff's test	-	+	-	-
	Hager's test	-	+	+	-
Amino acids and Proteins	Ninhydrin test	-	-	-	-
	Biuret test	-	-	-	-
	Millon's test	-	-	+	-
Carbohydrates	Molisch's test	-	-	+	+
	Fehling's test	-	-	+	+
Flavonoids	Shinoda's test	-	-	+	+
	$FeCl_3$ test	-	-	+	+
Glycosides	Borntrager's test	-	-	-	-
	Foam test	-	-	-	-
	Keller kiliani Test	-	-	-	-
	Sodium hydroxide reagent test	-	-	-	-
Steroids/ Triterpenoids	Salkowski's Reaction	-	+	-	-
	Liebermann Burchard's test	+	+	-	-
Tannins and phenolic compounds	Lead acetate solution test	-	-	+	+

(+) present, (-) absent.

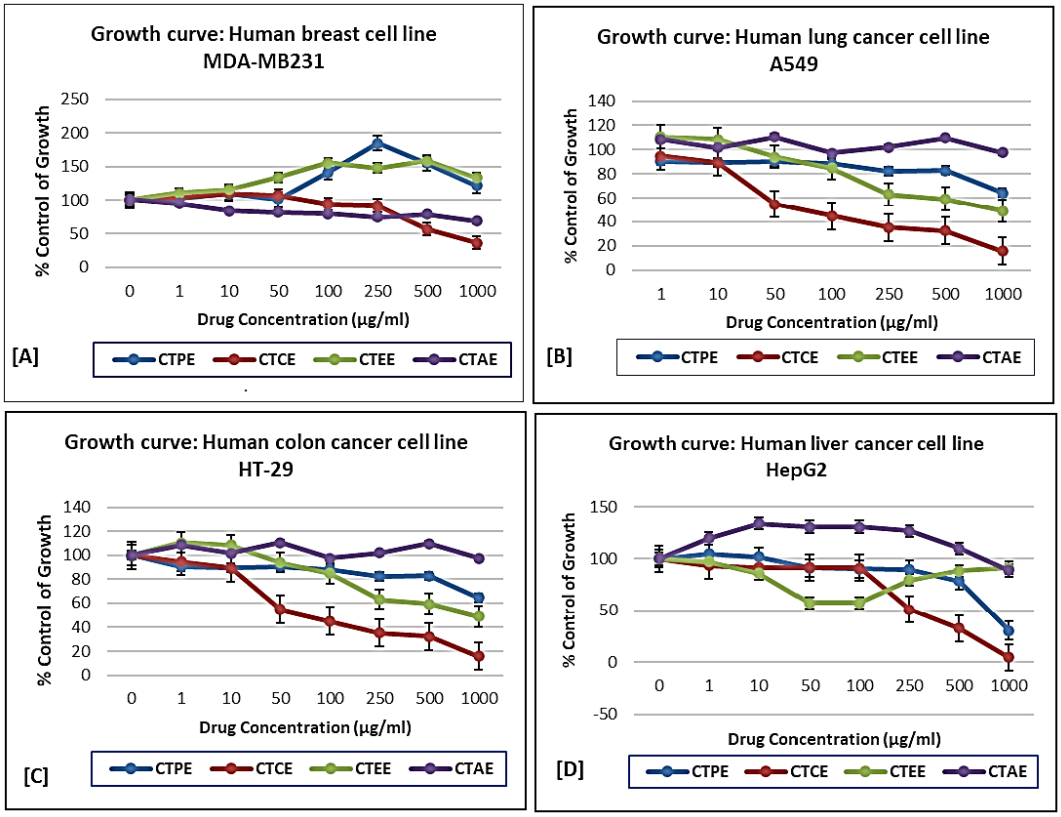

Figure 1: Cytotoxicity of *C. tamala* extracts in different cancer cell lines: (A) MDA-MB-231 (B) A549 (C) HT-29 (D) HepG2. The cells were exposed to different extract concentrations and cytotoxicity was assessed using MTT assay. Values were represented as mean±SEM.

Table 3: Cytotoxicity assay of different solvent extracts of *C. tamala*.

Plant	Cancer Cell lines	Sample name	Cytotoxicity activity IC_{50} (µg/mL)
Cinnamomum tamala leaves	MDA-MB-231	CTPE	>100
		CTCE	>100
		CTEE	>100
		CTAE	>100
		DTX	<10
	A549	CTPE	>100
		CTCE	>100
		CTEE	>100
		CTAE	>100
		DTX	<10
	HT-29	CTPE	>100
		CTCE	26.15
		CTEE	36.48
		CTAE	>100
		DTX	<10
	HepG2	CTPE	>100
		CTCE	>100
		CTEE	76.36
		CTAE	>100
		DTX	<10

All values are represented as mean±SEM of experiments carried out in triplicates (n=3). **IC_{50}:** (Half maximum inhibitory concentration) is the drug concentration that results in a 50% reduction in cell viability or growth. **DTX:** Docetaxel was used as Standard.

an antioxidant. The reduction in DPPH is an indicator of antioxidant activity. Plant materials possessing antioxidant compounds can quench the free radicals, visualized by colour change.[24] The findings showed that the CTCE extract contains active components, which have antioxidant activity and block free radicals. Antioxidants help to enhance the immunity and reduce the reactive oxygen species thus reducing the likelihood of developing some degenerative diseases and cancer.[27,28]

GC-MS of the chloroform extract of *C. tamala* leaves

GC-MS analysis was used to investigate the potential chemical components from the chloroform extract of *C. tamala* leaves (CTCE). The analysis revealed 50 constituents in the chromatograms (Figure 3(a)), with various peaks corresponding to each molecule present in the extract. The predicted compounds, Campesterol and γ-Sitosterol, with reported biological activities were identified by positive ionisation mode GC-MS analysis. Mass spectrum and structure of the identified phyto-components are also shown (Figure 3(b)) and (Figure3(c)).

DISCUSSION

Cancer is posing a threat to humans due to several environmental and genetic factors. The available drugs along with providing some relief in cancer also lead to liver or kidney damage, limiting their use in cure. Moreover, a single drug is not effective for cancers of different origin. In such a scenario the attempts to develop drugs with least adverse effects and maximum benefits is the need. Nature embraces many phytoconstituents as secondary metabolites that possess many therapeutic and beneficial effects. The diverse plant constituents have been used for ages in the treatment regime of several countries according to their habitat. In this continuation, a wide variety of plants have been investigated for their potential as anticancer drugs and have shown promising results. Around one-third of globally derived drugs from plant sources, comprising about 3000 species, have been recognized with anticancer potential.[29] The goal of this study was to investigate *Cinnamomum tamala* leaves for their potential benefits and therapeutic uses in different types of cancer cell lines. Previous studies with other solvent extracts and other plant parts have shown significant anticancer activities. The current work was drafted to explore the phytochemicals, antioxidant and anticancer activity of *C. tamala* leaves and active molecule identification from the most effective fraction by GC-MS.

The phytochemical screening showed the presence of alkaloids, carbohydrates, steroids/terpenoids and flavonoids in the various extracts. The yield was also calculated for each extract, with ethanolic extract having the highest (3.85% w/w) while least yield was found in ethyl acetate fraction (1.15% w/w). This shows the

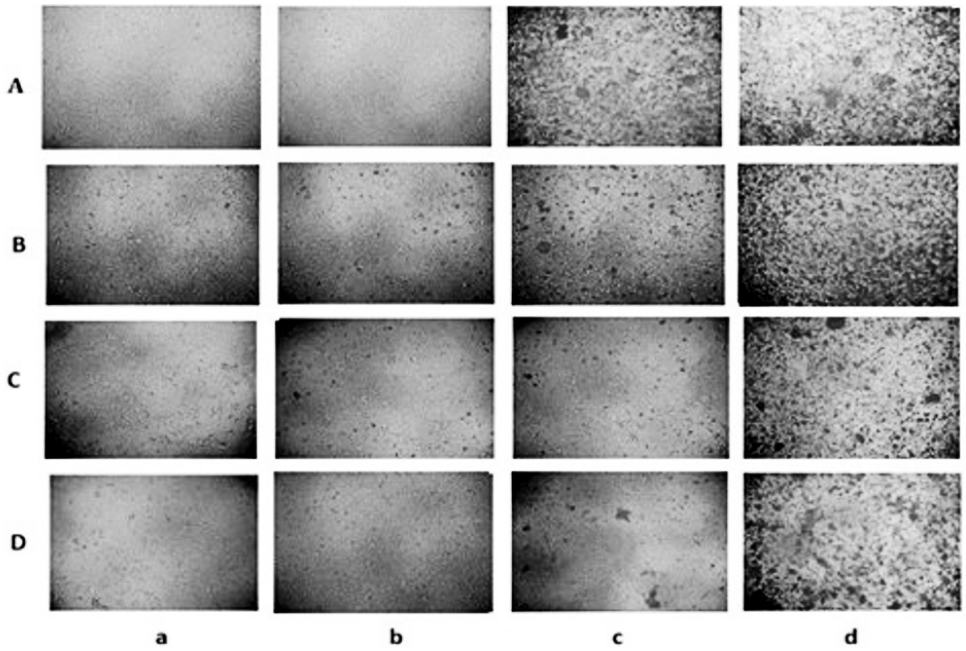

Figure 2: Microscopic images of [A] MDA-MB-231 (breast) [B] A549 (lung) [C] HT-29 (colon) [D] HepG2 (liver) cancer cells after treatment with *C. tamala* chloroform extract (CTCE) showing (a) Control cells (b) treatment with 10 µg/mL (c) treatment with 50 µg/mL (d) treatment with 500 µg/mL. At 24 hr treatment, morphological changes were seen under a phase-contrast microscope. Cells treated with CTCE showed some change in morphology and also reduced the proliferation rate of cancer cells in dose- and time- dependent manner.

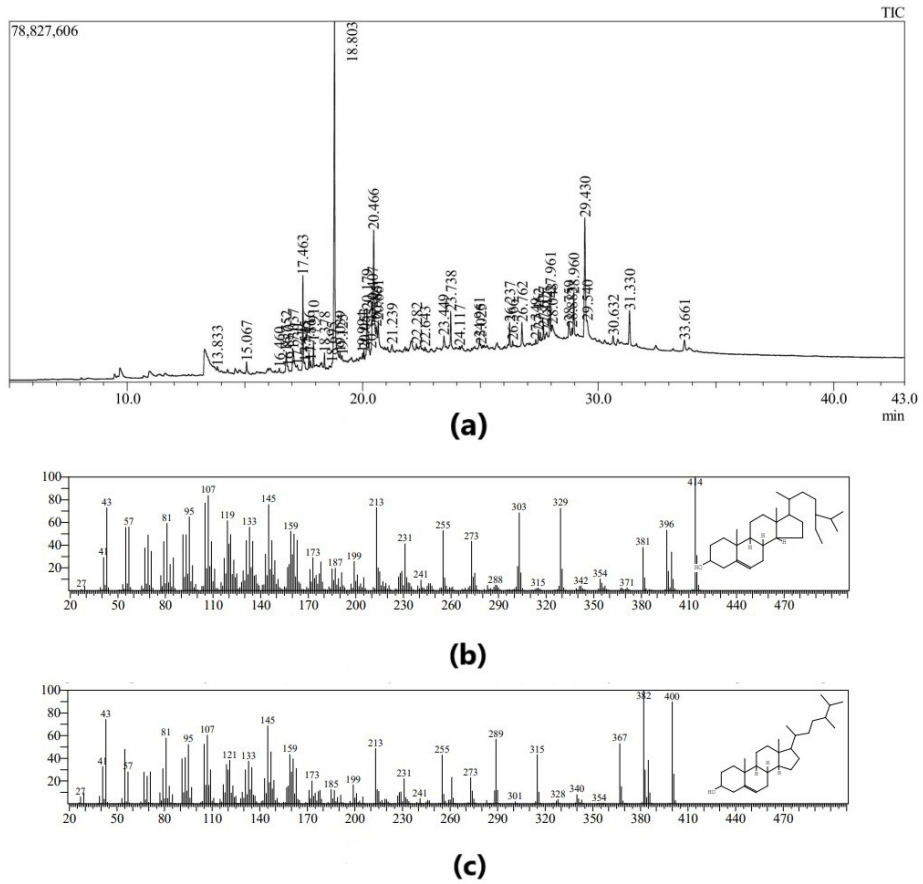

Figure 3: GC-MS analysis of chloroform extract (CTCE) of *C. tamala*. (a) Total ion current (TIC) chromatogram (b) Mass spectrum and structure of predicted molecule (γ-Sitosterol) with m/z: 329.30 *(c)* Mass spectrum and structure of predicted molecule (Campesterol) with m/z: 315.25.

solubility of particular phytochemicals in respective solvents. The collected extracts were later evaluated for the cytotoxic activity in cell lines (MDA-MB-231, A549, HT-29 and HepG2). The CTCE showed reduction in cancer cell growth in the HT-29 cell line with IC_{50} 26.15 µg/mL which was most significant among all other extracts. The CTEE was also found to exhibit cytotoxic activity with IC_{50} 36.48 µg/mL in the same HT-29 cell line. The cytotoxic activity depended on dose and exposure time of the extracts. The cytotoxicity on other cell lines either produced no effect or a higher dose was required for the significant effect. The CTCE presented better efficacy in HT-29 cell lines and was further evaluated for its antioxidant activity. The DPPH method was used for the analysis with ascorbic acid as reference. The IC_{50} value of 46.31±0.15 µg/mL was found for CTCE as compared to 27.38±0.16 µg/mL of the reference. These results indicated significant antioxidant capacity of the extract.

The cytotoxic and antioxidant studies proved CTCE to be a better extract, so GC-MS analysis was utilized to explore the active components present in it. According to the GC-MS interpretation, the predicted molecules, Campesterol and γ-Sitosterol were detected known to possess anticancer activity.[30-32] They belong to the class of phytosterols occurring naturally in plants. Both molecules are present in some dietary plants and provide beneficial effects when consumed in regular diets.[32] One of the predicted molecules, γ-Sitosterol acts by inhibiting the cell signalling molecule mTOR/Akt and can result in apoptosis or cell death.[31] Another identified molecule, Campesterol, is also known to have cytotoxic effects and is of therapeutic importance.[32] Thus, it may be concluded that the presence of these compounds may be the cause of the cytotoxicity that we observed on cancer cell lines in our study. Further studies for isolation of the specific molecules that are causing the cytotoxic action and also animal studies for making the research more lucid, the work is in progress in our laboratory.

CONCLUSION

The study demonstrated that among the various extracts of *C. tamala* leaves, chloroform extract possesses higher cytotoxicity particularly in colon cell lines and also shows good antioxidant activity. The plant leaves showed the presence of some phytoconstituents that have proven to be effective in some cancer models in other studies. The GC-MS of chloroform extract showed the presence of active constituents known to possess cytotoxic activity indicating it be a promising source of anticancer drugs in future and explore more areas for the studies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

C. tamala: *Cinnamomum tamala*; **CTPE:** *C. tamala* petroleum ether extract; **CTCE:** *C. tamala* chloroform extract **CTEE:** *C. tamala* ethyl acetate extract **CTAE:** *C. tamala* alcoholic extract; **GC-MS:** Gas Chromatography-Mass Spectroscopy; **TIC:** Total ion current.

SUMMARY

The study aimed to evaluate the cytotoxic activity of various solvent extracts of *Cinnamomum tamala* leaves on some cancer cell lines. The plant is known to possess various medicinal properties owing to the presence of a wide variety of phytoconstituents in form of secondary metabolites such as phenolics, mono and sesquiterpenes and flavonoids. The extracts were analysed for their cytotoxic effect on liver, breast, colon and liver cell lines. The chloroform extract (CTCE) showed the most significant IC_{50} value on colon cancer cell lines (HT-29). The GC-MS analysis of CTCE showed the presence of several bioactive compounds, among which campesterol and γ-sitosterol are reported to have cytotoxic activity in many studies. Further isolation and *in vivo* studies are in progress in our laboratory.

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