RESEARCH ARTICLE

In-vitro Anticancer Activity of Various Plant Extracts

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ABSTRACT

Herbal medicinal plants have been explored for a variety of pharmacological properties but still a large number of phytoconstituents are still unexplored. The manuscript aims to assess the anticancer property of *Leucas cephalotes* (Roth) Spreng*, Acalypha indica* L. and *Lantana camara* L. extracts on MDA-MB-231, A-549, PC-3 and Hep-G2 cell lines. The ethanol extract of the selected plants was explored for anticancer activity by SRB assay. Since *L. camara* exhibited promising activity, this plant's successive extraction in different extracts (n-hexane, chloroform, ethanol and hydroalcoholic) was further studied. All the extracts were screened for anticancer activity by SRB assay on MDA-MB-231 and A-549 cell lines. Additionally, the apoptotic assay was carried out on n-hexane and chloroform extracts on A-549 cell lines. GCMS also analyzed both extracts for characterization of the phytoconstituents. Obtained data indicate that n-Hexane and chloroform extracts of *L. camara* showed promising anticancer activity on MDA-MB-231 and A-549 cell lines. Both extracts also induced apoptosis in A-549 cell line. GC-MS data of *L. camara* extracts revealed the presence of 25 and 19 compounds in n-hexane and chloroform extract, respectively. The present study shows that *L. camara* has strong anticancer properties against lung cancer cell lines compared to breast, prostate, and hepatoma cancer cell lines.

Keywords: *Leucas cephalotes* (Roth) Spreng, *Acalypha indica* L., *Lantana camara* L., GCMS, Cytotoxicity.

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INTRODUCTION

The mortality due to cancer has doubled from 1990 to 2016 in the whole world. The incidence of new cases of cancer in India in 2018 was about 1.15 million, which is predicted to double by 2040.[1,2] Several chemotherapeutic agents are being used for the treatment, but the problem of selective toxicity and severe side effects persists. Thus, it is one of India's leading causes of devastating health expenditure. Hence, there is a need to look for alternative medicine which has fewer side effects and lowers financial hardship.

Ayurvedic classic works of literature have evidence of the variety of herbs for the purpose of cancer.[3,4] 80% of the medicine used before the 20th century was obtained from the medicinal plants' leaves, barks and roots. During that time crude botanicals were extracted in fluid like water, milk, and alcohol and the extract was prescribed to the patients. More than 25% of the drugs used are directly derived from plants and the other 25% are natural product models.[5] Natural products like vinblastine, vincristine, taxol, docetaxel and camptothecins are being used in chemotherapy.

India has Ayurveda as the oldest indigenous medicine system of herbal drugs, and most people are still utilizing traditional medication systems.[6] According to Indian Council of Medical Research (ICMR) report about 18% of the public still are prefer using traditional medicine for serious ailments mainly due to lack of side effects and low costs.[7] The plant contains multiple active components which often function synergistically, giving therapeutic benefits and reducing the risk of adverse effects.

L. cephalotes (Roth) Spreng is a member of the family *Lamiaceae*, a popular ayurvedic herb mostly found in south east Asian country and reported for its antidiabetic, anticancer, antihyperlipidemic, hepato-protective and antioxidant activities.[8,9] Similarly, a different part of *A.indica* L. has been explored to manage different ailments.[10] It possessed a rich source of antioxidants and also exhibited significant anticancer activity.[11] *L. camara* L. belongs to the family Verbenaceae also used to manage various human ailments, including skin-related diseases, malaria, tetanus and cancer.[12,13] The current study aims to carry out the phytochemical investigation of *L. cephalotes, A. Indica* and *L. camara* and their cytotoxicity study on MDA-MB-231 (breast cancer), A-549 (lung cancer), PC-3 (prostate cancer) and Hep-G2 (hepatoma cancer) cell lines.

MATERIAL AND METHODS

Plant Collection and Identification

In this study, the plants were selected based on the ethnobotanical knowledge derived from the various database and literature

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Table 1: Samples details of the selected plants were submitted in the herbarium

Plant	*Family*	*Sanskrit Name*	*Part used*	*Voucher Number*
Leucas cephalotes (Roth) Spreng	Lamiaceae	Dronapushpi	Whole Plant	UDPS-Dash-1001
Acalypha indica L.	Euphorbiaceae	Kuppi	Whole Plant	UDPS-Dash-1004
Lantana camara L.	Verbenaceae	Vanacchedi	Whole Plant	UDPS-Dash-1006

related to the traditional application of herbal products. This study selected three different plants to screen different extracts for anticancer activity. The plants *L. cephalotes* (Roth) Spreng*, A. indica* L. and *L. camara* L. were collected in and around Khordha district, Odisha, India. All the plants were definitively authenticated visually by Dr K.B. Satapathy of 'CUTM, Bhubaneswar, Odisha, India'. The specimen voucher was submitted at the herbarium of the Utkal University, Bhubaneswar, Odisha, India' (Table 1).

Physicochemical Parameters

The physiochemical parameters of the air-dried coarse powdered plant material were performed as per the WHO guidelines.[14]

Plant Extract

The whole plant was washed, dried under shade, and converted into coarse powder. The dried powder of each plant was shifted in to the soxhlet apparatus with ethanol as solvent. The obtained liquid extract was concentrated to a solid mass in a rotary evaporator (IKA Model RV 10D S96). The obtained solid mass was stored in desiccators till further use.

After a preliminary cytotoxicity study of ethanol extract of the selected plants, *L. camara* was further extracted successively with solvents viz, n-hexane, chloroform, ethanol and hydroalcoholic (ethanol and water in equal proportion) because the *in-vitro* cytotoxicity data revealed its promising cytotoxic activity against the tested cell lines.

Phytochemical Parameters

The estimation of successive extracts of only *L. camara* L. was carried out for the determination of major phytoconstituents by using the standard protocol.[15]

Cell Culture

MDA-MB-231, A-549, PC-3 and Hep-G2 cells were purchased from 'NCCS, Pune, India'.

Cytotoxicity Assay

The ethanol extract of three selected plants was first subjected to cytotoxicity assay for preliminary screening. Briefly, 90 µL of cell suspension of optimal density were seeded into 96 well culture plates. The working sample of all the plant extracts was prepared by dissolving in dimethyl sulfoxide (100 mg/mL) and further diluted with water to get different working concentrations such as 10 to 80 µg/mL. Each sample (10 µL) were transferred into the plate and incubated. Finally after the drying, sulphorhodamine B (50 µL) solution was transferred into the 96 well plates for the purpose of staining. Acetic acid (1%) was used to remove the unbound dye from the cells. The plate was dried and the stain was then solubilized by adding 100 µL of 10mM trizma base (pH 10.5). The absorbance of samples was determined at 540 nm.

Since *L. camara* exhibited better cytotoxic activity among all three tested plant species, so plant was further extracted successively with various solvents (n-hexane, chloroform, ethanol and hydroalcoholic) and each extract was again tested for cytotoxicity assay by using the same protocol.

Apoptotic Assay

Cytotoxicity data of both extract of *L. camara* showed the maximum value against the cell lines, therefore, the said extracts were further studied for apoptotic assay.

A549 fixed cell lines of lung cancer were seeded and *L. camara* extract (1-µg/mL) was transferred into the seeded cells for the exposure time of 2 hours. The fluorescence dye was used for the determination of apoptosis extent. The cell was washed thrice to remove excess of plant extract. Fluorescence images were captured by using a confocal microscope (Leica TCS SP5, Germany, Ex: 488 nm, Em: 525 nm). Total 10 different field analyses were done and fluorescence images of the cells were taken through the FITC channels.

Cell Morphology Study

For the determination of effect of plant extracts on cell morphology, A549 cells were used and treated with either dimethyl sulfoxide (DMSO, as control) or both of the plant extract (n-hexane and chloroform extract). The treatment dose of both the plant extract was similar to the *in-vitro* cytotoxicity study. After 24 hours of incubation, the morphological images of cell lines were captured using a contrast microscope. Obtained images were compared with the post-treatment images of cells.

GC-MS Analysis of *L. camara* Extracts

The study was performed using a Scion 436-GC (Bruker) coupled with a mass spectrometer (TRACE 1310/ISQ–LT; Thermo Scientific) equipped with a BR-5 MS capillary column. The subsequent conditions were used: flow rate 1.0 mL/min, oven temperature 110 to 280°C rate-10°C/min. For the detection of metabolites, NIST reference mass spectral library having more than 62000 patterns, was used.[16]

Statistical Analysis

All obtained data were recorded as mean value ± SD. ANOVA was used to evaluate the significant difference between the data by using Graph Pad Prism 8(San Diego, CA). This study's significance level was 5% with a value of $p<0.05$.

RESULTS

Physiochemical Parameters

Various physicochemical parameters of plant powder such as the loss on drying, ash values and extractive values

Table 2: The physiochemical analysis of *L. camara* powder

S. No	*Tests*	*Observation (%)*
1	Description	Pale green coloured dried coarse powder
2	Loss on drying at 105ºC	1.75
3	Total ash	7.36
4	Acid insoluble ash	1.48
5	Water soluble ash	0.84
6	Sulphated ash	2.13
7	Extractive value (n-Hexane)	8.76
8	Extractive value (Chloroform)	9.56
9	Extractive value (Alcohol)	11.02
10	Extractive value (Water)	14.32

were observed in the present study. The obtained data are represented in Table 2.

The percentage yields of ethanol extract of plants L. *cephalotes* (Roth) Spreng*, A. indica* L. and *L. camara* L. were found at 8.65, 9.37. 8.04 and 7.48, respectively.

Qualitative Analysis

The qualitative analysis of L.*camara* indicates the incidence of diversity of phytoconstituents like proteins, amino acids, carbohydrates, alkaloids, flavonoids and steroids in n-hexane, chloroform, ethanol and hydro alcohol extracts, Phenol and tannins were observed only in ethanol and hydro alcohol extract and saponins, phytosterols and glycosides were absent in all the extracts.

In-vitro Cytotoxic Assay

The assay was conducted to determine the potential cytotoxic activity of compounds affecting basic cellular functions. The current study was in accordance that ethanol extract of *L. cephalotes* and *A. indica* did not produce a significant effect on MDA-MB-231 and A-549 cell lines whereas *L. camara* exhibited a significant effect at 80 ug/mL (Figure 1) in both the cell lines. Among the three plants, *L. camara* showed a significant effect even at the lowest concentration (10 µg/mL) whereas the other two species have cytotoxic effects only at higher concentrations (80 µg/mL) (Figure 1). In PC-3 cell lines none of the plant extract exhibited a cytotoxic effect at any concentration. However, the response of *L. camara* was comparatively better at higher concentrations. In Hep-G2 cell line, only *L. camara* showed a cytotoxic effect at 80 µg/mL (Figure 1).

Further, in order to evaluate the cytotoxicity of *L. camara*, various successive extracts (n-hexane, chloroform, ethanol and hydroalcoholic) were tested on MDA-MB-231 and A549 cell lines. The test result revealed that n-hexane and chloroform extracts were effective against both cell lines (Figure 2). However, both extracts were more effective against the A549 cell lines.

L. camara Extract Induces Apoptosis

Since both the extracts of *L. camara* show the maximum outcome on A549 cell lines, therefore the ability of these

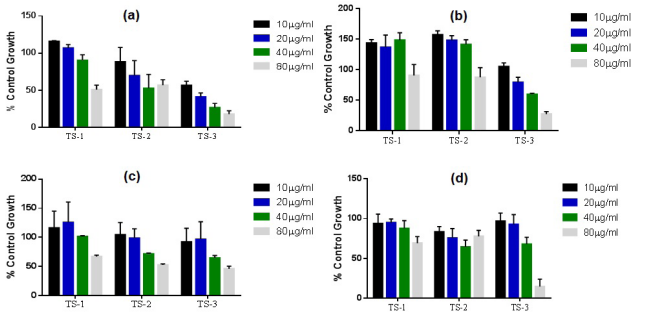

Figure 1: *In-vitro* cytotoxic activity of *L. cephalotes* (TS-1), *A. indica* (TS-2) and *L. camara* (TS-3) against MDA-MB-231 (a), A549 (b), PC-3 (c) and Hep-G2 (d) cell lines.

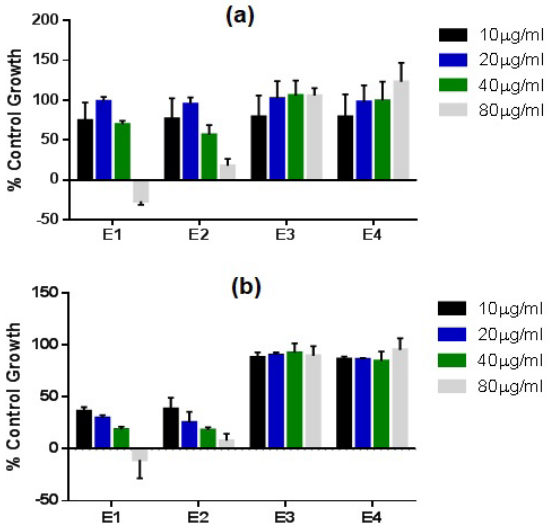

Figure 2: *In-vitro* cytotoxic assay of *L. camara* against MDA-MB-231 (a) and A549 (b) cell lines using different extraction solvents. (E1-n-Hexane, E2-Chloroform, E3-Ethanol and E4- Hydroalcohol).

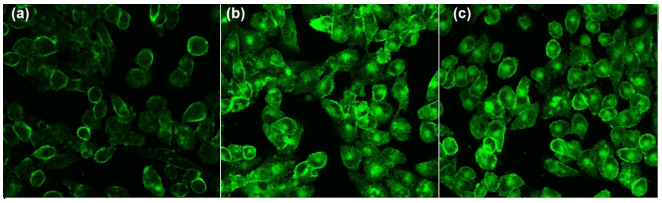

Figure 3: Fluorescent microscopy image of control (a), n-hexane extract (b) and chloroform extract (c) of *L. camara* inducing apoptosis in lung cancer cell line (A549).

extracts was checked to induce apoptosis. Both these extracts were observed to be effective in inducing apoptosis (green color) in the lung cancer cell line. However, apoptosis in the control group was not observed (Figure 3).

Cell Morphology Study

The obtained contrast microscope images clearly revealed the morphological changes in the A549 cell lines after the treatment with both *L. camara* plant extracts at all the treatment concentrations. The cells were shrinking and the sizes of cells were reduced as compared to the DMSO exposure (Figure 4).

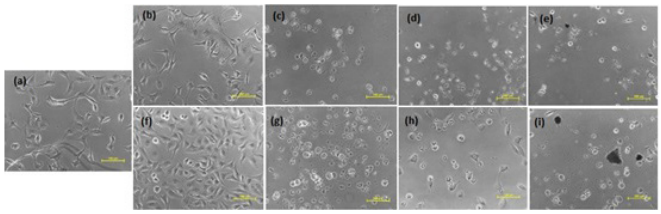

Figure 4: Cell morphology study after the treatment with DMSO (a), n-hexane *L. camara* extract at 10 (b), 20 (c), 40 (d), 80 µg/mL (e), chloroform *L. camara* extract at 10 (f), 20 (g), 40 (h), and 80 µg/mL (i).

Identification of Plant Metabolites by GC-MS

GC-MS studied the cytotoxic effects of *L. camara*'s N-hexane and chloroform extracts to characterize the various phytoconstituents. As 25 different compounds were found in n-hexane extract, including 1b,5,5,6a-tetramethyl-octahydro-1-oxa-cyclopropa[a]inden-6-one,cholestan-3-ol,2-methylene(3β,5α),2-methylenecholestan-3-ol, retinal, cis-13-eicosenoic acid etc. Similarly, 19 compounds were identified in chloroform extract which are Methane-oxybis-dichloro, phenol, 2,4-bis(1,1-dimethylethyl)-, cholestan-3-ol, 2-methylene-, (3β,5α)-, 2H-Pyran, 2-(7-heptadecynyloxy) tetrahydro, 2,5-octadecadiynoic acid, methyl ester etc. The GC-MS chromatograms of both the extracts are furnished (Figure 5). Possibly these compounds play potential role in the investigational therapeutic value of extracts

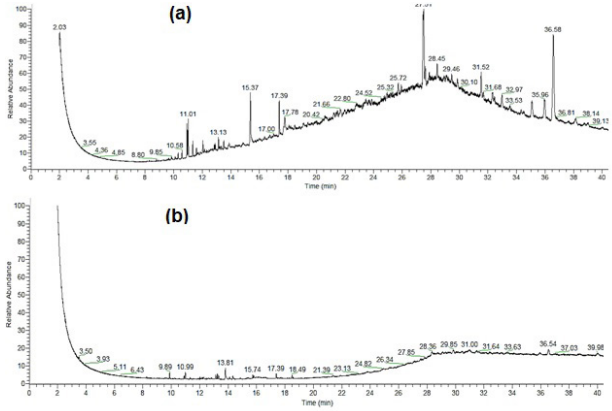

Figure 5: GC-MS analysis of *L. camara* extracts.

DISCUSSION

L. camara is an ornamental plant that belongs to the Verbenaceae family and has anti-cancerous, antihypertensive, anti-inflammatory, and antimicrobial properties.[17] According to the published report the ethanol extract of *L. camara* possess a cytotoxic effect against human lung cancer cell lines (A-549).[18] In the current work, the anticancer activity of *L. camara* extracts was observed on MDA-MB-231, A-549, PC-3 and Hep-G2 cell lines also. A successive extraction study further confirmed that among all the extracts, n-hexane and chloroform extracts showed a promising effect on MDA-MB-231 and A-549 cell lines even at the lowest concentration (10 µg/mL). The result of the apoptosis assay revealed that the extracts were effective in inducing apoptosis in A549 cell line. The result of the cytotoxic assay suggests the cancer-preventive effect of the plant extracts on A549 cell line.

GC-MS study of both extracts of *L. camara* has performed the identification of the presence of different phytochemicals. The study revealed the presence of caryophyllene oxide-hexadecanoic acid, β-sitosterol and 1-monolinoleoylglycerol trimethylsilyl ether in n-hexane extract, which has been reported to be anti cancerous in nature. Caryophyllene oxide is endowed with anti-cancerous, antioxidant, antiviral and analgesic activities. Caryophyllene oxide exhibits a cytotoxic effect against the majority of cancer cells by altering several pathways like MAPK, PI3K/AKT/mTOR/S6K1 and STAT3.[19] Keawsa-ard S *et al.* have reported anti-cancerous activity of n-hexadecanoic acid against human breast cancer, lung cancer, and oral cancer cell lines.[20] N-hexadecanoic acid strongly binds to DNA topoisomerase-I thus inhibiting the proliferation of cells.[21] Apart from its anti-cancerous effect, n-hexadecanoic acid also acts as a hypocholesterolemic, antioxidant, pesticide, nematicide and lubricant.[22] The anti cancerous property of β-sitosterol on cancer cells is owing to its ROS scavenging characteristics.[23] The anti cancerous effect of the compound has also been observed on multiple types of cancer cells.[24] *In-vitro* study of β-sitosterol on breast cancer cell line showed G0/G1 cell cycle arrest.[25] 1-monolinoleoylglycerol trimethylsilyl ether has an anticarcinogenic effect against hepatic cancer cell line.[26]

In the present study, n-hexane extract showed significant targeted growth inhibition on lung cancer cell line (A549) in comparison to chloroform extract, which is well evidenced from SRB assay and apoptosis data. GC-MS study of n-hexane extract revealed the availability of several phytochemicals having potential anticancer properties. Therefore, it may be concluded that the anticancer effect of n-hexane extract may be attributed to the presence of the constituents viz., caryophyllene oxide, n-hexadecanoic acid, β-sitosterol and 1-monolinoleoylglycerol trimethylsilyl. The study also suggests that *L. camara* can act as a source of potential anti-cancerous agents.[27]

CONCLUSION

The present study shows that *L. camara* has strong anticancer properties against lung cancer cell lines compared to breast, prostate, and hepatoma cancer cell lines. Along with cytotoxic properties, *L. camara* also induces apoptosis. GC-MS analysis has helped identify the phytochemicals present in n-hexane and chloroform extracts. Caryophyllene oxide, n-hexadecanoic acid, β-sitosterol and 1-mono-linoleoyl glycerol trimethylsilyl present in n-hexane extract has been reported to have anticancer properties. Therefore, *L. camara* can be considered as a potential source of anti-cancerous agents for lung cancer treatment. However, the anticancer activity of *L. camara* plant extract should be validated in a suitable animal model.

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determined against various human cell lines by the Advanced Center for Treatment, Research & Education in Cancer, and GCMS analysis was performed by the State Pollution Control Board in Bhubaneswar, Odisha.

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