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Original Article

Biotechnology Section

In-vitro Study of Macelignan as a Potential Anticancer Drug against Colorectal Cancer using HCT116 Cell Line

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BY-NC-ND

ABSTRACT

Introduction: Many recent studies have shown that lignans from many plant sources have an effective impact on cancer treatment and it is evident that many medicinal plants are rich in lignans. Genus *Leucas* is known for its medicinal use and is rich in lignans. Macelignan a polyphenolic derivative might play significant roles as clinically useful anticancer agents in treating Colorectal Cancer (CRC).

Aim: Isolation, characterisation and pharmacological profiling of bioactive compound lignan from *Leucas aspera* and *Leucas cephalotes* and to assess the anticancer potential using in-vitro methods using Human Colorectal Cancer (HCT116) cell lines.

Materials and Methods: This in-vitro study was conducted from August 2018 to January 2020 at The Oxford College of Engineering in Bengaluru, Karnataka, India. Anticancer potential of Macelignan was evaluated through 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Reactive Oxygen Species (ROS) measurement, cell cycle study, apoptosis analysis, and gene expression studies. One-way Analysis of Variance (ANOVA) was performed for the total phenolic content estimation and the results were expressed as mean±SD with n=3 trials.

Results: The MTT assay result indicated that macelignan has an IC50 value of 22.8 µM with 73% of cells showing inhibition, ROS production was enhanced 2.5-fold at a maximum concentration at 100 µM. Macelignan (12.5 µM and 25 µM) significantly prevented cell growth in G0/G1 and G2 phases of the cell cycle, while the apoptotic study showed that 12.5 µM and 25 µM macelignan induced early and late apoptosis in HCT116 cells with 21.28% and 19.17%, 21.54 % and 29.02% apoptosis at cellular level, respectively. This set of tests sought to examine the effect of macelignan on the *Caspase 3* gene expression in HCT116 cells by semi-quantitative Polymerase Chain Reaction (PCR). The study showed that *Caspase 3* expression was upregulated up to 1.98 and 2.87 folds when treated with macelignan.

Conclusion: The macelignan could serve as a potent drug derivative for the treatment of colon cancer with further study on the mechanism of action, structure-activity relation, toxicity profiling, bioavailability.

Keywords: Apoptosis, *Caspase 3*, Cell growth, Cytotoxicity, Human colorectal cancer, Reactive oxygen species production

INTRODUCTION

Cancer is one of the most serious health issues affecting the length and quality of human life and has been classified as one of the deadliest diseases affecting mankind worldwide. Limited success has been witnessed even with enormous effort put to cope with the disease. Since conventional therapeutic strategies do not meet the essential requirements for successful cancer therapy, the use of natural bioactive compounds isolated from medicinal plants such as *Leucas aspera* as anticancer agents has emerged as an alternative safe, inexpensive, and convenient method. Four of the most common cancers are lung, breast, prostate, and colon cancer [1].

Colorectal Cancer (CRC) is predominantly the 2nd most fatal cancer and the 3rd most widespread malignant tumour globally. A 2018 survey reported 1.8 million new CRC cases and 881,000 deaths, accounting for nearly 10% of new cancer cases and deaths on the global death scale [2]. Generally, CRC is characterised as an uncharacteristic growth on the internal lining of colon epithelial cells that are surgically removed upon early diagnosis [3]. The existing treatment for CRC consists of chemotherapy with solo drug fluoropyrimidine and numerous agent regimes including capecitabine, oxaliplatin, and irinotecan [4].

Leucas aspera Linn. (*L.aspera*) is a widely distributed herbaceous plant across the Indian subcontinent, which belongs to the family *Lamiaceae* and is an annual, branched plant. The taxonomic classification and anatomy of this plant are well documented through many research studies [5-8]. The plant is known to contain many potent metabolites like triterpenoids, oleanolic acid, urosolic acid, β-sitosterol, nicotine, sterols, glucoside, diterpenes, and phenolic compounds [8]. Indian traditional medicines Ayurveda and Siddha use this plant. The plant is reported to have pharmacologic activities like carminative, antihistaminic, antipyretic, and antiseptic. It is used to treat diseases like jaundice, anorexia, dyspepsia, fever, helminthic infestation, respiratory and skin diseases [9].

From the study on green synthesis of silver nanoparticles of *L.aspera*, it has been reported that these plants serve as potent herbs [10], and have been reported to contain many phytotoxic components [11].

Leucas cephalotes another species commonly called "Dronapushpi" in Sanskrit belonging to the family *Lamiaceae* as *L.aspera*, is a weed and grows in monsoon. Reportedly, two protostane-type triterpenoids named leucastrins A and B and oleanolic acid were isolated from *L.cephalotes* [12]. Triterpenoids β-sitosterol [13], stigmasterol [14], lupeol [15,16], labellinic acid isolated were also reported from *L.cephalotes* [17]. Aliphatic esters [18], essential oils [19,20], flavones [20] were other metabolites that have been successfully isolated from *L.cephalotes.*

Macelignan, a class of secondary metabolite classified as phytolignan, a polyphenolic derivative might offer new anticancer therapeutic ability and play significant roles as clinically useful anticancer agents in treating CRC [21]. In this context, the present study was undertaken to evaluate the anticancer efficacy of the macelignan isolated from *L.aspera* and *L.cephalotes* on CRC cell line HCT116, and a sincere attempt was made to provide scientific validation for the role of macelignan as a therapeutic lead molecule in treating CRC.

MATERIALS AND METHODS

This in-vitro study was conducted from August 2018 to January 2020 at the Oxford College of Engineering, Bengaluru, Karnataka, India. The experiment was carried out under a controlled laboratory environment, at the approved research centre and, this does not require ethical approval from regulatory committees.

Study Procedure

Collection of plant sample: The plants of *L.aspera* and *L.cephalotes* were collected from the Western Ghats region and Turahalli Forest of Karnataka, India. Taxonomical identification was performed and the samples were deposited with the Research and Development Centre, Department of Biotechnology, Oxford College of Engineering.

Preparation of plant extracts: Whole plants of *L.aspera* and *L.cephalotes* were washed, dried in shade, and pulverised with a blender. 100 gm of defatted plant residues were subjected to cold hydroethanolic extraction in ethanol: water (ratio 3:1), the samples were left to stand overnight and the extract was collected by vacuum filtration. The extract was concentrated and vacuum dried using a rotary flash evaporator (IKA, Germany).

Column chromatography: The polyphenols of both samples were separated using Sephadex LH-20 column chromatography. The column was mobilised by adding homogenised gel with a methanol solution concentration of 99.9%. Fractions containing polyphenols were pooled and subjected to High Performance Liquid Chromatography (HPLC) for purity [22].

Estimation of total phenolic content by Folin-Ciocalteu (FC) method: Phenolic content present in the extract reacts with the phosphomolybdic acid present in the FC reagent and forms a blue complex (molybdenum blue). Aliquots of the gallic acid standard (0.2-1.0 mL) and simultaneously 0.1 mL of crude extract and a sample eluted from column chromatography were taken and methanol was added to all tubes to increase the volume to 3.0 mL. Total 0.5 mL of Folin-Ciocalteu reagent was added to tubes and incubated for six minutes at Retention Time (RT) and 1.0 mL of 20% Na_2CO_3 was added and incubated for 1 hour in the dark. The developed blue colour was read at 650 nm. Gallic acid was used as the standard and total phenolics were expressed in Gallic Acid Equivalent (GAE)/100 mg [23,24].

High Performance Liquid Chromatography (HPLC): Sephadex LH-20 Column chromatography was used to separate polyphenols. The column was mobilised by adding homogenised gel at a concentration using 99.9% methanol solution. Polyphenol-containing fractions were combined and submitted to phenolic content estimate and HPLC purity testing (Pharmacia Corporation) to obtain pure lignan using the ODS-reverse phase column [25].

Gas Chromatography-Mass Spectrometry (GCMS) analysis: Gas Chromatography (GC) analysis was carried out using Agilent 6890N GC equipped with photon multiplier tube as detector coupled to front injector type 1079. The chromatograph was fitted with HP 5 MS capillary column (30 m×0.25 mm i.d., film thickness 0.25 mm). The injector temperature was set at 250°C, and the oven temperature was initially set at 70°C, held for 4 minutes then programmed to 200°C at the rate of 10°C/min and finally held at 200°C for 13 minutes. Helium was used as a carrier gas with a flow rate of 1.5 mL/min. 0.2 µL of the sample (diluted with methanol 1:10) was injected in the splitless mode. The mass spectrometer was operated in the electron impact mode at 70 eV. Ion source and transfer line temperature were kept at 250°C. The mass spectra were obtained by centroid scan of the mass range from 50-600 amu. The compounds were identified based on the comparison of their Retention Indices (RI), Retention Time (RT), mass spectra of WILEY, NIST library data of the GC-MS system, and literature data (Robert A, 2005), [25].

Cell lines and culture medium: Stock cells of HCT116 cell lines obtained from American Type Culture Collection (ATCC), were grown in modified Roswell Park Memorial Institute (RPMI) 1640 media with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 µg/ml) to confluent in CO_2 incubator at 37°C. The dissociation solution {0.2% trypsin, 0.02% EDTA, 0.05% glucose in Phosphate Buffered Saline (PBS)} was used to dissociate cell. The cellular viability was checked [26,27].

Cytotoxicity assay: The cytotoxicity activity of crude extract and purified bioactive macelignan was studied by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. HCT116 cell lines cultured at a concentration of 10^5 cells/well were seeded in a 96 well plate and incubated for 24 hours at 37°C. Doxorubicin and Dimethyl Sulfoxide (DMSO) were used as positive and negative controls. Further, the MTT assay kit (Himedia) guidelines were followed. The absorbance was read using a microplate reader at 590 nm [28]. Half maximal inhibitory concentration (IC_{50}) values were generated from the dose-response curves for different concentrations of test drugs [28].

Reactive Oxygen Species (ROS) measurement: Intracellular levels of ROS were determined using the 2', 7'- Dichlorofluorescein Diacetate (DCFDA) method. DCFDA is a ROS-sensitive fluorescence indicator. HCT116 cells at a concentration of 5×10^5 cells/well were seeded in a 96-well titer plate and after 24 hour of incubation 100 µL of test compound with varying concentrations were added and incubated for 24 hour. Treated samples were removed from the plates and washed with 1X PBS to which 100 µL of 25 µM DCFDA solution was added and incubated at 37°C for 30 minutes. The fluorescence was measured using a microplate reader at 485/535 nm [29].

Apoptosis analysis: Apoptosis is a cell death process with morphological and biochemical characteristics that appear at different stages. Apoptosis begins with varying kinetics depending on the cell type and ends with cell disintegration and the creation of apoptotic bodies. Several alterations on the surface of apoptotic cells have previously been identified, including the expression of thrombospondin binding sites, the removal of sialic acid residues, and the exposure of phospholipid-like phosphatidylserine (PS) [30,31]. Annexin V, a member of the annexins family of anticoagulant proteins, has shown to be a valuable tool in detecting apoptotic cells since it preferentially attaches to negatively charged phospholipids such as PS in the presence of Ca^{2+} and has a low affinity to phosphatidylcholine and sphingomyelin [32].

The HCT116 cells were subjected to Flow cytometry by Annexin V- Fluorescein Isothiocyanate (FITC) staining to determine the apoptotic cell distribution. A 1×10^5 cells per well were seeded in a 96 well plate. After 18 hours, the cells were treated with different concentrations of the test sample to induce apoptosis. After 24 hours of incubation, cells were pelleted. The pellet was rinsed in cold PBS and resuspended in 1 mL 1X binding buffer. A 500 µL of cell suspension was aliquoted with 5 µL Annexin V and 10 µL of Propidium Iodide (PI), 15 minutes of incubation was done at RT in dark, followed by flow cytometric analysis [31-33].

Cell cycle analysis: Cell cycle distribution was analysed by flow cytometry using PI staining. Briefly, 1×10^6 cells were seeded and cultured for 24 hours in a 96-well plate with 2 mL serum-free media. Cells were later treated with different concentrations of test compound and incubated for 24 hours. Cells were pelleted and washed in 2 mL of 1X PBS and were fixed in 300 µL of sheath fluid and 1 mL of chilled 70% ethanol was added. Cell pellets were stained with PI (20 µg/mL) in presence of RNase A (0.05 mg/mL) and incubated for 15 minutes in the dark. Determination of percentage of cells in various stages of the cell cycle in treated and untreated populations was done using Fluorescence-Activated Cell Sorter (FACS) Caliber (BD Biosciences, San Jose, CA) [34].

Gene expression studies: About 1×10^6 cells cultured with P35 were subjected to test sample followed with RNA isolation with TRIzol™ Reagent (In-vitro gen,) according to the manufacturer's instructions, and the extracted mRNA was further subjected to Reverse Transcription- PCR (RT-PCR). RT-PCR was carried out (Thermo Fischer VERITI Scientific system) to determine the levels of β-Actin mRNA and *caspase 3* expressions. The cDNA was synthesised from 2 µg of RNA using the Verso cDNA synthesis kit (Thermo Fischer Scientific) with oligo dT primer and the assay was performed as per the manufacturer's instructions [29]. The optimal cycles were selected for amplification of these genes experimentally such that it was in the exponential range and would not reach a plateau. Results were measured quantitatively using the optical density of the bands (computerised imaging program Image J). The values were normalised to β-Actin intensity levels.

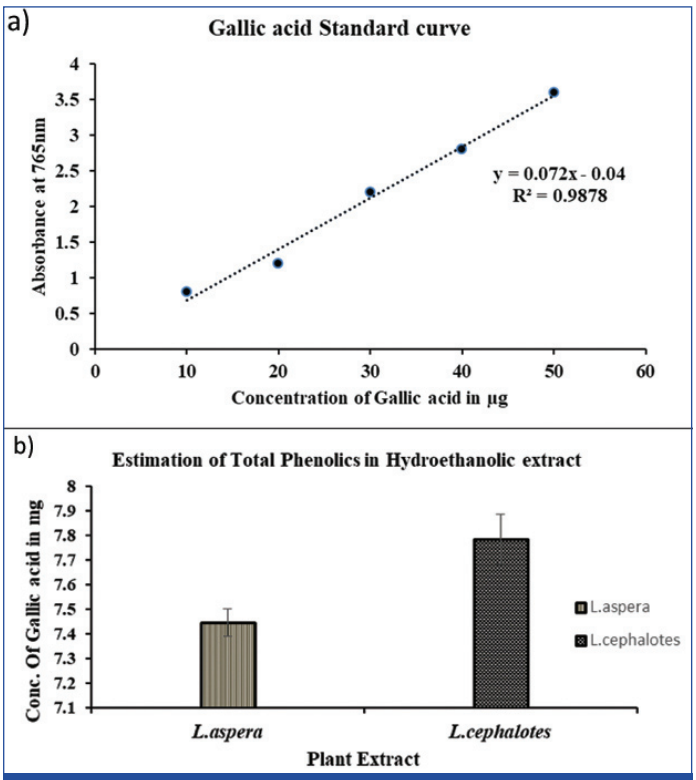
STATISTICAL ANALYSIS

One-way Analysis of Variance (ANOVA) was performed for the total phenolic content estimation and the results are expressed as mean±SD with n=3 trials. And non linear regression analysis using Graph Pad Prism 6 software was performed for the cytotoxicity study and graphical representation of the same has been incorporated in the results.

RESULTS

1. Total Phenolic Content

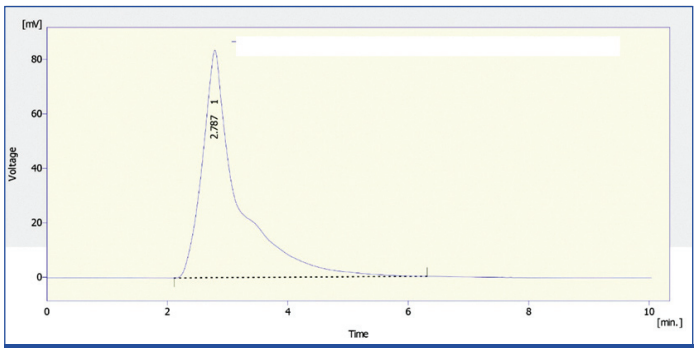
In the present study, on the total phenolic content of *L.aspera* and *L.cephalotes*, *L.cephalotes* showed the highest phenolic content of 7400±152.75 mg of GAE/100 g of plant extract in comparison to *L.aspera* which recorded 7133±152.75 mg of GAE/100 gm of plant extract [Table/Fig-1a,b]. The results of this study reveal that both plants possess a considerable amount of phenolic content that offers potential radical scavenging ability.



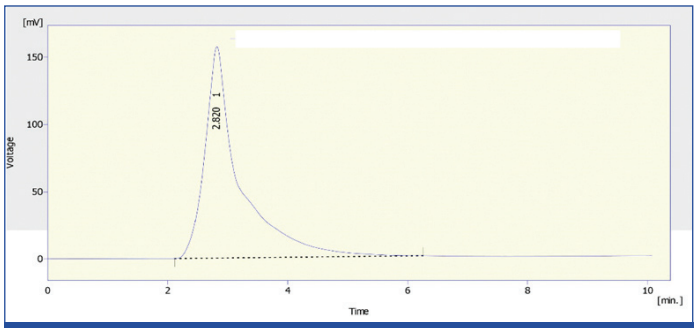
[Table/Fig-1]: a) Gallic acid standard curve for estimation of total phenolic content. b) The total phenolic content by FC method for hydroethanolic plant extract is statistically significant with p-value <0.05 and data expressed in mean±SD of 3 trials.

2. High Performance Liquid Chromatography

This analysis spectra of an eluted fraction of *L.aspera* and *L.cephalotes* showed the bioactive compound with different RTs as shown in [Table/Fig-2,3]. Various fractions were eluted by column chromatography and based on the chromatogram pattern the fractions were collected with similar Rf values and evaluated by GCMS. The hydroethanolic fraction of *L.aspera* and *L.cephalotes* yielded bioactive compounds when eluted with ethanol:water (3:1). The compounds obtained were fatty acid esters and phenolic derivatives which belong to various classes of flavonoids and lignans [Table/Fig-4-7]. One of the common compounds found in both the samples was Macelignan belonging to the lignan family of polyphenolics which has previously not been reported as isolated from *L.cephalotes* whereas the other compounds have been reported to be isolated from another family of *L.amiaceae*. As macelignan though isolated is the novel compound isolated from *L.cephalotes* which has not formed part of any previous anticancer study, an attempt to purify it was done, and its anticancer properties were evaluated against CRC using the HCT116 cell line.



[Table/Fig-2]: Chromatogram of *L.aspera* peak showing measurement of voltage (mV) vs time (min).



[Table/Fig-3]: Chromatogram of *L.cephalotes* peak showing measurement of voltage (mV) vs time (min).

Compound	Molecular Formula (MF)	Molecular Weight (MW) (gm/mol)	Retention Time (RT) (min)	Peak Area (PA) (%)
1,1-Cyclobutanedicarboxamide, 2-phenyl-N,N'-bis (1-phenylethyl)-	$C_{28}H_{30}N_2O_2$	426.3131	20.62	9.3
Di-(1,3,2)-oxazino (6,5-f:5',6'-H) quinoxaline, 2,3,4,5,6,7- hexahydro-3,6-bis (2-diethylaminoethyl)-10,11-diphenyl-	$C_{36}H_{46}N_6O_2$	86.00	16.17	43.6
Macelignan	$C_{20}H_{24}O_4$	328.00	17.85	21.3
Corynan-17-ol, 18,19-didehydro-10-methoxy-, acetate (ester)	$C_{22}H_{28}N_2O_3$	368.47	19.34	8.4
Cyclopentanepropanoic acid, 3,5-bis (acetyloxy)-2-(3,8-bis (acetyloxy) octyl)-, methyl ester	$C_{25}H_4O_{10}$	500.6	22.18	8.0
4-(4-Diethylamino-1-methylbutylamino)-1,2-dimethoxy-6-bromonaphthalene	$C_{21}H_{31}BrN_2O_2$	423.4	14.28	5.4

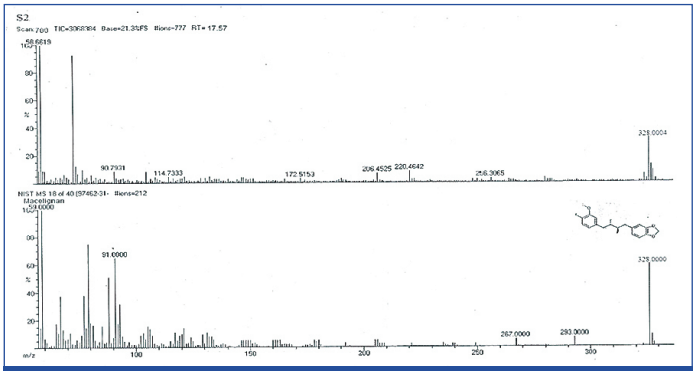
[Table/Fig-4]: Compounds detected in Gas Chromatography-Mass Spectrometry (GCMS) analysis of *L.cephalotes*.

3. Cytotoxicity Test

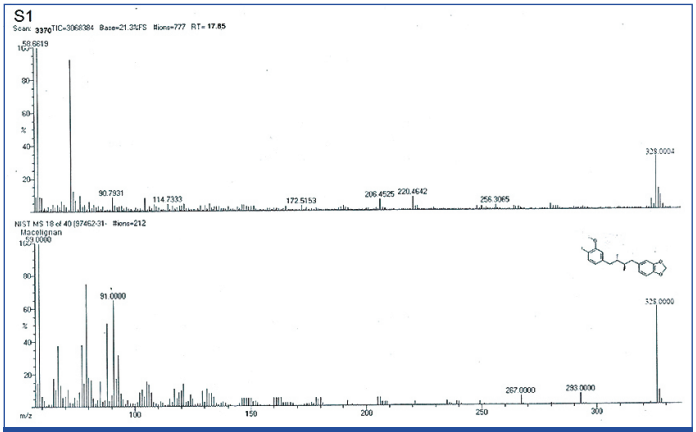
The cytotoxicity of the isolated compound on HCT116 cells was evaluated using the MTT test. The IC_{50} of a drug was ascertained by building a dose-response curve and an examination of the effect of

Compound	Molecular Formula (MF)	Molecular Weight (MW) (gm/mol)	Retention Time (RT) (min)	Peak Area (PA) (%)
Oxirane-2-carboxylic acid,3-(3,4,5 trimethoxyphenyl)-,methyl ester	$C_{13}H_{16}O_6$	268.26	22.55	23.1
Pentadecanoic acid methyl ester	$C_{16}H_{32}O_2$	256.4	16.63	100
Octadec-9-enoic acid	$C_{18}H_{34}O_2$	282.5	18.22	100
Macelignan	$C_{20}H_{24}O_4$	328.4	17.57	21.3
9-hexadecenoic acid	$C_{16}H_{30}O_2$	254.414	20.2	14.9

[Table/Fig-5]: Compounds detected in Gas Chromatography-Mass Spectrometry (GCMS) analysis of *L.aspera*.



[Table/Fig-6]: Gas Chromatography Mass Spectrometry (GCMS) chromatogram of *L. aspera* hydroethanolic extract showing six major peaks of active compounds and a peak at 17.57 indicate macelignan content.

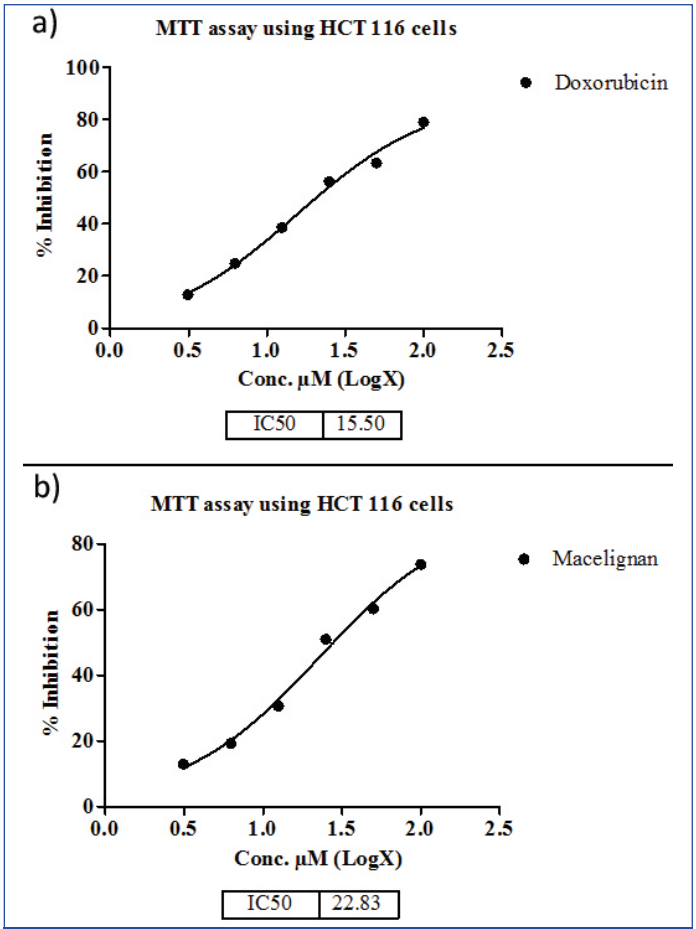


[Table/Fig-7]: Gas Chromatography-Mass Spectrometry (GCMS) chromatogram of *L. cephalotes* hydroethanolic extract showing 5 major peaks of active compounds and a peak at 17.85 indicate macelignan content.

different concentrations of antagonists on reversing agonist activity was made. The IC_{50} value of macelignan was found to be 22.83 µM while that of doxorubicin was 15 µM. From a non linear regression analysis based on the sigmoid dose-response curve (variable), IC_{50} values for cytotoxicity tests were obtained. Statistically, a non linear regression graph obtained using Graph Pad Prism 6 (Graph pad, San Diego, CA, USA) revealed that [Table/Fig-8a,b] about 73% of the cells were inhibited at a maximum concentration (100 µM) by macelignan against doxorubicin which showed 79% inhibition. Therefore, it can be considered that macelignan exhibited a cytotoxicity property as good as the standard drug.

4. Macelignan Exhibited Better Apoptosis Effect and Reactive Oxygen Species (ROS) Measurement

Flow cytometry was used for quantitative measurement of cellular apoptosis following annexin V-FITC/PI double staining. Annexin V was used as a marker to detect early apoptosis and PI to late apoptosis and necrosis. The cells treated with 25 µM of doxorubicin, induced early and late apoptosis in HCT116 cells with 48.43% and 3.71%, respectively as 12.5 µM and 25 µM of macelignan induced early and late apoptosis in HCT116 cells with 21.28% and



[Table/Fig-8]: Non linear regression schematic of cytotoxic effect of: (a) doxorubicin; (b) Macelignan, on the HCT116 cell line treated with varying µM concentrations.

19.17%, 21.54% and 29.02% apoptotic cells respectively. These conclusions are drawn upon observation indicated in the form of the cells scattered in the lower right and upper right region of the quadrant presented in the X-axis, as shown in [Table/Fig-9].

Sample	Percentage of cells at different apoptotic phases			
	Viable cells	Early apoptotic	Late apoptotic	Necrotic cells
Control	98.42	1.16	0.24	0.18
Macelignan 12.5 µM	58.35	21.28	19.17	1.20
Macelignan 25 µM	48.10	21.54	29.02	1.34
Doxorubicin 25 µM	46.20	48.43	3.71	1.66

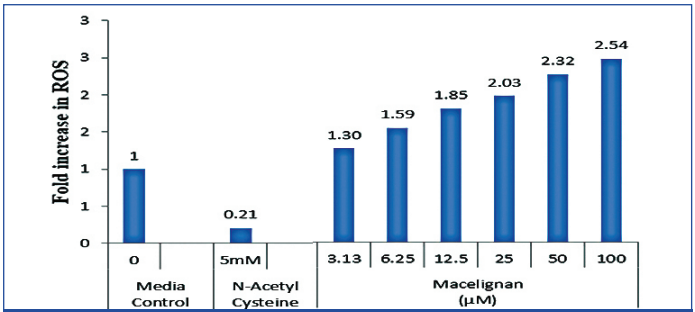
[Table/Fig-9]: Percentage of the cells that has undergone apoptosis in untreated, standard and test samples treated HCT116 cells.

The results clearly show the programmed cell death-induced effect of macelignan was comparatively stronger than the control.

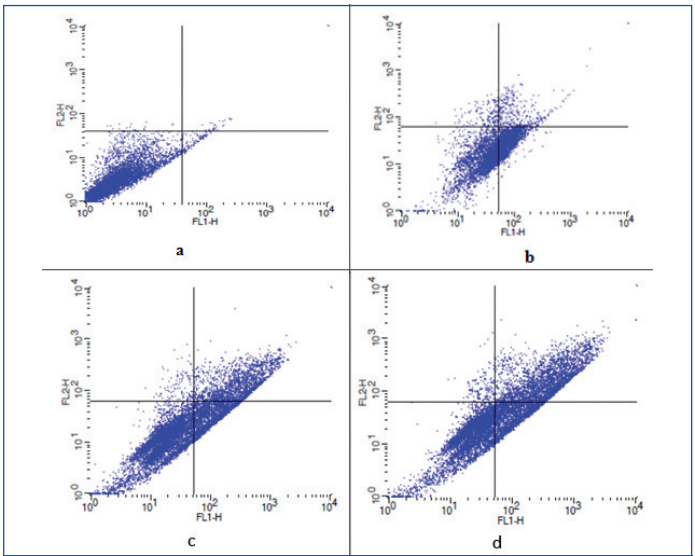
Intracellular ROS plays an important role in mediating cell death. The intracellular ROS levels in the HCT116 cells were measured using the DCFDA method. Measuring the fluorescence in terms of Relative Fluorescence Unit (RFU) at 485/535 nm, revealed macelignan enhanced the ROS production up to 2.5 folds at its maximum concentration (100 µM) in HCT116 cells [Table/Fig-10]. The results obtained indicate elevated levels of ROS which generally is a result of greater mitochondrial membrane potential. From our study, we infer that the 2.5-fold increase of ROS production strongly supports the cancer cell death through ROS dependent mechanism [Table/Fig-11].

5. Cell Cycle Arrest and Gene Expression Studies

The effect of macelignan on cell cycle distribution was calculated using flow cytometry. Macelignan (12.5 µM and 25 µM) stimulated arrest in G0/G1 phase and G2 phase. The percentage of cells arrested at different stages in comparison to standard doxorubicin



[Table/Fig-10]: ROS detection by DCFDA method, the graph illustrates the concentration of Macelignan vs. fold increase in ROS in the cells; culture media and N-Acetyl Cysteine was taken as control.

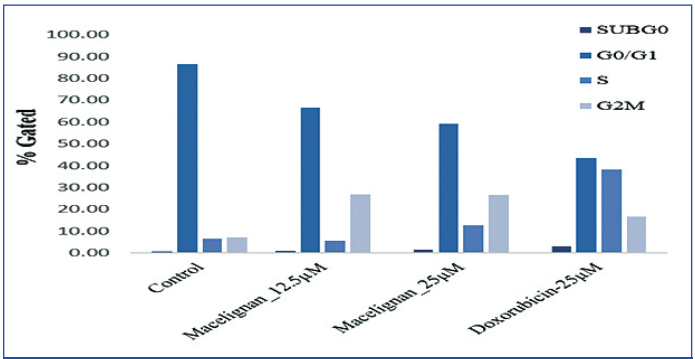


[Table/Fig-11]: Dot plots of HCT116 cells distribution due to apoptosis: (a) untreated cells; (b) treatment with doxorubicin 25 µM; (c) treatment with macelignan 12.5 µM; (d) treatment with macelignan 25 µM.

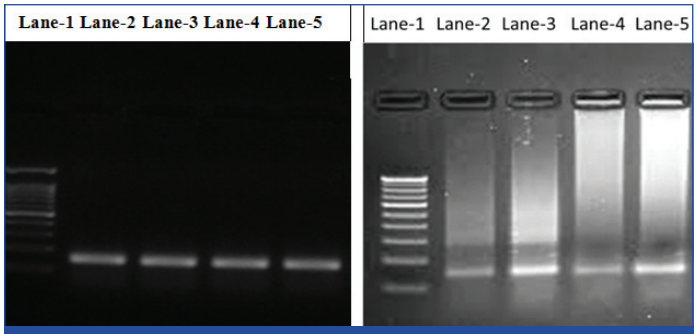
is shown in [Table/Fig-12]. A schematic of overlay representing cell cycle arrest with different concentrations of macelignan along with doxorubicin is indicated in [Table/Fig-13]. There is no direct study investigating the effect of the compound on *caspase 3* gene expression on HCT116, hence the effectiveness of cells was determined by semiquantitative PCR. β-Actin was used as a control to normalise the gene expression. The study revealed that the *caspase 3* expression was upregulated up to 1.98 and 2.87 folds in the treatment of macelignan [Table/Fig-14,15].

Samples	Percentage of cells arrested			
	Sub G0 Phase	G0/G1 Phase	S Phase	G2/M Phase
Control	0.10	86.51	6.53	7.18
Macelignan 12.5 µM	1.06	66.66	5.69	26.83
Macelignan 25 µM	1.68	59.34	12.65	26.70
Doxorubicin	3.07	43.47	38.40	16.83

[Table/Fig-12]: Percentage of cells arrested in HCT116 cells at different stages of cell cycle.



[Table/Fig-13]: Percentage of cells arrested in HCT116 cells at different stages of cell cycle.



[Table/Fig-14]: (i) Amplification of β-Actin gene in HCT-116 cells; (ii) Amplification of *Caspase 3* gene in HCT-116 cells. (Images from left to right).

Samples	Concentrations	Beta-Actin expression	*Caspase 3* expression
		Lane number	
DNA Ladder		1	1
Control	0	2	2
Doxorubicin	25 µM	3	3
Macelignan	12.5 µM	4	4
	25 µM	5	5

[Table/Fig-15]: Samples details in Lane.

DISCUSSION

Since ancient times, plants and their bioactive compounds such as phenols, terpenoids, flavonoids, sterols, and coumarins are in medicinal practices and several phytoconstituents from medicinal plants species inhibit the progression and development of cancer. Several isolated phytochemicals such as flavonoids, lignans, terpenes, saponins, vitamins, etc., play a significant role in inhibiting cancer cell-activating proteins, enzymes, and signalling pathways [35]. These phytoconstituents work on various mechanisms of signaling pathways such as apoptosis and cell cycle arrest at G2/M phase, anti apoptotic proteins *c-IAP* and *Bcl-2*, activation of pro-apoptotic protein such as p21, p53, *caspases 3*, and *caspase 9*, induction of ROS, inhibition of topoisomerase-1 and cyclooxygenase, etc., [36,37]. Novel anticancer drugs could, therefore, be useful in ameliorating damage with less severe side effects than the synthetic drugs currently available.

In the present study, a systematic screening approach has been followed to isolate and purify macelignan from *L.cephalotes* and *L.aspera*. The obtained compound thus may behave in a way as to induce apoptosis in cancer cells. Another study looked into the effect of macelignan on P-gp and cytochrome P450 enzyme (CYP3A4)-mediated drug metabolism, with the goal of finding a way to improve the bioavailability of paclitaxel, an anti-neoplastic medicine with anti-tumoural properties [38]. Several researchers have reported that macelignan could possess therapeutic properties and its subsequent isolation and screening from nutmeg have shown it to work as a potent biomolecule against obesity, diabetes and also as an antiaging molecule [39].

Studies by Lee KE et al., have reported macelignan, a natural lignan compound isolated from *Myristica fragrans* Houtt. (nutmeg), to possess antioxidant and anti-inflammatory activities when investigated on mechanisms of action in UV-irradiated human skin fibroblasts (Hs68) by RT-PCR, DCFDA assay, and ELISA [40]. A review summarised by Paul S et al., suggests, that macelignan has been shown to possess a spectrum of pharmacological activities, including anti-bacterial, anti-inflammatory, anti-cancer, anti-diabetes, and hepatoprotective activities; recent studies have also suggested that it could have neuroprotective activities [21].

This study was aimed at isolating and screening macelignan as a potential anticancer drug molecule. The results obtained on the cytotoxic effect of macelignan against HCT116 showed good effect with 73% of the cancer cell inhibition at a maximum concentration of 100 µM, which was significantly compared with standard doxorubicin which showed inhibition of 79%. The effect of macelignan on apoptosis and ROS measurement are also suggestive that, this molecule could potentially induce programmed cell death at 12.5 µM and 25 µM concentrations, which was found to be better than the standard drug. The present study also infers a 2.5 fold increase in ROS production which makes macelignan a favorable bioactive molecule. The present study showed that macelignan at concentrations of 12.5 µM and 25 µM induced cell cycle arrest in the G0/G1 phase and G2 phase, and upregulation of *caspase 3* gene up to 1.98 and 2.87 folds was observed.

Limitation(s)

Though the present study suggests that macelignan could be a lead derivative, as anticancer drug molecule for CRC type. However, further investigations such as in-vivo studies, detection of its bioavailability, preclinical and clinical studies and mechanism of action at molecular level are required to authenticate the claims. Furthermore, the plant under study should be mass produced and macelignan expression has to be enhanced for increased yield.

CONCLUSION(S)

The present study concluded that the isolated natural bioactive compound from the hydroethanolic extraction method from *L.cephalotes* was identified as macelignan based on the gas chromatography mass spectrometry characterisation. Based on the findings of this investigation, it is clear that the medicinal plant in question has a secondary metabolite with therapeutic potential. These plant's phytoconstituent and antioxidant profiles indicate that they can scavenge free radicals generated in the system and so can be utilised in the development of herbal formulations. The pure compound's anticancer potential could be regarded as a novel source for obtaining the lead and developing a new herbal medicine formulation. However, more clinical research on its safety profile, dose, and absorption is needed to aid in the development of natural anticancer medication.

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