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Research Article

EXPLORING THE THERAPEUTIC EFFICACY OF COUROUPITA GUIANENSIS EXTRACTS AGAINST BREAST CANCER: AN INVESTIGATION INTO ANTICANCER PROPERTIES

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ABSTRACT

Couroupita guianensis is a well-known multitherapeutic plant for its antimicrobial, anti-inflammatory, anti-nociceptive, and antioxidant properties. In this study, we investigated the anticancer potential of the crude extracts of leaf and bark of *C. guianensis* on human breast cancer MDA-MB-231 cell lines. The anticancer activity was investigated through MTT assay. Identification and diagnosis of bioactive compounds with anticancer effects were done by LC-MS/MS and NMR spectrometry. The MTT assay revealed that the chloroform and methanol extracts of the leaf have the highest potencial to inhibit MDA-MB-231 cell proliferation within 48 hours. According to our findings in this study, *C, guianensis* could be a successful anticancer candidate for inhibiting breast cancer cells.

Keywords: Anticancer, *Couroupita guianensis*, Breast cancer, MDA-MB-231, MTT assay, LC-MS/MS.

INTRODUCTION

Natural products have been an essential source of commercial medicines and drug leads. Screening crude plant extracts clears the way for discovering new bioactive compounds and elucidating their structures can open the door for new synthetic preparations. Anticancer agents are one of the most synthetic compounds from natural products in recent years.

Cancer is a multi-stage disease in which chemical, physical, environmental, metabolic, and genetic factors play a direct or indirect role in the formation and reduction of cancer. Breast cancer has the highest rates in women as the most frequent cancer among them. This number is increasing, especially in developing countries, where most cases are diagnosed late. Therefore developing novel anticancer agents has become an essential focus of experimental cancer research.

Couroupita guianensis, known by cannonball, is a deciduous tree in the flowering plant family *Lecythidaceae*. Cannonball is native to Central and South America, and it is cultivated in other tropical areas in the world because of its fragrant and beautiful flowers and its large and interesting fruits [1]. Medicinal uses have been reported for many plant parts of *C. guianensis*. Native Amazonians use extracts from several parts of the tree to treat hypertension, tumors, pain, and inflammation. It has been used to treat stomachache, skin conditions and wounds, the common cold, malaria, and toothache [2]. A novel bio-reducing agent, *C. guianensis* fruit extract, for instant green synthesis of gold nanoparticles has been identified. The results showed they could be employed for a wide spectrum of biomedical applications [3]. Leaves extracts of *C. guianensis* have an anti-inflammatory effect, partly due to a reduction on cell migration and an inhibition on cytokines and inflammatory mediators production [4]. In this attempt, the leaf and bark of *C. guianensis* were selected to screen for their anticancer activity against human breast cancer by using MDA-MB-231 cell lines.

MATERIAL AND METHODS

Plant material collection and authentication:

The plant material was collected from the Museum Garden Park located at Thiruvananthapuram Zoo, Thiruvananthapuram, Kerala, India. The plant was taxonomically identified and authenticated at the Herbarium, Department of Botany, University of Kerala, Thiruvananthapuram, Kerala.

Preparation of plant extract:

Leaf and bark of *C. guianensis* have been used for this study. Soxhlet extraction was done to fractionate the crude extracts of the powdered samples (300 g). Samples were extracted using solvents such as Hexane, Chloroform, and Methanol, respectively, each for 4.5 hours. The resulting extracts were concentrated and recovered using a rotary evaporator.

Cell lines and media:

The breast cancer cell lines MDA-MB-231 and normal 3T3 cell lines were collected from the National Center for Cell Science (NCCS), Pune, India. The cells were cultured in Dulbecco's Modified Eagle Medium

(DMEM) high glucose medium supplemented with 10% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic solution (Anti-Anti). Cells were maintained in the incubator with 5% CO_2 at 37°C.

MTT assay:

The *in vitro* anticancer activity of the extracts was performed on MDA-MB-231 cells using MTT assay as it is a sensitive method for evaluating cytotoxic activity. This assay is based on converting MTT to MTT–formazan by mitochondrial enzymes. 3T3 cell lines and MDA-MB-231 breast cancer cell lines were assessed to check the cytotoxic effect of *C. guianensis*. Cancer cells and normal 3T3 cells at a density of 5×10^4 were grown in DMEM media on 96 well plates and incubated at 37°C with 5% CO_2 for 24 hours to form a thin monolayer of cells attached to the wells. Plates were treated in triplicate with extracts from the concentration of 62.5, 125, 250, 500, and 1000 µg/ml for cancer cells to check the cytotoxic effect. After incubation for the specific time interval, the media have removed, and 100 µl of MTT solution was added to all 96 wells and incubated for two hours at 37°C, 5% CO_2 with 98% relative humidity. After two hours, each well was treated with 100 µl detergent (20% Sodium Dodecyl Sulfate in 50% DMSO) used to solubilize the crystals, which are formed by MTT solution. After incubating for another three hours, plates were read using spectrophotometric analysis at 570 nm to check the viability of cells by using the following formula to calculate the IC_{50} of the cells [5].

% of cell inhibition (death) = 100 – (Absorbance of sample / Absorbance of control × 100)

Thin Layer Chromatography (TLC):

The samples that have shown significant inhibition activity were used to conduct thin layer chromatography. TLC separation was carried out on silica gel 60 F254 plates of 0.25 mm thickness. The development of chromatograms was accomplished with different types of solvent systems. The chromatograms were allowed to dry after separation. The retardation factor of each band was calculated based on the ratio of the distance traveled by the solute to the distance traveled by the solvent. The separated bands on the TLC plate were observed under a UV-Visible spectrophotometer between 450 and 650 nm ranges. Prominent bands formed were recovered by scrapping from the adsorbent and reconstituting in methanol, followed by centrifugation at 14,000 rpm for 30 min so as to remove any silica from it. The supernatant was then stored in sterile glass vials under refrigeration.

LC-MS/MS spectrometry:

TLC-eluted fractions that had an inhibition effect on cancer cells were dissolved with methanol, and filtered with a 0.22 µm nylon membrane filter, using LC-MS/MS method. Analysis of a 10 mL aliquot of diluted sample was performed by LC-MS/MS (Shimadzu 8045, Japan) on a 1.9 µm C18 column at 40 C° using a Nexera X2 High Performance Liquid Chromatograph mass spectrometer interfaced with DUIS – ESI spectrometer.

FT-NMR spectrometry:

10 mg of sample was dissolved in Deuterated Methanol (MeOD) as the solvent to make a proper solution and used in the proton nuclear magnetic resonance experiment (H1-NMR) (Bruker, Avance III HD

400 MHz One Bay FT-NMR, USA). NMR spectrums have obtained in 1-15 minutes.

Statistical Analysis:

The IC_{50} value represents the minimal concentration of a drug that is required for 50% inhibition *in vitro*. The IC_{50} value was obtained from MTT assay and calculated using MS Excel. Data are expressed as mean ± standard deviation (STD), n=3. Statistical analysis was performed using one-way ANOVA test and Student's t-test, $P < 0.05$.

RESULTS AND DISCUSSION

Initial cytotoxicity test of extracts using MTT assay:

In the initial cytotoxicity test, we tested bark and leaf extracts fractionated with different solvents like; hexane, chloroform, and methanol. Anticancer activity was analyzed after 24, 48, and 72 hours. All the extracts of the bark and leaves showed non cytotoxicity effects on 3t3 normal cells. MTT assay results showed that the growth of MDA-MB-231 cells was inhibited significantly. All the hexane, chloroform, and methanol extracts of the leaf, hexane and chloroform extracts of the bark showed the cytotoxic activities after 24 and 48 hours (Table 1). The maximal cell inhibition observed after 48 hours with chloroform and methanol extracts of the leaf, 76.7±0.10 % and 73.5±0.10 %, respectively. At the same time, the other isolated extracts showed lower inhibitions.

		% cell inhibition in concentrations				
Extract	**Time**	**1000**	**500**	**250**	**125**	**62.5**
Leaf Hexane (LH)	24	44.7±0.12	31.3±0.10	21.3±0.10	11.8±0.10	9.3±0.07
	48	14.6±0.11	14.1±0.10	8.8±0.10	3.4±0.10	-
Leaf Chloroform (LC)	24	44.6±0.10	33.9±0.10	26.2±0.10	18.4±0.11	9.2±0.08
	48	**76.7±0.10**	62.2±0.09	48.7±0.10	36.3±0.10	26.8±0.10
Leaf Methanol (LM)	24	46.0±0.10	39.9±0.11	29.5±0.10	22.3±0.10	16.5±0.10
	48	**73.5±0.10**	58.3±0.10	45.9±0.10	36±0.10	30.7±0.11
Bark Hexane (BH)	24	38.7±0.10	29.7±0.10	25.2±0.11	22.7±0.11	20.5±0.10
	48	29±0.10	15.3±0.10	13.1±0.10	11.6±0.10	9.7±0.09
Bark Chloroform (BC)	24	42.6±0.10	40.3±0.10	36.6±0.10	34.9±0.10	32.7±0.10
	48	12.9±0.11	9.7±0.10	5.3±0.10	3.2±0.10	-
Bark Methanol (BM)	24	23.6±0.10	22.28±0.10	21.5±0.12	19.9±0.10	19.3±0.10
	48	19.9±0.10	17.8±0.11	13.5±0.10	10.6±0.10	9.9±0.10

Table 1: Percent cell inhibition of MDA-MB-231 cell line by leaf and bark extracts of *C. guianensis*. Data are expressed as mean ± SD (n = 3). Time is per hours. Concentration is per µg/ml.

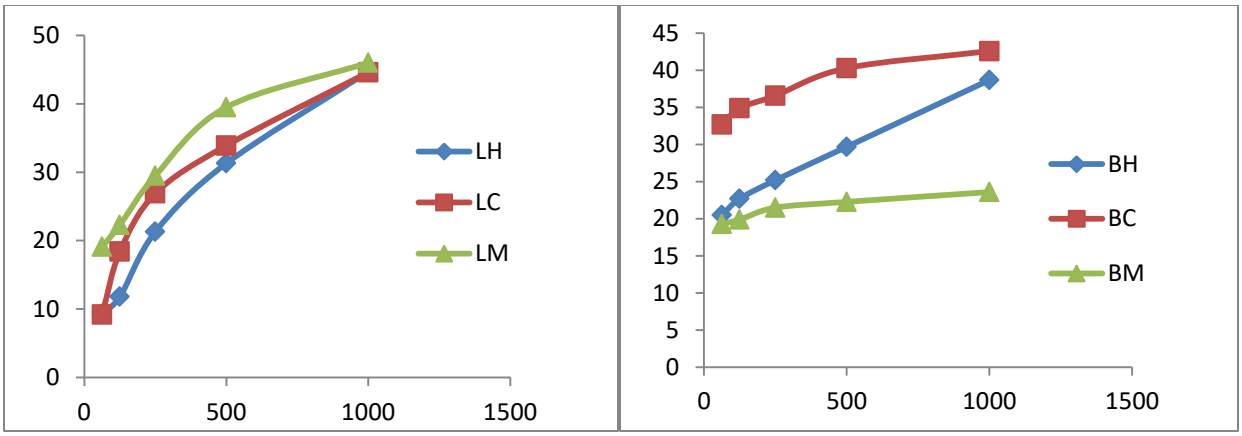

Figure 1: Inhibition of MDA-MB-231 cell line by leaf and bark extracts in 24 hours.

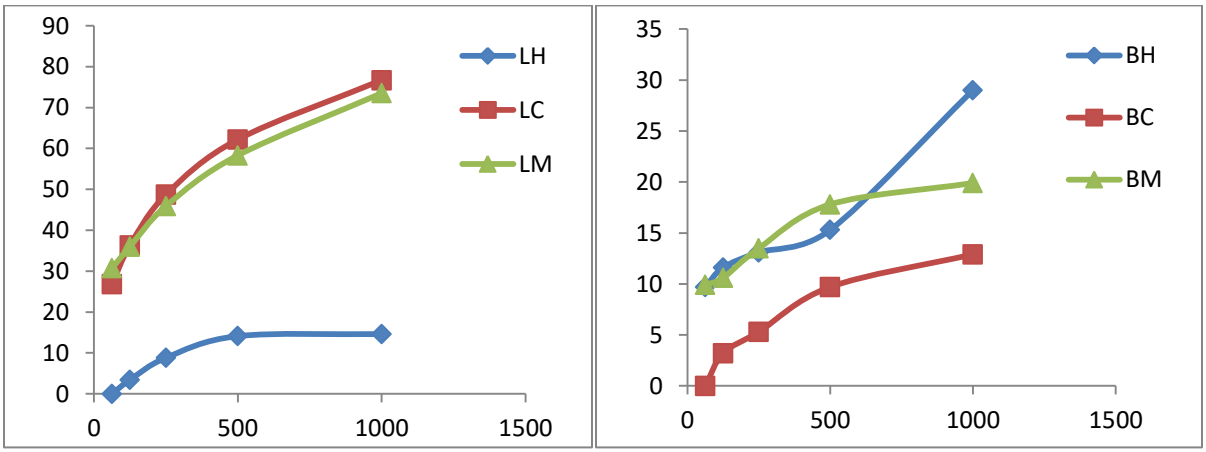

Figure 2: Inhibition of MDA-MB-231 cell line by leaf and bark extracts in 48 hours.

Anticancer activity of the potent extracts by MTT assay:

The cytotoxic activity of the most active fractions on the inhibition of the human breast cancer MDA-MB-231 cell line is presented in Table 2. Anticancer activity was analyzed after 48 hours. In the second cytotoxicity test, ten fractions of chloroform and methanol extracts of the leaf, which were isolated by the TLC method, got tested. Different inhibition percentages for different fractions were obtained. Fraction number 3 (F3) from the chloroform extract and fraction number 8 (F8) from the methanol extract showed the highest cytotoxicity effect of 75.8±0.10 % and 70.9±0.10 %, respectively (Fig 4). The IC_{50} value for F3 is 507.77 µg/ml, and for F8 is 543.50 µg/ml (Fig 3).

	% cell inhibition after 48 hours									
	leaf chloroform fractions					leaf methanol fractions				
con	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
1000		-	**75.8±0.10**	57.47±0.10	-	25.1±0.10	36.9±0.10	**70.9±0.10**	53.2±0.10	-
500	-	-	61.7±0.10	55.59±0.10	-	13.3±0.10	31.2±0.10	57.3±0.11	45.9±0.11	-
250	-	-	37.9±0.11	55.0±0.10	-	11.2±0.10	29.6±0.09	48.0±0.10	20.8±0.10	-
125	-	-	23.1±0.10	54.5±0.11	-	-	25.5±0.10	31.8±0.10	11.3±0.10	-
62.5	-	-	19.3±0.10	51.3±0.10	-	-	22.4±0.09	30.2±0.10	6.7±0.08	-

Table 2: Percent cell inhibition of the most active fractions.

Data are expressed as mean ± SD (n = 3). Concentration is per µg/ml.

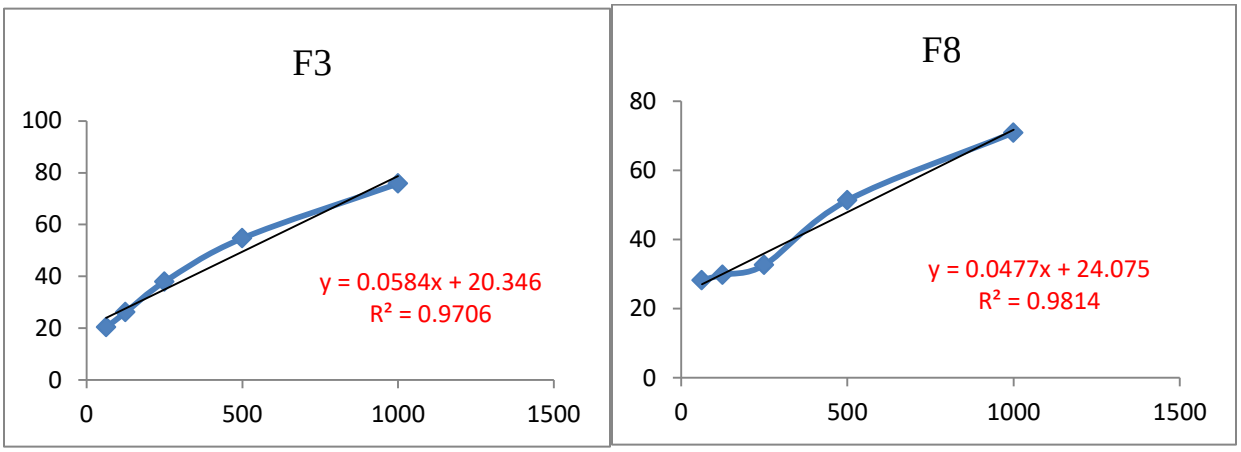

Figure 3: IC_{50} of F3 and F8

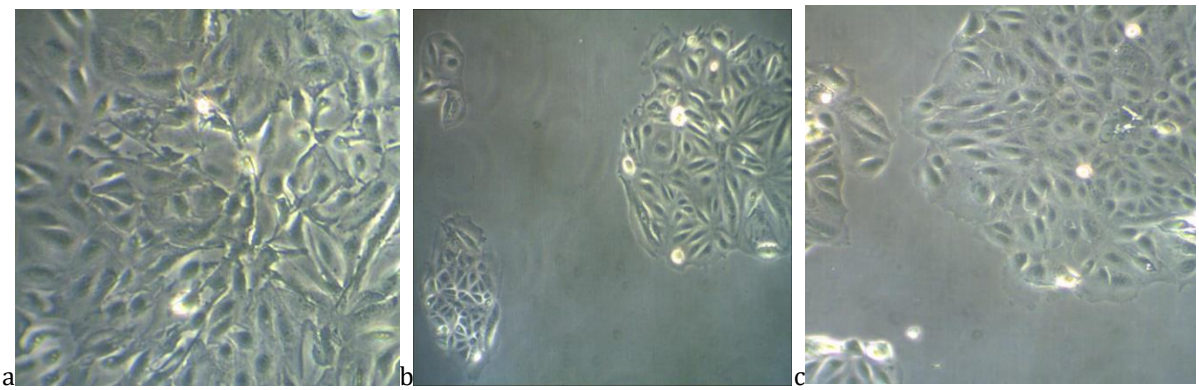

Figure 4: Microscopic images of MDA-MB-231 cells, a. 100% confluent, b. after 48 hours treatment by F3 (75.8±0.10 % of inhibition), c. after 48 hours treatment by F8 (70.9±0.10 % of inhibition).

LC-MS/MS spectrometry analysis:

The identification of the bioactive compounds of the extracts found by the MTT assay was conducted using LC-MS/MS spectrometry analysis. According to the MTT assay results, F3 and F8 were subjected to LC-MS/MS spectrometry analysis to identify the compounds. Specific terpenoids detected by LC-MS/MS analyses revealed several distinct peaks based on their retention times and mass to charge ratio. MS/MS analysis confirmed the structure of terpenoids.

The fragmentation of molecular ions at m/z=202.80 and its respective product ions are m/z=107.30/20.6.40/20.7.45/243.80/246.85/302.65 (Figure 5) assigned as Linichlorin A. Similarly, the MS2 spectra of ions at m/z=637.95 and its respective product ions are m/z=395.60/453.95/587.05/654.90/660.00/669.85/ attributed to Foetoside C (Figure 6).

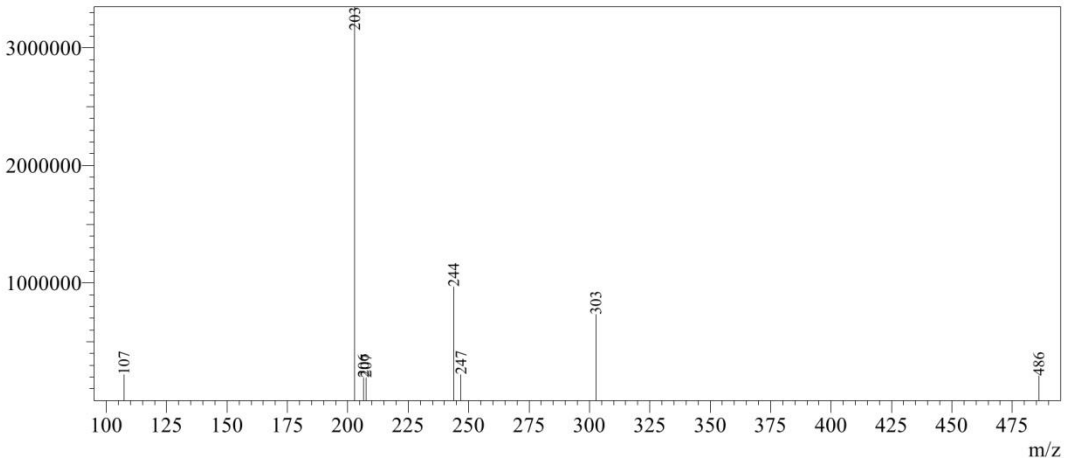

Figure 5: LC-MS/MS spectra of F3

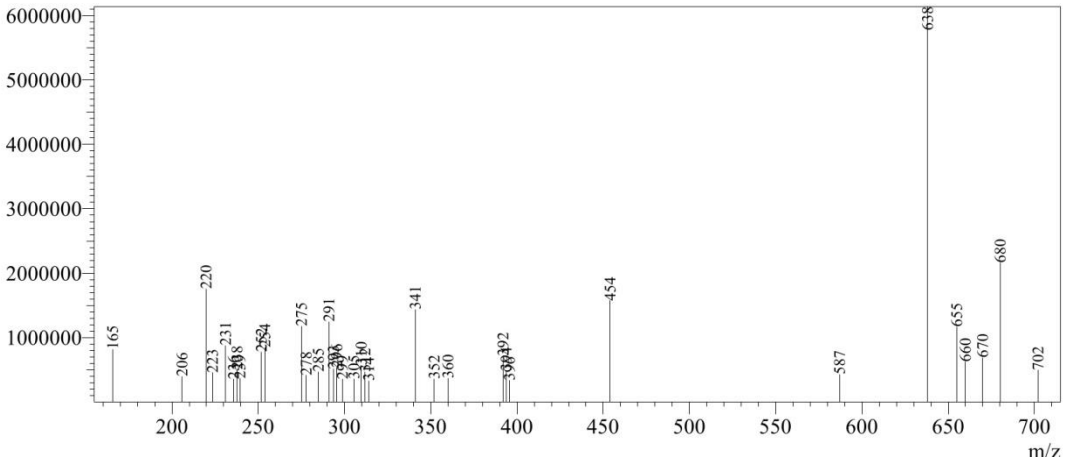

Figure 6: LC-MS/MS spectra of F8

NMR analysis:

The identification of the functional groups found in Fractions F3 and F8 was conducted using NMR analysis. The NMR spectra obtained from fraction F3 are presented in Figure 7. The broad peaks were located at 4.775 ppm (solvent), 3.601-3.817 ppm (Cl-C-H bands), 1.352 ppm (R2-CH2), 1.198-1.310 ppm (R2-CH2 or R-CH3), and 0.709 ppm (R-CH3). The NMR results acquired in this study were aligned with the structure of Linichlorin A. The NMR spectra obtained from fraction F8 are presented in Figure 8. The broad peaks were located at 4.775 ppm (solvent), 3.213-3.298 ppm (O-C-H bands), 1.358-1.389 ppm (R2-CH2), and 0.762-0.801 ppm (R-CH3). The NMR results were compared with the structure of Foetoside C and indicated the presence of trihydroxy and hydroxymethyl groups by the peaks.

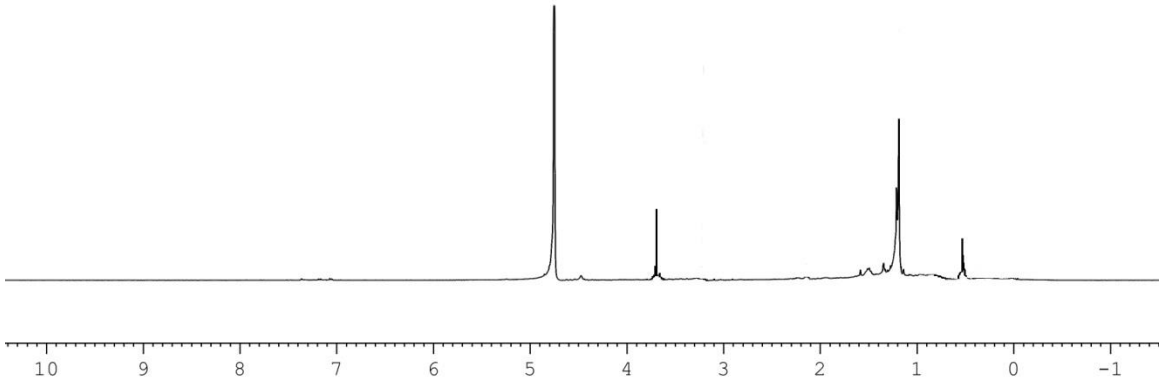

Figure 7: NMR spectra of Linichlorin A

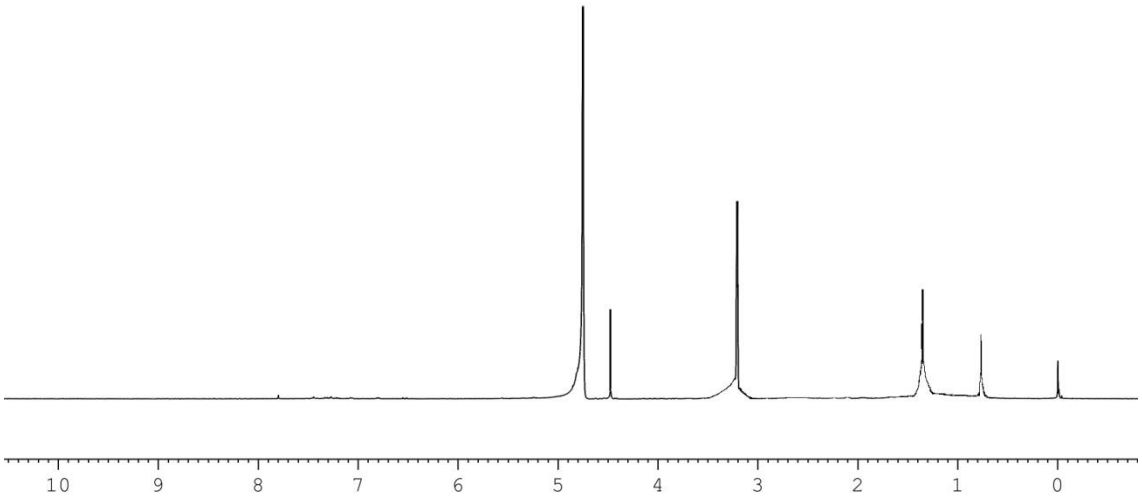

Figure 8: NMR spectra of Foetoside C

CONCLUSION

According to the results, the fractions F3 and F8, extracted from the leaf of *C. guianensis* by chloroform and methanol, respectively, have inhibition activity against MDA-MB-231 breast cancer cell lines. The results of LC-MS/MS and NMR analysis indicated the presence of active terpenoid compounds and their functional groups.

Linichlorin A is a sesquiterpenoid found in *Cousinia canescens*, *Saussurea elegans*, and other organisms. For the first time three sesquiterpene lactones, linichlorins A, B, and C were isolated from the aerial parts of *Centartrea linifolir* Vahl in 1977 [6]. According to the study done by Francisco Esteve and his colleagues in 2020, Linichlorin A was evaluated against the human leukemia cell lines HL-60, U-937, a specific U-937 cell line that overexpresses the anti-apoptotic Bcl-2 protein, and the human melanoma cell line SK-MEL-1. Linichlorin A was the most potent compound in terms of inducing growth inhibition in the four cell lines, and it was a potent apoptotic inducer in human U-937 leukemia cells, as determined by fluorescent microscopy and flow cytometry [7].

Fraction F8 extracted from the leaves of *C. guianensis* by methanol has shown a high inhibition effect on MDA-MB-231 cells. According to the LC-MS/MS and NMR analyses for this fraction, it appears that the compound is Foetoside C. Foetoside C is a triterpene glycoside found in *Thalictrum foetidum* in 1984 [8]. Triterpenoids are metabolites of isopentenyl pyrophosphate oligomers and represent the largest group of phytochemicals. There are more than 20,000 triterpenoids exist in nature [9]. Triterpenes are also highly effective agents in the prevention of diabetic complications. They have strong antioxidant activity and inhibit the formation of advanced glycation end products, implicated in the pathogenesis of diabetic nephropathy, neuropathy, embryopathy, and impaired wound healing. [10] Triterpenes have been described as antimicrobial, antiviral, anti-inflammatory, and antitumor agents, as well as immunomodulator compounds. Several of them are implicated in the resolution of immune diseases [11]. Triterpene alkaloids found in *Thalictrum foetidum L*, such as Foetoside C, have shown antitumorigenic activity [12].

In conclusion, it was observed that the plant *C. guianensis* contains a variety of secondary metabolites that hold strong anticancer potential based on the experiments performed, which add scientific evidence to conduct further studies, to investigate the lead compounds present in the plant, evaluate its anticancer potential on *in vivo* animal models and also to put forward an attempt to carry out trials on human beings in future.

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