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PHYTOCHEMICAL SCREENING, ANTIMETASTATIC AND ANTIMICROBIAL ACTIVITIES OF MOMORDICA BALSAMINA LEAF EXTRACTS

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Abstract- Medicinal plants are important sources of phytochemicals used for the treatment of various diseases including cancer. They are non-toxic, safe, less expensive and highly effective in management of diseases. Breast cancer is a major cause of death in women in most developing countries globally. Surgery, radiotherapy, immunotherapy and chemotherapy are the most widely used methods for breast cancer treatment. However, drug resistance, drug toxicity and cost of treatments are the major challenges in breast cancer management. Research on the use of alternative breast cancer therapy is highly important in other to eliminate such problems. The aim of this study was to investigate the antimetastatic and antimicrobial activities of Mormodica balsamina leaf extracts. Antimetastatic activity was evaluated on MDA-MB-231 breast cancer cell lines. Antimicrobial activity was tested using Staphylococcus aureus and Escherichia coli. The methanol extracts was further analysed for phytochemical composition using gas chromatography-mass spectrometry (GC-MS) and Fourier transform infrared spectroscopy (FTIR), thirteen phytoconstituents were detected with immense biological importance. For the first time, the extracts was found to possess antimetastatic activity on MDA-MB-231 cell line. Antimicrobial efficacy was also determined. Therefore, further research on Mormodica balsamina leaf extracts could lead to drug development.

1. INTRODUCTION

Cancer is a group of related diseases, breast cancer has become a global health problem because about 1.3 million women are been diagonised annually in the world, with almost 15% rate of cancer death[1]. The environment plays a major role in the incidence of breast cancer with about 10% related to genetic factors. Genetic factors are associated with high risk of breast cancer, however, recent researches have shown involvement of other forms of inheritance linked to epigenetic factors. Epigenetics are heritable characteristics associated with gene function and regulation other DNA sequence, it is therefore, a process whereby cell regulates the expression of a given gene. Epigenetics explain why cancer cells behaves different from normal cells due to abnormal gene expression levels [2].

The role of nutraceuticals which are natural plant products obtained from fruits and vegetables in breast cancer management have been studied using different in-vitro and in-vivo models to determine their various mechanism of action. Approximately, 50% of breast cancer patients and 37% of prostate cancer patients uses natural products from plants for cancer treatment. Over 75% of anti-cancer drugs in clinical trials are been isolated from natural products and their derivatives [3].M. balsamina is a nutraceutical plant which possess therapeutic and nutritional properties used for management of various diseases [4]. M. balsamina is associated with numerous medicinal properties such as: Anti HIV, anti-diabetic, analgesic, anti-inflammatory, anti-bacterial, anti-viral, anti-diarrheal and hepatoprotective activity. Research on effective and affordable anticancer agents is highly important to improve the quality of life of breast cancer patients and to decrease cost of treatment globally. Some plant-derived agents like the taxens (docetaxel and paclitaxel) have been approved by the Food and Drug Administration (FDA) for treatment of both localised and metastatic breast cancer. This study could serve as a strategy for a novel drug development against breast cancer by utilizing compound(s) present in Momordica balsamina leaf extracts.

2. MATERIALS AND METHODS

2.1 M. balsamina plant extracts preparation

Plant leaves were purchased from a local farmer at Babban giji damp, Kano state, Nigeria. They were identified at department of Plant Biology, Bayero University, Kano. Voucher specimen number BUKHAN0311 was deposited at Herbarium of plants, Bayero University, Kano. The plants were washed and dried under shade. Dried plants were powdered and dissolved in methanol (1:10) were used for extraction. The mouth was covered with aluminium foil and continuous agitation was carried out at 150rev/min on a shaker for 24hrs to completely extract all the active constituents. The solvent was filtered through Whatman no 1 filter paper[5]. The dried extract was kept at 4◦C. Stock solution of the plant extract

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(20mg/ml) was prepared by weighing the powder and dissolving in Dulbecco's modified Eagle's Medium (DNEM) which was filtered through 0.22µm sterile Millipore syringe filter.

2.2 Cell line and culture conditions

The cell lines used in this research is MDA-MB-231 highly metastatic breast cancer cell line are grown in Dulbecco's modified Eagle's Medium supplemented with 10% Fetal bovine serum (FBS) and 2mM L-glutamine. Cells were grown in a humidified incubator with 5% CO2, 20% O2 at 37◦C [6].

2.3 Cytotoxicity assay

Trypan blue assay was performed to determine the cytotoxic effect of M. balsamina crude extract on MDA-MB-231. 3×104 according to the procedure of [7]. Briefly, cells were seeded in 33mm Falcon tissue culture and allowed to settle overnight before treatment. Cells were treated with different concentrations of methanolic extracts of M.balsamina 60, 120. 240, 500 and 1000 µg/ml for 24 hours, treatment was carried out in triplicates, negative controls containing only media and cells 3×104 /ml were included. After 10 minutes of incubation at 37oC, 1ml of fresh cell culture media replaced Trypan blue solution, dead (blue coloured) as well as viable (clear) cells were counted under an inverted microscope at ×40 magnification. Percentage viability from three different dishes under same treatment condition was determined from 30 randomly selected fields of view using this following formula: % viability= (total number of live cells/ total number of cells) ×100.

2.4 Measurement of cell viability – MTT Tetrazolium assay

Briefly, 3×104 cells/well were seeded in 3 wells per condition in a 12 well plates and allowed to settle overnight at 37oC. After then, the cells were incubated with desired concentrations of M.balsamina extracts ranging from 60, 120. 240, 500 and 1000 µg/ml for 24 and 48 hours under same conditions. Wells containing culture media only (control wells) and treated wells were replaced with fresh ones after 24 hours. At the end of treatment time, 150µl of MTT was added and further incubated for 4 hours at 37oC after which MTT was removed and 890µl DMSO and 110µl glycine buffer were added to solubilize formazan. Spectrophotometric absorbance were taken at 570nm (630nm as reference wavelength) using Micro plate Reader (ELX 800™).

2.5 Minimum inhibitory determination by disc diffusion assay

This assay was performed according previous method described by [8] with modification. Mueller Hinton agar plates were inoculated with 1ml of each bacterial suspension containing 108 CFU within 15 minutes of bacterial standardization. Plates were dried before addition of extracts containing discs using flame sterilized forceps. Negative control discs containing methanol and positive control ciprofloxacin discs were included. Plates were incubated aerobically at 37oC for 18-24 hours [9]. Inhibition zones were measured after incubation, minimum inhibitory concentration was determined as lowest extract concentration that was able to inhibit bacterial growth. Results were interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI) protocol, (2007), for antimicrobial testing. Test was performed in triplicates and results are presented as means ± SEM ($n \geq 3$).

2.6 Minimum inhibitory concentration (MIC) determination by broth macrodilution method

Broth macrodilution was carried out according to the method of[10] with modifications. 1ml of each bacterial suspension already adjusted to 0.5 McFarland in Mueller Hinton broth was added to the wells of sterile microplates containing 1ml of two-fold serial dilutions (0.2-3.2mg/ml) of M.balsamina extracts in the same medium. Positive control wells contains nutrient broth and bacteria while negative control wells contains only nutrient broth (for sterility determination). The plates were incubated at the same conditions described above for 24 hours. Bacterial growth was confirmed by the appearance of turbidity. MIC values were recorded after 24 hours as the lowest concentration of extracts at which bacterial growth was inhibited.

2.7 Gas Chromatography Mass Spectrometric (GC-MS) analysis

Methanol crude extracts of M.balsamina was dissolved in methanol (1mg/ml). The solution was vortex appropriately and filtered through 0.22µm syringe filter. GC-MS analysis was carried out by injecting 1µl of the solution into GC-MS system. Analysis was carried out under the following conditions [11]using GCMS-QP2010 plus. Teknokroma TRB-5 ms column operating in electron impact [electron ionisation positive (ET+)] mode at 70eV was used. Helium (99.999%) as carrier gas at a constant flow rate of 1ml/min. Injection volume of 0.5ET (splitless). Injection temperature of 280oC with transfer line temperature of 300oC. Oven temperature of the system was set at 50oC (isothermal for 2 min), with 7oC increase per min. Mass spectra was analysed at 70Ev at a scan interval of 0.5s and full mass scan range from 25m/z to 1000m/z. Results were analysed using WILEY library by comparing mass spectrum of unknown compound with the spectrum of known compound in the library. Name, molecular weight, molecular formula were detected.

2.8 Fourier transform infrared spectroscopy (FTIR) of M.balsamina leaf extracts

FT-IR spectra was obtained by analysing the crude extract with Fourier Transform Spectrometry instrument, Shimadazu IR Prestige-21 Model at a frequency range of 4000-600cm-1. The functional groups of the compounds present in M.balsamina were identified.

3. RESULTS

3.1 Cell viability assay

The extracts was found to be cytotoxic after 60µgml concentration of the extracts. Increase in cytotoxicity was found to be dose dependent. Highest toxicity was observed at 1000µg/ml after treatment. IC50 value was found to 240µg/ml/. This work shows that the cytotoxic effects of the plant extracts on MDA-MB-231 was due to the presence of identified phytochemicals. The effects of cytotoxicity from phytochemicals obtained from medicinal plants against breast cancer cells have been studied either by inducing apoptic pathways or cell necrosis. Examples of these phytochemicals include: Epigallocatechin-3-gallate (EGCG) a catechin present in green tea [12], resveratrol a polyphenol [13], terpenes [14], flavonoids [15]. In this experiment, cytotoxicity was found to be concentration dependent.The figure below shows the cytotoxic effects of M.balsamina extracts on MDA-MB-231 cells.

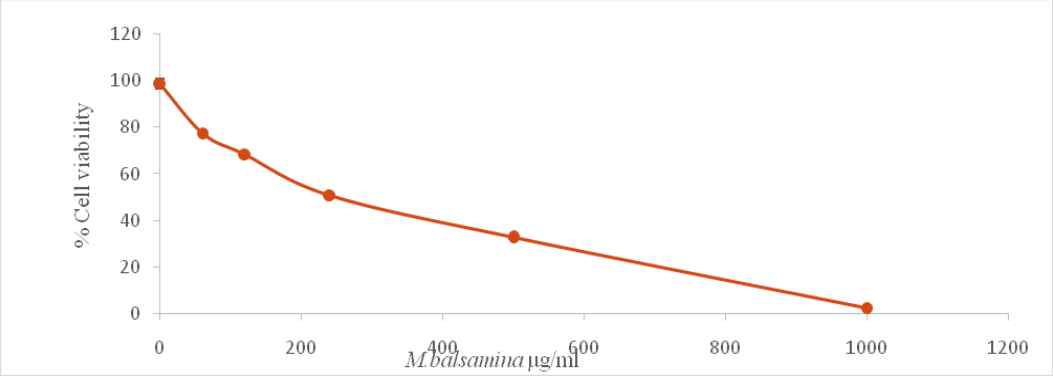

Figure 3.1 Cell viability dose dependent curves of methanol extracts of M.balsamina leaves after 24hrs.Results are represented as means ± SEM for all experiments done in triplicates (n=3×30).

3.2 Effects of methanol extracts of M.balsamina on cell proliferation

The antiproliferative effect of methanol extracts of M.balsamina on MDA-MB-231 as determined by MTT assay was observed in a dose dependent manner after 24 and 48 hours. There was no significant inhibition of cell growth at 60µg/ml in the MDA-MB-231 cells after 24hrs. But, at the end of 48 hours treatment, antiproliferative effect was observed at 60µg/ml. This shows that antiproliferative effect of M.balsamina extracts increases with time of exposure. Since the extract was cytotoxic on MDA-MB-231 cells, antiproliferative effect could be due to cell-cycle arrest as observed from other studies [16]. Similar results were obtained by [17], they observed that cell growth was decreasing with increase in concentration of Cymbopogon citratus extracts IC50 ≥ 200µg/ml, on MDA-MB-231 cell line. The mechanism of antiproliferative activities of many plants extract on breast cancer cell lines have been identified to be through inactivation of pathways in cell-cycle due to increase in kinase expression, Ras/MAPK cascade [18]. Antiproliferative effect was also observed in a concentration dependent manner on MDA-MB-231 cell line by induction of cell cycle arrest at G0/G1 phase using Euphorbia tirucalli extracts [16]. From the results of this research, it was observed that viable cell number was decreasing significantly as extracts concentration of M.balsamina increases compared to untreated cells (control). Cell number was found to be inhibited significantly as shown in Figure 3.2 and 3.3 when compared with control.

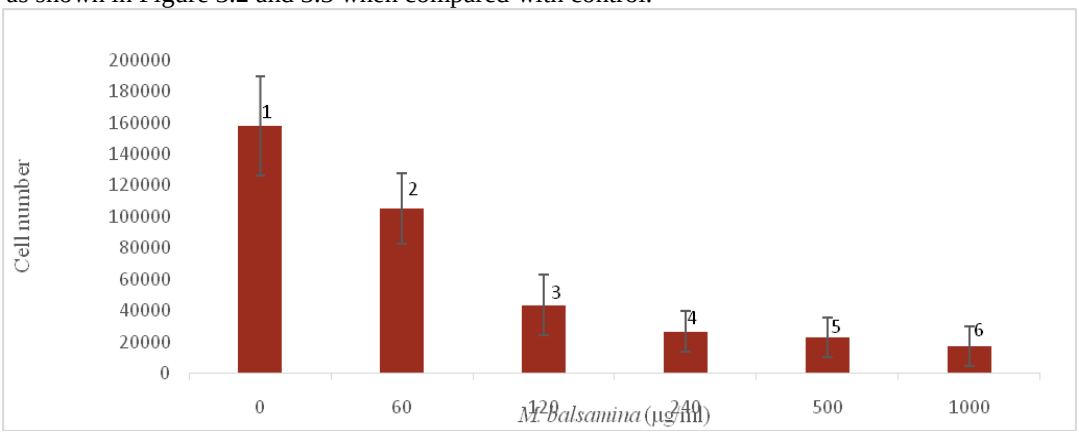

Figure 3.2 Dose-dependent effect of M.balsamina on cell number after 24hrs

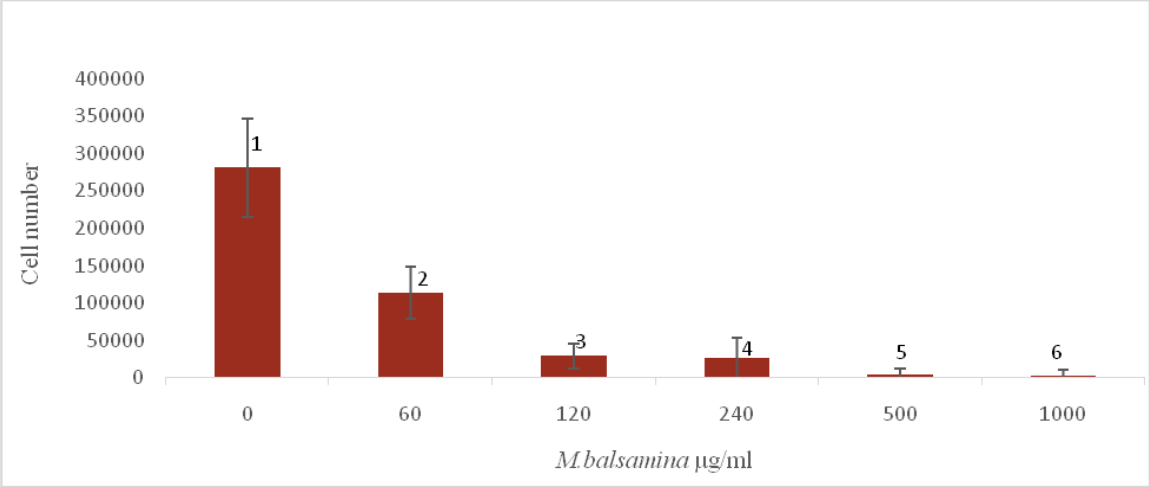

Figure 3.3 Dose-dependent effect of M.balsamina on MDA-MB-231 for 48hrs

3.3 Disk diffusion assay

The measurements of inhibition zones was carried out after 24 hours as shown in table 3.1. Inhibition zones were measured on agar plates inoculated with S.aureus and E.coli with extracts containing discs at 10mg/ml and 20mg/ml and without extract (0). Inhibition zone vary significantly when compared with that of ciprofloxacin (positive control). It was found to be 7-8mm for E.coli and 8.33mm for S.aureus. The extracts has less antibacterial activity on E.coli even at higher concentration (20mg/ml). The reason could be attributed to variation in diffusion pattern of the extracts due to differences in structure of cytoplasmic membrane of the bacteria, or the release of enzymes and toxins that could degrade antimicrobial agent, and also due to the structure of the extracts [19]. Disk diffusion assay is a simple and less expensive method of antimicrobial assay, but it is not a reliable assay for MIC determination of plants extracts because: MIC values obtained using disk diffusion assay are higher than that of broth dilution assays. Therefore, the marginal inhibition zones at low extracts concentrations will not justify the inefficiency of M.balsamina plants extracts [9].

Table 3.1Antimicrobial activity determined by disk diffusion assay

Microorganism	Zone of inhibition in mm			
	M.balsamina extracts (mg/ml)			Ciprofloxacin (5µg/ml)
	0	10	20	5
E.coli	-	7±0**	8±0**	36.3±1.155
S.aureus	-	8.3±0.578**	9.3±1.155**	24.3±0.578

Results are mean of MIC values ± SD of three measurements. – means no activity
**P< 0.005.

3.4 Broth macrodilution assay

The minimum inhibitory concentration was determined using (0.2-3.2mg/ml) concentrations of M.balsamina extracts. Minimum inhibitory concentration was recorded as the concentration at which no visible growth was observed as shown in table 3.2. S.aureus were more susceptible to the extracts with MIC value 0.4mg/ml. The MIC value for E.coli was 0.8mg/ml, two times the MIC value for S.aureus. Therefore E.coli were more resistant the reason been the cytoplasmic nature of the bacteria as described above. The results of this antimicrobial assay is in accordance with the work of [9] on the investigation of antimicrobial activities of plants extracts using both diffusion and dilution methods. They succeeded in getting MIC values from disk diffusion to be 3-30 times higher than values obtained using dilution method, regardless of the plant extract been analysed.

Table 3.2 Antimicrobial activity determined by broth macrodilution

Microorganism	M.balsaminaextracts (mg/ml)						
	0	0.2	0.4	0.8	1.6	3.2	Control
E.coli	+	+	+	-	-	-	-
S.aureus	+	+	-	-	-	-	-

Results were obtained from three experiments performed in triplicates

+ presence of turbidity
-absence of turbidity

3.5 Gas Chromatography Mass Spectrometric (GC-MS) analysis

GC-MS analysis of methanol extracts of M.balsamina identified the presence of twelve compounds with their respective molecular weight and area as shown by presence of various peak in the chromatogram. Compounds were identified by comparison of their mass fragmentation patterns with the mass fragmentation patterns of similar compounds in WILEY library.

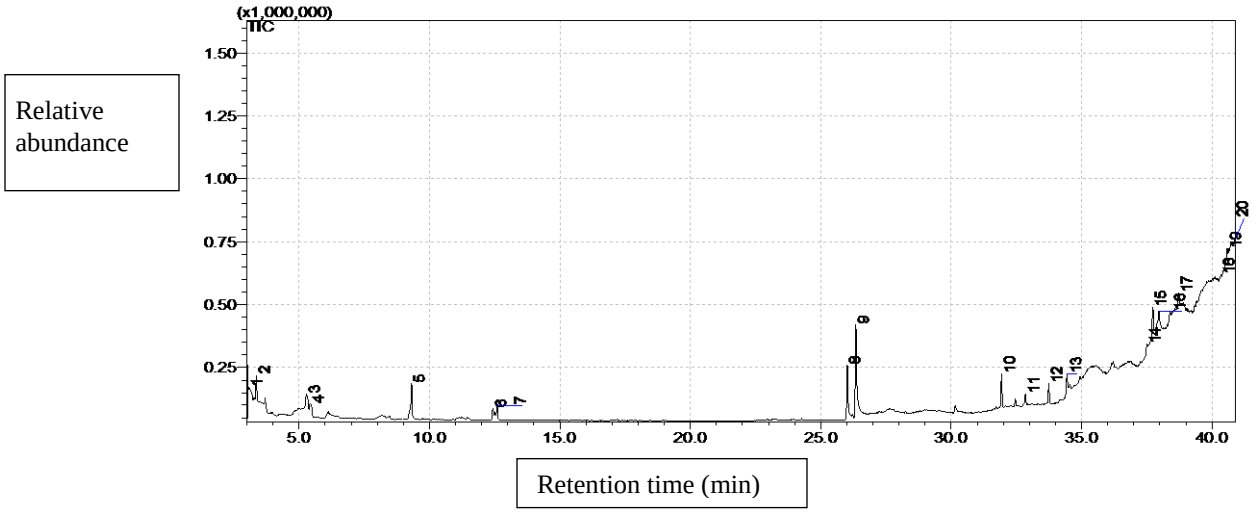

Figure 3.4GC/MS Chromatogram of Momordica balsamina leaf extracts

Phytochemical screening of methanol extracts of M.balsamina revealed the presence of some phytochemicals which could be responsible for antimetastatic effects on MDA-MB-231 cells as well as antimicrobial activities. The properties of various components are summarised in table 3.3. The major phytoconstituents with biological activities include: 1,3-Dioxane [20], 1,2-Benzenedicarboxylic acid, diethyl ester [21], and steric acid [22] are known for their antimicrobial activities. Another phthalate ester, 1,2-benzenedicarboxylic acid mono-2-ethyl ester have been shown to possess cytotoxic activity against some cancer cell lines including breast cancer cell line [21]. Similarly, another compound known to have antiproliferative and cytotoxic activity is a derivative of pregnane. They are known for their activity against cancer cells. Since they are steroids as such can be utilized for the management of some hormone related diseases. Pregnane have been found to have cytotoxic and antiproliferative activities against MCF-7 breast cancer cells [23]. The presence of these phytochemicals have confirmed the antimetastatic and antimicrobial properties of M.balsamina methanol leaf extracts.

Table 3.3Compounds identified in methanol extracts of M.balsamina by GCMS and their biological activities

	Peak RT(min)	Peak area	Compound detected	Molecular weight	Molecular formula	% peak area	Activity
1	3.075	1,159,959	1,3-Dioxane (CAS) m-Dioxane	88	C4H8O2	13.52	Antibacterial,antifungal, anti-viral, anticancer [20]
2	3.367	527,044	1,2,3-Propanetriol (CAS) Glycerol	92	C3H8O3	6.14	Food preservative and pharmaceuticals [24]
3	5.292	268,106	3-Nonenoic acid, methyl ester	170	C10H18O2	3.13	-
4	5.450	190,242	Pentanoic acid, pentyl ester (CAS) Amyl valerate	172	C1OH20O2	2.22	-
5	9.317	556,801	Octadecane, 6-methyl- (CAS) 6-Methyl octadecane	268	C19H40	6.49	-
6	12.433	154,037	Cyclohexane, 1,1'-[1-(2,2-dimethylbutyl)-1,3-propanediyl]bis- (CAS) Heptan, 1,3-dicyclohexyl-5,5-	292	C21H40	1.80	-

			dimethyl				
7	12.592	238,237	4-Octen-3-one (CAS) 3-Oxy-4-octene	126	C8H14O	2.78	-
8	26.017	725,143	1,2-Benzenedicarboxylic acid, diethyl ester (CAS) Ethyl phthalate	222	C12H14O4	8.77	Plasticizer [24],antimicrobial, anticancer [21]
9	26.350	1,454,368	1,2-Benzenedicarboxylic acid, diethyl ester (CAS) Ethyl phthalate	222	C12H14O4	16.95	-
10	31.933	397,131	Neophytadiene	278	C20H38	4.63	Antihelmeththic properties [24]
11	32.850	101,450	Pyrrolidine, 1-(1-pentenyl)- (CAS) 1-(1-Pyrrolidinyl)-1-n-pentene	139	C9H17N	1.18	-
12	33.742	211,484	Methyl 6-methyl heptanoate	158	C9H18O2	2.47	Treatment of luteal deficiency [25]
13	34.433	256,941	Octadecanoic acid (CAS) Stearic acid	284	C18H36O2	2.99	Antimicrobial [22]
14	37.508	134,171	14-beta-H-Pregna	288	C21H36	1.56	Anticancer [23].
15	37.733	350,396	14-beta-H-Pregna	288	C21H36	4.08	Anticancer [23].
16	37.958	159,556	14-beta-H-Pregna	288	C21H36	1.86	Anticancer [23].
17	38.717	173,795	14-beta-H-Pregna	288	C21H36	2.03	Anticancer [23].
18	40.342	303,762	14-beta-H-Pregna	288	C21H36	3.54	Anticancer [23].
19	40.583	212,600	14-beta-H-Pregna	288	C21H36	2.48	Anticancer [23].
20	40.858	975,961	14-beta-H-Pregna	288	C21H36	11.38	Anticancer [23].

3.6 Fourier transform infrared spectroscopy (FTIR) of M.balsamina leaf extracts

FTIR Spectroscopic technique have been used for identification of functional groups present in plant extracts [26]. In this study, the GC-MS results helped in revealing the compounds present in M.balsamina leaf extracts and their functional groups were identified by FTIR analysis. This research for the first time revealed the presence thirteen phytoconstituents in M.balsamina leaf extracts with their functional groups as shown in the table below.The presence of –OH stretch (3306 cm-1) is a characteristics of alcohol in the extracts, however, O-H bend of carboxylic acids was obtained at 921 cm-1. C-H of alkanes were present at 2918, 2879 and 1370 cm-1. C=O stretch of α,β-unsaturated esters were identified at 1707 cm-1. C-C stretch of aroamtics was obtained at 1585cm-1. Aliphatic amines were present at 1257, 1132, and 1045cm-1. =C-H bend of alkenes was obtained at 991 cm-1. And finally, C-H bend of para distributed aromatic functional group was obtained at 827 cm-1.

Table 3.4 FT-IR peak values and functional groups present in M.balsamina leaf extracts

Characteristic absorbance(s) in cm-1	Bond	Functional groups	Compound(s) identified
3306	O-H stretch	alcohols	1,2,3-Propanetriol
2918	C-H stretch	alkanes	6-Methyl octadecane
2879	C-H stretch	alkanes	6-Methyl octadecane
1707	C=O stretch	α , β- unsaturated esters	Methyl 6-methyl heptanoate, 3-Nonenoic acid, methyl ester, and Pentanoic acid, pentyl ester.

1585	C-C stretch	aromatics	14-beta-H-Pregna
1370	C-H rock	alkanes	6-Methyl octadecane and Heptan, 1,3-dicyclohexyl-5,5-dimethyl
1257	C-N stretch	Aliphatic amines	1-(1-Pyrrolidinyl)-1-n-pentene
1132	C-N stretch	Aliphatic amines	1-(1-Pyrrolidinyl)-1-n-pentene
1045	C-N stretch	Aliphatic amines	1-(1-Pyrrolidinyl)-1-n-pentene
991	=C-H bend	alkenes	4-Octen-3-one and Neophytadiene
921	O-H bend	Carboxylic acids	Octadecanoic acid
827	C-H bend	Para distributed aromatic	1,2-Benzenedicarboxylic acid, diethyl ester

4. CONCLUSION

In conclusion, the anti-metastatic activities of methanol extracts of M. balsamina leaves were evaluated. The extracts was found to be cytotoxic on MDA-MB-231 cell line with IC50 ≤ 240µg/ml after 24 hours of treatment. When assessing the antiproliferative effect, the extracts was able to decrease cell viability in a dose and time dependent manner. In addition, MIC values were determined for identification of antimicrobial activities on S.aureus and E.coli. In other to support the anti-metastatic and antimicrobial activities of the plant, phytochemical screening was carried out using GCMS and FTIR analysis. Thirteen biologically active compounds were identified which could be responsible for the anti-metastatic and antimicrobial properties of this plant extracts. Therefore, M.balsamina is an attractive plant for further studies which could lead to development of drug against breast cancer.

Further studies are required to determine the intracellular pathway(s) involved in the mechanism of cytotoxicity and antiproliferative activities. Emphasis on biological activities has been on phytochemical constituents, therefore, further research is required to identify and isolate possible compound(s) with potent activities on MDA-MB-231 cell line and other breast cancer cell lines.

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