New approach for production of saturated fatty acids in cassava cell cultures as antibreast cancer agent

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Background and objective
Cassava plant is one of the major economical crops, involved in many industrial applications and therapeutic purposes such as suppression of cancer cell activity. The present work targeted to assess saturated fatty acids and their derivatives in cassava cell cultures.
Materials and methods
Stem explants of *in vitro* growing plantlets were induced for calli on MS-medium supplemented with 1 mg/l naphthaleneacetic acid+0.5 mg/l benzylaminopurine. Medium containing 5 mg/l 2,4 dichlorophenoxyacetic acid (2.4-D) and 0.2 mg/l benzylaminopurine was selected for callus productivity. The extraction adequacy of aprotic polar (ethylacetate) and nonpolar (chloroform, *n*-hexane) solvents was analyzed and evaluated by gas chromatography–mass spectrometry.
Results and conclusion
Chloroform callus extract contained mostly fatty acid methyl esters and fatty acid propyl esters. In contrast, *n*-hexane extract contained higher amounts of fatty acid constituents in free form, such as palmitic acid (23.55%). Ethylacetate extract included the highest value of lauric acid (28.34%) in free form as well other fatty acids such as caprylic acid (14.525%), capric acid (2.53%), and enanthic acid (6.41%). Ethylacetate extract conferred the optimal efficiency to suppress the breast cancer cell prevalence (2.63 μg IC50), followed by hexanoic extract (3.44 μg IC50), and then chloroformic extract (6 μg IC50) recording the least value for cancer cell propagation.
Potential of cassava as one of the medicinal valuable plants should be promoted for health-boosting purposes.

Keywords:
cassava, cell cultures, gas chromatography–mass spectrometry

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Introduction

Cassava (*Manihot esculenta*) is a perennial woody shrub, one of dicotyledonous Euphorbiaceae family; it has been a fundamental crop for 800 million people and is one of the six essential crops all over the world [1]. Cassava is propagated through stem cuttings and slightly via seeds. Plants from seeds develop within a long period and are shorter and less vigorous compared with those from stem cutting [2]. Cassava plants are considered as an important economical crop for several countries, owing to it having starch assemblage amplitude, impedance to fewer nutrients, and tolerate drought in soils [3]. Additionally, cassava contains vitamin C, minerals, and carotenoids; besides, it is a source of clean energy for biofuel implementations [4].

Several research studies have examined tissue culture techniques using biotechnology methods to accelerate the propagation of cassava and establish plant recovery system for multipurpose applications. Ihemere [5] provided a material to farmers involving virus decontamination process. Callus is an essential material to culture embryos and acts as a successful opportunity in cassava propagation and germplasm storage [6]. Moreover, high callus-producing frequencies can be seen from stem and immature leaf explants [7]. Others improved a regeneration protocol of cassava [8]. Accomplishment was achieved regarding the effectiveness for cassava callus culture extracts to inhibit the breast carcinoma cells growth owing to their contents of phenolic compounds [9]. In this respect, many research studies have been conducted with a scope of interest regarding the vital role of fatty acids against cancer proliferation for several years. Fatty acids and their esters possess activity for reducing Ehrlich ascites cancer in mice owing to their antitumor efficiency [10]. Medium chain fatty acids (capric, caprylic, and caproic acids) have the potential mechanisms of anticancer activity for inhibition of

tumor cell vitality of breast, skin, and human colorectal cancer cells via apoptosis process by downregulating and upregulating genes of regulatory genes in the cell cycle [11]. The supplementation of daily diet with polyunsaturated fatty acids was beneficial for cancer prevention [12]. Fatty acid metabolites from *Oscillatoria* spp. have a significant pharmaceutical capability as anticancer activities [13].

Our work was biased toward exploiting the biotechnological methods for successful establishment of cassava callus cultures *in vitro*. The study examined the most suitable solvent to extract the highest amount of lipid contents, accompanied by screening and identification of fatty acid contents in these extracts by gas chromatography–mass spectrometry (GC-MS). These different extracts involving saturated fatty acids were directed to assess their performance on inhibition of breast carcinoma cell proliferation (MCF7).

Materials and methods

Plant material

Stem cuttings of cassava (*M. esculenta*) 25–35 cm in length with 5–7 nodes were obtained from the mature plants growing at the farm of Vegetables Research Department, Horticulture Research Institute, Agriculture Research Center, Ministry of Agriculture and Land Reclamation, Dokki-Giza, Egypt. All experiments were conducted in vitro and do not require ethics committee approval. Overall, 30 stakes of cassava stem cuttings were planted in 20-cm pots containing loamy soil fortified with peat moss and perlite as 1 : 1. Plants were transferred to a growth chamber and maintained at 24±2°C for 16-h photoperiod with fluorescent 45-µmol/s cool white light tubes and 8-h dark period.

In-vitro plantlets

New individual nodes were transferred to conical flask containing 150 ml of water with 20% v/v sodium hypochlorite solution with two drops of Tween-20 and then agitated at 200 rpm on a shaker for 45 min. Then, they were washed with sterile distilled water for three times in a laminar flow hood before culturing on MS basal medium supplemented with 30 g/l sucrose and solidified with 2.3 g/l of phytagel. Cultures were then transferred to a growth room and maintained for one month.

Callus cultures

Stem explants (5–10 mm in length) were excised from in-vitro growing plantlets and cultured horizontally on 50 ml of solidified MS-medium fortified with 1 mg/l naphthaleneacetic acid+0.5 mg/l benzylaminopurine (BAP) for callus induction.

Callus production

Cluster of calli (about 0.5 g) derived from the previous steps were sub-cultured on MS-medium supplemented with different concentrations of 2,4-D (4, 5, and 8 mg/l) combined with or without either 10 mg/l thiamine or 0.2 mg/l BAP.

Growth measurements of callus cultures

The cultures were implemented in triplicates and incubated in the culture room under light conditions (16-h photoperiod with fluorescent 45 µmol/s, cool white light tubes, and 8 h dark) at 24±2°C. For growth measurements, two parameters were calculated as follows:

(1) Growth rate (GR)=Pt–P0/10 (g/day).
(2) Growth value (GV)=Pt–P0/P0.

where Pt=weight (g) of calli at the end of every ten days, and P0=starting weight (g) of the callus.

Chemicals and sample preparation

Three organic absolute solvents were supplied by CARLO ERBA (Dasit Group, France) for extraction processes, namely, chloroform, *n*-hexane as nonpolar solvents, and ethylacetate as aprotic polar solvent (GC grade). Fresh stem calli of cassava plant about 50 g were submerged in 150 ml of chloroform, *n*-hexane, and ethylacetate for ~24 h.

Extraction of fatty acids and other phytochemical constituents

A Soxhlet apparatus was used for extraction as described before [14].

Separation of the mixture extract/solvent

Crude extracts were filtered using a 0.45-µm filter and then subjected to centrifugation at 15 000 rpm twice. The obtained supernatant was evaporated and concentrated by vacuum distillation using rotary evaporator device (Buchi, USA, R-210/215; Switzerland). Collection of the residues of extracts and re-solubility had been performed in the determinant solvents (chloroform, *n*-hexane, and ethylacetate) before keeping at 4°C for bending GC-MS analysis and biological studies.

Chromatographic separation by GC-MS for callus extracts

In this study, extracts were analyzed by GC-MS to identify aprotic polar and nonpolar metabolites and

evaluate their phytotoxic activity. Overall, 3 ml of chloroform, *n*-hexane, and ethylacetate extracts was evaporated to dryness and re-solubilized in 5 µl of the appropriate solvent for injection in GC-MS apparatus under the convenient conditions. The GC/MS analysis was performed using a Thermo Scientific, USA, Trace GC Ultra/ISQ_ Single Quadrupole MS, TG-5MS detection, with an electron ionization system with ionization energy of 70 eV. Helium gas was used as the carrier gas at a constant flow rate of 1 ml/min. The injector and MS transfer line temperature was set at 280°C. The oven temperature was programmed at an initial temperature 40°C (hold 3 min) to 280°C as the final temperature at an increasing rate of 5°C/min (hold 5 min). The quantification of all the identified components was investigated using a percent relative peak area. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system.

In vitro antibreast cancer assay

Each of chloroform, *n*-hexane, and ethylacetate extracts of cassava callus cultures was examined to assess their efficiency on inhibition of breast cancer cell proliferation (MCF7) using SRB method [15], with consideration measurements such as the viability suppression percentage for cancer cells and apoptosis assay by detection of DNA damage using agarose gel electrophoresis.

Cytotoxicity of callus culture extracts

Cytotoxicity of chloroform, *n*-hexane. and ethylacetate extracts derived from stem calli was examined at the National Cancer Institute, Cairo University Egypt, by SRB assay as described by Suresh *et al.* [15] using color intensity measurements by ELISA RADER at a wavelength of 570 nm. The main values of results were calculated as follows: survival fraction=OD (treated cells)/OD (control cells), and IC50 value=the concentration of tested extract required to produce 50% inhibition of cell growth, which was calculated using sigmoidal dose response curve fitting mode.

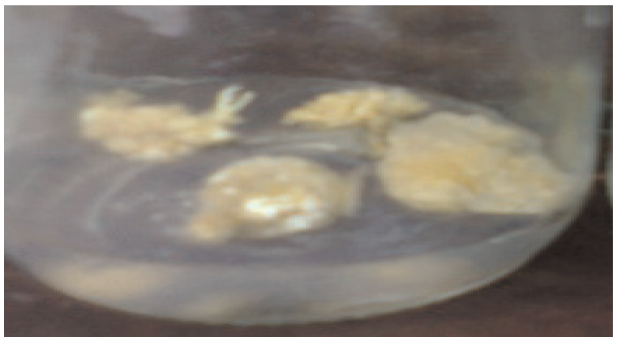
Apoptosis assay by detection of DNA fragmentation

Apoptosis measurement via DNA fragmentation was preceded according to the method of Gao *et al.* [16].

Statistical analysis

All calculated values were expressed as mean values±SE according to Snedecor and Cochran [17].

Figure 1



Callus induction of stem explants cultured on MS-medium containing 1 mg/l naphthaleneacetic acid+0.5 mg/l benzylaminopurine.

Results and discussion

Induction of callus cultures

In a previous study, we reported MS+1 mg/l naphthaleneacetic acid+0.5 mg/l BAP as the optimal medium for callus induction from stem explants [9]. Derived calli were sub-cultured on MS-medium contained 8 mg/l 2,4-D for callus production (Fig. 1).

Improvement of callus productivity by 2.4-D effectiveness

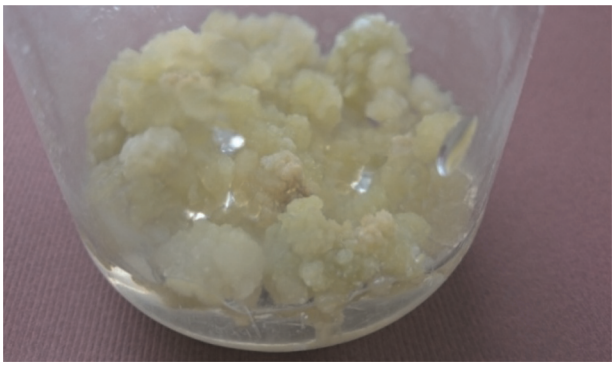
The results presented in Table 1 *showed* that MS medium modified with 5 mg/l 2,4-D+0.2 mg/l BAP gave the *highest fresh weight* and growth values (28.4 and 17.9 g, respectively). In case of growth rate, 4 mg/l 2.4-D and 4 mg/l 2.4-D+10 mg/l thiamine-HCl recorded maximum values (0.67±0.17 and 0.69±0.09, respectively) after 10 days of culture. Contrarily, among all tested concentrations, 8 mg/l 2,4-D gave the lowest growth rate value (0.37±0.06) after ten days of cultivation using 5 mg/l 2.4-D+0.2 mg/l BAP. The growth rate continuously increased, recording the highest value (1.01±0.29 g/day) at 30 days of culture (Table 1). No substantial differences were observed in stem calli proliferation using 4 mg/l 2.4-D or 4 mg/l 2.4-D+10 mg/l thiamine-HCl. The use of 5 mg/l 2.4-D+0.2 mg/l BAP was visually observed to be the best treatment in callus proliferation after 30 days of cultivation, as shown in Fig. 2. It could be recommended to use 5 mg/l 2.4-D+0.2 mg/l BAP for accessing the highest productivity of callus proliferation (Table 1). This result is in accordance with Sami *et al.* [18], who obtained the favored callus growth by adding 1.0 mg/l of 2,4-D concentration to 0.2 mg/l BAP in the third subculture. Our obtained results are partially inconsistent with those of Fletcher *et al.* [6], who produced a higher calli formation using 8 mg/l 2.4-D than other 2.4-D concentrations applied (12 and 15 mg/l) from leaf explants of local cassava varieties in Ghana [6].

Table 1 Effect of 2,4-D, BAP and thiamine added to MS-medium on fresh weight, growth value, and growth rate of stem calli after 40 days of culture

Treatments	Days of culture	Fresh weight (FW) (mean ±SE) (g)	Growth value (GV) (mean ±SE)	Growth rate (GR) (mean±SE) (g/day)
	10	8.2±1.818	4.467±1.213	0.67±0.17
	20	10.8±1.136	6.200±0.756	0.26±0.07
4 mg/l 2.4-D	30	13.1±1.343	8.00±0.576	0.015±0.015
	40	13.25±1.476	8.732±0.370	0.015±0.015
	10	9.4±1.476	2.85±0.224	0.69±0.09
4 mg/l 2.4-D+10 mg/l thiamine	20	12.95±1.213	4.765±1.520	0.36±0.18
	30	13.5±0.867	4.817±1.509	0.27±0.092
	40	14.6±0.557	4.867±1.498	0.11±0.056
	10	7.35±1.704	3.9±1.136	0.59±0.16
	20	16.05±1.361	9.700±0.906	0.87±0.04
5 mg/l 2.4-D+0.2 mg/l BAP	30	26.27±2.65	14.3±2.150	1.01±0.29
	40	28.4±1.74	17.932±1.167	0.20±0.09
	10	5.15±0.71	2.432±0.481	0.37±0.06
	20	7.9±1.56	4.267±1.05	0.26±0.07
8 mg/l 2.4-D	30	7.9±1.56	4.267±1.046	0.0
	40	7.9±1.56	4.267±1.046	0.0

Data represents means±SE of three independent frequencies. BAP, benzylaminopurine.

Figure 2



Stem calli grown on MS-medium supplemented with 5 mg/l 2.4-D +0.2 mg/l benzylaminopurine, after 30 days of cultivation.

GC-MS analysis of fatty acids and fatty acid derivatives in chloroform, hexane, and ethylacetate extracts of cassava calli

GC separated medium chain of fatty acids with their derivatives and then were identified by MS as the applied method for quantitative and qualitative analysis techniques of fatty acids constituents. Systematic and common name of fatty acids, retention time, molecular formula, molecular weight, and percentage of peak area are presented in Tables 2–4.

Identification of fatty acid composition in chloroform extract of cassava calli by GC-MS

The contents of fatty acids and their derivatives in calli as a result of extraction by chloroform solvent were separated and identified (Table 2). GC-MS analysis detected major saturated fatty acids such as propanoic acid derivatives ($C_8H_{16}O_2$), named isobutyl isobutyrate as the most abundant compound (9.39%) at retention time of 26.8, followed by decanoic acid derivatives ($C_{11}H_{22}O_2$), named capric acid methyl ester with concentration of 9.17% at retention time 31.44, and then nonanoic acid derivatives ($C_{10}H_{20}O_2$), named Pelargonic acid methyl ester, with concentration of 8.27% at retention time of 42.31. Butyric acid propyl ester ($C_{12}H_{24}O_3$), stearic acid methyl ester ($C_{19}H_{38}O_2$), and lauric acid ($C_{12}H_{24}O_2$) gave close values to each other (6.82, 6.21, and 6.11%, respectively) at retention times of 26.15, 37.14, and 26.15, respectively. However, both of myristonitrile ($C_{14}H_{27}N$) and enanthic acid, 9-decen-1-yl ester ($C_{17}H^{32}O^2$) recorded the least values (5.18 and 4.83%, respectively) at retention times of 41.70 and 46.44, respectively. Results obtained revealed that the constituents of fatty acids in chloroform callus extract were mostly derived as fatty acid methyl esters and fatty acids propyl ester except lauric acid, which was detected as free saturated fatty acid.

Identification of fatty acids composition in *n*-hexane extract of cassava calli by GC-MS

Fatty acid constituents and their derivatives in stem calli extract as a result of hexanoic extraction were detected and characterized by GC-MS (Table 3). Most of fatty acid compositions were frequently identified as free saturated fatty acids compounds in the hexanoic extract such as pentanoic ($C_5H_{10}O_2$), heptanoic ($C_7H_{14}O_2$), decanoic ($C_{10}H_{20}O_2$), and hexadecanoic ($C_{16}H_{32}O_2$) acids; other fatty acid constituents were separated into fatty acids methyl esters such octanoic

Table 2 Lipid profile characterization of saturated fatty acids and their derivatives in chloroformic extract of calli by GC-MS

Systematic name of the compound	Common name of the compound	Retention time (Rt)	Molecular formula	Molecular weight	% of peak area
2,2-Dimethyl-1-(2-hydroxy-1-isopropyl) propyl ester of isobutanoic acid	Butyric acid, propyl ester	26.15	$C_{12}H_{24}O_3$	216	6.82 ±0.403
Propanoic acid, 2methyl, 2methylpropyl ester	Isobutyl isobutyrate	26.84	$C_8H_{16}O_2$	144	9.39 ±0.711
Decanoic acid, methyl ester	Capric acid, methyl ester	31.44	$C_{11}H_{22}O_2$	186	9.17 ±0.221
Decanoic acid	Lauric acid	33.99	$C_{12}H_{24}O_2$	200	6.11 ±0.101
Octadecanoic acid, methyl ester	Stearic acid, methyl ester	37.14	$C_{19}H_{38}O_2$	298	6.21 ±0.299
Tetradecane nitrile	Myristonitrile	41.70	$C_{14}H_{27}N$	209	5.18 ±0.153
Nonanoic acid, methyl ester	Pelargonic acid, methyl ester	42.31	$C_{10}H_{20}O_2$	172	8.27 ±0.379
Heptanoic acid, 9-decen-1-yl ester	Enanthic acid, 9-decen-1-yl ester	46.44	$C_{17}H_{32}O_2$	268	4.83 ±0.110

Data represents means±SE of three independent frequencies. GC-MS, gas chromatography–mass spectrometry.

Table 3 Lipid profile characterization of saturated fatty acids and their derivatives in hexanoic extract of calli by GC-MS

Systematic name of the compound	Common name of the compound	Retention time (Rt)	Molecular formula	Molecular weight	% of peak area
Pentanoic acid	Valeric acid	20.92	$C_5H_{10}O_2$	102	10.98±0.512
Heptanoic acid	Enanthic acid	27.27	$C_7H_{14}O_2$	130	3.39±0.045
Decanoic acid	Capric acid	27.56	$C_{10}H_{20}O_2$	172	3.48±0.044
Octanoic acid, methyl ester	Caprylic acid, methyl ester	31.42	$C_9H_{18}O_2$	158	14.68±6.05
Hexadecanoic acid	Palmitic acid	33.52	$C_{16}H_{32}O_2$	256	23.55±6.13
Eicosanoic acid, methyl ester	Arachidic acid, methyl ester	37.12	$C_{21}H_{42}O_2$	326	2.76±0.032
Octadecanoic acid, 2-hydroxyethyl ester	Stearic acid, 2-hydroxyethyl ester	40.05	$C_{20}H_{40}O_3$	328	10.42±0.092
Decanol	Capric alcohol	46.42	$C_{10}H_{22}O_2$	158	5.73±0.030

Data represents means±SE of three independent frequencies. GC-MS, gas chromatography–mass spectrometry.

($C_9H_{18}O_2$) and eicosanoic ($C_{21}H_{42}O_2$) derivatives, fatty acids hydroxylethyl ester (FAHEs) such as octadecanoic derivatives ($C_{20}H_{40}O_3$), and fatty alcohol such as decanol ($C_{10}H_{22}O$). Considerable compound was detected in a free saturated fatty acid formula such as palmitic acid, with concentration of 23.55% at retention time of 33.52, followed by methyl ester derivatives such as caprylic acid methyl ester with concentration of 14.68% at 31.42. Both of valeric acid and stearic acid hydroxyethyl ester gave similar values (10.98% and 10.42%, respectively, at retention times 20.92 and 40.05, respectively). Slight amounts of capric alcohol ($C_{10}H_{22}O$), capric acid ($C_{10}H_{20}O_2$), enanthic acid ($C_7H_{14}O_2$), and arachidic acid methyl ester ($C_{21}H_{42}O_2$) have been determined (5.73, 3.48, 3.39, and 2.76%, respectively) at retention times of 46.42, 27.56, 27.27, and 37.12, respectively. It is manifested from our results that the highest value of fatty acids was achieved in a formula of free saturated fatty acids such palmitic acid and the lowest value was in a formula of methyl ester derivatives fatty acid such as arachidic methyl ester.

Identification of fatty acids composition in ethylacetate extract of cassava calli by GC-MS

Six saturated fatty acids were detected in ethylacetate extract at free formula with different concentrations at various retention times, such octanoic (C8H16O2), decanoic ($C_{10}H_{20}O_2$), heptanoic ($C_7H_{14}O_2$), dodecanoic ($C_{12}H_{24}O_2$), undecanoic ($C_{11}H_{22}O_2$), and nonanoic ($C_9H_{18}O_2$) acids (Table 4). On the contrary, conjugated fatty acids with ester linkage at various forms were achieved, for instance, anhydride derivatives of octanoic acid ($C_{16}H_{30}O_3$), methyl ester derivatives of hexanoic acid ($C_8H_{16}O_2$), ethyl ester derivatives of dodecanoic acid ($C_{16}H_{32}O_4$), hydroxyethyl ester derivatives of hexadecanoic acid ($C_{18}H_{36}O_3$), pentadecyl ester derivatives of hexanoic acid ($C_{21}H_{42}O_2$), and tridecyl ester derivatives of butanoic acid ($C_{17}H_{34}O_2$). The dominant free fatty acid in ethylacetate extract was lauric acid (28.34%) followed by caprylic acid (14.52%) then enanthic acid (6.41%) at retention times of 33.99, 21.47, and 27.69, respectively. Capric acid, undecylic acid, and pelargonic acid were gradually decreased (2.53, 2.30,

Table 4 Lipid profile characterization of saturated fatty acids and their derivatives in ethylacetate extract of calli by GC-MS

Systematic name of the compound	Common name of the compound	Retention time (Rt)	Molecular formula	Molecular weight	% of peak area
Octanoic acid	Caprylic acid	21.47	$C_8H_{16}O_2$	144	14.52±0.349
Decanoic acid	Capric acid	27.56	$C_{10}H_{20}O_2$	172	2.53±0.021
Heptanoic acid	Enanthic acid	27.69	$C_7H_{14}O_2$	130	6.41±0.16
Octanoic acid, anhydride	Caprylic acid, anhydride	28.71	$C_{16}H_{30}O_3$	270	3.89±0.055
Hexanoic acid, 5-methyl-, methyl ester	Caproic acid, 5-methyl-, methyl ester	31.44	$C_8H_{16}O_2$	144	3.10±0.011
Dodecanoic acid	Lauric acid	33.99	$C_{12}H_{24}O_2$	200	28.34±0.634
Undecanoic acid	Undecylic acid	34.28	$C_{11}H_{22}O_2$	186	2.30±0.073
Dodecanoic acid, 2(2hydroxyethoxy) ethyl ester	Diethylene glycol monolaurate	36.44	$C_{16}H_{32}O_4$	288	2.26±0.04
Hexadecanoic acid, 2-hydroxyethyl ester	Palmitic acid, 2-hydroxyethyl ester	40.15	$C_{18}H_{36}O_3$	300	18.84±0.744
Nonanoic acid	Pelargonic acid	41.37	$C_9H_{18}O_2$	158	1.83±0.02
Hexanoic acid, pentadecyl ester	Caproic acid, pentadecyl ester	45.06	$C_{21}H_{42}O_2$	326	3.52±0.06
Tridecyl, 2-methylpropanoate	Isobutyric acid, tridecyl ester	52.43	$C_{17}H_{34}O_2$	270	1.680.10

Data represents means±SE of three independent frequencies. GC-MS, gas chromatography–mass spectrometry.

and 1.83%, at retention times of 27.56, 34.28, and 41.37, respectively). The conjugated fatty acid constituents were diversely identified into multiderivatives such as palmitic acid hydroxylethyl ester, recording high value, 18.84% at retention time of 40.15, compared with the other derivatives of fatty acids, giving less amounts such caprylic acid anhydride, with concentration of 3.89% at retention time of 28.71, followed by caproic acid pentadecyl ester and caproic acid methyl ester, with concentrations of 3.52 and 3.10%, respectively, at retention times of 45.06 and 31.44, respectively; moreover, isobutyric acid tridecyl ester was detected at the least amount (1.68%) at retention time of 52.43. These results achieved that the nonconjugated fatty acids such as lauric acid recorded the highest value as a free saturated fatty acid, whereas capric acid recorded the lowest value.

As obvious from our achieved results in Tables 2–4, polarity differences of examined solvents for extraction possess a pronounced effect on lipid diversity profiles, which had been proved by GC-MS between free fatty acids and conjugated fatty acids derivatives. Chemical structures of fatty acid constituents for ethylacetate extract differed mostly than those for chloroform and hexane extracts, in which lauric acid, chemically known as *n*-dodecanoic acid, was more suitable to be extracted by ethylacetate, whereas isobutyl isobutyrate, chemically known as ester propanoic acid, and palmitic acids, chemically known as hexadecanoic acid, were more suitable to be extracted by chloroform and hexane, respectively. It could be concluded that the solvent polarity is a main reason for lipid profile diversity, where the structural differences of the detected fatty acids are affected by the efficacy of solvents' polarity, which thereby controls the extraction of various free fatty acids and fatty acid derivatives. The detected lipid profile by GC-Mass analysis in chloroform, hexane, and ethylacetate callus extracts exhibits 8, 8, and 12 peaks, respectively, of fatty acid structures as ion chromatogram, which compared with the spectra corresponding to the library equipment. In conclusion, our data revealed that ethylacetate as aprotic polar solvent is the ideal for more peaks (12 peaks) than chloroform and hexane as nonpolar solvents, which detected less peaks (eight of each). These investigations clearly recommended that ethylacetate is the most suitable of substantial fatty acids, in particular lauric acid (28.34%), as a component of triglycerides and monoglyceride derivative such monolaurate diethylene glycol (2.26%) as a monoester of lauric acid, and both of them have important medical applications. In our findings, the hexane extract comprised palmitic acid as the major compound of lipid profile, although it is recognized that palmitic acid registered controversial studies concerning its unhealthy effect potentials as approved by Denke and Grundy and Mancini *et al.* [19,20]. In this paper, the authors recommend ethylacetate to be the preferable solvent for most extractions of the lipid content owing to its higher safety in the extraction processes rather than either chloroform or hexane, which are less safe. These fulfillments are nearly similar to the achieved results by Nwabueze and Okocha [21], who mentioned that the solvent polarity for extraction may affect closely the extracted oil yield in seeds of African breadfruit, and also Arjuna and Somal [22], who preferred ethylacetate in lipid isolation as food grade solvent owing to lower toxicity. According to the previous investigations from our recent study, it could be concluded that the isolated lipids from the stem callus cultures of cassava in-vitro

plantlets contained a diversity of saturated fatty acid structures in free formulas. Authors in this survey did not speculate any degradation of fat content during their extraction, because the selected procedure for lipid extraction by the distillation using any of chloroform, hexane, and ethylacetate solvents is relatively a mild operation. This observation is in accordance with Kamenarska *et al.* [23] who identified some of fatty acids in a free structure in algae and expected no hydrolysis of lipids owing to the use of the distillation-extraction as a mild process.

Phytochemical analysis of chloroform, hexane, and ethylacetate cassava callus extracts by GC-MS

It is observed from this study that phytochemical structures accompanied lipid content during the extraction of fatty acid constituents and their derivatives. The obtained results are abbreviated in Table 5. Each solvent extracted some of phytochemical components from cassava stem callus extracts which had been detected by GC-Mass. For instance, seven compounds were identified in chloroform extract such terpineol (5.08%), paraffin (15.02%), organic phenols (11.03%), volatile oils (3.59%), isoparafin (3.63), hydrocarbon alkanes (3.13%), and alkylated dipyrrinones (2.53%). Likewise, seven compounds were identified in hexane extract such as organosilicon (2.81%), amyl nitrite (2.41%), sulfur compound (3.88%), phenols (7.03%), cholesterol (2.36%), alcohol (2.28%), and sugar alcohol (4.25%), whereas two compounds were identified in ethylacetate extract like phenols (2.24%) and esters of volatile compound (7.29%). Data in Table 5 reveal volatile oil in chloroform callus extract, which disappeared in both of hexane and ethylacetate callus extracts; however, sulfur compound was observed in hexane callus extract but was absent in either of chloroform or ethylacetate callus extracts; each of volatile oil and sulfur compounds were present at low concentrations (3.59 and 3.88%,

Table 5 Phytochemical constituents identified in different solvent extracts of Manihot esculenta stem calli by GC-MS

Solvent	Retention time (Rt)	Compound name	Compound nature	Molecular formula	Molecular weight	Peak area %
Chloroform	25.98	Cyclohexene-1-methanol, à,à,4trimethyl, (S)	Terpineol	$C_{10}H_{18}O$	154	5.08 ±0.04
	27.56	Dodecane	Paraffin	$C_{12}H_{26}$	170	15.02 ±0.703
	31.16	Butylated hydroxyl toluene	Lipophilic organic phenols	$C_{15}H_{24}O$	220	11.03 ±0.456
	32.87	Epiglobulol	Volatile oil	$C_{15}H_{26}O$	222	3.59 ±0.08
	33.62	Methylnonane	Isoparaffin	$C_{10}H_{22}$	142	3.63 ±0.021
	54.79	Docosane	Hydrocarbon alkanes	$C_{22}H_{46}$	310	3.13 ±0.090
	56.68	1,3,8trimethy,-4-butyl-5-ethyl-2(1-hydroxyethyl) –7-methoxy carbonyl-6,Ç-methylene carbonyl-porphine	Alkylated dipyrrinones	$C_{37}H_{44}N_4O_4$	608	2.53 ±0.012
Hexane	25.26	Dodecamethylcyclo hexasiloxan	Organosilicon compound	$C_{12}H_{36}O_6Si_6$	444	2.81 ±0.11
	27.55	Methylbutyl nitrite	Amyl nitrite	$C_5H_{11}NO_2$	117	2.41 ±0.02
	30.71	3-(5'-Chloro-1',3'diphenylpyrazol-4'-yl) 1-oxo(1H) pyrido[2,1-b]Benzothiazole-2,4carbonitrile	Aromatic heterocyclic sulfur compound	$C_{28}H_{14}C_1N_5OS$	503	3.88 ±0.12
	31.13	Methyl-2,6-di tertbutylphenol	Phenols	$C_{15}H_{24}O$	220	7.03 ±0.08
	35.62	19-hydroxy methyl-5à-cholest-1-ene	Cholesterol	$C_{27}H_{46}O$	386	2.36 ±0.035
	39.25	Cyclobutylsilane	Alcohols	$C_4H_{10}Si$	86	2.28 ±0.012
	41.31	Allo Inositol	Sugar alcohol	$C_6H_{12}O_6$	180	4.25 ±0.312
Ethylacetate	31.16	4-Methyl-2,6-di-tertbutylphenol	Phenols	$C_{15}H_{24}O$	220	2.24 ±0.023
	46.50	Methoxyacetic acid,2-tridecyl ester	Esters of volatile compound	$C_{16}H_{32}O_3$	272	7.29 ±0.423

Data represents means±SE of three independent frequencies. GC-MS, gas chromatography–mass spectrometry.

respectively). Paraffin as a versatile compound used in a variety of wellness, healthcare, and industrial settings was predominant in the chloroform extract (15.02%). Other phytochemical structures such phenols are present at different concentrations: 11.03% in chloroform extract, 7.03% in hexane extract, and 2.24% in ethylacetate extract. It could be summarized from Table 5 that the major component of phytochemical constituents was paraffin (15.02%) in chloroform extract, phenols (7.03%) in *n*-hexane extract, and volatile compound (7.29%) in ethylacetate extract. The presence of phenols' structures in all extracts affirms the vitality of cassava stem callus cultures against carcinogenesis as confirmed by Sabir *et al.* [24] who published that the existence of phenols in coconut could imply to be used for arthrosclerosis combat and cancer growth inhibition.

Comparitive assessment of the lipid content and other phytochemical constituents in chloroform, hexane, and ethylacetate cassava callus extracts

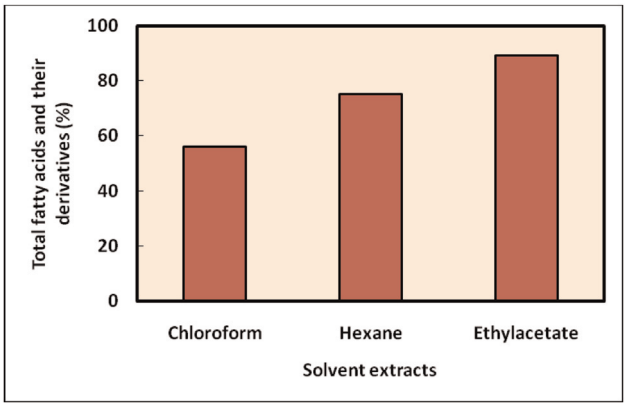
As can be seen in Fig. 3, the ethylacetate extract gave the highest extraction of lipid content (89.22%) with the least amount of phytochemical constituents (10.78%), whereas the chloroformic extract gave the minimum value of lipid content extraction (55.98%), with the highest amount of phytochemical constituents (44.02%). Meanwhile, the hexanoic extract recorded medium values in lipid content (74.99%) and phytochemical constituents (25.01%) compared with both of chloroform and ethylacetate extracts. Another significant aspect pointed out the ordering of ethylacetate, hexane, and chloroform solvents to be the first, the second, and the third, respectively, in fatty acid extraction from cassava stem callus cultures. Our results exhibited that lipid content varied in cassava callus extracts as a result of diversity of the used solvent efficiency in extraction process, which in turn are affected by the polarization of solvent which had been ranged from polar to nonpolar.

Based on the aforementioned results, it could be outlined that the derived extracts from cassava stem calli are considered as a main source of essential phytochemicals and beneficial fatty acid constituents with great role in therapeutic applications such as capric acid which possess antibacterial, antiprotozoal, antiviral, and anticancer influences [25], palmitic acid as strong antimicrobial [26], and lauric acid which have highly potent effectiveness to secrete insulin [27]. The existence of phenol structures in cassava callus extracts implies their antioxidant characteristics which are accountable for the medicinal significance, where the antioxidants decrease the process of free radicals' consistence or neutralize them to conserve the cell from oxidative damage [28]. Subsequently, potential of cassava as one of the medicinal valuable plants should be promoted for health-boosting purposes.

Bioactive effect of chloroform, hexane, and ethylacetate extracts of cassava callus cultures as antibreast cancer activity

This work was carried out by SRB method using the inhibition percent of carcinoma cells for breast and their IC50 values. Argumentative studies about the effectiveness of saturated fatty acids against tumor activity have reported. In this survey, authors provide a brief and overall update for the functional role of fatty acids and their derivatives for the three calli extracts in reduction of the breast cancer cell viability (Table 6). The tabulated data illustrate that ethylacetate extract highly inhibited the vitality of cancer cells (MCF7) to be IC50 of 2.63 μg/ml, whereas chloroform extract represented the lowest effect on cancer cell growth with IC50 value of 6 μg/ml; however, hexane extract affected slightly on cancer cell reduction, with IC50

Figure 3



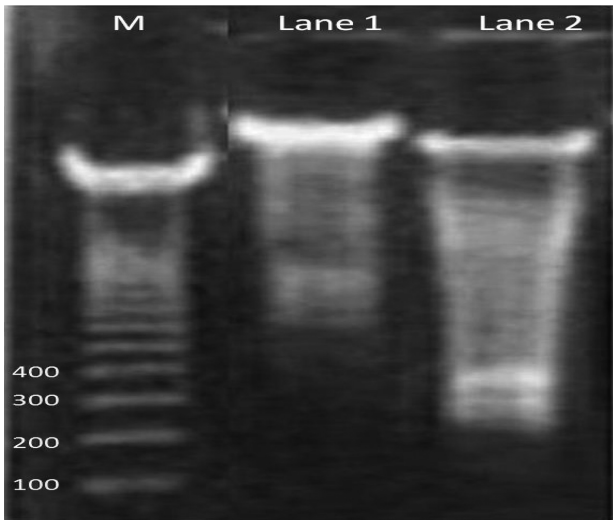
Total fatty acids and their derivatives (%) of chloroform, hexane, and ethylacetate calli extracts of cassava.

Table 6 Inhibitory impact of stem callus extracts of cassava plant on the tumor growth for breast cancer cells

Samples	Concentrations/μg					
	0	2.5	5	10	20	IC50
Chloroform extract						
SF%	100	93.8	52.8	38.1	19.6	6
Error	0.05	0.047	0.026	0.019	0.01	
IG%	0	6.21	47.2	61.9	80.4	
Ethylacetate extract						
SF%	100	54.7	23.1	9.86	5.09	2.63
Error	0.05	0.027	0.012	0.005	0.003	
IG%	0	45.3	76.9	90.1	94.9	
Hexane extract						
SF%	100	75.3	12.8	23.8	27.6	3.44
Error	0.05	0.038	0.006	0.012	0.014	
IG%	0	24.69	87.25	76.2	72.41	

value of 3.44 μg/ml. These results are in line with the acquired results in Fig. 3, which reported that ethylacetate solvent dissolved the major amounts of lipids followed by hexane solvent and then chloroform solvent. Hence, it could be deduced the vital role of lipids to raise the efficiency of cassava stem callus extracts for inhibition the breast cancer cell growth. Moreover, it should be taken into consideration the presence of lauric, capric, caprylic acids, and their derivatives with higher proportions in ethylacetate extract (Table 4) than in chloroform and hexane extracts (Tables 2 and 3). Those achieved results in Tables 2–4 are probably to be responsible for increment of the efficiency of ethylacetate extract to inhibit the growth of breast carcinoma cell lines (Table 6). Our findings are comparatively in congruence with data published before and reported the vital role of caprylic and lauric acids for improvement of cancer risk reduction [10,11,26].

Figure 4



Agarose gel electrophoresis of DNA extracted from MCF7 cells treated with extracts. M: DNA ladder(marker); lane 1: MCF7 tumor cells; lane 2: MCF7 cells treated with ethylacetate extract. All of wells were loaded with 20 μl extract.

Influence of the saturated fatty acids and their derivatives on induction breast cancer cells apoptosis

Programmed cell death, called apoptosis, is a normal physiological process that plays an important role in homeostasis and growth of the normal and cancer cells. In this study, the process of DNA fragmentation by ethylacetate extract of cassava stem callus cultures was examined using the isolated DNA from breast carcinoma cell patients to show their cracking for oligonucleotide fragments formation. DNA ladder is used to characterize between necrosis and apoptosis [29].

DNA-agarose gel electrophoresis was performed to detect DNA fragmentation (DNA laddering) by plant extracts. Such a phenomenon was observed in plant extract-treated MCF7 cells, which is consistent with the fact that nuclear fragmentation and condensation occur during apoptotic cell death (Fig. 4).

Overall results seem to indicate that exposure of MCF7 cells to 100 and 200 μg/ml of different plant extracts for 24 h may be effective in inducing apoptosis (Fig. 4).

Gao *et al.* [16] investigated resveratrol-induced DNA fragmentation in 32Dp210 leukemic cells. The compound-induced apoptosis in these cells with appeared by the induction of internucleosomal DNA fragmentation and the cleavage of procaspase-3 in resveratrol-treated cells [16,30].

Fig. 4 revealed that, the highest DNA fragments as a result of DNA cleavage process were detected in breast cancer cells which did not expose to ethylacetate extract (lane 1). On the contrary, the fragments of DNA which be expressed as DNA ladder patterns were slightly formed in breast cancer cells, which be incubated with ethylacetate extract as manifested in lane 2, showing little apoptosis in the isolated DNA. Our previous investigations in Table 5 and Fig. 4 indicated the hallmark efficiency of ethylacetate extract on breast cancer cells in terms of the cytotoxic effect of ethylacetate to suppress the cancer cell growth with less toxicity on their DNA fragmentation. Silva *et al.* [30] found that *Plinia edulis* leaves aqueous extract and *Mimosa caesalpiniifolia* leaves ethanolic extracts, respectively, led to cellular death, DNA degradation, and cytotoxicity in MCF7 cells via apoptosis mechanism. Our results demonstrated that ethylacetate extract of cassava callus acted as an anticancer agent against breast cancer by cell growth inhibition and apoptosis induction. Furthermore, it could be concluded that the ethylacetate extract of cassava stem calli did not induce any undesirable variation on the physiological processes for the human cells, which in turn lead to DNA degradation. This in line with Deders *et al.* [31], who assured that DNA cleavage at the intranucleosomal locations inducing apoptosis is controlled by physiological processes. Based on these achieved investigations, ethylacetate possesses more efficacies for saturated fatty acids extraction, high potent effect as anticancer activity, and less apoptosis induction for the cells compared with chloroform and hexane solvents.

Conclusion

In summary, the authors recommend to employ ethylacetate as aprotic polar solvent in lipid extraction from stem callus cultures extracts of cassava in vitro plantlets, owing to its efficiency for extraction of medicinally paramount fatty acids, with antibreast cancer factor. Potential of cassava as one of the medicinal valuable plants should be promoted for health-boosting purposes.

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Conflicts of interest

There are no conflicts of interest.

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