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Comparison the organic and inorganic solvents effect on phenolic compounds extraction and the activity against breast carcinoma cell lines from callus cultures of *Manihot esculenta*

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Abstract : Cassava (*Manihot esculenta*) is valuable plant including industrial and therapeutic potential, rich in essential nutrients for the body such as carbohydrates, vitamin A, vitamin C, iron, calcium, phosphorus and phenolic compounds. Ourstudy aimed to evaluate the efficiency of absolute organic solvent (methanol) and inorganic solvent (water) for phenols extraction from stem calli of Cassava and identification the phenolic compounds of their crude extracts (methanol and aqueous extracts) by HPLC. Examine the cytotoxicity of both of methanol and aqueous extractsagainst breast cancer cell lines (MCF7) were determined. Stem, leaf and petiole explants of *in vitro* plantlets were cultured in MS-medium to initiate calli, stem was chosen as the best explant for callus initiation frequency (74.88%)on medium containing 1mg/l NAA+0.5mg/l BA. Effect of 2,4-D and picloramat concentrations 8 and 16 mg/l of each were tested on callus production from stem explant, maximum value of calli mean production (76.51%) was achieved on the optimum selected medium for callus production (8 mg/l 2,4-D).Phenolic acid, flavonoids and cinnamic acid derivatives were identified in methanol and aqueous extracts by HPLC at 280, 320 and 360 nm of the UV chromatographs. At 280 nm, coumarin and gallic acid were mostly separated at concentrations 28.456 and 38.210 µg/ml of each in methanol and aqueous extracts, respectively. At 320 nm, gentisic acid and sinapic acid concentrations were 11.454 µg/ml and 0.448 µg/ml of eachin methanol and aqueous extracts, respectively. No detectable compounds at 360 nm in aqueous extract, but in methanol extract, rutin was identified at concentration 0.759 µg/ml.Total amount of the identified free phenolsrecorded 54.658 µg/ml in aqueous extract higher than value in methanol extract (50.547 µg/ml).Methanol extract gave IC50 of 1.43 µg/ml, while aqueous extract gave a potentialIC50 of 1.3 µg/ml. Our work was performed byDNA fragmentation and detection DNA content using flowcytometry of DNA in different groups, untreated- controland treated-Cassava aqueous extract were diploid, while the breast cancer cell line was aneuploid. The results of DNA electrophoresis demonstrated that DNA damage observed in breast cancer cell line intoxicated has been recovered with cassava aqueous extract protection and the treated MCF7 showed a single diploid peak.

Keywords : *Manihot esculenta*; Callus cultures; Methanol and aqueous extracts; Phenolic compounds;MCF7; DNA fragmentation and content.

Introduction

Common name of Cassava is *Manihot esculenta*, belongs to *Eupobiaceae* family as a perennial shrub. Despite its native country is South America Oriola and Raji[1], it islargely grown in Ghana and Africa as starchy tuber food for human and animal feed Kay[2]. Cassava is very important in third world regions for food security because it serves as starchy storage in terms of roots and leaves Koubala *et al*[3], providing food by farmers in small scale plantation for millions people Roca *et al*[4]. Beside that it tolerates high temperature, prolonged drought, opposite environmental conditions and enablesto establish after planting El-Sharkawy[5]. For that Cassava is consider a reliable crop, grown on peripheral soils giving adequate yields Le *et al*[6]. Cassava called life blood because of the consumption of its starchy tubers as staple starch, textile and pharmaceutical applicationsSrinivas[7]. Cassava is a potential source of proteins (21 - 39%), antioxidant constituents-Srinivas[7], Balamurugan and Anbuselvi[8], vitamins B1, B2 and C and minerals, iron, zinc, manganese, magnesium and calcium- Adewusi and Bradbury[9], Wobesto*et al*[10], Massey [11], also rich in micro and macronutrients-Fasuyi[12] and Chavez[13]. Indeed, Cassava production has risen worldwide due to a lot of different industrial applications such as quality food for human, animal feed, raw material and also medicinal applications like antidiabetic, anticarcinogen, antioxidant and antitumor potential El-Sharkawy[5], Montagnac *et al*[14], Suresh *et al*[15]. Cassava is considered a valuable plant including industrial and therapeutic potential, cassava in terms of the ethanolic and aqueous extracts involve alkaloids, anthraquinone, flavonoids, anthocyanosides, tannins and Saponins Anbuselvi and Balamurugan[16]. Cancer is a global health problem with an increasing number of sufferers each year. While the three main cancers are most often detected are lung cancer, breast cancer, and colon cancer. Cancer spread out in the developed countries and now is emerging in Asia Smith[17]. Cancer caused by chemical, metabolic, environmental and genetic factors, phenolic compounds display biological functions related to the carcinogenesis modulation Dai and Mumper[18]. Understanding the multi steps nature of prolonged tumorgenesis process has led to the realization that most malignancies can be fought on multiple fronts. Thus in addition to cancer therapy, cancer prevention has become an important approach to control cancer through consumption various fruits and vegetables to reduce the risk of malignant tumors. Cassava is rich in nutrients for instance, vitamins, carbohydrates, essential minerals such as iron, calcium, phosphorus as well as phenolic compounds. The damage of Cassava roots by infection either of fungi or cutting, tissues accumulate more phenols such as scopoletin, diterpenoid and diterpenoid in the infected regions Buschmann *et al*[19], Sakai and Nakagawa [20].Modern biotechnology was used for improving the traditional breeding in Cassava plants which have been hampered by several factors such as long reproduction period Hahn [21], apomixes and low flower production Nassar[22] and diversity in ploidy number Nassar[23] as well as the roots of Cassava, after harvest and during storage easily deteriorate Yi *et al*[24]. Alternative biotechnological methods begins to establish stable cultures of active callus in Cassava plants to can be genetically induced giving planting material, also calli play an important role for embryogenesis process to offer successful production of germplasm and generation storage Fletcher *et al*[25]. Cancer is an abnormal growth and proliferation of cells. It is a frightful diseasebecause the patient suffers pain, disfigurement and loss of many physiological processesVelraj and Sowmya[26]. Higher total phenols were found in Cassava in both of organically grown marrionberries and conventional fertilization method Asami *et al*[27].Phenolic compounds have antitumor activity to inhibit the growth of human breast (MCF-7), prostate (LNCaP, DU-145) oral (KB, CAL-27) and colon (HT-29,HCT-116) tumor cell lines Zhang *et al*[28] and Seeram *et al*[29]. The potential value of the tested compound to inhibit the human cancer cell growth is considered as indicator for in vivo anticancer agent Lieberman *et al*[30]. Biotechnology is one of the most efficient tools used in Cassava plants to reduce the toxicity of cyanogenic glucoside content-Le *et al*[6], although the toxicity of linamarinhad not been reported Cook [31], furthermore the hydrogen cyanide is considered the most powerful metabolic poison Keresztessy *et al*[32].According to Literature data showed by scholars that extraction efficiency of solvents isstrongly dependent on plant material, our work made attempts tocompileliteraturereporting quantification and pharmacological activitiesof phenolic compounds accumulation.Herein our current study was planned for producing calliof stem explants from *in vitro* plantlets of Cassavaand evaluation the best method for extraction of phenolic compounds using organic (methanol) and inorganic (water) solvents as well as report the preliminary investigations for the efficiency of the methanolic and aqueous extracts against breast cancer cell line, apoptosis rate was evaluated by detection DNA fragmentationlikewise DNA content was evaluated by flocytometric analysis.

Materials and Methods

1. Trial site and explants preparation.

This present study was conducted in Plant Biotechnology Department, Genetic Engineering and Biotechnology Division, National Research Centre, Giza, Egypt.Sterile stems, leaves and petioles derived from the intact *in vitro* plantlets of Cassava (*Manihot esculenta*) were cut into three segments each one piece of about 5 and 10 mm in sterilized Petri dishes. All stages of sterilization were done in thelaminar flow hood.

2. Callus culture conditions and Experimental design.

The excised segments were placed horizontally on Murashige and Skoog medium (MS-medium) supplemented with picloram (5 mg/l), NAA (0.1,1,5 mg/l), 2,4-D (5 mg/l) and BA (0.2, 0.5, 1 mg/l), GA3 (1 mg/l) with different combinations. The pH was adjusted to 5.8 before autoclaving at 121°C for 15 min.The cultures were done in triplicates and incubated in the culture room under controlled light regime (16 hrs photoperiod of fluorescent 45μ mol, cool white light tubes and 8 hrs dark)at 24±2°C for two weeks to observe callus formation. The initiated calli derived from stem explants grown on the best selected medium for callus initiation were sub-cultured on modified MS-medium including different concentrations of 2,4-D (8 and 16 mg/l) or picloram (8 and 16 mg/l) for increasing the productivity of callus proliferation.

2.1. Frequency of callus formation (%).

Frequencies of callus formation were calculated by the following equation:

Frequencies of callus formation (%) = (Explants produced callus / Total cultured explants) × 100.

3. Extract preparation.

Fresh samples of *in vitro* calli cultures of stem explantswere extracted using two solvents for extraction,absolute organic solvent (methanol) and inorganic solvent (water). Submerging 50g of fresh calli in 100ml of either of methanol or water, then the aqueous and methanol extracts were performed for a period of 24 hours followed by heating at 60°C for 3 hours. The sample was filtered through a 0.45 μm filter and subjected to centrifugation at 10.000 rpm. The supernatant evaporated and concentrated by vacuum distillation, the residues were re-dissolved in either of methanolor water, subsequently were stored at 4°C till further use for both of HPLC analysis and cytotoxicity assay.

3.1. Separation of phenolic compounds.

Separation of the phenolic compounds by HPLC analysis was performed for both of organic and inorganic extracts of Cassava callus cultures with help of Agilent Technologies 1100 series liquid chromatography equipped with an auto sampler and a diode-array detector. The analytical column was an Eclipse XDB-C18 (150 X 4.6 μm; 5μm) with a C18 guard column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 0.8 ml/min for a total run time of 70 min and the gradient programme was as follows: 100% B to 85% B min in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min and 0% B to 100% B in 5 min. The injection volume was 50 μl and peaks were monitored simultaneously at 280, 320 and 360 nm for the phenolic acid, cinnamic acid derivatives and flavonoids, respectively. All samples were filtered through a 0.45 μm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectra and compared with those of the standards.

4. Cell culture and *In vitro* anti-tumoral assay.

Effect of methanolic and aqueous crude extracts of Cassava stem calli were tested against breast cancer cell line viability. Anti-cancer testing using the SRB method with parameters such as the percentage of inhibition, apoptosis assay by detection DNA damage was assessed by agarose gel electrophoresis and DNA content analysis by flowcytometry.

4.1.   Cytotoxic assay.

The cytotoxicity of both of methanol and aqueous extracts from stem calli of *Manihot esculenta* was tested at the National Cancer Institute, Cairo University Egypt by SRB assay using the method of Suresh *et al*[33].Tumor cells used in this study were human cancer cells (MCF-7 human breast cancer cells),SRB is a bright-pink aminoxanthere dye with two sulfonic groups that bind to basic amino-acid residues under mild acidic conditions as the binding of SRB is stoichiometric the amount of dye extracted from stained cells is directly proportional to the cell mass, the assay depends on the ability of SRB to bind to protein components of cells that have been fixed to tissue-cultures plates by tri chloro acetic acid (TCA).Cells were plated in 96-multiwell plate (104 cells/well) and left for 24 hrs to allow attachment of cells to the wall of the plate, different concentrations (2.5,5,10,20µg/ml) of the tested extracts were added. The plates were incubated at $37°c$ for 48 hrs in atmosphere of 5% Co2, after that cells were fixed with TCA then washed and stained with SRB, excess stain was washed with acetic acid and attached stain was recovered with tris-EDTA buffer. The cytotoxic of methanol and aqueous extracts was carried out using color intensity measurements by ELISA RADER at wave length 570 nm. The main values of results were calculated as follows:- Survival fraction=O.D (Treated cells) /O.D(control cells), IC 50 values:The concentration of tested extract required to produce 50% inhibition of cell growth was calculated using sigmoidal dose response curve fitting mode.

4.2.   Apoptosis assay by detection DNA fragmentation

DNA was extracted from mature (normal cells) white blood cells and breast cancer cell line (MCF7)before and after treatment with the water crude extract. Nucleic acids extraction and detection for apoptosis was done according to "salting out extraction methods" of Gao *et al*[34].Cells were washed with PBS and then lysed in cold lysis solution (5 mmol/L of Tris, pH 7.4, 20 mmol/L of EDTA, 0.5% Triton X-100) for 20 min. Cell lysates were centrifuged at 27,000 g for 15 min, and DNA was extracted from the aqueous phase with phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) containing 0.1% (w/v) hydroxyquinoline. DNA was precipitated with 0.3 mol/L of potassium acetate and 2 volumes of cold 100% (v/v) ethanol. Agarose gel (3% w/v) electrophoresis was proceeded at 30 mA for 2 h followed by UV fluorescence was used to determine the degree of apoptotic DNA fragmentation. The picture of gel was taken via gel documentation system (BioRad).

4.3.   Flowcytometric studies.

FCM was used to determine DNA Ploidy by using Dako-Cytomter System. Standardization and initial alignment were performed according to the manufacturer's instructions. Solid tumor samples were mechanically dissociated to get a suspension of single cell nuclei and prepared for DNA analysis following the technique of Costa *et al*[35]. DNA Staining   was performed according to technique of Vindelov *et al*[36]. The DNA sample was considered "DNA diploid "if it showed a single G0/G1 peak with a CV ≤ 5%in histogram and whose position was confirmed by running of the lymphocyte sample at first. Nuclei sample was considered "DNA aneuploid"if it had an additional abnormal peak that represented at least 10% of the total population collected with an identifiable G2/M peak Vindelov *et al*[36]. The relative DNA content of the tumor was determined compared to the normal diploid control and obtained by calculating the ratio of the abnormal (aneuploid) G0/G1 peak and defined as DNA index (DI). The diploid tumors with normal DNA content have DI ≥ 0.95 & ≤ 1.03, aneuploid tumors with abnormal DNA content have DI > 1.03 or < 0.95.DI as suggested by Flyger*et al*[37].

5.   Statistical analysis.

The analyses were done in three independent replicates; concentration of the identified phenols within plant material was expressed asmean values ± SE according to Snedecor and Cochran[38].

Results

1.   Callus cultures formation of *Manihot esculenta*.

In the present study, an experiment for obtaining the highest productivity of callus cultures was intended in a two step-protocol including callus initiation and callus productionusing stem, leaf and petiole explants of *in vitro* plantlets from *Manihot esculenta*.

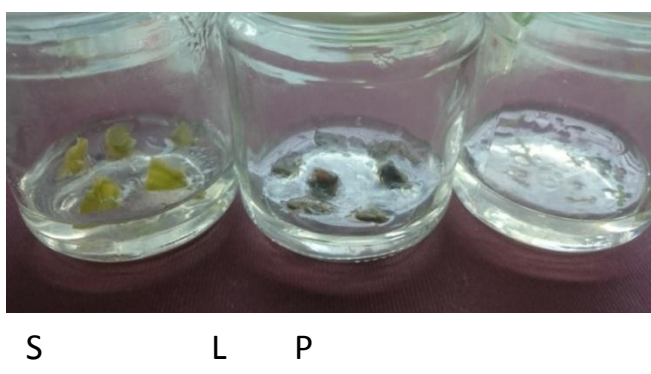### 1.2.   Frequency of *Manihot esculenta* callus initiation.

Stem, leaf and petiole explants of *in vitro* plantlets of *Mnihot esculenta* responded differentially to initiate calliby culturing on MS-medium supplemented with different combinations of picloram (5 mg/l), 2.4-D (5 mg/l), NAA (0.1, 1.0, 5 mg/l), BA (0.2, 0.5, 1.0 mg/l) and GA3 (1 mg/l). Table (1) reveals that MS-medium supplemented with 5mg/l NAA+0.2mg/l BA had no response of callus initiationfor all used explants, whereas the combinations of 5 mg/l picloram with 0.2 mg/l BA or with 5 mg/l 2.4-D + 5 mg/l NAA had poor performance for callus initiation frequency (23.64% and 22.25%, respectively)using single stem explants. Both of stem and leaf explants induced slightly callus frequency, 26.7% and 24.64%, respectively by culturing in MS-medium supplemented with 0.1mg/l NAA+1mg/l BA+1mg/l GA3. The best result for callus initiation frequencywas obtained with a culture in a MS-medium fortified with1mg/l NAA+0.5mg/l BAgivingsatisfactory performance using stem explants (74.88%) and less response usingleafexplants (25.44%). The formation of calli was successfully initiated using stem explants, partially using leaf explants andno response using petiole explants.These results are different from those of Fletcher *et al*[25]who induced callus from leaf, petiole and auxiliary buds of four Cassava cultivars recording that the leaf explants were superior in callus formation in all the Cassava varieties, contrary our obtained results which investigated that stems were the best explant for callus initiation. Nevertheless, stem explants produced relatively the same amounts of calli upon treatment with picloram independent or with 2, 4-D.

Table (1): Callus initiation frequency of stem, leaf and petiole explants of *Manihot esculenta* after 14days of cultivation.

Treatments	Frequency of callus initiation %		
	Stem	Leaf	Petiole
5mg/lpicloram+0.2mg/lBA	23.64 ± 1.148	0	0
5mg/lpicloram+5mg/l2.4-D+5mg/lNAA	22.25 ± 1.096	0	0
5mg/l NAA+0.2mg/lBA	0	0	0
1mg/lNAA+0.5mg/lBA	74.88± 0.352	25.44 ± 0.672	0
0.1mg/lNAA+1mg/lBA+1mg/l GA3	26.7± 0.699	24.64 ± 0.462	0

Values are mean ± Standard Error of three replicates.

Leaf explants showed delayed browning callus initiationcompared with callusing of stemexplantswhich appearedyellow to green in color and performed better as initial explants for healthy callus initiation, whereas petiole explants failed to yield callus as shown in Fig.(1).

Fig. (1) Initiated calli of stem (S),leaf (L), petiole (P) explants of *Manihot esculenta* cultured on MS-medium containing 1 mg/l NAA+0.5 mg/l BA after 14 days of culture.

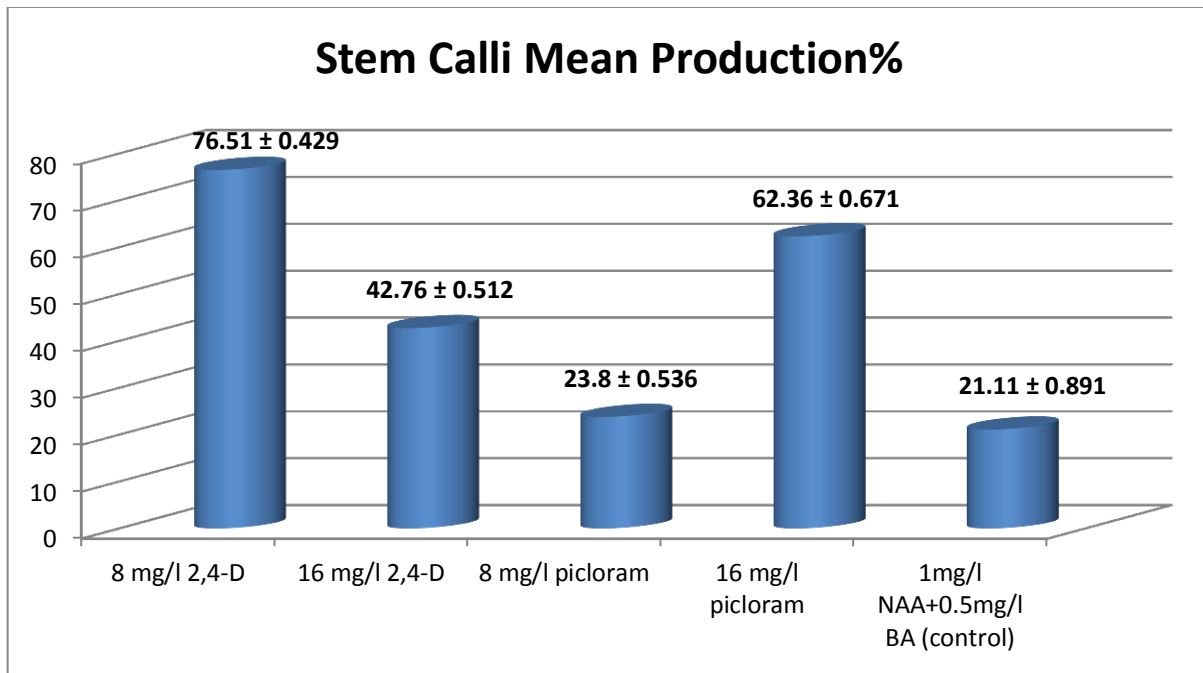### 1.3. Callus production of *Manihot esculenta*.

As previously observed, the medium which composed of MS-medium supplemented with 1 mg/l NAA+0.5 mg/l BA was selected to be the suitable medium for callus initiation of stem explants, the initiated calli were routinely subcultured on that medium, but no proliferation of callus was obtained (Fig. 2).

Fig. (2) Stem calli of *Manihot esculenta* cultured on medium fortified with 1 mg/l NAA+0.5 mg/l BA.

Similar to the frequency of callus initiation which was influenced by the type of explants, different types and concentrations of auxin registered significant interaction for callus production using stem explants. Therefore, a factorial design with 1 mg/l NAA+0.5 mg/l BA as a control and MS-medium supplemented with 8 and 16 mg/l 2,4-D or 8 and 16 mg/l picloram was used (Fig.3).Response to callus productionof stem explants varied between different treatments, high concentration of picloram (16 mg/l) produced 62.36% of callusmean production more than2.4-D (42.76%) at the same concentration (16 mg/l), whereas low concentration of picloram (8 mg/l)recorded the minimum mean value of calli (23.80%) in line with the control which gave slightly callus (21.11%). In contrast 2.4-D recorded satisfiedmean value of callus production (76.51%) at concentration 8 mg/l. Significant differences with respect to callus production of stem explants were observed in the presence of high concentration of picloram (16 mg/l) and low concentration of 2.4-D (8 mg/l) compared to the control, while no significant differences were observed for callus production using low concentration of picloram (8 mg/l)but were observed slightly using high concentration of 2.4-D (16 mg/l).The results in Fig. 3 manifest the most appropriate auxinto be more effective than control and other treatments for enhancement callus production from stem explants of *Manihot esculenta* was to be 2.4-Dat 8 mg/l concentration. Therefore, it could be concluded that there was a generally increased tendency of callus production with the decreasing concentration of 2.4-D and increasing of picloram concentration as manifested in Fig. 4.These findings were ensured by Fletcher *et al*[25]who found that 8 mg/l 2,4-D concentration was visually the best in callus formation across the cassava cultivars butusing leaf explants where leaf cells have thin cell walls which are less lignified Schädel *et al*[39] and less recalcitrant to differentiation in culture medium due to their high responsiveness to phytohormonesSan-José *et al*[40],conversely, our results displayed that stem explants generated callogenesis significantly when compared with leaf explants. On the other hand, our results are in disagreement with both of AdilaMeloVidal *et al*[41]who obtainedhigher frequency ofcalli from cassava using 8 mg/l picloram and Fletcher *et al*[25]who recorded that12 mg/l 2,4-D concentration gave the highest value of calli using bark explants from cultivar *Afisiafi* of Cassava plant.

Fig. (3) Stem calli mean production of *Manihot esculenta* cultured on medium containing2,4-D and picloram with different concentrations after three weeks of culture.Data represents means ± SE of three independent experiments.

As previously mentioned in Table (1), the treatment using combination of 2,4-D with picloram decreased the frequency of callus initiation compared with the treatment using NAA with BA (control).Nevertheless the data in Fig. 3illustrates that the independent auxins evaluation of both of 2,4-D and picloram increased the mean production of calli, irrespective of the used concentrations of eachrelatively to the treatment including NAA with BA. Callus production percentages wereascendingranged from 23.8 % to 76.51 % for stem explants using 2,4-D and picloramat concentrations8 and 16 mg/l. These investigations are in agreement with Marigi *et al*[42] who obtained the highest frequencies of callus induction (>98%) for both cassava cultivars (KMM and TMS60444), when 2,4-D and Picloram were used within 14 days of culture.On the other hand, sami *et al*[43]induced the highest calli using zeatinor 2,4-D at 0.5 mg/l for each from leaf explant (100%) of Hibiscus syriacus L. Fig 4illustrateshigh mean production of callus by culturing stem calli on media fortified with 2,4-D at each of 8 and 16 mg/l orpicloram at 16mg/l, meanwhile picloram at 8 mg/l concentration gave poor performance for callus production. The obtained results are confirmed by Marigi *et al*[42]who indicated that callus formation in Cassava cultivar (KMM) varies with the type and concentration of auxins used.

Fig. (4) Stem calli of *Manihot esculenta* grown on MS-media containing 2,4-D and picloram with different concentrations after three weeks of culture.

Note:(S1) Stem calli of *Manihot esculenta* inoculated on 8 mg/l 2,4-D.**(S2)** Stem calli of *Manihot esculenta* inoculated on 16 mg/l 2,4-D. **(R1)** Stem calli of *Manihot esculenta* inoculated on 8 mg/l picloram. **(R2)** Stem calli of *Manihot esculenta* inoculated on 16 mg/l picloram.

2. Identification of phenols and flavonoids with HPLC-UVin methanol and aqueous extracts derived from stem calli of *Manihot esculenta*.

Extraction and analysis of Cassavaphenolic Compounds were achieved using differenttwo solvents varying from organic solvent such as methanol and inorganic solvent such as water from stem calli of *Manihot esculenta*. Three wavelengths such as 280, 320 and 360 nm of the UV chromatographs are examined for the presence of various phenol compounds that were separated and identified by comparison with available standards. HPLC separations allowed to identifying 6 phenols at 280 nm, 4 phenols at 320 nm and 2 phenols at 360 nm in both of methanol and aqueous extracts for the stem calli of the studied plant. Concentrations of phenols, flavonoids and cinnamic acid derivatives with retention time at the three signals (280, 320 and 360 nm) were summarized in Table (2).Phenolic acid, flavonoids and cinnamic acid derivatives as hydrophilic phenols were found in both of methanol and aqueous extracts in different significant. HPLC-UV separates some of phenolic acids at 280 nm such as gallic and vanillic at different concentrations of each, inmethanol extracts recording 2.591µg/ml and 5.497µg/ml, respectivelyand in aqueous extractsrecording 38.210µg/ml and 4.565µg/ml, respectively. Nevertheless, protochuic and syrngiconly occurred in aqueous extracts withclose values, 3.460 µg/ml and 3.787µg/ml, respectively, and it has been shown that gentisic was detected singlyin methanol extracts at 320 nm recording 11.454 µg/ml. HPLC analysis revealed clear differences in the cinnamic acids and their derivatives at 320 nmbetween methanol and aqueous extracts where, ferulic acid content was higher in methanol extracts (0.460 µg/ml) than in aqueous extracts (0.184 µg/ml). Contrary, sinapic acid content recorded value, 0.448 µg/ml in aqueous extracts higher than value, 0.159 µg/ml in methanol extracts. On the other hand, caffeic acid was detected only in methanol extracts, 1.000 µg/ml. High concentration of coumarin content (28.456 µg/ml) was achieved at 280 nm in methanol extracts while that it was reduced in aqueous extracts to concentration, 1.566 µg/ml.Some of flavonoids concentration such as rutin, kaempferol at 360 nm and Catachine at 280 nm were differentially responded where, rutin and kampferol were not detected in aqueous extracts while recorded 0.759 µg/ml and 0.171 µg/ml, respectively in methanol extracts. Concretely, Catachine disappeared completely in methanol extracts and gave 2.438 µg/ml in aqueous extracts.

Table (2) Concentration of the identified free phenols (µg/ml, mean ± S.D)within methanol and aqueous extracts of *Manihot esculenta* stem calli using HPLC- UV
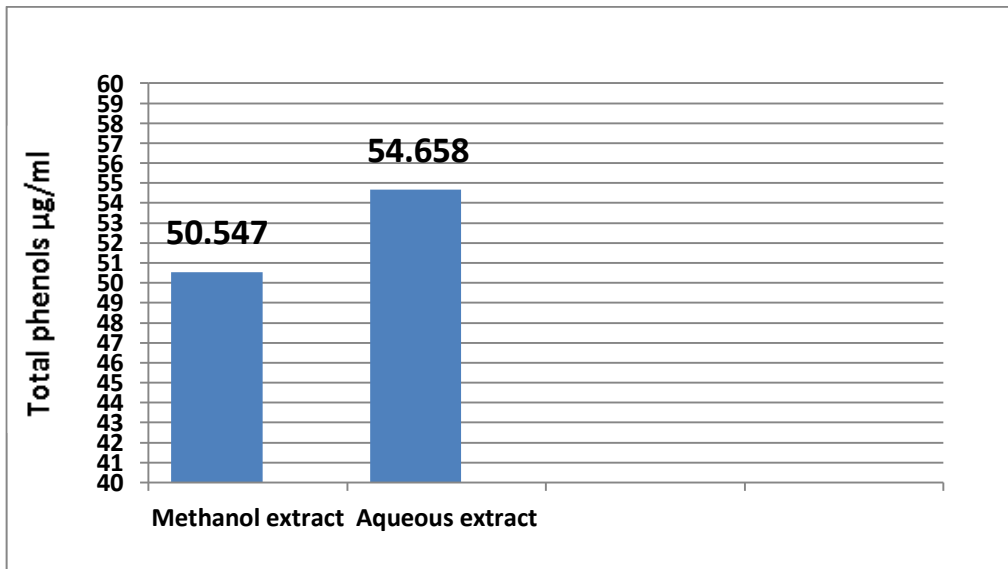
Wave lengths of UV/nm	Chemical structures	Phenols	Retention time	Methanol extracts µg/ml	Aqueous extracts µg/ml
280	Phenolic acid (C6-C1)	Gallic acid	5.50	2.591 ± 0.351	38.210 ± 0.615
	Phenolic acid (C6-C1)	Protochuic acid	9.60	ND	3.460 ± 0.271
	Flavanol (C6-C3-C6)	Catachine	18.20	ND	2.438 ± 0.399
	Phenolic acid (C6-C1)	Syrngic acid	22.40	ND	3.787 ± 0.564
	Phenolic acid(C6-C1)	Vanilic acid	24.10	5.497 ± 0.556	4.565 ± 0.734
	Benzopyrone lactone (C6-C3)	Coumarin	36.40	28.456 ± 0.544	1.566 ± 0.412
320	Phenolic acid(C6-C1)	Gentisic acid	16.70	11.454 ± 0.574	ND
	Hydroxycinnamic acid(C6-C3)	Caffeic acid	20.90	1.000 ± 0.110	ND
	Hydroxycinnamic acid (C6-C3)	Ferulic acid	31.90	0.460 ± 0.071	0.184 ± 0.059
	Hydroxycinnamic acid (C6-C3)	Sinapic acid	33.30	0.159 ± 0.050	0.448 ± 0.049
360	Glycoside of flavonoid quercetin (C6-C3-C6)	Rutin	35.90	0.759 ± 0.057	ND
	flavonol (C6-C3-C6)	Kaempferol	46.10	0.171 ± 0.055	ND

Data represents means ± SE of three independent experiments

Data are shown in Table (2) summarize the Predominant phenols withinboth ofmethanol and aqueous extracts are gallic, vanillic, coumarine, frulic and sinapic, taking into account that protochuic, catachine and

syrngic did not detected in methanol extracts conversely gentisic, caffeic, rutin and kampfrol did not detected in aqueous extracts. When comparing between methanol and aqueous extracts at the major compound of free phenols, it is appeared that methanol extracts contained mostly coumarine and aqueous extracts contained mostly gallic acid. Comparatively total content of the identified phenolic compounds between methanol and aqueous extracts derived from stem calli of *Manihot esculenta*, it was noted that the aqueous extracts contained 54.658 µg/ml of the total identifiedfree phenolshigher than in methanol extracts which contained 50.547 µg/ml as illustrated in Fig. (4

Fig. (4)Total amount of the identified free phenols in methanol and aqueous extracts from *Manihot esculenta* stem calli

Water as inorganic solvent was more efficient solvent for phenolic compounds extraction than methanol as absolute organic solvent, therefore it could be recommended to extract and resolve more phenols from stem calli of *Manihot esculenta* by water as a favorable solvent for the extraction. This result is similar agreement with those of Sultana *et al*[44]who obtained the higher phenolic contents and extract yields using aqueous organic solvents, as compared to the respective absolute organic solvents, Tomsone *et al*[45] who revealed that the best solvents for phenolic isolation from Horseradish Roots was ethanol and ethanol / water solutions and Spigno *et al*[46] who combined water with ethanol to improve the extraction rate of phenolic compounds. Therefore, it could be advised to use water asthe most efficient solvent for phenols extraction from stem calli of *Manihot esculenta* which giving the biggest amounts of total phenols. Furthermore, the extraction by water as inorganic solvent increased gallic acidto 38.210 µg/ml and decreased coumarin content to 1.566 µg/ml, conversely methanol as organic solvent showed the best value of coumarin content(28.456 µg/ml) and exerted slightly gallic acid (2.591 µg/ml).In summary, our results clearly showed thattotal amounts of free phenols and the composition of phenolic acid, flavonoids and cinnamic acid derivatives of Cassava extracts is significantly affected byusing both ofwater and methanol solvents for the extraction.These findings are in accordance with data published earlier by Michiels *et al*[47]who reported that the extracting solvents properties significantly affected on total phenolics content (±25% variation) and antioxidant capacity(up to 30% variation) in fruits and vegetablesand influenced the natural antioxidants content using different extractionsolvents, taking into consideration that Phenolic compounds are usually mainly responsiblefor the antioxidant properties of fruits and vegetables Hervert-Hernández D, *et al*[48].Also our investigations are confirmed by Agbangnan *et al*[49] who extracted a high yield of phenolics using water from sorghum leaf and Verma *et al*[50] who reported that phenolics extraction from wheat bran requires 80% aqueous ethanol.Subsequently, it is appeared that both of water and methanol were the most efficient solvents for extraction phenolic compounds Boeing *et al*[51].Different investigations between our results with those obtained by other researchers about the best solvent for phenols extraction support the scientific truth which reveal that the optimum recovery of phenolics is different from one sample to the other and relies on the type of plant and its active compounds Khoddami *et al*[52].Where Nourian *et al*[53] found that the methanolic extract had the highest phenolic content which showed that methanol can be an efficient solvent for extracting phenols in comparison with ethyl acetate and dichloromethane.Nevertheless,the obtained data from

our study refer to the acceptability of using water as the best inorganic solvent for resolving and extraction phenolic compounds rather than the absolute methanol as organic solvent.This may be due to low efficiencyof solvation provided by methanol compared to water, although both of water and methanol are most efficient solvents for phenols extractionbased on their capability as proton donors Boeing *et al*[51].

3. Cell culture and *In vitro* anti-tumoral assay.

Cancer is still one of the most serious causes of death worldwide with no major progress in reducing its morbidity and mortality, three main cancer is most often detected are lung cancer, breast cancer and colon cancer.Cassava is rich in a lot of nutrients such as phenolic compounds, for that Cassava is consumed as anti-cancer vegetables for human body. However, not much research on the anti-cancer activity of cassava, especially in breast cancer had been done.

3.1. Cytotoxic effect and IC50 values of methanol and aqueouscrude extracts from stem calli of Manihot esculenta on breast carcinoma cell lines.

Aim of this part of our study is to investigate the anti-cancer activity of both of aqueous and methanol crude extracts which derived from stem calli of *Manihot esculenta* against the viability of the breast carcinoma cell line (MCF7). The study was evaluated by measuring cell growth inhibition/cytotoxicityas shown in Table (3) using SRB assay method with parameters such as the percentage of inhibitionfor breast cancer cells and IC50 values.

Table (3)Cytotoxic effect of methanol and aqueous crude extracts from stem calliof *Manihot esculenta* on breast cancer cell lines (MCF7)
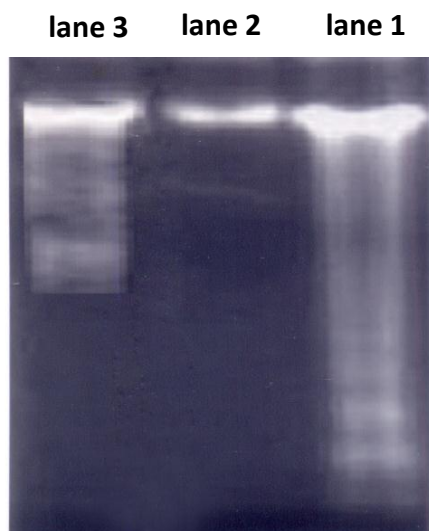
Samples		Concentrations µg/ml					
		0	2.5	5	10	20	IC50
Methanol extracts	S.F%	100	24.7	19.6	18.2	15.8	1.43
	Error	0.05	0.012	0.01	0.009	0.008	
	I.G%	0	75.3	80.35	81.77	84.23	
Aqueous extract	S.F%	100	4.935	4.871	19.9	12.13	1.3
	Error	0.05	0.002	0.002	0.01	0.006	
	I.G%	0	95.06	95.13	80.1	87.87	

Each of methanol and water as organic and inorganic solvents, respectively were used to resolveand extract phenols from stem calli of *Manihot esculenta*, and determine the cytotoxic effects of the methanol and aqueous crude extracts on MCF7. Two experimental samples were testedat a concentration of 2.5 to 20 µg/ml, methanol crude extract showed cytotoxicactivity against breast cancer to be IC50 of 1.43 µg/ml, whereas aqueous crude extract represented effects slightly higher on breast cancer with IC50 value of 1.3 µg/ml. Although waterresolvedmore effective compounds from Cassava stem callias the optimal inorganic solvent for the extraction process than using methanol as organic solvent, but both of methanol and aqueous extracts recorded relative values close each other for inhibition the growth of breast cancer cells.This achievement is relatively in accordance with Graidist *et al*[54] who revealed that methanol was more effective than dichloromethane to extract the effective compounds from *Piper cubeba* seeds, where the dichloromethane crude extract demonstrated a weak effect on all cell lines of breast cancer compared to methanol crude extract. Also Saleh and Emara[55]showed that the Methanol extracts of Balanitis aegyptiaca cell suspension showed *in vitro* cytotoxicity against two differenthuman cancer cell lines such as liver (Hep-G2), and prostate (PC-3). Where as in case of PC-3 cell line treated with plant extract at 3.125 ug showed maximum activity 98.47 decreasing to 26.74 % for 100%.Beside that our results differed with the obtained data by Mat Akhir *et al*[56]whocleared that the ethanolic extract of *Ficus deltoidea* plant affected significantly to inhibitthe growth of human ovarian carcinoma cell Linecompared to the aqueous extract. Furthermore, it could be referred to the most efficient for inhibition the breast cancer cell growth was usingaqueous crude extract in comparison withmethanol crude extract. This investigationmay be due to that, aqueous extract contained the biggest amount of gallic acid comparable methanol extract as mentioned in Table (2). Gallic acid play a great role as anticarcinogenic Nayeem *et al*[57], additionally that gallic acid could be considered as a promising compound for new drug development. In another study by Karamaæ *et al*[58] and Kaur*et al*[59], gallic acid has been proved to have potential

preventiveand therapeutic effects in various diseases, neurodegenerative disorders, cardiovascular diseases, in aging and cancer. Subsequently,our investigations recommended that water is applied for valuable extraction process as the mostappropriate solvent to extractmore phenolic compounds content and resolve the mostgallic acid that leads to suppress significantly the growth of breast carcinoma cell lines (MCF7).

3.2.   DNA Fragmentation on the treated cells.

Apoptosis is a programmed cell death that can occur by a variety of internal or external stimuli, and these signals are controlled by two distinct pathways. One is an extrinsic pathway (death receptor pathway), and the other is an intrinsic pathway in which mitochondria are involved Chia-Jung [60]. In present study, we have shown analysis of DNA fragmentation induced by aqueous stem calli extracts of *Manihot esculenta* in three cell lines was examined by isolation of DNA from breast cancer cell line (MCF7) patients for showing their degradation into oligonucleotide fragments forming a clear laddering pattern of apoptosis. To characterize cells apoptosis induced by breast cancer cell line (MCF7), the DNA ladder was examined as shown in (fig. 5). Lane 1 expressed MCF7 cell line without aqueous extract, lane 2 expressed DNA from normal cellincubated with the aqueous extract, and lane 3 expressed MCF7 cell line treated with aqueous extract. DNA fragmentation assay was used to determine whether the action of aqueous extract derived from stem calli of *Manihot esculenta* was associated with apoptosis or not. Aqueous extracts of Cassava stem calli cultures have potent pro apoptotic effects in tumor cells as shown in Fig.(5). Lane 1 DNA ladder characteristic of apoptosis and DNA fragmentation. Lane 2 control was set in the DNA ladder assay without any fragmentations. Lane 3 shown traces of apoptosis were present in DNA extracted from the treated MCF7 with cassava aqueous extract. DNA-agarose gel electrophoresis was performed to detect DNA fragmentation (DNA laddering) by aqueous extract, which is consistent with the fact that nuclear fragmentation and condensation, occurs during apoptotic cell death. The highest cleavage of DNA forming fragmented DNA was found on MCF7without exposure to aqueous extract, on the other handladder pattern of the fragmented DNA was not detected in normal cell line treated with aqueous extract, and meanwhileMCF7 treated with aqueous extract expressedslightly formation of a DNA ladder. The ladder pattern of the fragmented DNA can be used to distinguish between apoptosis and necrosis according to Saraste and Pulkki[61] and Elmore[62]. Specific DNA cleavage is due to the activation of endogenous endonuclease that cleaves at the exposed linker regions between nucleosomes, but necrosis associates with the random form of DNA cleavage as demonstrated by Darling [63]. Considerable that formation of DNA ladder indicated to the cytotoxic effect of aqueous extractfor inhibition the growth of breast cancer cell line and might be extended to normal cell line through apoptosis.The shown data in fig (5) exhibit highly increment of DNA fragmentation for untreated breast cancer cell line compared to the decrement of DNA fragmentation which be occurred for the treated breast cancer cell line, whilst DNA was intact and DNA fragmentation was not detected in normal cell line.
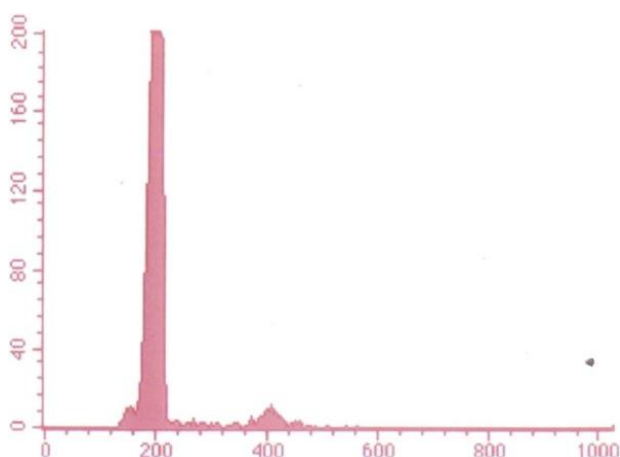

Fig.(5)Gel electrophoresis of DNA fragments derived from MCF7,before treatment as positive control (lane 1), aqueous extract was used to treat normal cell lineas negative control (lane 2), aqueous extract was used to treat MCF7(lane 3).
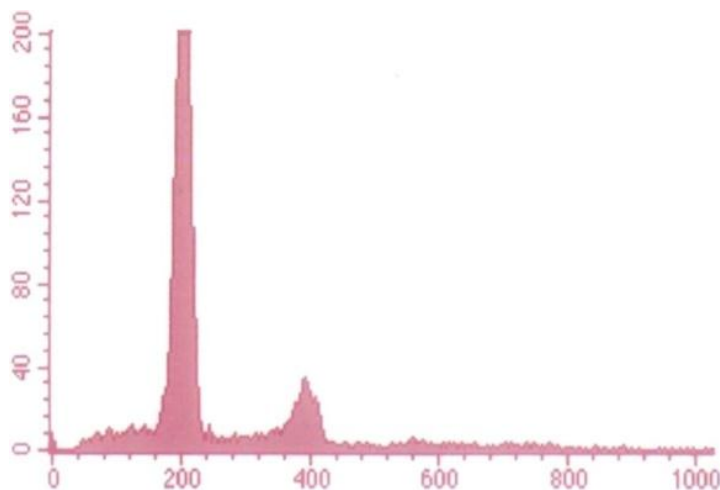
The obtained investigations in Table (3) and Fig (5) revealed that aqueous extract showed cytotoxic effect to inhibit cell growth of breast carcinoma cell line (MCF7) and lower toxicity for DNA fragmentation in breast cancer cell line without appearing any apoptosis induction for normal cell line. It is worthy to highlight thataqueous crude extract seemed to be safe for normal cells as well as the hallmark of aqueous crude extract for MCF7 apoptosis that decrease the cleavage of the nuclear DNA forming less ladder pattern of the fragmented DNA. The cellular DNA cleavage at the intranucleosomal sites undergoing apoptosis induced under physiological conditions producing DNA fragmentation as reported by Deders *et al*[64].The results outlined above are in a good agreement with those of Mat Akhir*etal*[56]who did not observed DNA fragmentation in human ovarian carcinoma cell lineusing aqueous extract of *Ficus deltoidea*, where the cleavage of the internucleosomal DNA did not produce ladder pattern for the aqueous extract treated cells but ethanolic extract could cause DNA degradation. In contrast our achievements differed with the obtained results by Carvalho*etal*[65] and Silva *et al*[66] who found that both of an aqueous extract of *Plinia edulis* leaves and ethanolic extract of *Mimosa caesalpiniifolia* leaves caused cell death, cytotoxicity and DNA fragmentation for MCF-7 cells througha mechanism using the apoptosis pathway.

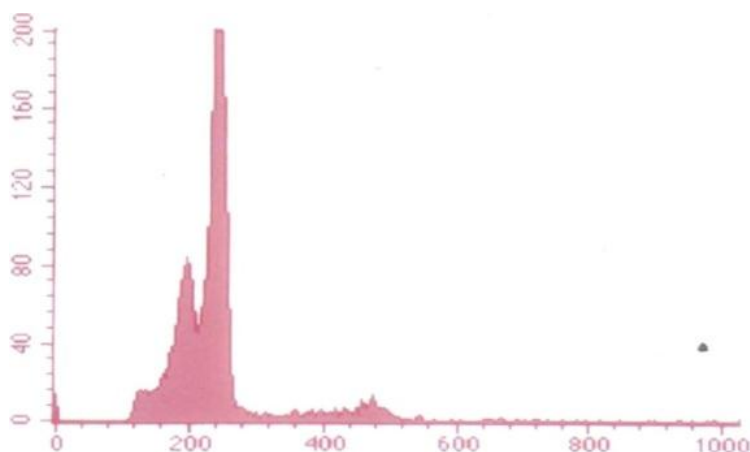### 3.3. DNA content by flowcytometry on the treated cells.

In the flowcytometric analysis, the normal DNA as acontrol treated group showed a single diploid peak. Similarly, the MCF7 treated group showed a single diploid peak as well. Regarding the group of MCF7 without treated showed abnormal aneuploid peak, an additional abnormal peak was detected in all of MCF7 samples (aneuploid cells) along with the diploid one. All samples of control cell line treated with aqueous extract of cassava showed a single diploid peak. Breast cancer cell line (MCF7) treated group with aqueous extract of Cassava showed a diploid DNA peak in all of samples with disappearance of the abnormal aneuploid peak that was detected in the MCF7 treated group. However, 15% of liver samples showed aneuploidy (Figure 6,7 and 8)**.**DNA Flowcytometry is a semi-quantitative method for rapid, accurate and quantitative analysis of the DNA ploidy, Proliferative activity and the distribution of cells in the different phases of the cell cycle Shapiro [67].The flow cytometric results showed that MCF7 with cassava aqueous extract treated group showed a single diploid peak similar to that of normal cell line treated with cassava aqueous extract group, while breast cancer cell line (MCF7), an additional aneuploid peak was detected. The present result is in agreement with other reports that showed the aneuploid pattern in majority of cases bearing adenocarcinoma of the extra hepatic bile ducts Brunt and Kraemer[68] and Hsu *et al*[69].

Fig. (6) Showing a diploid peak at channel number 200 with a G2/M cell population at channel number 400 and low   S-phase fraction (< 7%)

Fig.(7)Showing diploid population with high SPF (>10%)

Fig. (8)Showing diploid (D) peaks and aneuploid (A) peaks

Conclusion

Our work was planned to compare the capability of methanol as organic solvent and water as inorganic solvent for resolving and extraction phenolic compounds from callus cultures of Cassava which is obtained by sub-culturing calli of stem as a favorable explant on the best selected medium for callus production (8 mg/l 2,4-D). Water is safe and more appropriate solvent than methanol solvent to resolve and extract the most amount of phenolic compounds particularly gallic acid. Aqueous extract affected positively to inhibit the viability of breast carcinoma cell line(MCF7) without causing DNA fragmentation for normal cell line.The flowcytometry of normal DNA and MCF7 treated with cassava aqueous extract group showed a single diploid peak. It is concluded from this study that the predominant antibreast cancer activity effect for aqueous crude extract from Cassava stem callus cultures due to including mostly gallic acid which play a vital role against cancer activity. Upon *Manihot esculenta* is a good starting point for the search for plant-based medicines.

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