Anticancer Agents from Non-Edible Parts of *Theobroma cacao*

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**Abstract**

In order to establish the medicinal properties as anticancer agents from the non-edible of cocoa plant parts, the studies on fresh of non-edible cocoa plant parts such as cocoa leaf (CL), cocoa bark (CB), cocoa husk (CH), cocoa shell unfermented (CSUF), cocoa shell fermented (CSF), cocoa root (CR), cocoa cherelle (CC) & cocoa pith (CP) were performed by extracting in methanol solvent, screening against various type of cancer cell lines, purified using bioassay guided fractionation and identified active compounds using gas chromatography-mass spectrometry (GC-MS). Results revealed that the cocoa leaf extract presented the highest antiproliferative activity against breast cancer cell line (MCF-7) with IC₅₀ value of 41.43 ± 3.26 µg/ml and selected for further purification using bioassay guided fractionation. The hexane partitioned fraction of cocoa leaf showed the highest anticancer activity with IC₅₀ value about 66.7 ± 7.95 µg/ml. Hexane partitioned fraction was further purified using flash column chromatography and divided into 3 steps such as fractionation-1, fractionation-2 and fractionation-3. Sample fraction II/F7 from fractionation-2 was selected for chemical characterization using GC-MS due to the highest anticancer activity with IC₅₀ value was 6.36 ± 0.71 µg/ml and generated 10 major active compounds with synergistic effect against MCF-7. Nine compounds were known compounds and one compound was unknown compound. *Theobroma cacao* is still an underutilized plant and its bean has been used traditionally as source of food and medicine. The consumption of cocoa leaf as non-edible part could provide health benefits especially to treat human cancer in the future.

**Keywords:** *Theobroma cacao*; Anticancer activity; Bioassay guided fractionation; Natural product; Plant extracts; Cancer cell

**Introduction**

*T. cacao* was a genus *Theobroma* and classified together with the genera *Herrania*, *Giauzuma*, and *Cola* in the family Sterculiaceae. *T. cacao* was originated from ancient Central America where the civilizations of Maya and Aztecs cultivated it for its seeds (beans), which was used for drinking called chocolatl, a precursor to the modern chocolate [1]. Peoples from Olmec and Mayan believed that cacao bean had a divine origin and belief it as “food of the gods”. Based on the scientific name of *Theobroma*, Theo is meaning “food” and Broma is meaning “gods” [2,3]. More than 100 medicinal uses for cocoa were described in manuscripts produced from over 3000 years ago to the present and cocoa has been used to treat anemia, mental fatigue, tuberculosis, fever, gout, kidney stones, and even poor sexual appetite [4]. Cocoa phytochemical contains various health effects, including improved heart function, relief of angina pectoris, and stimulant of the nervous system, facilitated digestion, improved kidney and bowel function [4]. Recent investigation on Panama’s Kuna Indian population revealed that Kuna Indians living on the islands, and heavy consumers of cocoa, had significantly lower rates of heart disease, cancer, and diabetes mellitus compared to those on the mainland, who do not consume cocoa [5]. This was suggesting that cocoa might be useful in the prevention of heart disease, cancer, and diabetes mellitus. Principally, cocoa contains high active compounds such as theobromine, flavonoids(-)-epicatechin, (+)-catechin and their dimers procyanidins B2 (PB2) and B1, although other polyphenols such as quercetin, isoquercitrin (quercetin3-O-glucoside), quercetin 3-O-arabinose, hyperoside (quercetin 3-O-galacto side), naringenin, lutelenin and apigenin have also been found in minor quantities [6].

Cancer chemopreventive activity of cocoa has been reported that cocoa liquor procyanidins significantly reduced the incidence and multiplicity of lung carcinomas and also decreased thyroid adenomas developed in male rats [7]. Selected procyanidins present in cocoa inhibit tumorigenesis, tumor growth, and angiogenesis [8]. Consumption of cocoa or dark chocolate can also decrease the burden and efficacy of epigenetic carcinogens [9]. Procyanidin-enriched cocoa seed extracts caused G2/M cell cycle arrest and 70% growth inhibition in Caco-2 colon cancer cells [10]. On the other hand, cocoa-derived pentamer procyanidin caused G0/G1 cell cycle arrest and selective growth inhibition in human breast cancer cells [11]. It has been also found that cocoa procyanidins reduce vascular endothelial growth factor activity and angiogenic activity associated with tumor pathology [8]. Fundamental mechanisms of carcinogenesis are associated with ROS, and population studies have demonstrated that people with a regular intake of foods containing antioxidants, such as vegetables, fruits, tea, or soy products, display a lower incidence of various types of cancer [9,12]. It can be postulated, therefore, that consumption of cocoa or chocolate, which have high antioxidant activity, could be beneficial in decreasing the damage from genotoxic and epigenetic carcinogens, and inhibiting the complex processes leading to cancer. Prevention of cancer through the diet is receiving increasing interest, and cocoa because of its polyphenolic compounds has become an important potential chemopreventive and therapeutic natural agent. Cocoa and its main polyphenols have been reported to interfere at the initiation, promotion and progression of cancer. Cocoa flavonoids demonstrated that influenced several important biological activities in vitro and in vivo by their free radical scavenging ability or through the regulation of signal transduction pathways to stimulate apoptosis, inhibit inflammation, cellular proliferation, apoptosis, angiogenesis and metastasis [13]. Nevertheless, these molecular mechanisms of action are not completely characterized and many features remain to be elucidated. The objective for this study is to purify and identify...
the anticaner agents from non-edible parts of *Theobroma cacao* with potent anticaner activity.

**Material and Methods**

**Plant materials**

The fresh of non-edible cocoa plant parts such as cocoa leaf (CL), cocoa bark (CB), cocoa husk (CH), cocoa shell unfermented (CSUF), cocoa shell fermented (CSF), cocoa root (CR), cocoa cherelle (CC) & cocoa pith (CP) were collected from Research Field of Malaysian Cocoa Board at Ranau, Sabah, Malaysia. Plant samples collected during peak season of cocoa fruit on April. The fresh cocoa plant parts were harvested, rinsed under tap water and air dried followed by oven dried at 40°C for 5 days. Then, the samples were grinded using commercial blender (Waring). Each 5 g powder plant part was extracted by soaked in 200 ml methanol for 3 days at room temperature. The mixture was then filtered using a clean muslin cloth and then, Whatman No. 1 filter paper. The filtrate was then evaporated to dryness using a rotary evaporator attached to a vacuum pump. Extract was stored at 2 to 8°C until further used. To test the biological activity, 10 mg of dried crude extracts were dissolved in 1 ml dimethyl sulfoxide (DMSO) to produce 10 mg/ml concentration of stock solution. This stock solution of crude extracts were later mixed with culture media (DMEM or RPMI 1640) to achieve the desired concentration of 1000, 100, 10, 1, 0.1, 0.01 and 0.001 μg/ml using 10 fold serial dilutions and stock solution of fractions were started with desired concentration from 100 to 0.0001 μg/ml using 10 fold dilutions.

**Human cell lines and cell culture preparation**

Cancer cell lines used in this study were breast with estrogen receptor positive (MCF-7), breast with estrogen receptor negative (MDA-MB-231), liver (HepG2), colon carcinoma (HT-29), lung (A549) and cervical (HeLa) cell lines. Normal cell line was liver (WRL-68). Colon (HT-29) and breast with estrogen receptor negative (MDA-MB-231) cancer cell lines were cultured in 89% Modified Eagle Dulbecco’s Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% penicillin/Streptomycin. While, breast with estrogen receptor positive (MCF-7), liver (HepG2), cervical (HeLa) and lung (A549) cancer cell lines were cultured in 89% RPMI 1640 with 10% Fetal Bovine Serum (FBS) and 1% penicillin/Streptomycin. Both of these cancer cell lines were cultured in 89% RPMI 1640 with 10% Fetal Bovine Serum (FBS) and 1% penicillin/Streptomycin. While, breast with estrogen receptor negative (MDA-MB-231), liver (HepG2), colon carcinoma (HT-29), lung (A549) and cervical (HeLa) cell lines. Normal cell line was liver (WRL-68). Colon (HT-29) and breast with estrogen receptor negative (MDA-MB-231) cancer cell lines were cultured in 89% Modified Eagle Dulbecco’s Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% penicillin/Streptomycin. While, breast with estrogen receptor positive (MCF-7), liver (HepG2), cervical (HeLa) and lung (A549) cancer cell lines were cultured in 89% RPMI 1640 with 10% Fetal Bovine Serum (FBS) and 1% penicillin/Streptomycin. Both of these cancer cell lines were cultured at 37°C at a humidity of 95% and 5% CO2 for 3 days.

**Media, chemicals and reagents**

Silica gel 60 (particle size 0.040-0.063 mm, 230-400 mesh ASTM) was purchased from Merck, Germany, Thiazolyl Blue Tetrazolium Bromide (MTT), dimethyl sulfoxide (DMSO), Trypan blue (0.4%) and phosphate buffered saline (PBS) were purchased from Sigma Chemical Co. USA. Roswell Park Media Institute (RPMI) 1640, penicillin/streptomycin (100x), trypsin-EDTA (1x) and fetal bovine serum mycoplex (FBS) were obtained from PAA, GMBH, (Germany). Hexane, methanol, dichloromethane, ethyl acetate and chloroform used were of the highest pure grade obtained from Fisher, UK. Iodine resublimed was purchased R& M Chemicals, Essex, UK.

**MTT assay**

Antiproliferative effect of cocoa extracts and fractions were determined in vitro against cancer cell lines using the microtitration colorimetric method of MTT reduction [14] with minor modification. The tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is used to determine cell viability in assays of cell proliferation and cytotoxicity. Briefly, cells were harvested from maintenance cultures in the exponential phase and counted by a hemocytometer using trypan blue solution after the cells reached 80-90% confluency. The cell suspensions were dispensed (100 μL) in triplicate into 96-well plate cultures at optimized concentration about 1.0×10^5 cells/mL for each cancer cell line. After 24 hours incubation at 37°C, 100 μL cultured medium was removed from the well and 100 μL fresh medium containing series concentrations of cocoa plant part extracts were added to each well and incubated for another 48 hours. The RPMI 1640 used as negative control. After treatment period, the medium in each well was aspirated and replaced with 20 μl of 5 mg MTT working solution (MTT) stock solution mixed with medium to attain a final concentration of 0.5 mg/mL MTT powder was dissolved in PBS to form a stock solution of MTT (5 mg/mL). The stock solution was filter-sterilized through a 0.22 mm filter and stored at -20°C. The cells were incubated at 37°C for 4 hours and then the medium was aspirated and replaced with 100 μl DMSO to dissolve the formazan crystals formed. The culture plates were shaken for 5 min and the absorbance (OD) of each well was read using Elisa Reader at 570 nm with 630 nm as the reference wavelength. The dose-response curves of the cocoa fractions were fitted by means of the computer programme Graph Pad Prism 5.02 (Graph Pad Software, San Diego, CA, USA) and IC_{50} (the concentration of sample needed to reduce a 50% absorbance relative to the vehicle-treated control) values were calculated.

**Plant extraction and fractionation preparation**

Extraction process were followed based on Pan et al.; Ludwiczuk et al. and Forgo et al. [15-17] methods with some modification. The dried and ground active cocoa plant part (1 kg) of *Theobroma cacao* was extracted with methanol (10 L) by soaking in room temperature for 3 days. After filtration using white cloth, the liquid phase was pooled and concentrated by evaporated under vacuum using rotary evaporator at 40°C to yield a methanolic crude extract of cocoa leaf. The methanolic crude extract was extracted by successive liquid-liquid partition with hexane (250 ml) followed by dichloromethane (250 ml) and lastly methanol (250 ml) to yield 3 partitioned fractions. The hexane, dichloromethane and methanol partitioned fractions were obtained after filtered using vacuum filter and evaporated under reduced pressure. The partitioned fractions with potent antiproliferative activity were subjected to flash column chromatography based on Lai et al. and Ode et al. [18,19] methods with some modification to separate the extract into its component fractions. Silica gel 60G was used as the stationary phase while varying solvent combinations of increasing polarity were used as the mobile phase. In the setting up of column chromatography, the lower part of the glass column was stocked with glass wool with the aid of glass rod. The slurry prepared by mixing silica gel 60 G (fractionation-1), 30 g (fractionation-2) and 10 g (fractionation-3) with hexane of 250 ml respectively and poured down carefully into the column. The column was left open to allow free flow of solvent into a conical flask below. The set-up was seen to be in order when the solvent drained freely without carrying either the silica gel or glass wool into the tap. At the end of the packing process, the tap was locked. The column was allowed 24 h to stabilize, after which, the clear solvent on top of the silica gel was allowed to drain down to the silica gel meniscus. The wet packing method was used in preparing the silica gel column. The sample was mixed with silica gel 5.0 g (fractionation-1), 3 g (fractionation-2) and 1 g (fractionation-3)
Bioassay guided fractionation and isolation

The antiproliferative effect of active non-edible methanolic plant extracts of Theobroma cacao was tested against susceptible cancer cell lines using the MTT cell viability assay to confirm the presence of anticancer activity. The methanolic extract was then partitioned in hexane, dichloromethane and methanol solvents and the resultant partitioned fractions were again tested for their antiproliferative activity. Partitioned fractions that demonstrated more than 50% growth inhibition as determined by comparison of the IC50 values were then subjected to separation using silica gel chromatography, affording a series of fractions. The chemical composition of each fraction was evaluated by using thin layer chromatography (TLC) and visualized with UV light and with the reference standards. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test material were identified. The active extracts and fractions were set aside and not subjected to further study in this work.

Phytochemical screening

Preliminary phytochemical analysis methanolic cocoa leaf extract and hexanolic cocoa leaf partitioned fraction of Theobroma cacao with high anticancer activity was carried out based on 5.0 g of dried powder material to identify the presence of some phytochemical constituents that usually exhibit biological activities such as alkaloids, flavonoids, saponins, tannins, triterpines & steroids. All samples were analyzed in the Laboratory of Phytochemistry, Forest Research Institute of Malaysia, Kepong, Malaysia.

Gas chromatography-mass spectrometry (GC-MS)

Most active fraction was analyzed using GC-MS and carried out by following the method of Hema et al. [20] with some modification. GC-MS analysis were performed using a QP2010 Plus Shimadzu system and gas chromatograph interfaced to a Mass spectrometer (GC-MS) equipped with a SGE BPX5, fused silica capillary column (30m x 0.25 mm ID x 0.25 µm), composed of 100% Dimethyl poly siloxane). For GC/MS detection, an electron ionization system with ionizing energy 70 eV; a scan interval of 0.5 seconds and mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

Statistical analysis

All determinations were expressed in mean ± SEM. The MTT assays for each sample were carried out triplicates at three different days. The IC50 values were calculated using Graph Pad Prism version 5.02. Significant differences between means were determined by Tukey HSD test. A significant difference was considered at the level of p<0.05.

Results and Discussion

Screening cocoa plant parts for antiproliferative activity

All the cocoa extracts against various cancer cell lines were evaluated based on MTT assay values and curves of percentage viability of treated cells were plotted against extracts concentration. Based on anticancer screening activities using MTT assay illustrated in Figure 1 that the extracts against cancer cell lines with IC50 20 to 100 µg/ml were produced from cocoa leaf (41.43 ± 3.260 µg/ml) against MCF-7, bark (71.97 ± 9.27 µg/ml) against MCF-7, husk (62.23 ± 14.90 µg/ml) against MCF-7, shell (unfermented) (65.03 ± 4.17 µg/ml) against MCF-7, shell (fermented) (71.40 ± 12.11 and 68.90 ± 10.18 µg/ml) against HeLa and HepG2 respectively, root (76.40 ± 13.81 µg/ml) against MCF-7 and cherelle (67.80 ± 9.36 and 68.90 ± 11.41 µg/ml) against HeLa and MCF-7 respectively considered moderately active. Other cocoa extracts against cancer cell lines with IC50 values of 100 - 1000 µg/ml considered weakly active. The small number of IC50 value means more potent anticancer activity as stated by Atjanasuppat et al. [21]. MTT assay is a typical method and common use in determination of anticancer activities. The cleavage of tetrazolium ring in MTT involves the mitochondrial succinate dehydrogenase and depends on the activity of the respiratory chain and the redox state of the mitochondria responsible for the production of cell energy. The exposure of cancer cells to cocoa extracts induced a significant reduction in the conversion of MTT, which means a cellular disintegration and cytotoxicity reflected from the parallel dose and time dependent decrease of the absorbance measured [22]. Based on IC50 value, cocoa leaf extract was the most potent anticancer activity against MCF-7 with IC50 value.

Figure 1: Anticancer activity (IC50 values) of eight cocoa plant part extracts (Theobroma cacao) that used in the treatment of Lung (A549), cervical (HeLa), liver (HepG2), colon (HT-29), breast estrogen receptor positive (MCF-7) and breast estrogen receptor negative (MDA-MDB-231) cancer cell lines.
Note: a,b,c : The results of IC\textsubscript{50} values indicates significant difference between six cancer cell lines, P<0.05. Results are means ± SEM of triplicates experiments.

**Table 1**: Cocoa plant part extracts of *Theobroma cacao* leaves with potent anticancer activities (IC\textsubscript{50} values) against various cancer cell lines.

<table>
<thead>
<tr>
<th>Cancer Cells</th>
<th>Cocoa Extracts</th>
<th>IC\textsubscript{50} values (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung (A549)</td>
<td>Shell (Fermented)</td>
<td>520.67 ± 19.06\textsuperscript{a}</td>
</tr>
<tr>
<td>Cervical (HeLa)</td>
<td>Cherelle</td>
<td>67.80 ± 9.36\textsuperscript{b}</td>
</tr>
<tr>
<td>Liver (HepG2)</td>
<td>Shell (Fermented)</td>
<td>68.90 ± 10.18\textsuperscript{c}</td>
</tr>
<tr>
<td>Colon (HT-29)</td>
<td>Shell (Unfermented)</td>
<td>163 ± 18.58\textsuperscript{c}</td>
</tr>
</tbody>
</table>

Breast estrogen receptor positive (MCF-7) Leaf 41.43 ± 3.28\textsuperscript{b}

Breast estrogen receptor negative (MDA-MB-123) Pith 360.00 ± 34.77\textsuperscript{c}

**Figure 2**: Diagram shows the bioassay guided fractionation process comprised the preparation of crude methanolic extract of *Theobroma cacao* leaf, liquid-liquid partitions, fractionation-1, fractionation-2 and fractionation-3. Samples preparations were carried out using rotary evaporator at 40°C. TLC results were used to combine the fractions. Dry weight basis and antiproliferative activity were indicated in parentheses.
In this study, initial purification process were performed on crude methanolic extract of Theobroma cacao leaf in order to improve for the selective antiproliferative bioactive fraction using cell line antiproliferation assay as a guide [28]. The sequential solvent partitioning in three different polarity solvents were used to further fractionate the cocoa leaf extract. Hexane partitioned fraction, dichloromethane partitioned fraction and methanol partitioned fraction were obtained and showed significant antiproliferative bioactivity on breast cancer cell line (MCF-7) tested by MTT assay Figure 3. However, the non-polar solvent of hexane was demonstrated the most effective activity against MCF-7 with IC50 value of 66.67 ± 7.95 µg/ml compared to dichloromethane and methanol. Solvent partitioning is important to obtain active compounds in the active extract that distributed according to their polarity and extensively used for fractionation following bioactivity guided fractionation [29]. Thus, the hexane partitioned fraction was for further fractionated by flash column chromatography with elution based upon solvents of increasing polarity. About 66 eluted fractions (I/F1-F66) from fractionation-1 were collected and chromatographed in thin layer chromatography (TLC) by to generate 9 combined fractions (II/F1-F9). Nine combined fractions were tested against MCF-7 and revealed that combined fraction II/F4 showed most potent antiproliferative activity at IC50 value of 7.58 ± 1.29 µg/ml Table 2. The fraction II/F4 was selected due to significantly difference (P<0.05) of IC50 value compared to other fractions illustrated in Table 3 for further purification by eluted again in solvent with increasing polarity generated about 45 eluted fractions (I/F1-F45) from fractionation-2 and chromatographed in TLC to generate 7 combined fractions (II/F1-F7). Seven combined fractions were tested against MCF-7 and revealed that combined fraction II/F7 showed most potent antiproliferative activity at IC50 value of 6.36 ± 0.71 µg/ml compared to other fractions. Lastly, the fraction II/F7 from fractionation-2 was selected due to significantly difference (P<0.05) of IC50 value compared to other fractions illustrated in Table 3 for further purification using flash column chromatography and eluted in series of solvents with increasing polarity to generate 10 combined fractions (II/F1-F10) from fractionation-3. The 10 combined fractions were tested against MCF-7 and revealed that fraction II/F8 showed most potent antiproliferative activity at IC50 value of 43.30 ± 2.83 µg/ml (Table 4). However, based on the pattern of results presented here, it is possible that antiproliferative activity in fraction II/F7 (fractionation-2) is derived from combination of compounds and this fraction was selected for identified anticancer agents using GC-MS due to synergistic effect against breast cancer cell line (MCF-7) and also showed significantly difference (p<0.05) of IC50 value compared to fraction II/F8 (fractionation-3) displayed in Table 5. In the future study, the fractional inhibitory concentration index (FICI) method could be applied to determine the level of synergy and antagonism [28]. In this study, the cytotoxic effects for hexane partitioned fraction, fraction (II/F7) from fractionation-1, fraction (II/F8) from fractionation-2 and fraction (II/F8) from fractionation-3 against normal embryonic liver cell line (WRL68) were presented in Table 5. The IC50 value of hexane partitioned fraction, fraction (II/F4) from fractionation-1, fraction (II/F4) from fractionation-2 and fraction (II/F8) from fractionation-3 against embryonic liver cell line (WRL68)
therapeutic activities. Plant based natural compounds can be obtained in medicinal properties especially useful in pharmaceutical and alkaloids compounds Table 6. The presence of various phytochemicals strongest precipitation with 3 + found in hexanolic cocoa leaf fraction contained saponins, flavonoids, triterpenes, steroids and compounds. Phytochemicals of hexanolic cocoa leaf partitioned steroids and showed absent of alkaloids and hydrolysable tannins compounds. Phytochemicals of hexanolic cocoa leaf partitioned fraction contained saponins, flavonoids, triterpenes, steroids and showed absent of alkaloids, condensed tannins and hydrolysable tannins compounds. The result also indicated that steroids showed strongest precipitation with 3 + found in hexanolic cocoa leaf partitioned fraction. But, for both samples showed the absent of alkaloids compounds Table 6. The presence of various phytochemicals active compounds identified are known to have beneficial importance in medicinal properties especially useful in pharmaceutical and therapeutic activities. Plant based natural compounds can be obtained from any part of the tree such barks, leaves, roots, flowers, fruits, seeds and others [31,32]. This important information on phytochemical screening will be helpful as a primary platform for further study on pharmacological aspect in cocoa leaf extract. Alkaloids were not detected in this study plant. Alkaloids normally related with medicinal uses for centuries and one of their common bioactivity properties is their phytotoxicity and their absence in this plant able to lower the risk of poisoning by the plant [33] and this literature supported the results in Table 5 where cocoa leaf extract showed not toxic to normal cell line (WRL68) based on IC_{50} values. Saponins and flavonoids were presence in both samples. Saponins are a mild detergent responsible for various pharmacological and therapeutic effects such as used in hypercholesterolemia, hyperglycemia, antioxidant, anticancer, anti-inflammatory and weight loss, etc. [32]. However, flavonoids have been shown to exhibit the wide range of biological activities like antimicrobial, anti-inflammatory, antiangiogenic, analgesic, antiarrhythmic, cytostatic and antioxidant properties. In human diet, flavonoids could reduce the risk of various cancer diseases [34]. Tannins are antiviral, antibacterial, anticancer and certain tannins are also able to inhibit HIV replication, sensitivity and diuretic [35]. Hydrolysable and condensed tannins were negative for methanolic cocoa leaf extract and hexanolic cocoa leaf partitioned fraction but positive for condensed tannins in methanolic cocoa leaf extract. The possible reason could be added is due to mode of extraction process. But the absent for both tannins in hexanolic cocoa leaf partitioned fraction will not reduce the medical efficacies of Theobroma cacao leaf. Triterpenes proved the antioxidant effect and has been shown that ursoic acid, oleanolic acid and other triterpenoids were efficient as antimicrobial, anti-inflammatory, anticancer, antiplatelet aggregation, anti-Mycobacterium Tuberculosis and anti-HIV/AIDS [36]. Finally, steroids showed strongest precipitation in hexanolic cocoa leaf partitioned fraction suggests the ability of this plant play a role as hypercholesterolemic effects, antiinflammatory, anti-influenza, antipyretic, antifungal, hypotensive, muscle relaxant activities, anticancer and antiviral agents [37].

### Identification of anticancer agents by GC-MS

The bioactive compounds in selected cocoa leaf fraction (II/ F7) from fractionation-2 Table 3 with high anticancer activity were identified using GC-MS analysis. Data generated from GC-MS was interpreted based on database of National institute Standard and Technology (NIST). The chromatogram of the GC-MS analysis was presented in Figure 4. The GC-MS analysis results clearly showed that about 10 major compounds (>1% area) existed in active fraction (II/F7) of Theobroma cacao leaf. The components of the fraction II/F7 such as compound name, retention time (RT), area percentage (%), molecular weight (g), compound formula and similarity percentage (%) were summarized in Table 6. The major compounds with synergistic effect for anticancer activity were dimethyl sulfoxide (1.26%), 2-Cyclohexene-1-one, 4-(1-methyl) (1.53%), phenol,2,4-bis(1,1-dimethylethyl) (16.26%), 7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(2-methoxyxiranyl)

### Table 6: Phytochemical constituents of methanolic cocoa leaf extract and hexanolic cocoa leaf partitioned fraction of Theobroma cacao.

<table>
<thead>
<tr>
<th>No.</th>
<th>Test</th>
<th>Phytochemical Screening</th>
<th>Methanolic Cocoa Leaf Extract</th>
<th>Hexanolic Cocoa Leaf Partitioned Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Saponins</td>
<td>1 +</td>
<td>1 +</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Flavonoids</td>
<td>1 +</td>
<td>1 +</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Hydrolysable tannins</td>
<td>2 +</td>
<td>1 +</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Condensed tannins</td>
<td>2 +</td>
<td>1 +</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Triterpenes</td>
<td>2 +</td>
<td>3 +</td>
<td></td>
</tr>
</tbody>
</table>

For saponins: 1 +, 1-2 cm froth; 2 +, 2-3 cm froth; and 3 +, >3 cm froth.
For flavonoids, tannins, triterpenes, and steroids: 1 +, weak colour; 2 +, mild colour; 3 +, strong colour.
For alkaloids: - and 1 +, negligible amount of precipitate; 2 +, weak precipitate; and 3 +, strong precipitate.

### Table 5: Antiproliferative activity of hexane partitioned fraction, fractionation-1 (II/ F4), fractionation-2 (II/F7) and fractionation-3 (II/F8) against breast cancer cell line (MCF-7) and liver normal cell line (WRL68).

<table>
<thead>
<tr>
<th>Sample name</th>
<th>IC_{50} (µg/ml)</th>
<th>IC_{50} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane partitioned fraction</td>
<td>66.67 ± 7.95</td>
<td>651.33 ± 5.36</td>
</tr>
<tr>
<td>Fractionation-1 (II/F4)</td>
<td>7.58 ± 1.29</td>
<td>51.70 ± 3.33</td>
</tr>
<tr>
<td>Fractionation-2 (II/F7)</td>
<td>6.36 ± 0.71</td>
<td>44.0 ± 1.76</td>
</tr>
<tr>
<td>Fractionation-3 (II/F8)</td>
<td>43.30 ± 2.83</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Note: *Values are means of three replicate samples (n=3). Data is presented as mean ± SEM.
(2.43%), hexadecanoic acid, methyl ester (7.00%), Octadecanoic acid, methyl ester (7.34%), Hexadecanoic acid, 2-hydroxyethyl ester (4.24%), Octadecanoic acid, 2-hydroxyethyl ester (2.39%), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (1.45%) and lastly unknown (33.08%). The unknown compound existed in this fraction II/F7 with highest percentage of area and needs further structural identification. The active compounds identified in fraction II/F7 of Theobroma cacao leaf are medicinally valuable and possess various pharmaceutical applications mentioned in Table 7. The presence phenol,2,4-bis(1,1-dimethylylethyl) attributes to the antioxidant and antibacterial [38]. Compounds such as and octadecanoic acid, methyl ester have been reported possessed antioxidant, hypercholesterolemic and pesticide [38] and for hexadecanoic acid, methyl ester possessed antiinflammatory, antiandrogenic cancer preventive, dermatitigenic ypocholesterolenic, 5-Alpha reductase inhibitor, anemiagenic insectifuge and flavour [39]. Compound hexadecanoic acid, 2-hydroxyethyl ester possessed antioxidant, hypercholesterolemic, nematicide, antiandrogenic and 5-Alpha reductase inhibitor [40]. Compound octadecanoic acid, 2-hydroxyethyl ester possessed anti androgenic, hypercholesterolemic, nematicide, 5-Alpha reductase inhibitor, antiacen, anti-inflammatory, antiandrogenic, cancer preventive and dermatitigenic [41] and lastly, compound hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester possessed hemolytic, pesticide, flavour and antioxidant. For unknown compound need further chemical structure confirmation using nuclear magnetic resonance (NMR).

Conclusion

Plants as sources of natural active compounds have remarkable pharmaceutical and the therapeutic potentials for human health. Plants are the great sources of secondary metabolites with various biological activities contributed by variety of phytochemical properties. In the present study, cocoa leaf of *Theobroma cacao* was the best extract as anticancer agent due to potent antiproliferative activity against breast cancer cell line (MCF-7) compared to other cocoa plant parts. Phytochemical screening of crude methanolic cocoa leaf extract reveals the presence of various secondary metabolites like flavonoids, saponins, tannins, triterpene, polyphenolics and steroids but absent in alkaloids. The strategy of bioactivity guided fractionation and isolation was used to purified and identify the most active anticancer isolated from cocoa leaf of *Theobroma cacao* by GC-MS analysis. In future, further study on unknown compound presented in active fraction II/F7 from fractionation-2 presented the highest antiproliferative activity with synergistic effects and 10 major chemical constituents were identified from cocoa leaf of *Theobroma cacao* by GC-MS analysis. For unknown compound need further characterization using nuclear magnetic resonance (NMR) and isolation and identification of individual constituents also needed where the bioactive compounds could serve as natural resources for anticancer drug development research.

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