

Anti-oxidative and cytotoxic attributes of phenolic rich ethanol extract of *Musa balbisiana* Colla inflorescence

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ABSTRACT

Inflorescence of *Musa* species is one of the most commonly consumed vegetables in Southeast Asian region. In the present study, chemical composition and antioxidant potential of the ethanolic extract of inflorescence of *Musa balbisiana* Colla (MbCi) were evaluated. In addition, the extract was also subjected to cytotoxicity testing on a panel of human cancer cell lines. The ethanolic extract of inflorescence of *Musa balbisiana* Colla was evaluated for antioxidant activity using 1,1-diphenyl-2-picryl-hydrazyl assay (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) assays. The extract was chemically characterized for total phenolic (TPC) and total flavonoid (TFC) contents and by gas chromatography-mass spectrometry (GC-MS) analysis. Cytotoxicity was evaluated by MTT cell viability assay. The extract showed moderate antioxidant activity in all the antioxidant assays. Chemically, the extract was found to possess high total phenolic (92.01 ± 0.40 µg gallic acid equivalent/mg extract) but low flavonoid (4.852 ± 0.04 µg quercetin equiv/mg extract) contents. In cell viability assay, MbCi extract showed selective cytotoxicity towards HT-29 cell line. Morphological observations show that MbCi has apoptosis inducing nature. GC-MS analysis has revealed the presence of 22 compounds, mainly belonging to steroids, fatty acids and long chain aliphatic compounds, which in part may be responsible for observed antioxidant and cytotoxic activities of ethanol extract. Our study revealed that MbCi has chemotherapeutic potential activity that warrants further investigation.

INTRODUCTION

Plants from genus *Musa* contribute as the fourth most important food in the world today. *Musa* species are natives to Indo-Malaysian, Asian and Australian tropics (Nelson *et al.*, 2006). Inflorescence of *Musa* species (banana) is much appreciated vegetable in Malaysia. The inner part of inflorescence as well as lower soft inner part of pseudostem is widely used for cooking (Vimala *et al.*, 2003). Decoction of half ripe fruit of *Musa balbisiana* Colla (MbC) is used in the

treatment of dysentery. In India, exudates from rhizomatous stem of MbC are used for the treatment of pinworm infection (Kalita and Deb, 2004), while the tablets prepared from the fresh and dried seed paste of MbC are used as contraceptives (Das *et al.*, 2014). Decoction from the pith of *Musa* species is used in the treatment of congestive heart failure and hypertension by Temuan tribe of Peninsular Malaysia (Azliza *et al.*, 2012). *Musa* species are studied extensively for their pharmacological activities. Kumar *et al.* (2012), reported that flavonoid leucocyanidin significantly increase the thickness of the mucous membrane layer of the stomach and improved gastric ulcer in comparison with antacid. Aqueous extract from the roots of MbC produced significant size reduction in albumin induced hind paw edema in Wistar rats in a dose dependent manner (Ibegbu *et al.*, 2012).

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Fresh dried pulp of the fruit was reported to possess *in vitro* as well *in vivo* antioxidant activity in a dose dependent manner (Mudoj *et al.*, 2011). Antioxidant activity of eight Malaysian bananas were reported by Sulaiman *et al.* (2011), who found that the chloroform extract of dried pulp possessed good antioxidant activity. The authors also reported weak correlation between the antioxidant activity and total phenolic content of the samples. Vilela *et al.* (2014) identified GC-MS of dichloromethane extracts of several *Musa* species and reported the presence of lipophilic phytochemicals. Hitherto, there is no data available on the antioxidant activity and cytotoxicity of inflorescence of *Musa balbisiana* Colla (MbCi). Therefore, the present study was designed to investigate the phytochemical composition, antioxidant capacity and cytotoxicity of MbCi.

MATERIALS AND METHODS

Plant materials

Inflorescence of MbC was collected from Balik Pulau hilly area, Penang, Malaysia. A voucher specimen (11559) was deposited in Herbarium Unit at the School of Biological Sciences, Universiti Sains Malaysia.

Preparation of MbCi extract

The fresh inflorescences were cut into smaller pieces, crushed and triturated in mortar and pestle by addition of small amount of ethanol and made into paste. This mass was further extracted by maceration for 6 days with absolute ethanol. The extracts were filtered using whatman filter paper and concentrated using rotavapor (Heidolph Instruments; Schwabach, Germany). Then, the extracts were stored in sealed vial at 4 °C until biological testing. All determinations were done in triplicate and absorbencies were measured using microplate reader (TECAN Infinite Pro® M200, Switzerland).

Chemical characterization

Total phenolic content

Total phenolic content was determined using Folin–Ciocalteu reagent following the method described by Kumaran and Joel Karunakaran (2007), using gallic acid as a reference standard. The assay was carried out by mixing Folin–Ciocalteu reagent, sodium carbonate, standard/extract sample and distilled water in a test tube in a ratio of 5:15:1:79 to the final volume of 1000 µL. The tubes were incubated for 2 hour at room temperature and an aliquot (200 µL) of each mixture was transferred into 96-well microplate. The amount of test sample was substituted by distilled water in blank. Absorbencies were taken at 765 nm. The results are expressed as µg gallic acid equivalent/mg dry extract.

Total flavonoid content

Total flavonoid content was determined by the aluminum chloride method as described by Orhan *et al.* (2011) using quercetin as a reference standard. For the assay, the standard or extract solutions (100 µL) were mixed with of 10% (w/v)

aluminum chloride (20 µL), 1 mol/L sodium acetate (20 µL) methanol (300 µL) and distilled water (560 µL). After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. The results are expressed as µg quercetin equivalent/mg dry extract.

Gas Chromatography Mass Spectrometry (GC-MS) analysis

The chemical composition of MbCi was determined using Agilent GC-MS system consisting of a gas chromatograph (Agilent 6890) coupled to a mass spectrophotometer (Agilent 5973; inert mass selective detector). Separation was achieved on a HP-5 MS column of 30 m length, 0.25 mm diameter consist of film thickness 0.25 µm. The injector was set at 70 °C for 2 minutes and steadily increased 20 °C up to 285 °C. Helium was used as the carrier gas with the flow rate of 20 mL per minute. An amount of 2 µL of sample was injected. Transfer line was maintained at 250 °C. The mass spectrophotometer was operated at 1717.6 eV. The total run time was 47.75 minutes. The identification of compounds was done by using NIST 02 library.

Antioxidant assays

DPPH scavenging assay

Free radical scavenging activity was determined using 2,2-Diphenyl-2-Picrylhydrazyl (DPPH) as described by Al-Mansoub *et al.* (2014). A 100 µL of the extract sample (0.78 – 200) µg/mL dissolved in DMSO were mixed with 100 µL of DPPH (200 µmol/L) dissolved in methanol, and the reaction mixture was incubated at room temperature for 30 min. Ascorbic acid was used as a reference standard. The absorbance was measured at 517 nm. The results are expressed as IC₅₀.

ABTS radical scavenging activity assay

ABTS radical scavenging activity was measured by the modified ABTS cation decolorization assay as described by Al-Mansoub *et al.* (2014) as described by Re *et al.* (1999). ABTS radical cation (ABTS^{•+}) solution was prepared by mixing of 14 mM ABTS and 4.9 mM potassium persulfate (K₂S₂O₈) dissolved in deionized water in equal volumes. This solution was allowed to react in the dark place at room temperature for 16-20 h before use. Then, 1 mL of stock ABTS^{•+} solution was then diluted with 40 mL of deionized water to yield an absorbance equals to 0.70 ± 0.02 at 734 nm. In Brief, to 180 µL of ABTS radical solution, 20µl of sample extract (3.13 – 400) µg/mL were added. Ascorbic acid was used as a reference standard. The absorbance of ABTS^{•+} sample extract/standard was taken at 734 nm. The results are expressed as IC₅₀.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out by method of Benzie and Strain (1996) as developed by Griffin and Bhagooli (2004). FRAP working solution was prepared by mixing (300 mmol/L) acetate buffer, pH 3.6 (10 mmol/L) TPTZ in (40 mmol/L) HCl and (20 mmol/L) FeCl₃ in a ratio of 10:1:1. An amount of 150 µL of

working solution was added to 50 μL standard/extracts. Blank was done in the same way using methanol instead of test solutions. The reaction mixture was incubated for 8 min then readings were taken at 600 nm. Ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) was used as reference standard and the results are expressed as nmol Fe^{+2} equivalent/mg dry extract.

In vitro anticancer assays

Cell viability assay

Cytotoxicity of MbCi was tested on EA.hy926 (human normal endothelial cells) and four cancer cell lines namely, MCF-7 (human breast cancer ATCC[®] HTB-22), HeLa (human cervical carcinoma ATCC[®] CCL-2), HT-29 (human colorectal adenocarcinoma ATCC[®] HTB-38) and HCT 116 (human colorectal carcinoma ATCC[®] CCL-247) using MTT assay [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide] following the protocol described by Asif *et al.* (2016). MCF7 and HeLa cells were cultured in Dulbecco's Modified Eagle medium (DMEM) (Gibco[®] Invitrogen) whereas HT-29 and HCT-116 were cultured in Roswell Park Memorial Institute medium (RPMI) (Gibco[®] Invitrogen) supplemented with fetal bovine serum (10%) and penicillin-streptomycin (1%) (Gibco[®] Invitrogen). Cells were maintained at 37 °C in a water saturated atmosphere containing 5% CO_2 . Counting of cells was done using a Neubauer hemocytometer under light microscope by using trypan blue (Gibco[®] Invitrogen) staining method. Cells of 5,000 to 10,000 densities per well seeded in a 96 well plate and treated with different concentrations (100-3.125 $\mu\text{g/mL}$) of ethanolic extract dissolved in DMSO (0.5% in final concentration). 5-fluorouracil (5-FU) was used as the reference standard and 0.5% DMSO was used as the negative control. The treated cells were incubated for 48 hours. The absorbance was read using microplate reader (TECAN infinite Pro[®] M200, Switzerland) at 570 nm using 620 as reference wavelength. The results were presented as percent viability.

Morphological analysis

Changes in the morphology of cells treated with MbCi were studied following well established method of Ebrahim *et al.* (2014) with some modifications. In brief, overnight seeded cells (5×10^5) were incubated with IC_{50} values of MbCi for 48 hours in a 6-well tissue culture plate. At the end of treatment period, the medium was discarded and cells were washed once with PBS. The changes in the morphology of treated cells were observed using inverted phase contrast microscope at 10 \times magnification.

Statistical analysis

Data are presented as mean \pm standard error of mean (SEM). The minimum inhibitory concentration (IC_{50}) was

calculated from the linear regression equations of dose response curve for each experiment. All statistics analyses were carried out using SPSS software (20.0 version).

RESULTS

Chemical characterization of ethanol extract

The extract was chemically characterized by colorimetric and GC-MS methods.

Total phenolic and flavonoid contents

MbCi was revealed to have higher total phenolic content (92.02 ± 0.40 μg gallic acid equiv./mg extract) and low total flavonoid content (4.85 ± 0.05 μg quercetin equiv./mg extract) (Table 1).

GC-MS analysis

The GC-MS analysis has shown the presence of 22 compounds in the extract; among these 16 compounds were found to be with more than 90% of similarity with NIST 02 library. These identified compounds belong to the class of steroids, long chain unsaturated and saturated hydrocarbons, esters and fatty acids.

On the other hand, compounds with less similarity index while found to be in major proportion could be predicted as the polyphenolics by considering the higher TPC value of the extract. The extract was enriched with major sterols stigmasterol, beta-sitosterol, and campesterol, in descending order of abundance. However, the quantitative analysis of the tested extract revealed that the major dominant peak corresponds to Z-12-Pentacosene (8.23%), followed by stigmasterol (7.33%), 10-Heneicosene (5.73%), and beta-sitosterol (5.23%) (Table 2, Figure 1).

Antioxidant Assays

Finding of the present study shows that MbCi has moderate antioxidant activity in DPPH, ABTS free radical scavenging, while ferric reducing antioxidant power of the MbCi in FRAP assay demonstrated good activity.

The antioxidant results of MbCi in these respective assays are shown in Table 1.

The DPPH and ABTS IC_{50} values of MbCi (IC_{50} value is the concentration of the sample required to inhibit 50% of radical) were 64.24 ± 3.09 and 76.23 ± 2.20 $\mu\text{g/mL}$. The results were compared to reference standard ascorbic acid which were found to be 5.41 ± 0.41 $\mu\text{g/mL}$ and 3.49 ± 0.05 $\mu\text{g/mL}$, for the DPPH and ABTS assays, respectively. In addition, the MbCi showed good antioxidant activity in FRAP test with a value of 70.08 ± 12.86 nmol Fe^{+2} equiv./mg extract.

Table 1: Antioxidant activity of ethanolic extract of *Musa balbisiana* Colla inflorescence.

Sample	Total phenolic (μg gallic acid equiv./mg extract)	Total flavonoid (μg quercetin equiv./mg extract)	DPPH IC_{50} ($\mu\text{g/mL}$)	ABTS IC_{50} ($\mu\text{g/mL}$)	FRAP (nmol Fe^{+2} equiv./mg extract)
MbCi	92.02 ± 0.40	4.85 ± 0.05	64.24 ± 3.09	76.23 ± 2.20	70.08 ± 12.86
Vitamin C (Standard)	-	-	5.41 ± 0.41	3.49 ± 0.05	-

Values are expressed as mean \pm SEM (n=3).

Table 2: GC-MS profile of ethanolic extract of *Musa balbisiana* Colla inflorescence

S. No.	Compound	Retention time	Area %	Molecular formula	Molecular weight	Similarity index
1	Hexadecanoic acid, methyl ester	10.37	3.89	C ₁₇ H ₃₄ O ₂	270.450	99
2	9,12- octadecanoic acid (z,z)-methyl ester	11.13	3.02	C ₁₉ H ₃₄ O ₂	294.472	99
3	Heptadecanoic acid, 16-methyl-, methyl ester	11.21	1.47	C ₁₉ H ₃₈ O ₂	298.503	94
4	Linoleic acid ethyl ester	11.40	0.79	C ₂₀ H ₃₄ O ₂	308.498	99
5	9-Tricosene, (Z)-	11.74	0.74	C ₂₃ H ₄₆	322.611	99
6	Heptadecane	11.83	0.64	C ₁₇ H ₃₆	240.468	96
7	10-Heneicosene	12.51	5.73	C ₂₀ H ₃₄	310.600	99
8	Hexadecane,2,6,10,14-tetramethyl-	12.60	3.67	C ₂₀ H ₄₂	282.547	96
9	Z-12-Pentacosene	13.31	8.23	C ₂₅ H ₅₀	350.665	91
10	Eicosane	13.40	0.49	C ₂₀ H ₄₂	282.547	92
11	1-Nonadecene	14.29	4.21	C ₁₉ H ₃₈	266.505	95
12	17-Pentatriacontene	15.63	2.45	C ₃₅ H ₇₀	490.930	93
13	Vitamin E	16.53	1.98	C ₂₉ H ₅₀ O ₂	430.706	98
14	Campesterol	17.80	2.79	C ₂₈ H ₄₈ O	400.680	99
15	Stigmasterol	18.15	7.33	C ₂₉ H ₄₈ O	412.691	91
16	Beta-sitosterol	18.92	5.23	C ₂₉ H ₅₀ O	414.706	99

Compounds having similarity index with NIST02 library more than 90 were considered for reporting

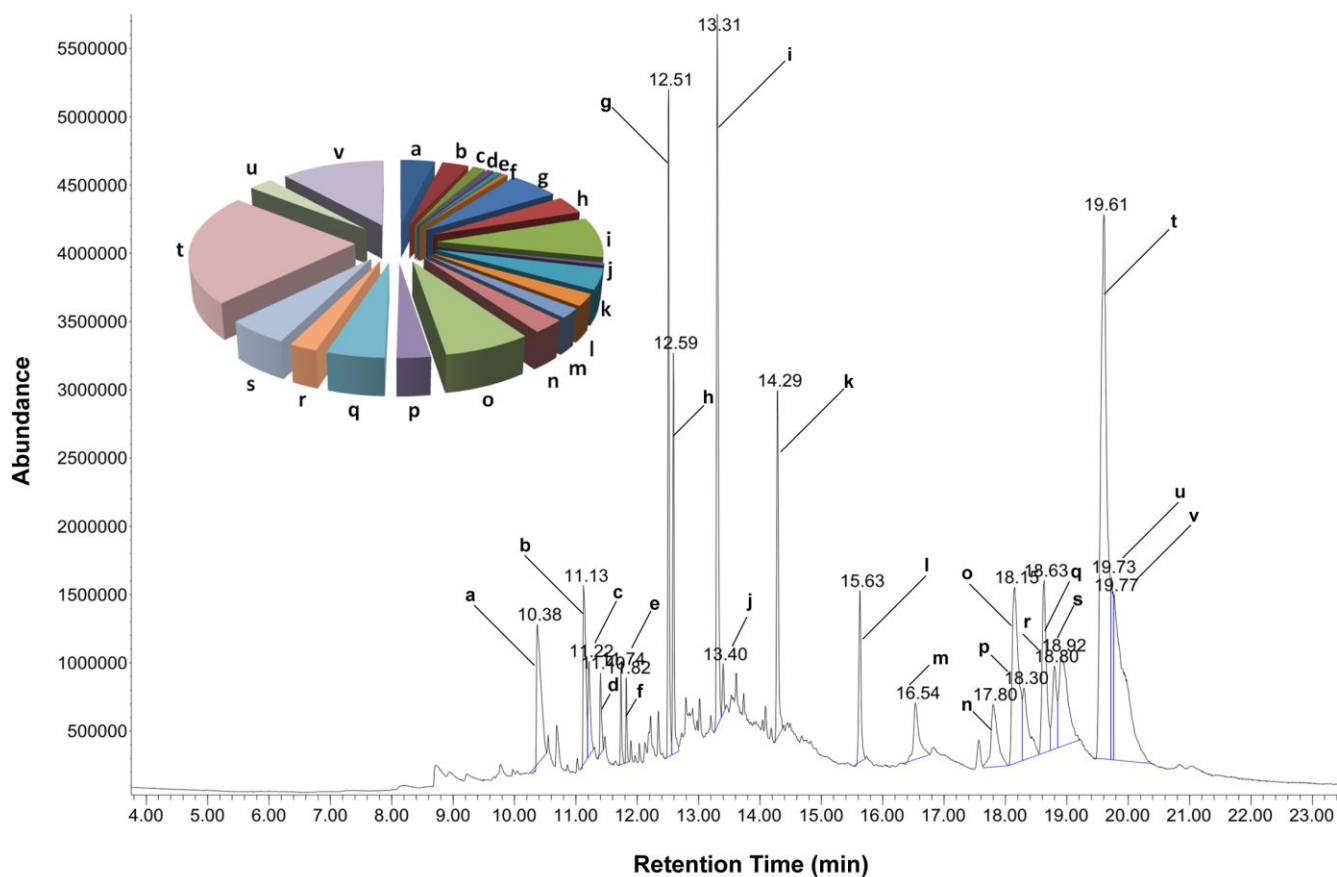


Fig. 1: Gas chromatography-mass spectrometry (GC-MS) chromatogram, and the pie chart depicts the percentage of each phytochemical present in the ethanolic extract of *Musa balbisiana* Colla inflorescence. The identification of compounds was done by using NIST 02 library. Where, i = Z-12-Pentacosene (8.23%), o = stigmasterol (7.33%), g = 10-Heneicosene (5.73%), and s = beta-sitosterol (5.23%).

Table 3: Cytotoxicity of ethanolic extract of *Musa balbisiana* Colla inflorescence against panel of cell lines

Cell lines	IC ₅₀ (µg/mL)
MCF-7 Breast cancer	61.81 ± 0.64
HCT 116 Colon cancer	39.89 ± 1.63
HT-29 Colon cancer	5.25 ± 0.26
HeLa Cervical cancer	114.08 ± 6.12
EA.hy926 Normal human endothelial	66.64 ± 3.56

Values are expressed as mean ± SEM (n=3).

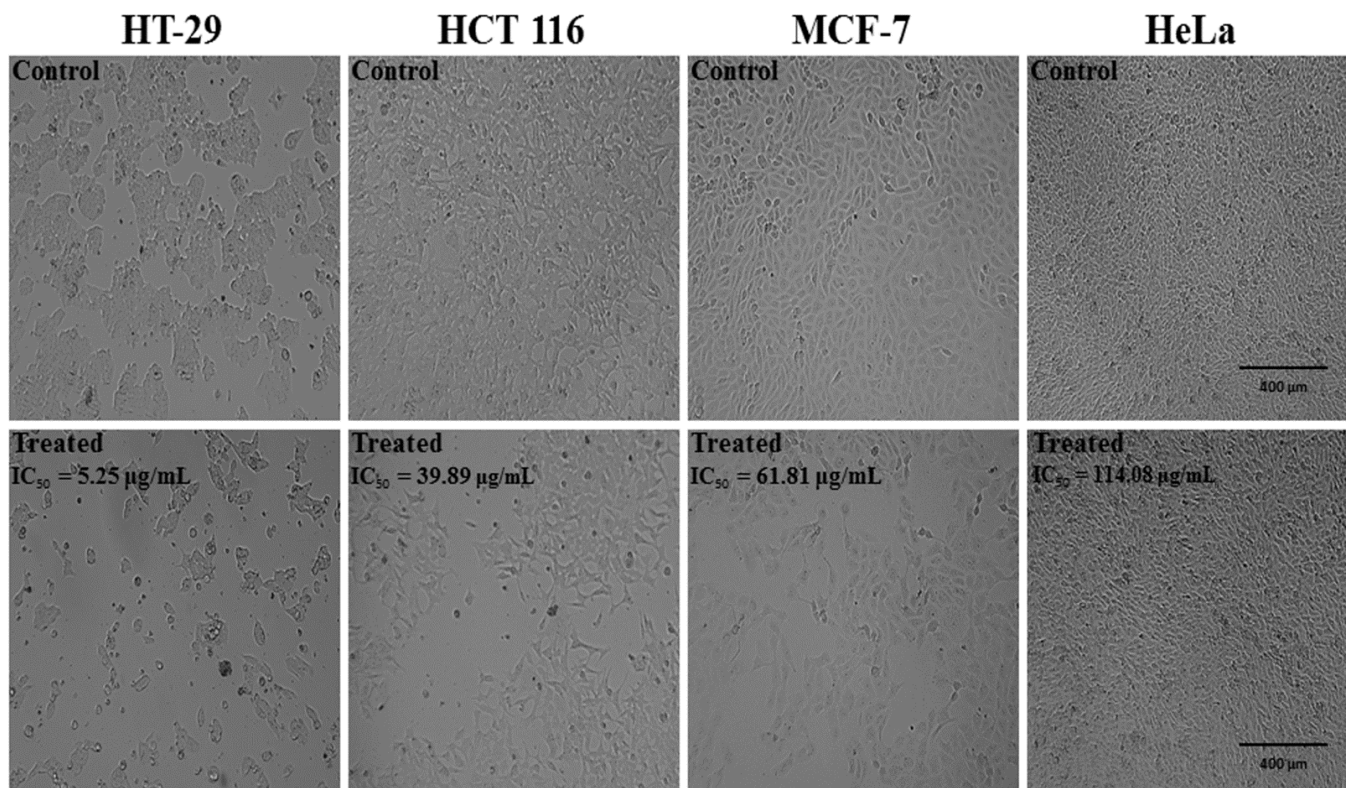


Fig. 2: Cytotoxicity of ethanolic extract of *Musa balbisiana* Colla inflorescence towards a panel of human cancer cell lines after 48h of treatment. Photos were taken at 10× magnification (scale bar 400 µm).

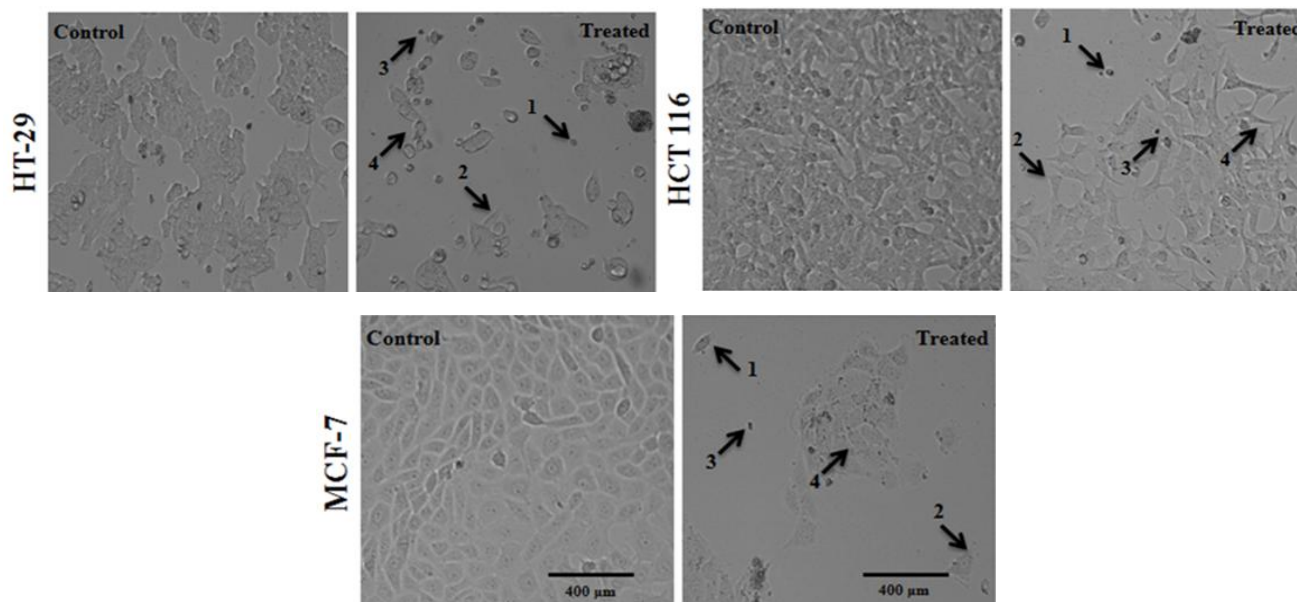


Fig. 3: The morphological changes observed in human cancer cell lines treated with ethanolic extract of *Musa balbisiana* Colla inflorescence. Where 1 = cell shrinkage, 2 = membrane blebbing, 3 = dead cells and 4 = loss contact with neighboring cells. Photos were taken at 10× magnification (scale bar 400 µm).

In vitro anticancer assays

Cell viability assay

The extract showed selective cytotoxicity on two colorectal cancer cell lines, namely HT-29 and HCT 116. Meanwhile moderate cytotoxicity was found on MCF-7 human breast cancer cell line and the extract was found relatively non-toxic on HeLa cells (Table 3, Figure 2). Treatment of MbCi for 48 h exhibited no cytotoxic activity towards normal human endothelial cells (EA.hy926) with IC_{50} of 66.64 ± 3.56 μ g/mL.

Morphological analysis

Monitoring of MbCi extract treated cells under phase contrast microscope after 48 h showed significant morphological changes compared to control cells (0.5% DMSO). The MbCi induced typical apoptotic changes in the morphology of all HCT 116, HT-29 and MCF-7 cells. Figure 3 highlights the morphological changes induced in human cancer cell lines as a result of exposure to the extract of MbCi (10 \times magnification).

DISCUSSION

Development of cancer involves three main phases namely, initiation, promotion and progression phases. The currently employed treatment strategy which mainly includes radiation, chemotherapy, immunosuppression and surgery have limited prospective as indicated by high morbidity and mortality rate. Thus, there is a need for new treatment strategies towards the treatment of cancer. Chemoprevention is one such major strategy which involves use of pharmacological, dietary biofactors, phytochemicals and even whole plant extracts to prevent, arrest or reverse the cellular and molecular processes of carcinogenesis. Several recent studies have proven that various dietary phytochemicals are significantly effective in controlling, inhibiting the carcinogenesis. Research has also effectively proven that total plant extract possess significant effect over single compound mainly because of synergistic effects of cocktail of various metabolites and multiple points of intervention in chemoprevention. These phytochemicals found to act through different mechanisms such as inhibition of genotoxic effects, increased antioxidant and anti-inflammatory activity, inhibition of proteases and cell proliferation, protection of intercellular communications to modulation of apoptosis and signal transduction pathways (Shafi Sofi *et al.*, 2013). With this background and based on previous literature available on dietary sources, *Musa balbisiana* Colla was not only used as food source but also possess traditional uses as anticancer agents. The ethanolic extract from inflorescences of *Musa balbisiana* Colla was selected for its antioxidant and anticancer activity on various major cell lines in our current study.

In the present study, ethanol extract of *Musa balbisiana* inflorescences was tested against a panel of human of cancer cell lines. Finding of study shows that ethanol extract was selectively active towards human colorectal carcinoma HT-29 cell line. The order of selectivity was HT-29 > HCT 116 > MCF-7 > HeLa

respectively. In addition, the MbCi has no cytotoxic activity towards normal human endothelial cells (EA.hy926). Morphological observations reveal that ethanol extract of MbCi induced typical apoptotic changes i.e., cell shrinkage, membrane blebbing and loosening of contact with each other. Finding of our study are in line with other research reports where similar type of apoptotic changes was induced in cancer cells as a result of exposure to natural product (Shafi *et al.*, 2008).

It is a fact that a single assay cannot represent the behaviour of free radicals and antioxidants in a living system. Therefore, we performed an array of assays in the present study (i.e. DPPH, ABTS and FRAP models) to investigate the oxidant scavenging potential of ethnaol extract. Our findings indicated good antioxidant activity of MbCi in the FRAP model, and moderate activity in the DPPH and ABTS models, respectively. These assays could provide a more precise description of antioxidant activity, which indicates that the pharmacological effects of MbCi might be due to multiple antioxidant mechanisms.

In an attempt to establish a link between the antioxidant effects of MbCi and their polyphenolic contents, we further estimated the phenolic and flavonoid contents of MbCi by adopting common methods reported in the literature. Several lines of evidences suggest that phenolics act as good free radical scavengers and capable of reducing the oxidative stress. Therefore, phenolics act not only as antioxidants but also have beneficial effect as anticancer agents (Surh *et al.*, 2008; Yang *et al.*, 2008). Polyphenolic compounds are abundant in plants and may associated to provide protection against several chronic diseases (Ovaskainen *et al.*, 2008).

Epidemiological studies revealed that high dietary intake of polyphenols is linked with decreased risk of a range of diseases including cardiovascular disease and some forms of cancer (Hu, 2011). Plant-derived phenolic compounds such as phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes and tannins have shown promising results in different antioxidant and anticancer models (Huang *et al.*, 2009). The phenolic compounds is mainly attributed to their redox properties and may either act as an efficient radical scavengers, ion chelators, such as iron and copper (Bilušić Vundać *et al.*, 2007), or may exert the anticancer effects and inhibit cancer cell growth through a variety of mechanisms (Vauzour *et al.*, 2010).

Detailed chemical characterization by GC-MS reveals that MbCi also contains steroidal compounds. Compounds with steroidal pharmacophore are reported to possess cytotoxicity through several mechanisms (Gupta *et al.*, 2013). On the other hand, phytosterols are triterpenes and important structural components of plant membranes (Moreau *et al.*, 2002). It was demonstrated that the most common dietary are β -sitosterol, campesterol and stigmasterol may offer protection from the colon, breast and prostate cancer (Awad *et al.*, 2000). Moreover, early studies reported that β -sitosterol inhibits the growth of HT-29 human colon cancer cells and alters membrane lipids by activating the sphingomyelin cycle (Awad *et al.*, 1996; Awad *et al.*, 1998). Woyengo *et al.* (2009) reported that the phytosterols seem

to act through several mechanisms of action, including inhibition of carcinogen production, cancer-cell growth, angiogenesis, invasion and metastasis, and through the promotion of apoptosis of cancerous cells. Phytosterols including β -sitosterol, stigmasterol and campesterol detected in the MbCi extract exhibited the most abundant sterol compounds in the diet. It has been established that diet significantly impacts one's risk for cancer disease (Grattan, 2013). Previous studies have been reported that triterpene sterol stigmasterol compound has a moderate radical scavenger activity. In addition, the stigmasterol was the most potent as an anticancer agent towards WiDr cells lines (Sahidin *et al.*, 2014; Sahidin *et al.*, 2015).

The results of our phytochemical analysis reveal that the cytotoxicity of the extract is mainly due to the synergistic effect of class of secondary metabolites, which present in high content of polyphenols and compounds with steroidal nucleus. The ethanolic extract of MbCi has also shown the presence of minor amount of fatty acids, long chain saturated and unsaturated hydrocarbons. Studies have revealed that polyunsaturated fatty acids and their metabolites are capable of inducing apoptosis, cell cycle arrest, capable of attenuating cyclooxygenase enzyme (COX) levels and also act as free radical scavengers in cancer cell lines (Xu *et al.*, 2014).

CONCLUSION

In conclusion, the findings of the present study suggest that the ethanolic extract of inflorescence of *Musa balbisiana* Colla possess moderate antioxidant activity and promising cytotoxicity on HT-29 and HCT-116 colorectal human cancer cell lines, while moderate cytotoxicity on MCF-7 breast cancer cell line. The MbCi extract has no cytotoxicity on HeLa cervical cancer on normal human endothelial (EA.hy926) cell lines. This activity may be due to its high total phenolic and steroidal contents. Thus, further investigations to isolate pure compounds for its potential anticancer mechanisms are warranted.

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