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Ethyl acetate partition obtained from the methanol extract of *Muntingia calabura* leaf exerts effective *in vitro* antiproliferative activity against the HT-29 colon cancer

[La partición de acetato de etilo obtenido del extracto metanólico de la hoja de *Muntingia calabura* ejerce una actividad antiproliferativa *in vitro* eficaz contra el cáncer de colon HT-29]

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Abstract: Methanol extract of *Muntingia calabura* L. leaf ($MEMC_L$) has been shown to exert the antiproliferative activity against the HT-29 (human colon adenocarcinoma) cell line. To further investigate on the medicinal potential of this plant, $MEMC_L$ was sequentially partitioned to obtain the petroleum ether, ethyl acetate and aqueous partitions, which was then tested against the HT-29 cell line and also subjected to the *in vitro* anti-inflammatory study. The most effective partition was also subjected to the phytoconstituents analysis using the UHPLC-ESI-MS. Findings showed that the ethyl acetate partition (EAP) exerts the most effective antiproliferative activity (IC_{50} = 58.0 ± 12.9 µg/mL) without affecting the 3T3 normal fibroblast cells, exhibits the highest anti-inflammatory effect when assessed using the lipoxygenase (> 95%) and xanthine oxidase (> 70%) assays, and contained various types of polyphenolics. In conclusion, *M. calabura* exerts apoptotic-mediated antiproliferative activity, partly via the anti-inflammatory action and synergistic action between the polyphenolics.

Keywords: *Muntingia calabura*; Ethyl acetate partition of methanol extract; HT-29 cells; Antiproliferative activity; Flavonoids.

Resumen: Se ha demostrado que el extracto metanólico de hoja de *Muntingia calabura* L. (MEMCL) ejerce actividad antiproliferativa contra la línea celular HT-29 (adenocarcinoma de colon humano). Para investigar más a fondo el potencial medicinal de esta planta, MEMCL se dividió secuencialmente para obtener el éter de petróleo, el acetato de etilo y las particiones acuosas, que luego se probó contra la línea celular HT-29 y también se sometió al estudio antiinflamatorio *in vitro*. La partición más eficaz también se sometió al análisis de fitoconstituyentes utilizando UHPLC-ESI-MS. Los resultados mostraron que la partición de acetato de etilo (EAP) ejerce la actividad antiproliferativa más efectiva (IC_{50} = 58.0 ± 12.9 µg/mL) sin afectar las células de fibroblastos normales 3T3, exhibe el mayor efecto antiinflamatorio cuando se evalúa usando la lipoxigenasa (> 95%) y ensayos de xantina oxidasa (> 70%), y contenían varios tipos de polifenoles. En conclusión, *M. calabura* ejerce una actividad antiproliferativa mediada por apoptosis, en parte a través de la acción antiinflamatoria y la acción sinérgica entre los polifenoles

Palabras clave: *Muntingia calabura*; Fracción con acetato de etilo del extracto de metanol; Células HT-29; Actividad antiproliferativa; Flavonoides.

INTRODUCTION

Globally, cancer is the second leading cause of morbidity and mortality among the non-communicable diseases after cardiovascular disease (Bray *et al*., 2018) with 11.5 million numbers of cancer deaths is projected in 2030 from 7.1 million in 2002 (Mathers & Loncar, 2006). Among the various types of cancer, colorectal cancer (CRC) is listed as the third most commonly diagnosed cancer in males and the second in females with 861,000 deaths and 1.8 million new cases recorded in 2018 (Rawla *et al*., 2019). Various factors contribute to the progress of colorectal cancer, which include lack of physical activity, excessive alcohol consumption, extreme intake of high-fat, low-fiber diets, long-term diets high in red meat, and diseases (i.e. diabetes or inflammatory bowel diseases) (Donovan *et al*., 2017). With the advancement in medical fields, various screening approaches could be used to prevent CRV, which include stool tests, radiographic imaging, and colonoscopy, to detect the presence of adenomatous polyps, which are precursor lesions to colon cancer (Niederreiter *et al*., 2019). On the other hand, the standard treatment for cancer is mostly based on using antineoplastic drugs, chemotherapy, radiotherapy and surgery (Huang *et al*., 2017). In addition, antiangiogenic agents are also applied in the treatment and control of cancer progression (Comunanza & Bussolino, 2017). Treatment of CRC, in particular, depends on the stage involved with a combination of surgery and chemotherapy being the options for stage IV and the recurrent CRC (Mishra *et al*., 2013).

Despite advancement in the field of cancer treatment, each treatment approach is associated with unwanted or adverse side effects (Nurgali *et al*., 2018). For examples, the surgical treatment of colon cancer has been associated with the risk of local repetition and a great mortality (Line-Edwige *et al*., 2009) whereas radiotherapy and chemotherapy with drugs can expose the patients to many unwanted side effects (Carnesecchi *et al*., 2002). Moreover, the effectiveness of chemotherapy in killing active cells such as cancerous cells that grow and divide more rapidly than other cells is also overshadowed by their ability to kill or damage active healthy cells (Chen *et al*., 2000). This is further worsened by the fact that although early diagnosis and treatment of cancer provide advantages in increasing the individual probabilities of survival, most of the publics in developing countries are restricted from having access to the effective and modern diagnostic methods and facilities (Farmer *et al*., 2010). Due to this restriction, WHO have estimated that approximately 80% of the world population have shifted to the use of traditional medicine, particularly phytotherapy or phytomedicine, as their new approach to treating cancer (Pan *et al*., 2014; Xiang *et al*., 2019).

Traditionally, medicinal plants have been used since a long time ago to reinstate the capability of the body to shield, control, and heal itself, helping to maintain a physical, mental, and emotional well-being with many scientific studies demonstrated the healing effects of plants specifically on cancer diseases, particularly CRC (Ekor, 2014; Xiang *et al*., 2019). As a result of their therapeutic potential against various types of cancer, which have been attributed to the presence of antioxidant and anti-inflammatory activities, numerous medicinal plants have been proposed for use in the cancer prevention and therapy (Greenwell & Rahman, 2015). Since the link between oxidation and inflammation with cancer development has been greatly acknowledged (Crawford, 2014), it is suggested that any plants with antioxidant and anti-inflammatory activities might also possess anticancer activity, including against CRC (Reuter *et al*., 2010). Interestingly, plant like *Muntingia calabura*, exerts remarkable antioxidant and anti-inflammatory activities (Mahmood *et al*., 2014), and is, therefore, postulated to also possess anti-colon cancer potential that is worth investigated. Our preliminary screening revealed the in vitro antiproliferative potential of aqueous, chloroform and methanol ($MEMC_L$) extracts of *M. calabura* leaves against several cancer cell lines with only the aqueous and methanol extract demonstrated antiproliferative against the HT-29 (human colorectal adenocarcinoma) cell line with the recorded IC_{50} that was <30 µg/mL (Zakaria *et al*., 2011) suggesting their potential to be developed as an anti-colon cancer agent. Concurrent with the antiproliferative findings, $MEMC_L$ followed by the aqueous extract of *M. calabura* was found to exhibit a remarkable free radical scavenging activity when assessed using the DPPH- and superoxide anion (SOA)-radical scavenging assays and contained a very high total phenolic content (TPC). Later, Nasir *et al*. (2017), reported on the anticarcinogenic activity of $MEMC_L$ against azoxymethane-induced colon cancer in rats as proven by a reduction in the total aberrant crypt formation. Moreover, the authors also reported that the extract modulates the colon tissue endogenous enzymatic antioxidant defence markers such as

catalase (CAT), and glutathione (GSH) superoxide dismutase (SOD) while reducing the oxidant marker, namely malondialdehyde (MDA) levels.

To further investigate on the anticolon cancer potential of *M. calabura*, $MEMC_L$ was further partitioned into the petroleum ether-, ethyl acetate- and aqueous-extract, before being subjected to the *in vitro* antiproliferative investigation against the HT-29 cancer cell line.

MATERIAL AND METHODS

Chemicals and reagents for:

Anti-inflammatory study

Lipoxygenase from glycine max (soybean), linoleic acid, xanthine oxidase from bovine milk, xanthine, nordihydroguaiaretic acid (NDGA), allopurinol, potassium phosphate (K3 PO4), sodium phosphate (Na_3PO_4), dimethyl sulfoxide (DMSO) and methanol (MeOH) were procured from Sigma-Aldrich (St. Louis, MO, USA).

Antiproliferative study

Cell culture medium (Dulbecco's modified eagle's medium (DMEM)), fetal bovine serum (FBS), trypsin-EDTA, penicillin and streptomycin were procured from Gibco BRL (Life Technologies, Paisley, Scotland). 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ethanol, Griess reagent, and Triton X-100 were acquired from Sigma-Aldrich Corp (St. Louis, MO, USA).

UHPLC-ESI analysis

Acetonitrile and formic acid (LC-MS grade) were procured from Fisher Scientific (Kuala Lumpur, Malaysia). Reverse osmosis Milli-Q water (18.2 MΩ) (Millipore, Billerica, MA, USA) was used to prepare all solutions and for dilution purposes. Chemical standards (i.e. catechin, ferulic acid, gallic acid and chlorogenic acid) were purchased from Sigma Aldrich (St. Louis, MO, USA). Methanol/water, (v:v, 1:1) was used to dilute the standards to 10 mg/mL before being filtered through 0.22 µm membranes for the LC-MS analysis.

Collection of plant materials and the preparation of $MEMC_L$

The leaves of *M. calabura* were collected from their natural habitat around the Universiti Putra Malaysia (UPM), between July and December 2012. The plant was previously identified by Dr. Shamsul Khamis, a certified botanist from the Institute of Bioscience, UPM, and a voucher specimen (SK 2200/13) has been deposited in the Herbarium of the Laboratory of Natural Products, Institute of Bioscience, UPM.

The matured-, dried-leaves were ground into a coarse powder followed by the process of maceration in methanol (MeOH) (1:20, w/v) for 72 h at room temperature. The maceration process was performed in triplicates and the supernatant collected from each maceration process was pooled together before being evaporated under reduced pressure at 40ºC. The dried MeOH extract of *M. calabura* leaves ($MEMC_L$) obtained (14.3% yield) were then subjected to fractionation process (Zakaria *et al*., 2016).

Preparation of various partitions of different polarity from $MEMC_L$

Dried $MEMC_L$ was partitioned by suspending the weighed extract in MeOH followed by the addition of distilled water (dH_2O) to afford an aqueous MeOH solution. Partitioning of the aqueous MeOH solution was sequentially initiated using solvents of different polarity, namely petroleum ether (PE; non-polar) and ethyl acetate (EA; intermediate polar), to yield the petroleum ether (PEP) and ethyl acetate (EAP) partitions, respectively. The process of partitioning using each solvent was carried out in triplicates. The solution left after PE and EA partitioning represents the aqueous (polar) partition (AQP) of $MEMC_L$ was also collected. The collected PEP and EAP were separately evaporated at 40°C under reduced pressure to obtain the respective dry partition, whereas the collected AQP was kept at −80°C for at least 2 days and then subjected to the freeze-drying process (4°C; under reduced pressure) to obtain the dried aqueous partition. Lastly, all dried partitions were prepared to the required concentrations by dissolving each of them in a 10% DMSO prior to subjection to the antiproliferative study (Zakaria *et al*., 2016).

In vitro anti-inflammatory assays

Lipoxygenase assay

A mixture of 160 µL of sodium phosphate buffer (0.1 M, pH 8.0), 10 µL of the respective test solution (e.g. partitions (PEP, EAP or AQP) or nordihydroguaiaretic acid (NDGA; reference standard) and 20 µL of soy bean lipoxygenase solution were prepared and kept at 25ºC for 10 min. Then, 10 µL of linoleic acid was added to the mixture solution to initiate the reaction. Prior to the reaction, the respective test solution was prepared by dissolving them in MeOH. All the reactions were performed in triplicates using a 96- well plate and

absorbance was measured at 234 nm (Ul-Haq *et al*., 2004).

Xanthine oxidase assay

A mixture of 10 µL of the respective test solution (e.g. partitions (PEP, EAP or AQP) or allopurinol (reference standard)), 130 µL potassium phosphate buffer (0.05 M, pH 7.5) and 10 µL of the xanthine oxidase solution was prepared and incubated (25°C) for 10 min. Xanthine solution was added to the mixture to start the reaction (conversion from xanthine to hydrogen peroxide and uric acid). Prior to the reaction, the respective test solution was dissolved in DMSO. All the reactions were performed in triplicates using a 96- well plate and absorbance was measured at 295 nm (Noro *et al*., 1983).

Evaluation of antiproliferative activity

Cell lines and culture conditions

The HT-29 and 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% FBS, 100 µg/mL streptomycin and 100 U/mL penicillin. These cells were seeded at density of $1x10^5$ cells/mL and sustained in a humidified atmosphere (37ºC) containing 5% CO_2.

Sample preparation for cell culture

PEP, EAP and AQP were dissolved in 1 mL of DMSO and further diluted in cell culture medium until six final concentrations (0, 6.25, 12.5, 25, 50 and 100 µg/mL) were obtained. All partitions were filtered through the 0.22 µm filters before being subjected to cell culture treatments.

MTT assay

Cells ($1x10^5$ cells/mL) were seeded into each well of the 96 well-plate for 24 h and further treated with the sample extracts for another 72 h. After incubation of HT-29 cell with different concentrations of the respective *M. calabura* partition, 20 µL of MTT solution was added to each well followed by 100 µL of DMSO to dissolve the formazan before the plate was incubated (37ºC) for 4 h. The viability of cells treated with the respective partition was spectrophotometrically measured at 570 nm and the IC_{50} value was determined (Bagheri *et al*., 2018).

Acridine orange (AO) and propidium iodide (PI) double staining

An acridine orange (AO) and propidium iodide (PI) double staining assay was carried out to distinguish the morphologic features of apoptosis in the HT-29 cells following pretreatment with EAP by means of a fluorescent microscope (Olympus BX51, Japan). HT-29 cells were plated at a density of 5×10^4 cells/mL in a T-25 culture flask and then treated with the serially diluted concentrations of EAP for 24 h before being harvested and stained with equal volume (10 µg/mL) of AO and PI dyes. The treated and untreated cells were then observed under a UV-fluorescent microscope within 30 min to study the cell morphology (Bagheri *et al*., 2018).

UHPLC–ESI analysis of $MEMC_L$ and EAP

The UHPLC-ESI-MS system and conditions used to analyze the phytoconstituent of EAP was similar to the previously described procedure (Zakaria *et al*., 2016). Briefly, a Dionex 3000 UHPLC system (Thermo Fisher Scientific, USA) comprised of an Autosampler attached to a column oven, a tray compartment cooler, and a binary pump with built in solvent degasser. A BEH C18 UHPLC column, 100 mm x 2.5 µm, 1.7 µm (WATERS) together with the mobile phases consisting of 0.1% formic acid in water (pH 2.5; labelled as A) and 0.1% formic acid in acetonitrile (pH 4.0; labelled as B) were used to complete the chromatographic separation at a flow rate of 0.3 mL/min. The gradient was modulated as follows: Starting gradient was 10% B for 5 min until B reached 20%, followed by 60% B after another12 min (t = 17 min) before reaching the isocratic elution at 90% B at 3 min. The gradient was allowed to reach the initial condition before being kept for 2 min as a re-equilibration step. The injection volume and column temperature were maintained at 10 µL and 40 °C, respectively. The UHPLC system was attached to a linear ion-trap-Orbitrap mass spectrometer Q Exactive (Thermo Fisher Scientific, U.S.A) furnished with an electrospray ionization (ESI) source. The mass detection was carried out in a range of 150-1500 m/z. The ESI source was ran in a negative ion mode under the conditions described as follows: source voltage: 3.2 kV; sheath gas: 35 arbitrary units; auxiliary gas: 15 arbitrary unit; sweep gas: 10 arbitrary unit; capillary temperature: 320°C, and; nitrogen (>99.98%): used as the sheath, auxiliary and sweep gas. Instrument control and data acquisition were performed with Chameleon 6.8 software and Xcalibur 2.2 software (Thermo Fisher Scientific).

Statistical analysis

Data were expressed as mean values ± SD. Statistical

analysis was performed using the one-way analysis of variance (ANOVA) followed by the Dunnett's *post hoc* test as provided by the GraphPad Prism version 5. Data with $p<0.05$ was considered as statistically significant.

RESULTS

In vitro anti-inflammatory activity of various partitions of MEMC$_L$

Of the three partitions, EAP was found to possess the most effective anti-inflammatory activity against the action of LOX and XO as indicated by its ability to exert the highest percentage of inhibitory effect against LOX (> 95% inhibition) and XO (> 70% inhibition). PEMC and AQMC only showed remarkable anti-inflammatory action against LOX (100% and > 80% inhibition, respectively), but not XO assay (Table No. 1). NDGA and allopurinol were found to exert high percentage of inhibitory activity against the respective LOX (> 99%) or NO (> 97%) assay. Statistically, EAP exerted significantly ($P < 0.01$) lower inhibitory effect against the XO activity when compared against the standard drug, allopurinol, but, demonstrated insignificant ($P > 0.05$) different in inhibitory effect against the LOX when compared to the NDGA.

Table No. 1
Percentage of inhibition showed by *M. calabura* partition extracts in xanthine oxidase and lipoxygenase assays

Sample	Xanthine oxidase (%)	Lipoxygenase assay (%)
Allopurinol (reference standard of XO)	97.58 ± 0.32^{a}	-
NDGA (reference standard of LOX)	-	99.86 ± 0.14^{a}
PEMC	8.67 ± 2.80^{b} (L)	100.00 ± 0.0^{a} (H)
EAMC	72.81 ± 2.52^{c} (H)	95.54 ± 4.46^{ab} (H)
AQMC	21.54 ± 4.95^{d} (L)	84.40 ± 7.85^{b} (H)

All values are expressed as mean ± SEM.
[a,b,c,d] Data with different superscript in the respective column differed significantly at $p<0.05$.
Note: H, high (71–100%); M, moderate (41–70%); L, low (0–40%); NA, not active (Zakaria *et al*., 2014)

The effect of various partitions of MEMC$_L$ on HT-29 and 3T3 cells viability

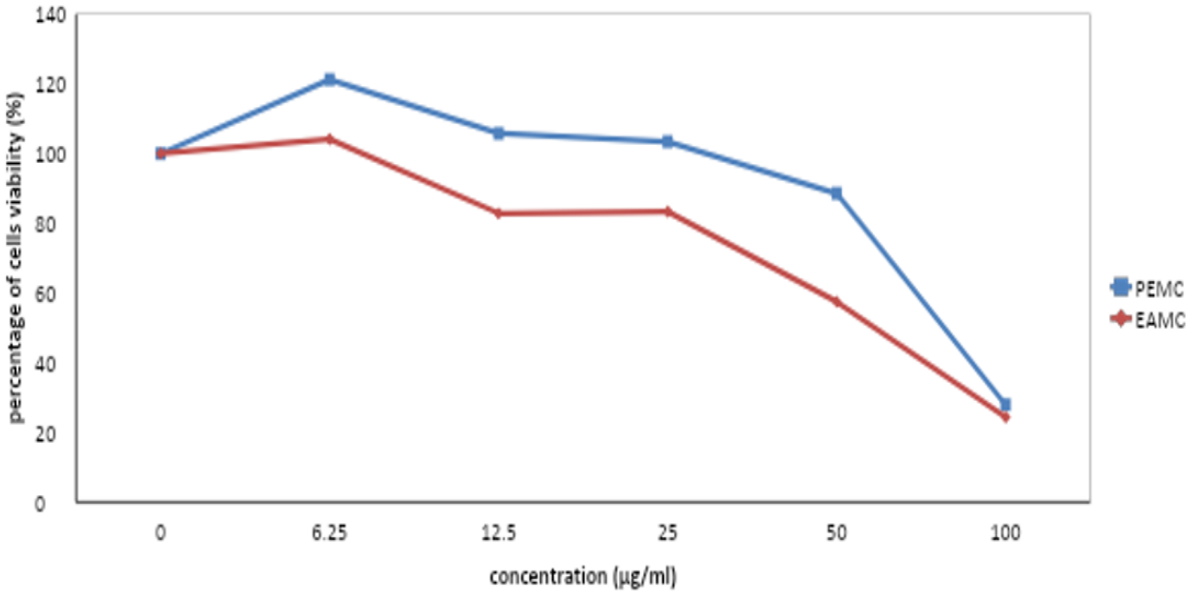
Of the three partitions, EAP demonstrated the most potent ($p<0.01$) cytotoxic effect against HT-29 cells followed by PEP indicated by the former lowest IC_{50} value recorded (IC_{50} = 58.0 ± 12.9 µg/mL) in comparison to the latter (IC_{50} = 82.0 ± 3.3 µg/mL) (Table No. 2). In addition, EAP also significantly ($p<0.01$) inhibited the growth of colon cancer cells in a concentration-dependent manner. Based on this finding, EAP was further tested on normal cell line (3T3) and demonstrated no antiproliferative effect against the 3T3 cells, thus, suggests that EAP is safe towards normal cells and selective towards colon cancer cells (Figure No. 1A and No. 1B). Due to this, EAP was selected for the rest of the experiment.

Table No. 2
IC50 value of *M. calabura* partition extracts (PEMC, EAMC and AQMC) towards HT-29 cell line

Sample	HT-29 cells [IC_{50} (µg/mL)]	3T3 cells [IC_{50} (µg/mL)]
PEMC	82 ± 3.3^{a}	-
EAMC	58 ± 12.9^{b}	> 100
AQMC	> 100	-

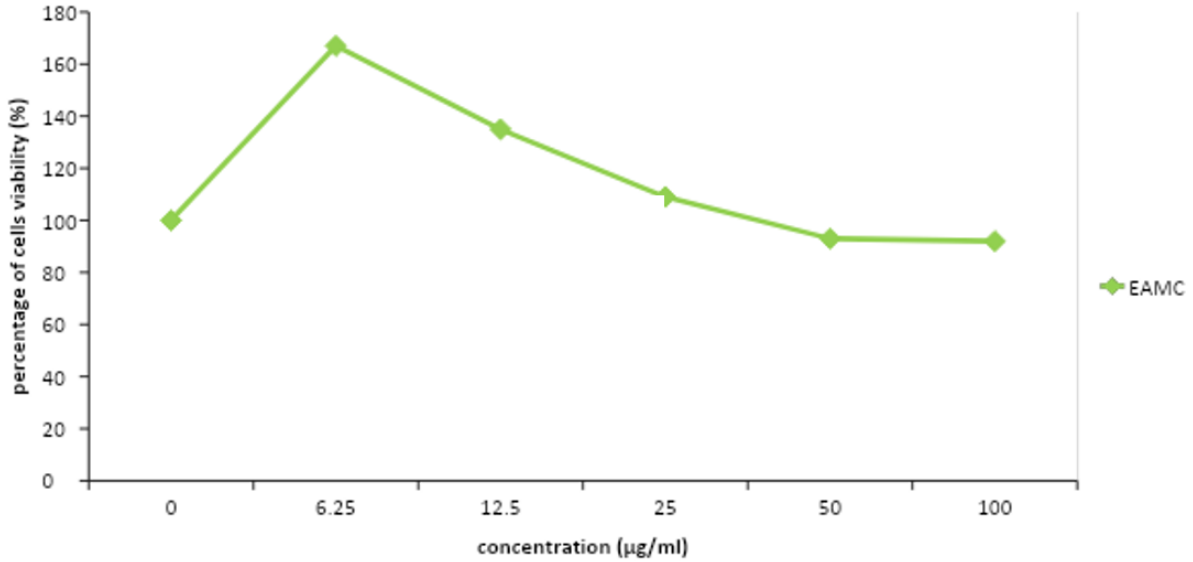
The IC_{50} is average value of three independent experiments and data shown are means ± SD
[a,b]Data with different superscript in the respective column differed significantly at $p<0.05$

Figure No. 1A



Cytotoxic effect of EAMC and PEMC towards colon cancer cell line (HT-29) after 72 h incubation as determined by the trypan blue dye exclusion method. Each data point represents the mean of three independent experiments. Value was expressed as mean ± SD. * indicates *p*<0.05 when compared to control (untreated cells). Note: AQMC was found to cause >100% inhibition

Figure No. 1B



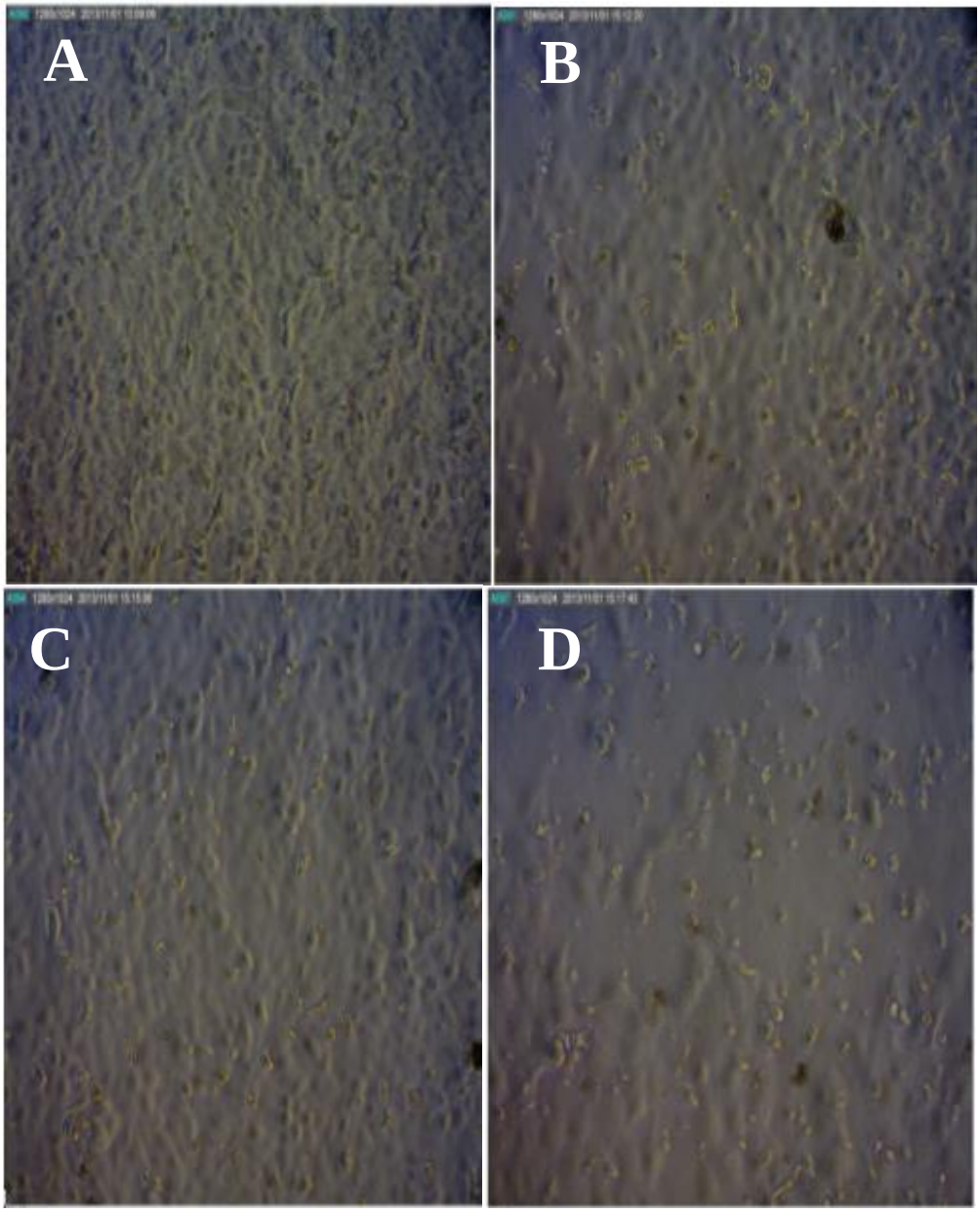
Cytotoxic effect of EAMC extract towards mouse embryonic fibroblast cell line (3T3) after 72 h incubation as determined by the trypan blue dye exclusion method. Each data point represents the mean of three independent experiments. Value was expressed as mean ± SD

Effect of EAP on the morphological changes of HT-29 cells: Microscopic observation

Morphological changes of HT-29 cells following treatment with EAP were examined under inverted light microscope. The results showed that the treated cells were detached from substratum in the presence of EAP, but not the control (untreated) cells as it spread evenly on the substratum.

Cell population was decreased with the increased concentration of EAP as illustrated in Figure No. 2 (inverted microscope) and Figure 3 (phase contrast), respectively. The morphological alteration of cells observed under phase contrast field revealed the changes associated with apoptosis such as membrane blebbing, cell shrinkage and apoptotic bodies.

Figure No. 2



Morphological changes of HT-29 cells treated with different concentrations of EAMC for 72 h and observed under inverted microscope. A) Control (untreated cells), B) HT-29 cells treated with 30 µg/mL of EAMC C) HT-29 cells treated with 60 µg/mL of EAMC D) HT-29 cells treated with 90 µg/mL of EAMC. (200 x magnification). Reduce in cell population was noticed with the increase in the concentration of treatment as compared to the control

Figure No. 3

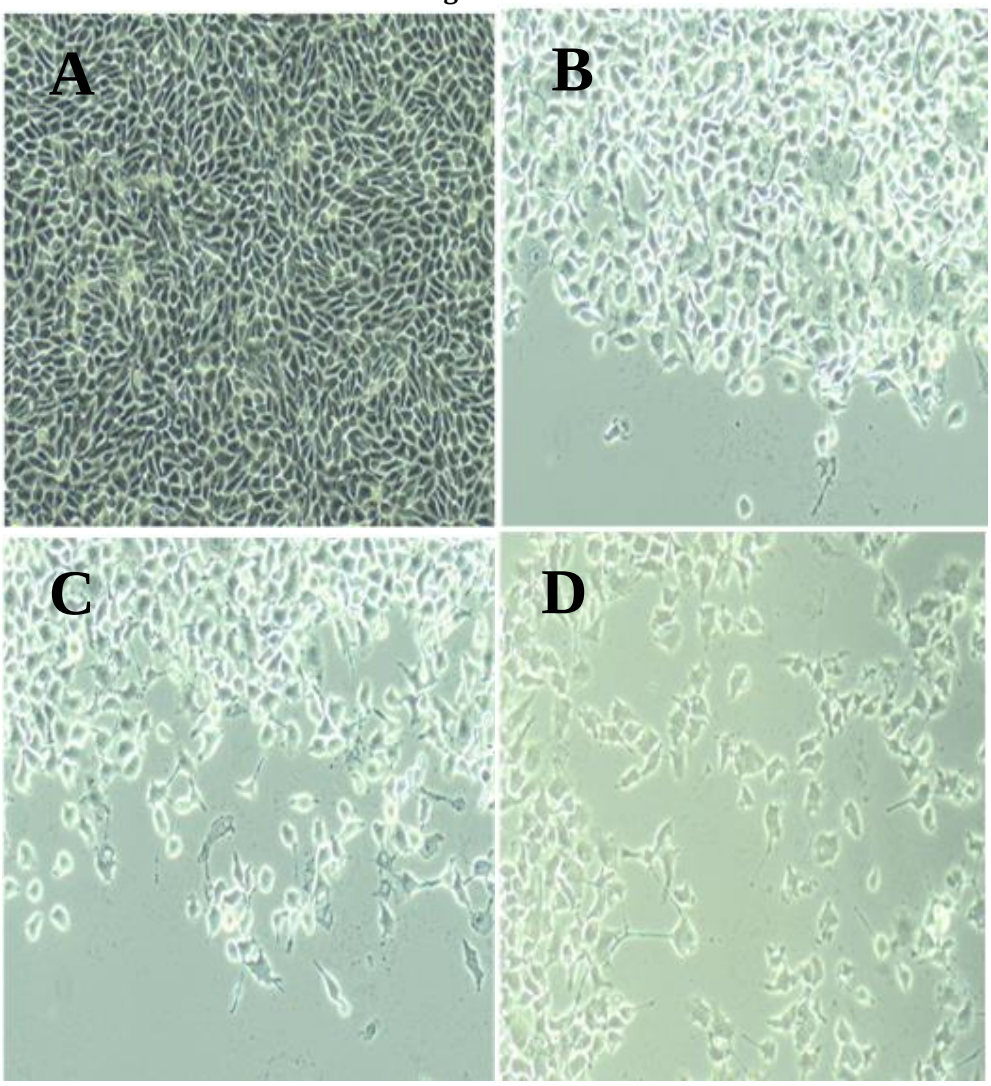


Figure No. 3A

Morphological changes of HT-29 cells treated with different concentrations of EAMC and observed under phase contrast field. A) Control (untreated cells), B) HT-29 cells treated with 30 µg/mL of EAMC C) HT-29 cells treated with 60 µg/mL of EAMC D) HT-29 cells treated with 90 µg/mL of EAMC. (200 x magnification). Some cells detached from the substratum after treated with MEMC as compared to the control

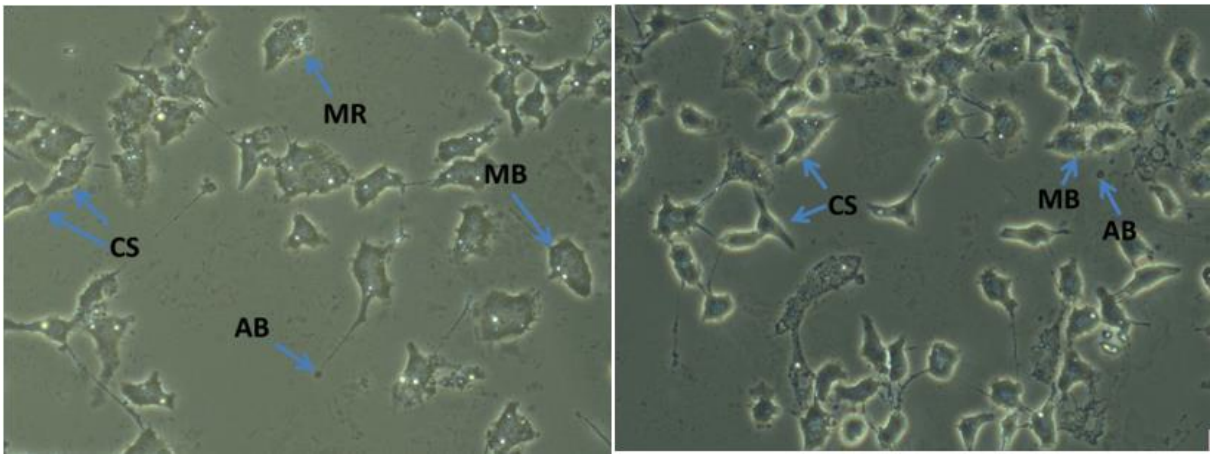


Figure No. 3B

Effect of various doses of EAMC against HT-29 cells using phase contrast field. A) Exposure of EAMC for 72 h to HT-29 cells at concentration of 60 µg/mL. B) Exposure of EAMC for 72 h to HT-29 cells at concentration of 90 µg/mL. Both Figure A and B showed the characteristic of apoptosis; 1) membrane blebbing (MB), 2) cell shrinkage (CS), and 3) apoptotic bodies (AB). (400 x magnification)

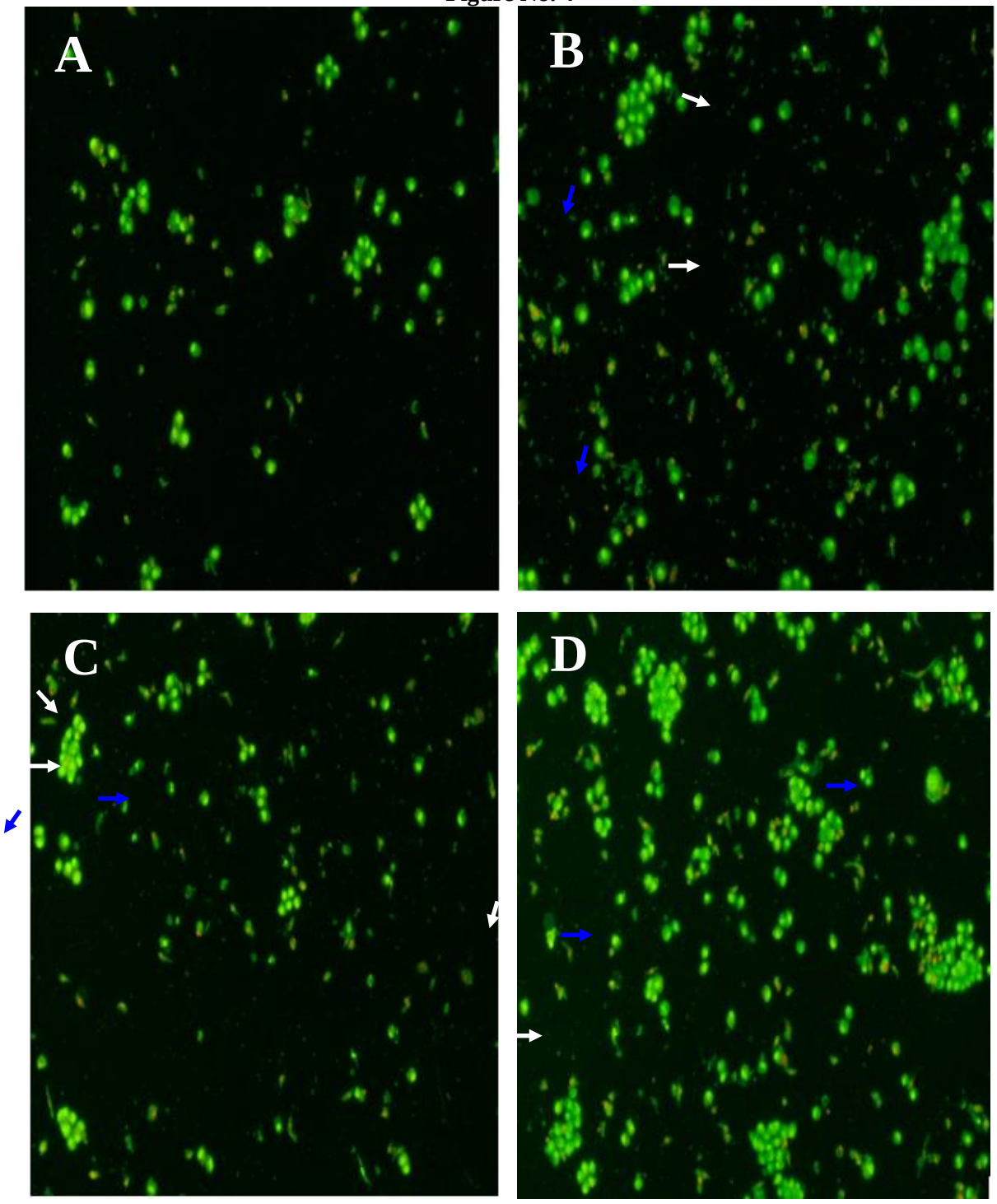
AO/PI dual staining assay

The results obtained from the AO/PI double staining are shown in Figure No. 4. In general AO/PI staining can distinguish between viable, apoptotic and necrotic cells. Viable cells with intact DNA and nucleus were found to give a rounded and green-colored nuclei, while cells that have undergone apoptosis contained fragmented DNA with green-colored nuclei. On the other hand, late apoptotic and necrotic cells appeared orange and red cells. Some affected cells exhibited characteristics of apoptosis like membrane blebbing and membrane rupture after being treated with EAP.

Phytochemicals identification in MEMCL and EAP

The respective phytochemical analyses of MEMCL and EAP using the UHPLC-ESI-MS method has been carried out earlier and published elsewhere (Zakaria et al., 2016; Zakaria et al., 2019). Based on the reports, 18 bioactive compounds were found to be present in both samples and are listed in Table No. 3, Interestingly, some of these bioactive compounds, namely gallic acid, protocatechuic acid, ferulic acid, myricetin, quercetin, pinocembrin, kaempferol and chyrsin (Ko et al., 2005; Nasr Bouzaiene et al., 2015; Kumar et al., 2007; Cho & Park, 2013; Yang et al., 2016; Rosman et al., 2018; Saifullah et al., 2018; Song et al., 2019), have been reported to exert antiproliferative activity against the HT-29 cells.

Figure No. 4



Morphological alteration of HT-29 cells treated with EAMC for 72 h at the concentrations of (A) 0 mg/ml, (B) 30 µg/mL, (C) 60 µg/mL, (D) 90 µg/mL view under fluorescence microscope. The treated cells showed

characteristics of apoptosis such as cell membrane blebbing (white arrow), cell membrane rupture (blue arrow) and necrotic cell

Table No. 3
Phytoconstituents identified in MEMCL and EAP using the UHPLC-ESI-MS method. Several of these phytoconstituents have been reported to possess anticancer activity

No.	Compounds identified in $MEMC_L$[a] and EAP[b]	Report on the antiproliferative effect against HT-29 cells
1	Gallic acid	Rosman *et al*.
2	Protocatechuic acid	Saifullah *et al*.
3	Ferulic acid	Nasr Bouzaiene *et al*.
4	Quercitrin-2″-*O*- gallate	-
5	Pentagalloyl-hexoside II	-
6	Kaempferol-3-*O*-galactoside	-
7	Myricetin	Ko *et al*.
8	Isoferulic acid	-
9	Afzelin-*O*-gallate	-
10	Quercetin	Yang *et al*.
11	Quercetin dimer	-
12	Pinocembrin	Kumar *et al*.
13	Kaempferol-3-*O*-glucoside	-
14	Rhamnetin	-
15	Pinobaksin	-
16	Kaempferol	Cho *et al*.
17	Chyrsin	Song *et al*.
18	Kaempferide	-

[a]Reported by Zakaria *et al*. (2019); [b]Reported by Zakaria *et al*. (2016)

DISCUSSION

In the earlier study, $MEMC_L$ was demonstrated to possess *in vitro* antiproliferative activity against the HT-29 colon cancer cells (Zakaria *et al*., 2011). This was followed by another study that revealed the anticarcinogenic activity of $MEMC_L$ against azoxymethane-induced colon cancer model in rats (Md Nasir *et al*., 2017). In an attempt to further investigate on the anticolon cancer potential of *M. calabura* leaves, the dried crude $MEMC_L$ was further partitioned sequentially with solvents of different polarity to obtain the PEP, EAP and AQP, which were then subjected to the *in vitro* antiproliferative study against the HT-29 colon cancer cells.

It is generally known that cancer cells have unique features that enable them to survive, such as limitless replication, ability to avoid cell death and metastasis (Hanahan, 2011). Besides, tumor microenvironment likes inflammation also triggers the carcinogenesis process (Grivennikov *et al*., 2010). Evidence showed in certain cases, inflammation occurred at the early stage of carcinogenesis (Vendramini-Costa & Carvalho, 2012). Inflammation provides molecules such as angiogenic factors and enzymes that facilitate survival of cancer and modify the extracellular matrix (Hanahan, 2011). In view of the relationship between cancer and inflammation (Bellik *et al*., 2013), the *in vitro* anti-inflammatory potential of various partitions of $MEMC_L$ was determined against two inflammatory-related pathways, namely LOX and XO, prior to the in vitro anticolon cancer study. The results obtained show that EAP possesses the most effective anti-inflammatory activity against both the LOX and XO pathways, whereas PEP and AQP only demonstrated effective activity against the LOX pathway. Interestingly, the ability of EAP to exert in vitro anti-inflammatory activity is concurrent with previous

reports that demonstrated the ability of *M. calabura* extracts to attenuate inflammation *in vivo* (Zakaria *et al*., 2007; Jisha *et al*., 2019) and *in vitro* (Balan *et al*., 2015). The difference in anti-inflammatory intensity demonstrated by the three partitions of $MEMC_L$ could result from the dissimilarity of phytoconstituents and different types of solvent extraction used (Khanapur *et al*., 2014).

Other than inflammation, a relationship between cancer and oxidative stress has been widely acknowledged (Noda & Wakasugi, 2001). Reactive species, comprise mostly of reactive oxygen species (ROS), are produced as a result of metabolic reactions that take place in the mitochondria of eukaryotic cells. At low concentrations, reactive species are used by normal cells in signal transduction before being eliminated from the body. However, cells with greater metabolism like cancer cells tend to demand for high concentrations reactive species to sustain their high proliferative rate (Reuter *et al*., 2010; Khanapur *et al*., 2014). Therefore, it is suggested that by monitoring and regulating the level of reactive species in the body, the amount of injury caused to the cells could be controlled as well be prevented, which is what seen in cancer therapy. In the treatment of cancer, generally used radiotherapeutic and chemotherapeutic drugs affect tumor outcome via the modulation of reactive species. Interestingly, earlier investigation by Balan *et al*. (2015), has demonstrated that EAP contained a higher total phenolic content and demonstrated high antioxidant activity when assessed using various antioxidant models (e.g. DPPH- and superoxide anion-radical scavenging assays, and ORAC assay). In line with the above-mentioned ROS modulation potential of chemotherapeutic drugs, it is plausible to suggest that EAP will also demonstrate *in vitro* anti-colon cancer activity via its remarkable antioxidant and free radical scavenging effects, in addition to the anti-inflammatory activity.

Based on the above-mentioned anti-inflammatory findings and further supported by the previous report on its significant antioxidant and free radical scavenging activities, EAP was chosen for further in vitro anticolon cancer investigation. The results obtained show that EAP exerted the most effective *in vitro* antiproliferative activity against the HT-29 cells indicated by the lowest IC_{50} value recorded in comparison to PEP with AQP showed no antiproliferative activity at the tested dose range. EAP was also found to inhibit the growth of HT-29 colon cancer cells in a concentration-dependent manner, but demonstrated no antiproliferative effect against the 3T3 cells, suggesting that EAP is selective in its cytotoxic effect towards the cancer cells while being safe towards the normal cells. Morphological changes examination of HT-29 cells treated with EAP revealed that the treated cells were detached from substratum in comparison to the untreated control cells that spread evenly on the substratum while inverted or phase contrast microscope observations revealed the decrease in healthy cell population with increased presence of apoptotic cells as indicated by the presence of membrane blebbing, cell shrinkage and apoptotic bodies (Elmore, 2007). Furthermore, the AO/PI double staining of EAP-treated HT-29 cells further confirmed the abundant presence of late apoptotic (orange-colored) and necrotic (red-colored) cells as well as decrease number of viable cells (green-colored). The ability of this staining to differentiate apoptotic cells from necrotic cells is attributed to the AO dye ability to bind to the double stranded DNA in living cells and gives the green appearance, but binds to single stranded DNA in dead cells to give the orange appearance (Foglieni *et al*., 2001). On the other hand, PI is only taken up by the non-viable cells as their membrane rupture and enable this.

Damaged cells are eliminated from the body through an orderly and effective form of programmed cell death known as apoptosis, which can be incited via signals from within the cell, for examples genotoxic stress, or by extrinsic signals (i.e. binding of ligands to cell surface death receptors) (Pfeffer & Singh, 2018). Apoptosis is multifaceted and includes various signalling pathways, and is generated in a cell either via the caspase-mediated intrinsic or extrinsic pathways. Activation of these pathways will, together, initiate the effector apoptotic caspases leading eventually to the morphological and biochemical cellular changes, characteristics of apoptosis. In precancerous lesions where DNA damage is likely to occur, the initiation of apoptosis can help to eliminate the possibly harmful cells, thus hindering tumor growth (Kiraz *et al*., 2016). It is well acknowledged that alteration in the process regulated by the apoptotic cell death machinery is a hallmark of cancer and, is accountable for tumor growth and progression, as well as for tumor resistance to therapies. Currently, most of the anticancer drugs applied at the clinical level take advantage of the intact apoptotic signaling pathways to cause cancer cell death. Hence, flaws in the death pathways may result in drug resistance, which limits the

effectiveness of therapies (Kiraz *et al*., 2016; Pfeffer & Singh, 2018). Interestingly, the ability of EAP to induce apoptosis as seen in the present study seems to suggest its potential to be used as lead in the future anticolon cancer drug development.

Previous phytochemical analysis carried out on *M. calabura* leaves revealed the presence of several types of flavonoids with some of them proven to possess antiproliferative activity (Chen *et al*., 2005). Concurrently, our preliminary phytochemical screening of *M. calabura* leaves also demonstrated the presence of flavonoids in addition to tannins, saponins and steroids (Zakaria *et al*., 2011). The vital roles of flavonoids particularly in cancer chemoprevention and chemotherapy have been well acknowledged whereby the inhibition of cancerous cell growth relies on their antioxidant activity, particularly the ability to scavenge free radicals (Ren *et al*., 2003; Elsyana *et al*., 2016). Other mechanisms of anticancer action demonstrated by the flavonoids include the activation of apoptosis of cancer cells (Hertzog & Tica, 2012) and the downregulation of pro-inflammatory gene, such as nitric oxide synthase (NOS) and cyclooxygenase-2 (COX-2) (Kim *et al*., 2004). The recent phytochemical analysis of EAP by means of the UHPLC-ESI-MS system revealed the presents of various flavonoid-based bioactive compounds (Zakaria *et al*., 2016) of which 18 phytoconsituents were also found in its parent crude extract, $MEMC_L$. Interestingly, 8 of these compounds, namely gallic acid (Rosman *et al*., 2018), protocatechuic acid (Saifullah *et al*., 2018), ferulic acid (Nasr Bouzaiene *et al*., 2015), myricetin (Ko *et al*., 2005), quercetin (Yang *et al*., 2016), pinocembrin (Kumar *et al*., 2007), kaempferol (Cho & Park, 2013) and chrysin (Song *et al*., 2019), have been reported to demonstrate in vitro anticolon cancer activity. These compounds were believed to work synergistically to produce the antiproliferative effect against HT-29 cells as seen in the present study. Various mechanisms of apoptosis-mediated action could be proposed to play role in the EAP-induced apoptosis against HT-29 cells based on the presence of these bioactive compounds. Ferulic acid triggers apoptosis of HT-29 cells by inhibiting the matrix metalloproteinases-2 (MMP-2) enzyme activity, which is the key factor in the metastatic process of the cells (Chen *et al*., 2005; de Souza Rosa *et al*., 2018). Quercetin, on the other hand, induces apoptosis of the HT-29 cells various modes of action, which include characteristic changes in nuclear morphology, activation of caspases-3 and -9, collapse of mitochondrial membrane potential, upregulation of pro-apoptotic Bax, and downregulation of antiapoptotic Bcl-2 and Bcl-XL (Li & Gao, 2013); up-regulation of the apoptosis-related proteins, such as AMP-activated protein kinase (AMPK), p53, and p21 (Kim *et al*., 2010), and; reduction of the protein expression level of phosphorylated-Akt but increased protein degradation of CSN6, which affect the expression levels of Myc, p53, B-cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein (Ren *et al*., 2003). In addition, kaempferol causes apoptosis of the HT-29 cells by downregulating the activation of PI3K/Akt and ERK-1/2 pathways via the inhibition of IGF-IR and ErbB3 signaling (Lee *et al*., 2014a) and via the events associated with the activation of cell surface death receptors and the mitochondrial pathway (Lee *et al*., 2014b). According to Lee *et al*. (2014b), these might include increasing the levels of cleaved caspase-9, caspase-3, caspase-7, cleaved poly-(ADP-ribose)-polymerase, Bik, mitochondrial Bad and membrane-bound FAS ligand; increasing the caspase-8 activity, mitochondrial membrane permeability and, cytosolic cytochrome c concentrations, and; reducing the levels of Bcl-xL proteins, uncleaved caspase-8, intact Bid, Akt activation and Akt activity. Lastly, chrysin was also demonstrated to cause a minor cytotoxic effect against HT-29 cells (Lin *et al*., 2018), but no attempt was made to determine the possible mechanism of action involved. Although no reports have been published with regard to the apoptotic effect of gallic acid, myricetin and pinocembrin on HT-29 cells, their apoptotic effect on other type of cancer cells have been well documented (Suresh Kumar *et al*., 2007; Kim *et al*., 2014; Subramanian *et al*., 2016).

In conclusion, *M. calabura*'s partition, EAP, exerts antiproliferative activity against HT-29 cells via the apoptotic mechanisms, which could be attributed to the synergistic action of several flavonoid-based bioactive compounds proven earlier to exert *in vitro* anticolon activity.

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