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Articulo Original / Original Article

Anticarcinogenic activity of methanol extract of *Melastoma malabathricum* leaves is attributed to the presence of phenolics compounds and the activation of endogenous antioxidant system

[Actividad anticancerígena del extracto metanólico de hojas de *Melastoma malabathricum* atribuida a la presencia de compuestos fenólicos y a la activación del sistema antioxidante endógeno]

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Abstract: *Melastoma malabathricum* (*M. malabathricum*) extracts have been reported to exert various pharmacological activities including antioxidants, anti-inflammatory and antiproliferative activities. The objective of the present study was to determine the anticarcinogenic activity of its methanol extract (MEMM) against the azoxymethane (AOM)-induced early colon carcinogenesis in rats. Rats were randomly assigned to five groups (n=6) namely normal control, negative control, and treatment (50, 250 or 500 mg/kg of MEMM) groups. Colon tissues were harvested for histopathological analysis and endogenous antioxidant system determination. MEMM was also subjected to HPLC analysis. Findings showed that MEMM significantly (p<0.05) reversed the AOM-induced carcinogenicity by: i) reducing the formation of aberrant crypt foci (ACF) in colon tissues, and; ii) enhancing the endogenous antioxidant activity (catalase, superoxide dismutase and glutathione peroxidase). Moreover, various phenolics has been identified in MEMM. In conclusion, MEMM exerts the *in vivo* anticarcinogenic activity via the activation of endogenous antioxidant system and synergistic action of phenolics.

Keywords: *Melastoma malabathricum*; Methanol extract; Colon cancer; Azoxymethane; Aberrant crypt foci

Resumen: Se ha informado que los extractos de *Melastoma malabathricum* (*M. malabathricum*) ejercen diversas actividades farmacológicas, incluidas actividades antioxidantes, antiinflamatorias y antiproliferativas. El objetivo del presente estudio fue determinar la actividad anticancerígena de su extracto de metanol (MEMM) contra la carcinogénesis de colon temprana inducida por azoximetano (AOM) en ratas. Las ratas se asignaron al azar a cinco grupos (n=6), a saber, los grupos de control normal, control negativo y tratamiento (50, 250 o 500 mg/kg de MEMM). Tejidos de colon fueron recolectados para análisis histopatológico y determinación del sistema antioxidante endógeno. MEMM también se sometió a análisis de HPLC. Los hallazgos mostraron que MEMM invirtió significativamente (p<0.05) la carcinogenicidad inducida por AOM al: i) reducir la formación de focos de criptas aberrantes (ACF) en los tejidos del colon, y; ii) potenciar la actividad antioxidante endógena (catalasa, superóxido dismutasa y glutatión peroxidasa). Además, se han identificado varios fenólicos en MEMM. En conclusión, MEMM ejerce la actividad anticancerígena *in vivo* mediante la activación del sistema antioxidante endógeno y la acción sinérgica de los fenólicos.

Palabras clave: *Melastoma malabathricum*; Extracto en metanol; Cáncer de colon; Azoximetano; Focos de criptas aberrantes

INTRODUCTION

Melastoma malabathricum L. is a shrub commonly found in the tropical climate regions, particularly the Southeast Asian countries including Malaysia. Known as 'Senduduk' to the Malay, *M. malabathricum* has gained a herbal status in the Malay folklore as well as the Indian, Chinese and Indonesian (Koay, 2008). Traditionally, various parts of *M. malabathricum* has been used in the Malay, Chinese and Indian traditional cultures to treat different types of disorders such as cuts and wounds, sore legs, hemorrhoids, toothache, stomachache, diarrhea, dysentery, flatulence, leucorrhea and infection during confinement (Zakaria *et al*., 2006).

Scientifically, various parts of *M. malabathricum* soaked in different solvent systems have been cited to exert various pharmacological activities. With regards to the methanol extract of *M. malabathricum* leaves (MEMM), pharmacological activities such as antiproliferative, antioxidant and antiulcer have been cited by Joffry *et al*. (2012). After 2012, several new studies have demonstrated the potential of *M. malabathrium* to exert antioxidant and hepatoprotective (Mamat *et al*., 2013), anti-diabetic, antioxidant and anti-hyperlipidemic (Kumar *et al*., 2013), chemopreventive (Kooi *et al*., 2014), anticoagulant (Khoo *et al*., 2014), gastroprotective (Zakaria et al., 2015), anti-obesity (Karupiah & Ismail, 2015), antinociceptive (Jaios *et al*., 2016), anti-inflammation (Kumar *et al*., 2016), and chemomodulatory (Verma *et al*., 2016).

Various phytoconstituents have been isolated and identified from different extracts of various parts of *M. malabathricum* (Mohd. Joffry *et al*., 2012). Focusing only on reports on MEMM, Mohd. Joffry *et al*. (2012), has reported that the phytochemicals screening revealed the presence of saponins, flavonoids, tannins, triterpenes and steroids. Further analyses using the chromatographic techniques lead to the isolation and identification of ursolic acid, 2-hydroxyursolic acid, asiatic acid, 2,5,6-trihydroxynaphtoic carbonic acid, methyl-2,5,6-trihydroxynaphtalene carbonate, glycerol-1,2-dilinolenyl-3-*O*-*β*-D-galactopyranoside, glycerol 1,2-dilinolenyl-3-*O*-(4,6-di-*O*-isopropylidene)-*β*-D-galactopyranoside, kaempferol-3-O-(2″,6″-di-O-p-trans-coumaroyl)-β-glucoside, rutin, quercitrin, and quercetin from MEMM whereas *β*-sitosterol, *α*-amyrin, uvaol, quercetin, quercitrin, rutin, and sitosterol-3-*O*-*β*-D-glucopyranoside has been identified in the hexane fraction of MEMM (Pongprom *et al*., 2003).

Since *M. malabathricum* leaves, particularly MEMM, possess significant antioxidant and anti-inflammatory activities as mentioned above, the plant's leaves/its extract was earlier proposed to also possess a chemopreventive activity based on the well-acknowledged link between oxidative stress, inflammation and carcinogenesis (Valko *et al*., 2007; Lu, 2007). Interestingly, in our earlier investigation using the *in vitro* model, MEMM was found to exert cytotoxic activity against the HT-29 colon cancer cell line (Kamsani *et al*., 2019). Taking this finding into consideration, the present study was designed to investigate on the potential of MEMM to attenuate colon carcinogenesis using the *in vivo* azoxymethane (AOM)-induced colon cancer in rat model.

MATERIALS AND METHODS

Plant Sample Collection

M. malabathricum leaves were collected between June and July 2013 from its natural habitat near the area of Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia and a voucher specimen (SK 2199/13) has been deposited in the herbarium of the Institute of Bioscience, UPM.

Preparation of MEMM

Detailed procedure to prepare MEMM can be found in a report by Kamsani *et al*. (2019). Briefly, the dried leaves powder of *M. malabathricum* (200g) was macerated in methanol (1:20 (w/v)) for 72 hours. The supernatant was then collected and the residue was macerated in methanol again. Overall, the procedure of maceration was repeated three times wherein the supernatant was collected, pooled together, filtered and evaporated to obtain the dried methanol crude extract. At the end of the evaporation process, the amount of crude dried MEMM obtained was 36.6 g and the percentage of yield was approximately 18.3%.

In vivo Anticarcinogenic Study

Animal Management and in vivo Anticarcinogenics study

The animal ethics approval was obtained from the International Animal Care and Use Committee, Faculty of Medicine and Health Sciences, UPM (IACUC Ethical approval no. UPM/FPSK/PADS/BR-UUH/00488). Detail procedures used to handle and acclimatize the animals in the animal units has been described elsewhere (Jaios *et al*., 2016). Thirty (30) male Sprague-Dawley rats (4 weeks of age; weighed

between 90 g to 150 g) were acclimatized for 2 weeks and then randomly divided into five groups (n=6) wherein Group 1 was intraperitoneally (i.p.) administered with normal saline (Vehicle 1 – used to dissolve AOM (Sigma Chemical Co., St. Louis, MO)) (normal group) and Group 2-5 were administered (i.p.) with carcinogenic agent (15 mg/kg AOM) (Ghafar *et al*., 2012). The administration of normal saline or AOM was performer for two consecutive weeks (Week 2 and Week 3). On Week 4, Group 2 was orally (p.o.) treated with 8% Tween 80 (vehicle 2 – used to dissolve MEMM) whereas Group 3, 4 and 5 were treated (p.o) with 50, 250 or 500 mg/kg of MEMM, respectively. The administration of test solutions occurred for 8 weeks starting from Week 5 in the volume of 10 mL/kg of body weight. The body weight and the food intake of all rats were recorded weekly for the whole duration of the experiment. Each group and the respective treatment given are as illustrated in Figure No. 1.

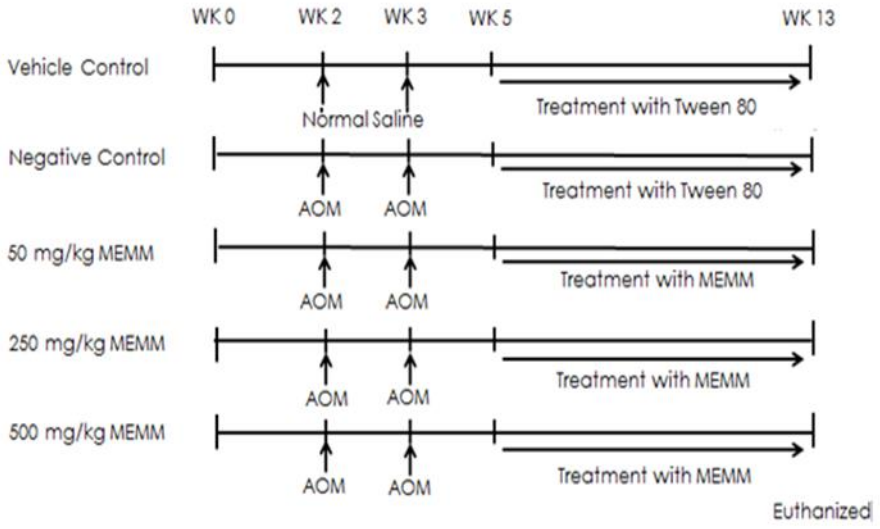

Figure No. 1

Experimental design of the study. The rats were randomized into five groups. Group 1 was not injected with normal saline, whereas group 2, 4, 5 and 6 were injected with 15 mg/kg AOM for 2 consecutive weeks (Wee k2 and 3) followed by the test solutions administration for 8 weeks starting on Week 5. The experimental groups were as follows: vehicle control (group 1), negative control (group 2), and treatment groups (group 3-5) that received the respective 50, 250 or 500 mg/kg MEMM

Colon sample collection

At the end of experimental period (Week 13), animals were anesthetized with ketamine (100 mg/kg; intramuscular (i.m.)) and xylazine (16 mg/kg; i.m.) to allow blood collection via cardiac puncture. Following the blood collection, each rat was euthanized by cervical dislocation and the colon was excised, opened longitudinally before being rinsed with normal saline, and then weighed. The opened colon was then separated into two halves of equal width of which half of the colon was pinned on a board with flat mucosal on the top, fixed in 10% formaldehyde overnight before being stained with haematoxylin and eosin (H&E) to allow histopathological examination and quantification of aberrant crypt foci (ACF). On the other hand, the remaining of the colon tissues were homogenized for antioxidant study.

Histopathological examination of rats colon tissue and enumeration of ACF

The detail procedures related to tissue sectioning and haematoxylin and eosin (H&E) staining were described elsewhere (Al-Henhena *et al*., 2014). Briefly, the fixed tissues were embedded in paraffin wax and sectioned using the rotary microtome to obtain tissue sections of 4 μm thickness, which were later stained with using an autostainer. Lastly, slides of stained tissues were examined under a light microscope.

The colon tissues were separated into three portions (proximal, middle and distal) whereby each

portion of the colon was examined and the total incidence of ACF was counted. Generally, ACF can be distinguished from normal crypts based on the morphological crypt seen under microscope: (1) darker in stain; (2) the size is enlarged and elongated; (3) thick epithelial lining; and (4) often had oval or slit-like lumen (Md. Nasir *et al*., 2017). The formed ACF can also be categorized based on their multiplicity per focus: a) small (1-2 crypts per focus); b) medium (3-4 crypts per focus) and c) large (>5 crypts per focus) (Papanikolaou *et al*., 2000).

Hematological and biochemical analysis

The effects of oral administration of MEMM on certain hematological parameters (e.g. hemoglobin (Hb), total red blood cell (RBC) count, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), total white blood cell (WBC) count, neutrophils (Neutro), monocytes (Mono), lymphocytes (Lymp) and eosinophils (Eosin)) and biochemical parameters (e.g. alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), creatinine (Crea), urea and total bilirubin (TBil)) of AOM-treated rats were examined using an automated hematology or chemistry analysers, respectively.

Antioxidant study

Once colons have been stored in -80°C, they were then homogenized in 1 mL of cold PBS and centrifuged at 4000 rpm for 25 minutes at 4°C. Supernatant was transferred into a new centrifuge tube for the assays.

Measurement of superoxide dismutase (SOD) activity

SOD assay was performed as described by Superoxide Dismutase Assay Kit. Briefly, 20 µL of SOD standard was dissolved in 1.98 mL of Sample Buffer as the stock solution. 200 µL of diluted Radical Detector and 10 µL of each standard with different final SOD activity (0, 0.005, 0.010, 0.020, 0.030, 0.040 and 0.050 U/mL) and 10 µL of samples were pipetted into a 96-well plate. Reactions were initiated by mixing with 20 µL of diluted Xanthine Oxidase and incubated on a shaker for 30 minutes at room temperature. Absorbance was measured with ELISA reader at wavelength of 450 nm. Amount of SOD from samples were determined based on graph generated by the SOD standard curve.

Determination of catalase (CAT) activity

Catalase assay was performed as described by Catalase Assay Kit. Briefly, 10 µL of Catalase Formaldehyde Standard was dissolved in 9.99 mL of diluted Sample Buffer as the stock solution. 100 µL of diluted Assay Buffer, 30 µL of methanol and 20 µL of each standard with different final concentration (0, 5, 15, 30, 45, 60 and 75 µM formaldehyde), 20 µL of diluted Catalase (Positive Control) and 20 µL of samples were pipetted into a 96-well plate. Reactions were initiated by mixing with 20 µL of diluted Hydrogen Peroxide and incubated on a shaker for 20 minutes at room temperature. Next, 30 µL of Potassium Hydroxide was added to terminate the reactions and followed by 30 µL of Catalase Purpald (Chromogen). Plate was incubated on a shaker for another 10 minutes at room temperature. Finally, 10 µL of Catalase Potassium Periodate was added and incubated again for 5 minutes at room temperature on a shaker. Absorbance was measured with ELISA reader at wavelength of 540 nm. Amount of catalase from samples were determined based on graph generated by the formaldehyde standard curve.

Establishment of glutathione (GSH) activity

GSH assay was performed as described by Glutathione Assay Kit. Briefly, GSH standards were prepared by dissolving GSSG Standard in MES Buffer. 50 µL of each standard with different final concentration (0, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 µM GSSG) and respective equivalent total GSH (0, 0.5, 1.0, 2.0, 4.0, 8.0, 12.0 and 16.0 µM) were pipetted into a 96-well plate. 50 µL of samples were pipetted into designated sample wells. Assay cocktail was prepared by mixing 11.25 mL of MES Buffer, 0.45 mL of reconstituted Cofactor Mixture, 2.1 mL of reconstituted Enzyme Mixture, 2.3 mL of HPLC-grade water and 0.45 mL of reconstituted DTNB. Next, 150 µL of the freshly prepared Assay Cocktail was added to each well and incubated on a shaker in the dark. Absorbance was measured with ELISA reader at wavelength of 405 nm at 5 minutes interval for 30 minutes. Amount of GSH from samples were determined based on graph generated by the GSSH concentration slope.

Investigation on the phytoconstituents of MEMM

HPLC analysis of MEMM

MEMM phytocontents was analysed using the HPLC procedure according to the detailed method described by Zakaria *et al*. (2015). In the present study, the HPLC profile of MEMM was established at the

wavelength of 366 nm, the range of λ_{max} value of those detected peaks were analysed, and compared to several known standard flavonoids.

High-resolution UPLC-ESI-HRMS analysis of MEMM

Phytochemicals constituents of MEMM was earlier analysed using the UHPLC-ESI-MS methods and the detailed procedure has been described elsewhere (Kamsani *et al*., 2019). Several phenolic-based phytoconstituents have been identified and will be discussed in the Results section.

Statistical analyses

Statistical analyses were performed using Graph Pad Prism version 5.02. Data were expressed as means ± Standard Error Mean (SEM) analysed using one-way analysis of variance (ANOVA) and differences between groups were determined using Dunnett's Multiple Comparison Test with $p<0.05$ as the limit of significance.

RESULTS

Effect of MEMM on body weight and relative organ weight of AOM-induced colon cancer rats

As shown in Figure No. 2, there was an increase in body weight of rats with increase in experimental period in Group 1 and this observation was also seen in Group 3, Group 4 and Group 5. In comparison. Group 2 (negative control) demonstrated body weight lost starting from Week 7 until the end of the experiment (week 13). Moreover, there were no significant differences in relative organ weight among the treatment groups when compared to Group 1 (Table No. 1).

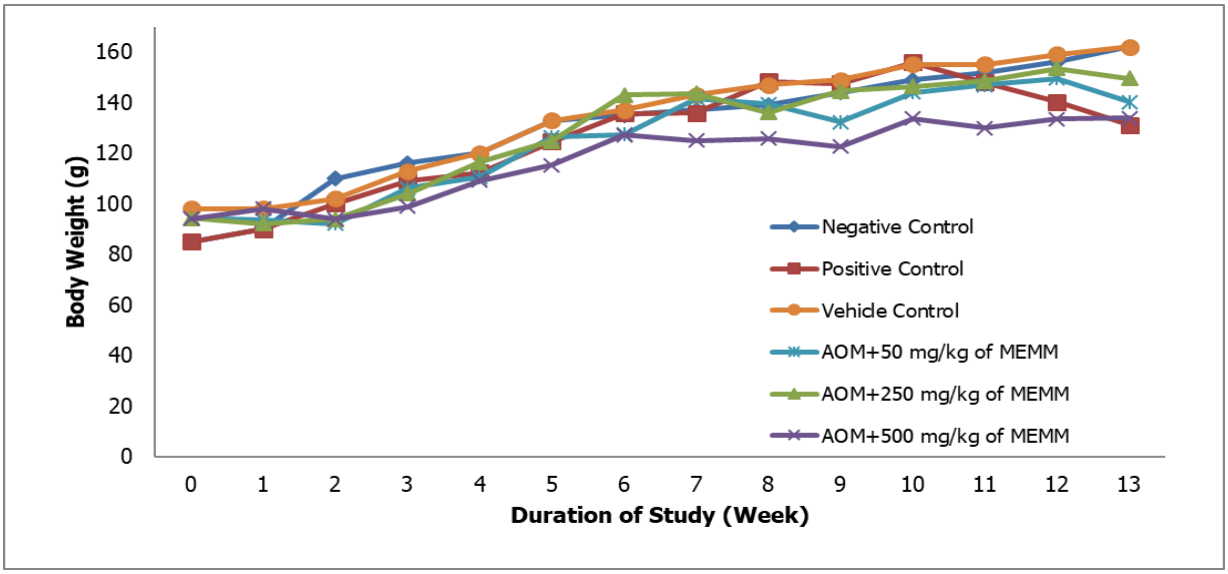

Figure No. 2
Body weight of AOM-induced Sprague-Dawley rats for chemopreventive study. Groups: Negative control (injected with normal saline); Positive control (injected with AOM); Vehicle control (treated with Tween 80); Treatments (injected with AOM, treated with 50 mg/kg of MEMM, 250 mg/kg of MEMM and 500 mg/kg of MEMM)

Effect of MEMM on hematological and biochemical parameters in AOM-induced colon cancer rats

As shown in Table No. 2, all hematological parameters of Group 2 showed no significant differences ($p<0.05$) when compared to Group 1). Treatment with MEMM, at all doses, also demonstrated insignificant changes in those parameters except for creatinine and triglyceride levels.

In addition, Table No. 3 also demonstrated that the biochemical parameters of Group 2 did not differ significantly against Group 1. In comparison, all doses of MEMM also did not cause any significant changes in biochemical parameters assessed when compared to Group 2.

Table No. 1

Relative organ weight of AOM-induced Sprague-Dawley rats for chemopreventive study. Groups: Negative control (injected with normal saline); Positive control (injected with AOM); Vehicle control (treated with Tween 80); Treatments (injected with AOM, treated with 50 mg/kg of MEMM, 250 mg/kg of MEMM and 500 mg/kg of MEMM)

	Organs (g)					
Group	**Spleen**	**Liver**	**Heart**	**Kidney**	**Lung**	**Colon**
Negative control (normal saline	0.26 ± 0.05	2.88 ± 0.31	0.44 ± 0.05	0.80 ± 0.08	0.80 ± 0.09	0.88 ± 0.08
Positive control (AOM)	0.32 ± 0.02	3.26 ± 0.26	0.62 ± 0.14	0.92 ± 0.12	1.20 ± 0.07*	1.08 ± 0.11
Vehicle control (Tween 80)	0.24 ± 0.04	2.76 ± 0.35	0.37 ± 0.03	0.74 ± 0.06	0.80 ± 0.05	0.84 ± 0.13
AOM + 50 mg/kg of MEMM	0.20 ± 0.01	2.83 ± 0.10	0.37 ± 0.01	0.74 ± 0.01	0.97 ± 0.26	0.97 ± 0.04
AOM + 250 mg/kg of MEMM	0.21 ± 0.02	2.99 ± 0.09	0.34 ± 0.01	0.74 ± 0.03	0.89 ± 0.04	0.88 ± 0.05
AOM + 500 mg/kg of MEMM	0.23 ± 0.03	2.83 ± 0.14	0.37 ± 0.03	0.80 ± 0.05	0.80 ± 0.05	1.14 ± 0.03

Values are expressed as means ± S.E.M

***Significant different as compared to control, $p<0.05$**

****Significant different as compared to control, $p<0.05$**

Table No. 2

Hematology profiles of chemopreventive study on AOM-induced Sprague-Dawley rats. Groups: Negative control (injected with normal saline); Positive control (injected with AOM); Vehicle control (treated with Tween 80); Treatments (injected with AOM, treated with 50 mg/kg of MEMM, 250 mg/kg of MEMM and 500 mg/kg of MEMM)

Parameters	**Negative control (Normal saline)**	**Positive control (AOM)**	**Vehicle control (Tween 80)**	**AOM + 50 mg/kg of MEMM**	**AOM + 250 mg/kg of MEMM**	**AOM + 500 mg/kg of MEMM**
RBC (x1012/L)	8.00 ± 0.52	8.32 ± 0.09	8.50 ± 0.39	8.42 ± 0.16	9.50 ± 0.43*	8.17 ± 0.33
Hb (g/L)	160.25 ± 9.45	140.75 ± 1.65	157.75 ± 8.85	167.75 ± 3.54	169.75 ± 9.75	147.00 ± 5.23
PCV (L/L)	0.39 ± 0.02	0.39 ± 0.00	0.42 ± 0.02	0.42 ± 0.01	0.41 ± 0.02	0.39 ± 0.02
MCV (fL)	48.31 ± 1.57	46.50 ± 0.65	49.05 ± 0.65	49.65 ± 1.37	42.75 ± 1.11**	47.00 ± 0.71
MCHC (g/L)	416.11 ± 8.65	363.00 ± 5.76*	378.71 ± 14.08	402.23 ± 11.89	418.75 ± 12.30	383.25 ± 11.21
WBC (x109/L)	7.10 ± 0.45	7.28 ± 0.84	7.45 ± 1.14	5.10 ± 0.70	9.63 ± 1.45	3.69 ± 0.67
Neutro (x109/L)	2.30 ± 0.40	2.21 ± 0.36	2.37 ± 0.43	1.61 ± 0.33	3.45 ± 0.60	1.05 ± 0.35
Lymp (x109/L)	4.07 ± 0.10	4.29 ± 0.67	4.22 ± 0.62	3.12 ± 0.36	4.88 ± 1.11	2.32 ± 0.29
Mono (x109/L)	0.42 ± 0.07	0.38 ± 0.03	0.46 ± 0.07	0.21 ± 0.04	0.59 ± 0.14	0.18 ± 0.04
Eosin (x109/L)	0.14 ± 0.07	0.22 ± 0.07	0.25 ± 0.04	0.07 ± 0.01	0.42 ± 0.06**	0.08 ± 0.03
Thrombo (x109/L)	334.25 ± 272.96	786.50 ± 158.03	700.75 ± 40.56	834.75 ± 266.57	953.75 ± 162.11	83.00 ± 9.98
P. Prot (g/L)	85.00 ± 1.00	73.00 ± 2.52	78.50 ± 2.06	77.75 ± 1.80	83.00 ± 9.98	83.00 ± 2.08

Values are expressed as means ± S.E.M

***Significant different as compared to control, $p<0.05$**

****Significant different as compared to control, $p<0.05$**

Table No. 3
Biochemical profiles of chemopreventive study on AOM-induced Sprague-Dawley rats. Groups: Negative control (injected with normal saline); Positive control (injected with AOM); Vehicle control (treated with Tween 80); Treatments (injected with AOM, treated with 50 mg/kg of MEMM, 250 mg/kg of MEMM and 500 mg/kg of MEMM)

Parameters	Control (Tween 80)	50 mg/kg of MEMM	250 mg/kg of MEMM	500 mg/kg of MEMM
ALT (U/L)	52.58 ± 4.62	57.65 ± 10.89	55.88 ± 5.78	59.08 ± 7.10
ALP (U/L)	136.80 ± 25.87	151.50 ± 15.88	224.80 ± 37.50	236.50 ± 16.83*
AST (U/L)	170.30 ± 16.42	235.60 ± 38.06	224.10 ± 49.70	223.60 ± 45.40
Tbil (umol/L)	1.58 ± 0.33	0.10 ± 0.00**	0.43 ± 0.33*	0.10 ± 0.00**
Creat (umol/L)	61.00 ± 2.12	57.75 ± 2.60	54.75 ± 1.80	58.50 ± 2.50
Urea (mmol/L)	7.15 ± 0.93	7.33 ± 1.07	5.43 ±0.30	5.75 ± 0.30

Anticarcinogenic activity of MEMM against AOM induced Colon Cancer in rats

Effect of MEMM on crypt multiplicity in AOM-induced Colon Cancer Rats

Photomicrographs of AOM-induced rat colonic mucosa tissue stained with H&E following pretreatment with MEMM is shown in Figure No. 3A-E. The crypt multiplicity can be categorized according to the following category: small (1-2 crypts per focus); medium (3-4 crypts per focus) and large (>5 crypts per focus). Rats in Group 2 (Figure No. 4B) that received (AOM + 8% Tween 80) demonstrated 100% incidence of ACF formation in comparison to Group I (Figure No. 4A), which showed no presence of ACF. Interestingly, MEMM, at the dose of 50, 250 and 500 mg/kg, significantly attenuated ACF formation in comparison to Group 2 as indicated by decrease in the number of ACF formed (Figure No. 4C-E). Crypt multiplicity of 4, 5 and more than 5 were significantly lower in the extract-treated groups.

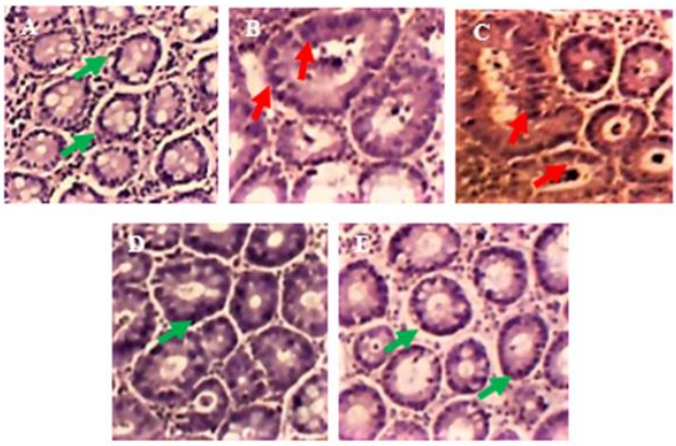

Figure No. 3
Effects of MEMM on histological sections of AOM-induced ACF
All sections were cut parallel to the muscular layer. (A) Normal colon mucosa showing circular shape of the crypts and nuclei lining the crypt, basal nuclear polarity and absence of stratification. (B) AOM treated group showed elongated ACF with narrow lumen, marked nuclear atypia (elongated and stratified nucleus), loss of basal nuclear polarity and increases proliferation with decreases in goblet cells (C) 50 mg/kg MEMM + AOM showed elongated ACF and nuclei, slight decreases in goblet cells and nuclei showed slightly nuclear stratification (D) 250 mg/kg MEMM + AOM showed less elongated ACF and non-stratified nuclei, slight decreases in goblet cells and ACF with dysplastic mucosa lining (E) 500 mg/kg MEMM + AOM showed ACF with slightly rounded shape and without stratified nuclei, slightly decreased in number of goblet cells and proliferation of nuclei (H & E stain 100×)

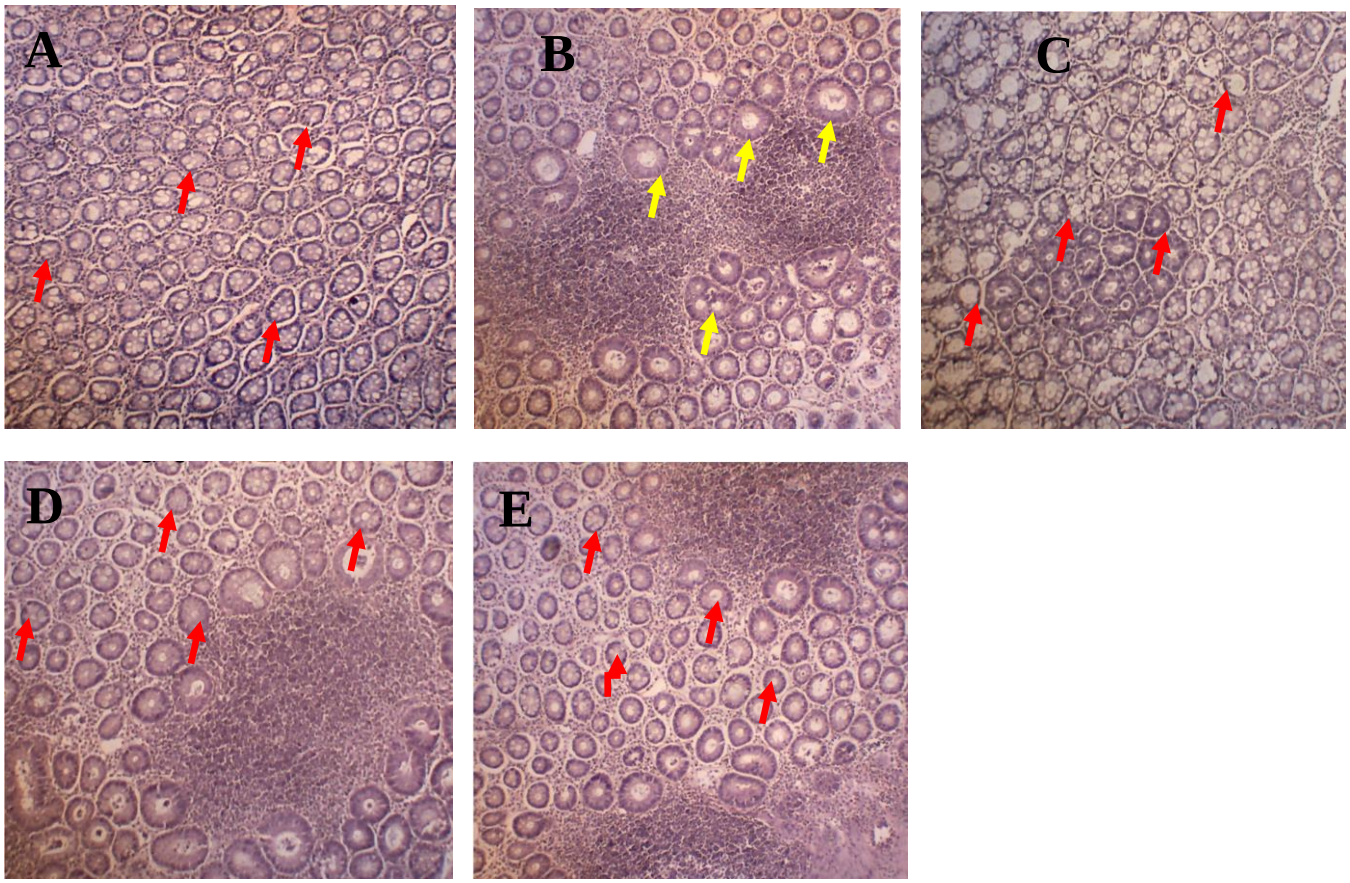

Figure No. 4
Cross-section of the rat colon stained with hematoxylin and eosin. (A) Normal group with normal crypts, (B) AOM group (C) 50 mg/kg MEMM-treated group, (D) 250 mg/kg MEMM-treated group, (E) 500 mg/kg MEMM-treated group (100× magnification). Red-coloured arrows indicate the normal colon cells with round nuclei in normal and treated groups while yellow-coloured arrows indicate the ACF cell with elongated nuclei and depletion of mucus in AOM induced group.

Effect of MEMM on the incidence of ACF in AOM-induced colon cancer rats

Table No. 4 showed the incidence of ACF in colons harvested from AOM-induced rats following pretreatment with MEMM. The number of ACF in the proximal, middle and distal part were higher in Group 2, but significantly reduced after pretreatment with MEMM. Moreover, a relatively higher incidence of ACF was observed within the distal colon regardless of the treatment concentrations.

A significantly lower (p<0.05) incidence of ACF (approximately 51% inhibition) was observed in Group 5 when compared to Group 2 while Group 3 and Group 4 recorded approximately 23 and 29% inhibition, respectively.

Effect of MEMM on SOD, CAT and GSH activities in AOM-induced colon cancer rats

Table No. 5 shows the effect of MEMM on the activities of SOD, CAT and GSH in colon tissue of AOM-treated rats. Group 2 demonstrated a significant (p<0.05) decrease in SOD and GSH activities when compared to the normal control group. Interestingly, pretreatment with MEMM, at all concentrations, reversed the toxic effect of AOM indicated by the increase in SOD and CAT activities when compared against Group 2 whereas only the 250 and 500 mg/kg MEMM significantly (p<0.05) reversed the activity of GSH when compared to Group 2.

Phytocontituents of MEMM

HPLC profile of MEMM

The HPLC profile of MEMM was established at 366 nm and revealed the present of two major peaks detected at the retention time of 21.047 (Peak 1) and 23.601 (Peak 2) mins (Figure No. 5A). Further analysis on the range of λ_{max} value of both peaks demonstrated that Peak 1 and Peak 2 have a λ_{max} value ranging between 254.3–357.7 and 255.5–349.4 nm, respectively, suggesting the presence of flavonoid-based phytoconstituents (Figure No. 5B). On the other hand, Figure No. 5C shows comparison made between the chromatogram of MEMM against that of quercitrin and rutin, which revealed the presence of at least quercitrin in the extract.

Table No. 4
Incidence of ACF in AOM-induced male Sprague-Dawley rats. Groups: Negative control (injected with AOM); Treatments (injected with AOM, treated with 50 mg/kg of MEMM, 250 mg/kg of MEMM and 500 mg/kg of MEMM)

Group	ACF Count				
	Proximal Colon	**Middle Colon**	**Distal Colon**	**Total**	**Reduction compared to Positive Control (%)**
Negative Control	28.00 ± 1.58	24.75 ± 4.44	43.25 ± 5.65	96.00 ± 11.67	
AOM+50 mg/kg of MEMM	23.50 ± 4.01	15.50 ± 0.65	35.25 ± 1.25	74.25 ± 5.91	88
AOM+250 mg/kg of MEMM	23.00 ± 1.08	12.25 ± 1.44	32.50 ± 1.04	67.75 ± 3.56	61
AOM+500 mg/kg of MEMM	16.50 ± 2.72*	9.00 ± 2.68	21.75 ± 1.18*	47.25 ± 6.58	37

Values are expressed as means ± S.E.M
***Significant different as compared to control, $p<0.05$**
****Significant different as compared to control, $p<0.05$**

High-resolution UPLC-ESI-HRMS analysis of MEMM

Based on the phytoconstituents analysis carried out earlier on MEMM using the UHPLC-ESI-MS method, bioactive compounds such as gallocatechin, quercetin-3,4-diglucoside, quercetin, *p*-coumaric acid, procyanidin A, and epigallocatechin have been identified.

DISCUSSION

According to Lund *et al*. (2006), various experimental investigations have demonstrated that environmental factors play vital role in most of the cases of colorectal cancer, thus, suggested that this type of cancer is preventable. Later, Halliwell *et al*. (2007), reported on findings that showed the remarkable potential of naturally occurring phenolics in fruits or vegetables as a combination to prevent cancer cell growth in comparison to a single compound while Lee *et al*. (2017), reported on studies that demonstrated the noteworthy ability of higher intake of fruits or vegetables to protect against colorectal cancer. In support of those reports, Guizani *et al*. (2013), have reported based on the various epidemiological reviews that high intake of antioxidant rich diet may reduce the risk of oxidative stress-related disorders, including cancer. Taking these reports into accounts, the high antioxidant *M. malabathricum* leaves is expected to possess a potential anticancer activity against colorectal cancer, thus, warrant further investigation.

Table No. 5
Antioxidants activities in colon tissues of rats subjected to MEMM treatments

Group	Antioxidant Assays		
	SOD (U/g tissue)	CAT (U/g tissue)	GSH (mM/g tissue)
Negative Control (Normal Saline)	2.46 ± 0.06	1.82 ± 0.00	37.64 ± 1.45
Positive Control (AOM)	0.77 ± 0.01#	0.79 ± 0.01#	21.74 ± 6.44#
AOM+50 mg/kg of MEMM	4.40 ± 0.29*	0.95 ± 0.01*	31.25 ± 3.84
AOM+250 mg/kg of MEMM	4.87 ± 0.07*	1.40 ± 0.01*	37.10 ± 0.70*
AOM+500 mg/kg of MEMM	5.60 ± 0.32*	1.81 ± 0.04*	45.29 ± 0.55*

Values are represented as mean ± SEM
Values with * are significantly different as compared to control

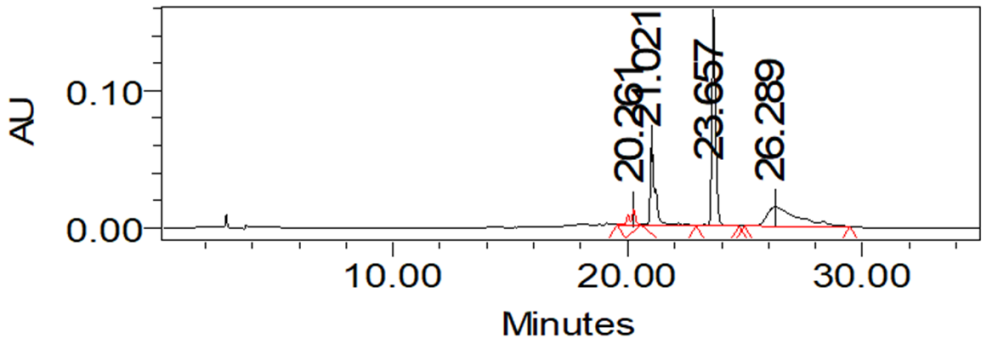

Figure No. 5
HPLC profile of MEMM. (A) HPLC chromatogram of MEMM at 366 nm. (B) The UV spectra analysis of two peaks detected in MEMM using HPLC at 366 nm. Peak 1 and Peak 2 were detected at the retention time of 21.047 and 23.601 mins, respectively. Each peak was detected in the λmax region that range between 254.3 – 357.7 and 255.5 – 349.4 nm, respectively. Based on the recorded λmax value, each of the peak was is suggested to represent flavonoid-based bioactive compounds. (C). The HPLC chromatogram of MEMM shows the presence of quercitrin (1), but not rutin (2,) at 366 nm

The present study is a continuation of the previous report on MEMM, which has been shown earlier to possess anti-inflammatory and antioxidant activities (Mamat *et al*., 2013). The link between oxidation, inflammation and carcinogenesis has been well acknowledged (Valko et al., 2007; Lu, 2007). By-products of oxygen metabolism, such as reactive oxygen species (ROS) (Abele, 2002), when present in excess can cause damage of cell, tissue and organ functions, which if not overcome can lead to carcinogenesis (Lu, 2007). Thus, it is plausible to suggest that any compounds with antioxidant and/or anti-inflammatory activities may also possess a potential to attenuate carcinogenesis. Taking this suggestion into account, it is believed that MEMM could be one of the new potential sources of anticarcinogenic agents (Lin *et al*., 2008; Mendonca *et al*., 2019). In earlier studies, MEMM was subjected to the subacute and subchronic toxicity, and cytotoxicity studies. The results obtained demonstrate that MEMM did not show any signs of toxicity upon subacute and subchronic oral treatment into rats as indicated by no record of mortality and irregular behavior with no change in body weight as well as

food and water intake. Moreover, no changes in haematological parameters were also observed in both studies. Furthermore, the in vitro cytotoxic study of MEMM against HT29 colon cancer cell line showed that MEMM exerted a cytotoxic effect against the colon cancer cell line under the inverted and phase-contrast microscopes and further confirmed by the acridine orange/propidium iodide (AOPI) staining (Kamsani *et al*., 2019). The ability of MEMM to attenuate HT29 cancer cell growth leads to the need to investigate on the potential of MEMM to inhibit the progression of colon cancer *in vivo*. One of the suitable in vivo colon cancer models is the AOM-induced colon cancer in rats.

AOM-mediated carcinogenesis process includes mutagenicity, initiated by chromosomal damage and initiation of micronuclei cell formation (Yaduvanshi *et al*., 2012), resulting in colon cells morphological changes indicated by the development of ACF and DNA damage in proliferating cells (Chen & Huang, 2009). According to Chen & Huang (2009) AOM-induced carcinogenesis demonstrated similar pathogenesis to that of normal human sporadic colon cancer in the sense that both types of cancer lead to the development of similar types of lesions called ACF. ACF is early morphological changes observed in rodents after administration of colon-specific carcinogen such as AOM. Following the administration of AOM, carcinogenesis process is mediated through mutagenicity initiated by chromosomal damage and induction of micronuclei (MN) cells (Bhatia & Kumar, 2013) leading to morphological changes associated with ACF development and DNA damage in proliferating cells (Yaduvanshi *et al*., 2012). ACF, assumed as preneoplastic lesions comprising of thick, large crypts, are categorized by an increase in the size of the crypts, the epithelial lining and the pericryptal zone, and share many morphological and biochemical characteristics with tumors, including a comparable increase in cell proliferation can be observe in AOM-induced rat's colon tissue sample upon staining with methylene blue (Ochiai *et al*., 2005).

From the results obtained, the normal colon tissue exerted normal circular crypts with and nuclei lining the crypt, basal nuclear polarity and absence of stratification. On the other hand, the administration of AOM causes morphological changes to the normal crypts leading to the formation of ACF, which can be distinguished by their elliptical shape, large size and dark staining (Rodrigues *et al*., 2002). Pre-administration of MEMM suppresses the number of ACF in a dose-dependent manner when compared to the negative group (AOM-treated) and help to prevent the progression of preneoplastic crypts into malignant neoplasia. This finding suggests that the extract plays a vital role in inhibiting formation of ACF and causing constant and rapid reduction of ACF in MEMM-treated groups. Interestingly, the effectiveness of MEMM as a chemopreventive agent is further supported by its non-toxic effect, which is suggested since the extract did not cause any significant adverse changes in the hematological and biochemical parameters. Most notably, the rats fed with MEMM showed no adverse effects on the food consumption and the animal growth rate (data was not shown).

According to Waly *et al*. (2014), AOM induced ROS formation in colonic cells, which then induces colon cancer in rats through a mechanism that is interceded by depletion of GSH, a major intracellular antioxidant, and the impairment of total antioxidant capacity in colonic cells of rats. GSH undergoes oxidation to the disulfide form (GSSG) and oxidized form, which scavenges reactive oxygen species (ROS). According to Waly *et al*. (2011), more than 90% of the total glutathione pool in healthy cells and tissues were in the reduced form (GSH) with less than 10% existed in the disulfide form (GSSG). It has been well acknowledged that a reduced GSH/GSSG ratio indicates oxidative stress, a state under which GSH, antioxidant enzymes (glutathione peroxidase, SOD, and CAT), and dietary antioxidants (vitamin C and E, β carotene and selenium) are not counterbalancing the reactive oxygen species (ROS), and later induce cellular damage by carcinogenic pathogenesis. In the present study, MEMM increased the activities of internal antioxidant (i.e. GSH) and antioxidant enzymes (i.e. SOD and CAT); thus, justify the decreased formation of ACF as described earlier. This observation suggests that the phytochemicals present in MEMM could act directly as antioxidants or indirectly by activating the internal antioxidant system to decrease the ROS level, which was elevated due to the mucosal damage caused by AOM.

AOM administration has been associated with the presence of marked inflammatory effect, suggesting that the AOM-induced oxidative stress is partly mediated by an inflammatory response (Waly *et al*., 2014). Concurrent with the above suggestion, the inflammatory process is a well-known part of the cell's natural defense against tissue damage and is commonly associated with oxidative stress (Lowes *et*

al., 2013). Based on the association between AOM action, inflammatory effect and oxidative stress, it is plausible to suggest that the ability of MEMM to reverse the carcinogenic action of AOM, other than due to its action to reduce oxidative stress, could also be due to the extract anti-inflammatory potential. This suggestion is in coexisting with previous reports on the anti-inflammatory activity of *M. malabathricum* (Zakaria *et al*., 2006; Ismail Suhaimy *et al*., 2017).

Oxidative stress triggers carcinogenesis through various mechanisms such as inducing DNA injury, triggering persistent inflammation, and de-regulating apoptosis to name a few (Kaur *et al*., 2018). Other than the anticancer drugs such as tamoxifen, 5-fluorouracil, doxorubicin etc., drugs like synthetic non-steroidal anti-inflammatory drugs (NSAIDS) have also been identified as potential colorectal cancer chemopreventive agents since expressive alteration of several vital molecules associated with inflammation contributed significantly to the colorectal tumor progression. Nevertheless, most of these synthetic agents are associated with unwanted and sometimes fatal side effects. In the last couple of years, much of the scientific interest has been enticed to the discovery of new, safe and effective chemopreventive agents from natural sources. Plant based chemopreventive agents have been shown either to interrupt or end carcinogenesis by interfering with one or multiple cellular pathways and increasing evidence supports the efficacy of phytoconstituents, such as those with anti-inflammatory activity, to prevent and treated colorectal cancer. The presence of various phenolic compounds such as gallocatechin, quercetin-3,4-diglucoside, quercetin, *p*-coumaric acid, procyanidin A and epigallocatechin in MEMM has been established using the UHPLC-ESI-MS. Some of these compounds, such as quercetin (Gibellini *et al*., 2011), *p*-coumaric acid (Sharma *et al*., 2019), procyanidin A (Lee, 2017), epigallocatechin (Wang *et al*., 2017) have been shown to exert chemopreventive action in rats. On the other hand, compounds like gallocatechin (Plumb *et al*., 2002), quercetin-3,4-diglucoside (Zielinska *et al*., 2008), quercetin (Xu *et al*., 2019), *p*-coumaric acid (Shen *et al*., 2019), procyanidin A (Packer *et al*., 1999) and epigallocatechin (Nikoo *et al*., 2018) have also been proven to possess antioxidant activity. Moreover, compounds like gallocatechin (Pérez-Mendoza *et al*., 2019), quercetin (Li *et al*., 2016), *p*-coumaric acid (Zhu *et al*., 2018), procyanidin A (Bak *et al*., 2013), epigallocatechin (Khalatbary & Ahmadvand, 2011) have also been reported to possess anti-inflammatory activity with quercetin-3,4-diglucoside (Lopes *et al*., 2020) reported to show the highest affinity and favorable interaction with the COX-2 structure when assessed using the molecular docking approach indicating its high anti-inflammatory potential. Thus, it is proposed that these compounds work synergistically to contribute towards the observed chemopreventive action of MEMM.

CONCLUSION

In conclusion, MEMM exerted chemopreventive activity against AOM-induced colon cancer in rats, possibly via the synergistic action of several chemopreventive-bearing phenolics, which work partly through the activation of antioxidant and anti-inflammatory mechanisms.

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