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Methanolic extract of *Moringa oleifera* leaves mediates anticancer activities through inhibiting NF-κB and enhancing ROS in Ehrlich ascites carcinoma cells in mice

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ABSTRACT

Cancer is one of the vital causes of morbidity and mortality worldwide and recognized as the second most leading cause of death. Considering the side effects and high cost of synthetic anticancer drugs, plant might act as a source of novel anticancer agents with better host safety. In this investigation, Moringa oleifera leaves methanolic extract (MOLME) was subjected to evaluate its anti-proliferative effect with molecular mechanism in Ehrlich ascites carcinoma (EAC) cells in Swiss albino mice. MOLME inhibited EAC cell growth approximately 64% at the dose of 200mg/kg body weight. DAPI stained cells had shown nuclear condensation and fragmentation in treated cells. Up-regulation of pro-apoptotic gene, *Bax* and tumor suppressor gene, *p53*; and down-regulation of anti-apoptotic gene *Bcl-2* confirm the induction of EAC cells followed by MOLME treatment. Moreover, MOLME inhibited NF-κB activity and enhanced ROS generation. Results suggest that the anticancer effect of MOLME is associated with the induction of apoptosis through activation of p53 and reciprocal expression of Bax and Bcl-2. The apoptosis might be triggered by the inactivation of NF-κB and generation of ROS. GC-MS analysis revealed that MOLME contains several bioactive compounds, thus it is assumed that the anticancer properties could be attributed by the active leads and could be potential resource of chemotherapeutics.

INTRODUCTION

Despite the advancement in understanding the molecular basis, detection and treatment of cancer, mortality is still high and there is still not a proper treatment to eradicate the growth of tumor [1] Cancer claims over six million lives every year and remains one of the deadliest threats of human survival [2]. To block the development of cancer, a better therapeutic option with less adverse effects is of great importance. Now-a-days, using synthetic or natural agents (alone or synergistically) is an extremely promising way to countervail the growth of tumor [3,4]. However, in the developing countries like Bangladesh, it is difficult to get the expensive synthetic drugs adequately and always has some serious side effects [5].

Therefore, because of having less or no side effects and universal availability, naturally occurring or plant derived anti-cancer agents are getting more and more attention in the pharmacological evaluation for cancer therapy [6]. The plant used in this study is *Moringa oleifera* (Moringaceae), very common all over the Indian subcontinent including Bangladesh. It is known as miracle tree and an excellent source of vitamins, minerals and proteins [7]. Every part of the

plant is toothsome and considered as 'natural nutrition' of the tropical area [8]. Moreover, *Moringa oleifera* has been reported to being the panacea of several maladies including hypertension, liver disease, inflammation and hypercholesterolemia [9, 10, 11]. Some important metabolites like quercetin, zeatin, campesterol, sitosterol, kaempferol etc., which are attributed to various medicinal uses of *M. oleifera* [10]. A recent study has shown significant antibacterial activity of silver nanoparticles from leaf extract of *Moringa oleifera* [12]. Several studies have also shown that aqueous extract of *M. oleifera* possesses anti-proliferative activity against human alveolar and cholangiocarcinoma cell lines [13, 14]. However, in vivo growth inhibitory effect of MOLME against Ehrlich ascites carcinoma (EAC) cells has not been reported elsewhere. We hypothesized that MOLME might inhibits EAC cell proliferation by inducing apoptosis.

Therefore, the purpose of the present study was to evaluate the in vivo anti-proliferative activity of MOLME against EAC cells in mice model. Here, we report that MOLME inhibited EAC cell growth by inducing apoptosis through inactivation of NF-κB and generation of intracellular ROS.

MATERIALS AND METHODS

Chemicals

Methanol was purchased from Duksan, South korea; 2′,7′-dichlorodihydrofluoresce in diacetate (DCFH-DA) were purchased from Sigma Aldrich, MO, USA; Agarose and Tris were purchased from MP Biomedicals, LLC, USA; RNA extraction kit was purchased from Favorgen, Taiwan; RT PCR kit was purchased from Promega, Madison-Wiscosin, USA. All other chemicals and reagents were of analytical grade.

Preparation of *Moringa oleifera* leaves methanolic extract (MOLME)

Preparation of extract was carried out using the method as described previously [11]. Matured leaves of *Moringa oleifera* were collected from the local area and authenticated by Department of Botany, University of Rajshahi, Bangladesh. The fresh leaves were washed in tap water and dried in shed. After complete drying, leaves were grinded into coarse powder. About 150 gm of the powder was soaked in 500 ml of methanol. After 24 h of stirring, dissolved portion of the plant sample was separated. About 100 ml methanol was further added and previous step was repeated again. The resulting extract was filtered through Whitman No.1 filter paper and then was centrifuged with a speed of 10000 rpm for 10 minutes. Afterwards, the solvent was evaporated through a rotary evaporator (HAHNSHIN S&T Co., Ltd, South Korea; Model no: HS-2005S-N) and the residue was kept in refrigerator until use.

Test animals

Adult Swiss albino mice aged 6 to 8 weeks (25±4g body weight) were collected from the animal house of Department of Pharmacy, Jahangirnagar University, Savar, Bangladesh. Mice were housed in polypropylene cages in well-ventilated rooms, with maximum of six animals in a cage under hygienic conditions. Standard food and drinking water were given *ad libitum* at natural day night cycle.

Ethical clearance

Permission to use mice model in this study was approved by the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC) for experimentation on Animal, Human, Microbes and Living Natural Sources (128/320-IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Cell line

EAC cells were collected with the courtesy of Department of Pharmacy, Jahangirnagar University, Savar, Bangladesh. The cells were maintained as ascites tumor in Swiss albino mice by weekly intraperitoneal (i.p.) inoculation of $1x10^6$ cells per mouse.

Determination of average tumor weight

Survival time and tumor weight was determined as described by our previous study [15]. Animals were divided into 5 groups, consisting of 4 mice in each and inoculated with 1.0×10^6 cells/mice on the day '0'. After 24 h of inoculation, treatment (i.p.) with MOLME was started on group 1, 2 and 3 at doses of 50,100 and 200 mg/kg/mice/day respectively and continued for 10

days. The tumor weight of each mouse was measured after every 48 h. This process was continued for 20 days after EAC cell inoculation.

Determination of cell growth inhibition

Determination of in vivo tumor growth inhibition was carried out by the method as described in the previous study [16]. At first, $1x10^6$ EAC cells were inoculated in each mouse of every group on day '0'. Treatment was started after 24 h of EAC cells inoculation and continued for 5 days. Groups 1 to 3 were applied with MOLME at doses 50, 100 and 200mg/kg body weight per day per mouse respectively. In every case, the volume of the test solutions injected (i.p.) was 0.1ml/day per mouse. Group 4 received standard bleomycin (0.3 mg/kg, i.p) and were considered as positive control. Whereas Group 5 were used as control receiving solvent only (0.98% NaCl). Mice of each group were sacrificed on day 6 and the total intraperitoneal tumor cells were harvested by normal saline (0.98% NaCl). Viable cells were first identified with Trypan blue (0.4%) and then counted with a haemocytometer under an inverted microscope (XDS-1R, Optika, Italy). Total numbers of viable cells in every animal of the treated groups were compared with those of the control group.

The cell growth inhibition was calculated using the following formula: %Cell growth inhibition = (1- Tw / Cw) x 100

Where, Tw = Mean of number of tumor cells of the treated group of mice and Cw = Mean of number of tumor cells of the control group.

Observation of morphological appearance and nuclear damage of EAC cells by DAPI staining

MOLME induced cellular apoptosis was investigated by the method as described previously with slight modification [17]. Morphological observation of both treated and untreated EAC cells were studied using a fluorescence microscope (Olympus IX71, Japan). At first, EAC cells were collected from the mice receiving MOLME at the dose of 200mg/kg body weight and saline (non-treated control) and stained with 25 µl (1mg/ml) of DAPI (4′,6-diamidino-2-phenylindole) at 37°C for 20 minutes. Then the cells were washed with phosphate buffer saline (PBS) with low light intensity and re-suspended in PBS for observation of morphological changes under the fluorescence microscope.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Expression of proliferation related gene such as p53 and Bcl-2, Bax and NF-κB was studied by PCR, where GAPDH, a housekeeping gene, was used as control. Total RNA from both treated and non-treated EAC cells was isolated using RNA extraction kit following manufacturer's guidelines. The cDNA was prepared through reverse transcription PCR (polymerase chain reaction) using 2 µl total RNA, 1µl of each forward and reverse primer, 1µl dNTPs, 2µl $MgCl_2$, inhibitor 0.50µl and 1µl of reverse transcriptase and 4 µl reaction buffer. The sequences of primer used in the experiment are shown in Table 1. Each 10 µl of PCR reaction mixture contained 5µl of master mix, 1µl each of forward and reverse primer, template 1 µl and 2µl of nuclease free water. The PCR products of these genes and GAPDH were electrophoresed in 1.0% agarose gel. The gels were stained with EtBr (ethidium bromide) and visualized in UV-trans illuminator.

Table 1. The sequence of primers used for PCR amplification.

Gene name	Primer sequence References
GAPDH	Forward: (5′-GTGGAAGGACTCATGACCACAG-3′) Reverse: (5′-CTGGTGCTCAGTGTAGCCCAG-3′)
p53	Forward: (5′-CACAAAAACAGGTTAAACCCAG-3′) Reverse: (5′-AGCACATAGGAGGCAGAGAC-3′)
Bcl-2	Forward: (5′-GTGGAGGAGCTCTTCAGGGA-3′) Reverse: (5′-AGGCACCCAGGGTGATGCAA-3′)
Bax	Forward: (5'-GGCCCACCAGCTCTGAGCAGA-3') Reverse: (3'-GCCACGTGGGCGTCCCAAAGT-5')
NF-kB	Forward: (5'-AACAAAATGCCCCACGGTTA-3') Reverse: (3'-GGGACGATGCAATGGACTGT-5')

Measurement of reactive oxygen species (ROS) generation

The generation of ROS positive EAC cells were examined using 2′,7′-dichlorodihydrofluoresce in diacetate (DCFH-DA). The cells were first collected from control mice and treated mice on day six of tumor inoculation and then washed with PBS at 1200 rpm for 2 minutes. Then the cells were incubated with 10µl of 50µM DCFH-DA at 37°C for 30 minutes in the dark. The cells were washed with PBS again and maintained in 1ml culture medium and finally ROS generation was assessed using a fluorescence microscope at excitation and emission wavelengths of 485 nm and 530 nm, respectively [18].

Gas chromatography–mass spectrometry (GC-MS) analysis

GC-MS analysis of active compounds from methanolic extract of *M. oleifera* leaves was carried out by Varian GC (Model Varian CP-3800, USA) with MS (Model: Varian saturn-2200) spectrometer equipped with a flame ionization detector and capillary column with VF-5 ms (30m×0.25mm, 0.25µm). The instrument was operated in electron impact mode at ionization voltage (70 eV), injector temperature (250°C), and detector temperature (280°C). The carrier gas used was helium at a flow rate of 1 ml/minute and about 1 µl of the sample was injected. The temperature program for the column was from 40°C (1 minute) to 310 °C at a rate of 10°C/minute and then held at 10 minutes. The chemical compounds and measurements of peak areas were identified by GC/MS NIST LIBRARY (NIST 05).

Statistical Analysis

Data are expressed as mean ± SD (Standard Deviation). Data have been analyzed with one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using SPSS software of 16 version. P<0.05 was considered to be statistically significant.

RESULTS

MOLME reduced tumor weight in EAC-bearing mice

Treatment of MOLME on mice previously inoculated with EAC cells, resulted in the inhibition of tumor weight. In case of control group, the tumor weight was increased by 16.11 gm in 20 days when compared to the normal mice. On the contrary, treatment with MOLME caused significant inhibition of tumor weight. For the doses 50 mg/kg, 100 mg/kg and 200 mg/kg the tumor weight was increased by 10.55 gm, 7.33 gm and 5.77gm, respectively, in 20 days which were significantly lower from untreated control mice. Tumor weight of treated and untreated mice is given in Figure 1.

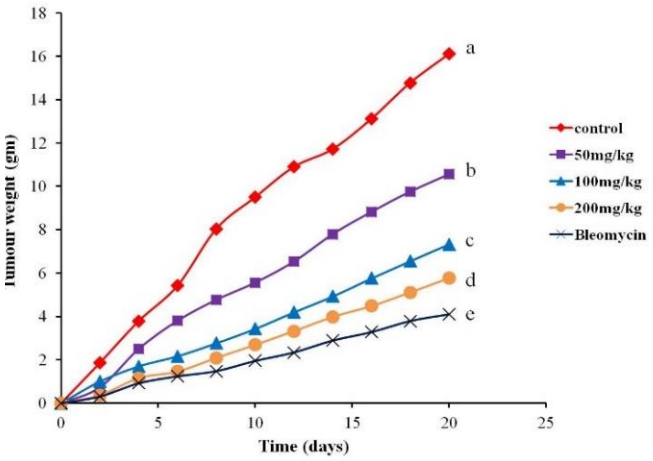

Figure 1. Tumor weight of EAC bearing mice treated with MOLME and bleomycin. Results are shown as mean ± SD (standard deviation) (n=4). Mean with different lowercase letters are significantly different at P < 0.05 by Duncan's multiple-range test.

MOLME significantly decreased EAC cells proliferation

Effects of MOLME at the doses of 50, 100 and 200 mg/kg/day and bleomycin (0.3 mg/kg/day) on EAC cell growth on day 6 are shown in Figure 2. Treatment with MOLME resulted in maximum cell growth inhibition at the doses of 100 and 200 mg/kg (i.p.), as they showed 41.15% and 64.62% inhibition, respectively.

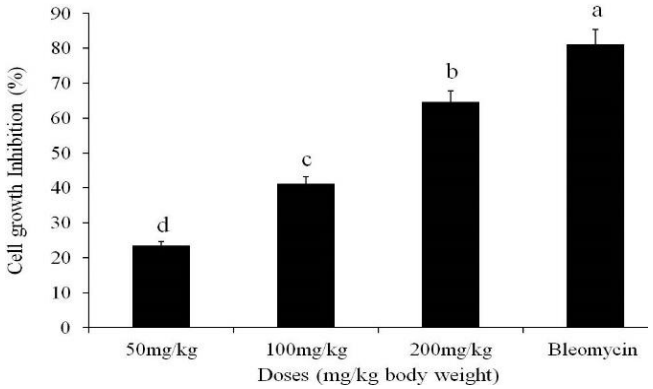

Figure 2. Effects of MOLME on EAC cell growth inhibition. Data were expressed as mean ± SD (n=4). Mean with different lowercase letters are significantly different at P < 0.05 by Duncan's multiple-range test.

MOLME induced morphological changes of EAC cells

Morphological changes of EAC cells collected from both control and treated mice were examined by DAPI staining. The morphological alteration from both control and treated cells under fluorescence and optical microscope were marked by arrow which is shown in Figure 3. Under microscope, round, regular, and normal shaped nucleus were observed in cells from control mice. On the contrary, irregular, fragmented, and condensed nucleuses were found in treated cells.

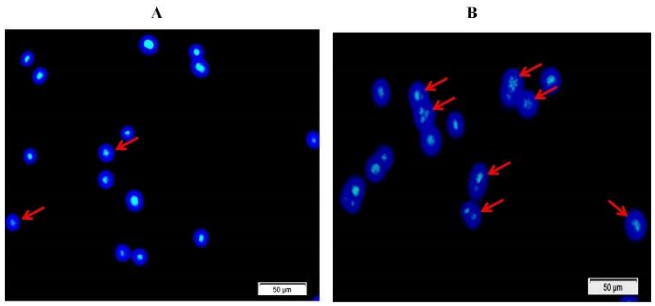

Figure 3. DAPI staining of EAC cell. (A) represents fluorescence and optical microscopic of untreated EAC cells, whereas (B) represents MOLME treated cells. Under microscope, normal and round shape nucleuses as well as cells appeared where cells from MOLME treated mice showed the nucleuses and cells having apoptotic properties which are indicated by arrows.

Induction of apoptosis in EAC cells by MOLME

It was found that among the three doses 200mg/kg was more effective against EAC cells. Therefore, it was chosen to evaluate the gene expression levels. As it is well established that various genes play crucial roles in apoptosis, we examined whether or not MOLME affected the expression of pro-apoptotic genes like *p53*, and *Bax* and the expression of anti-apoptotic gene *Bcl-2*. Both the control and treated cells showed the identical bands of similar expression level at 475 bp position in the agarose gel that is shown in Figure 4. Another gene namely p53, a tumor suppressor gene, and it's up regulation was found in MOLME treated EAC cells. In Figure 4, the expression of p53 gene was higher compared to control at the 458 bp position on the gel. Consistently, the expression of pro-apoptotic gene Bax was increased at the 479 bp level in MOLME treated EAC cells compared to control EAC cells. On the other hand, the Bcl-2 is an anti-apoptotic gene, and its altered expression plays a critical role in regulating the cell's apoptosis. Interestingly, the expression of Bcl-2 gene was lower compared to control at the 304bp position on the gel which is shown in Figure 4. The results of gene amplification study reported that the treated mice showed up regulation of p53 and pro-apoptotic gene Bax mRNA level and down regulation of Bcl-2 mRNA level (when compared with their respective control) which indicated that the experimental extract work to cause mitochondrial mediated apoptosis of EAC cells.

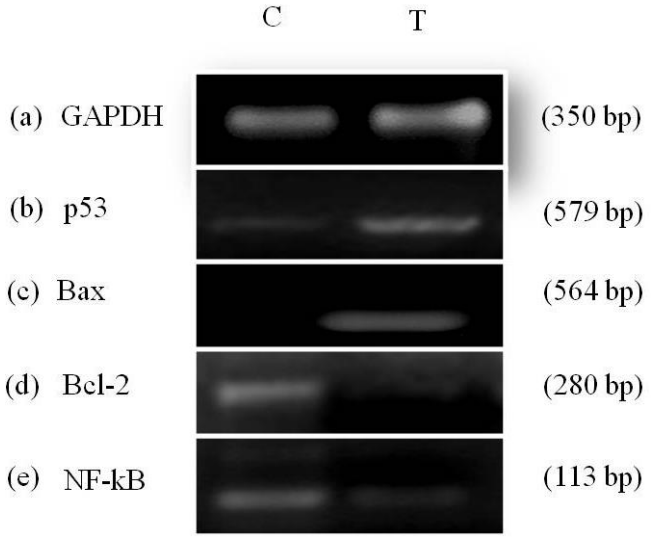

Figure 4. Induction of apoptosis and Inactivation of NF-κB in EAC cells. Amplification of cell proliferation related genes *p53, Bax, Bcl-2*, and *NF-κB* and control gene *GAPDH*. PCR reaction products separated on agarose gel stained with ethidium bromide. T RNA from EAC cells of MOLME treated mice and C RNA from EAC cells of MOLME untreated mice. (a) Expression of *GAPDH* in case of both control and treated EAC cells, (b) Upregulation of *p53* gene in treated cells in compare to control, (c) Upregulation of *Bax* gene in treated cells in compare to control, (d) Downregulation of *Bcl-2* gene in case of treatment compared to control, and (e) Down-regulation of *NF-κB* gene in treated cells compared to untreated control.

Inactivation of NF-κB in MOLME treated EAC cells

NF-κB is involved in cell proliferation and survival and in the induction of apoptosis [19, 20]. To analyze if the effects of MOLME on inhibited cell growth are related to NF-κB activity, the expression of NF-κB in EAC cells treated with MOLME was determined. Interestingly, it was found that MOLME significantly decreased the level of the NF-κB expression relative to the control (Figure 4). This result indicates that inhibiting NF-κB activity might be involved in the inhibition of EAC cell growth by MOLME.

Generation of intracellular ROS in EAC Cells

DCFH-DA is a common fluorescent probe that was used for detecting ROS in MOLME treated EAC cells. The DCFH-DA first hydrolyzed to DCFH by

intracellular esterase, which is then oxidized by reactive species and originates a fluorescent compound 2, 7- dichlorofluorescein (DCF), whose fluorescence intensity is proportional to the levels of ROS. As shown in Figure 5, the untreated control cells displayed little green fluorescence, while treatment with MOLME resulted in much stronger signals by producing the stronger intensity.

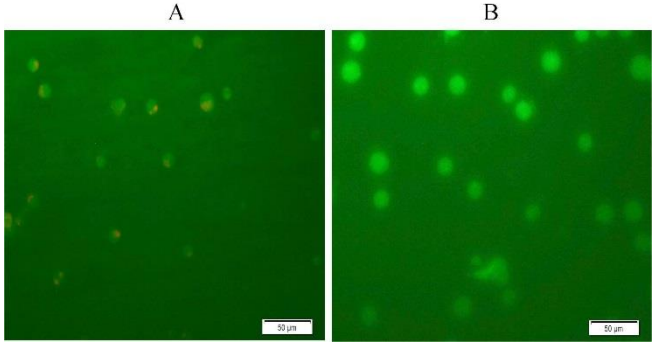

Figure 5. Effects of MOLME in intracellular ROS generation in EAC cells. Observation of cells by fluorescence microscope after incubation with DCFH-DA. (A) Control EAC cells, (B) MOLME treated cells.

Chemical composition of MOLME

GC/MS analysis of MOLME enabled the identification of 42 compounds (Table 2) (Figure 6) of different chemical families. The compounds were account for 96.74% of total plant extract. The major identified compounds are 2,4-Imidazolidinedione, 5-[3,4-bis[(trimethylsilyl)oxy] phenyl]-3-methyl-5-phenyl-1-(trimethylsilyl)- (13.73%), psi.,.psi.-Carotene, 3,3',4,4'-tetradehydro-1,1',2,2'-tetrahydro-1-hydroxy-1'-methoxy- (9.2%), Canthaxanthin (5.51%), Cephalotaxine, 11-(acetyloxy)-, acetate (ester), (11.alpha.)- (5.86%), Methyl (Z)-5,11,14,17-eicosatetraenoate (7.23%), Hexadecanoic acid, methyl ester (9.9%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (4.46%), 3,8,12-Tri-O-acetoxy-7-desoxyingol-7-one (3.85%), and prominent compounds were Spiro[9,9']difluorene, 2,2'-(2,5,8,11-tetraoxadodecane-1,12-diyl)-, 3,9.beta.;14,15-Diepoxypregn-16-en-20-one, 3,11.beta.,18-triacetoxy-, 3,8,12-Tri-O-acetylingol 7-phenylacetate, Pregn-4-ene-3,11-dione, 17,20,21-tris [(trimethylsilyl)oxy]-, 3-(O-methyloxime), (20S)- etc.

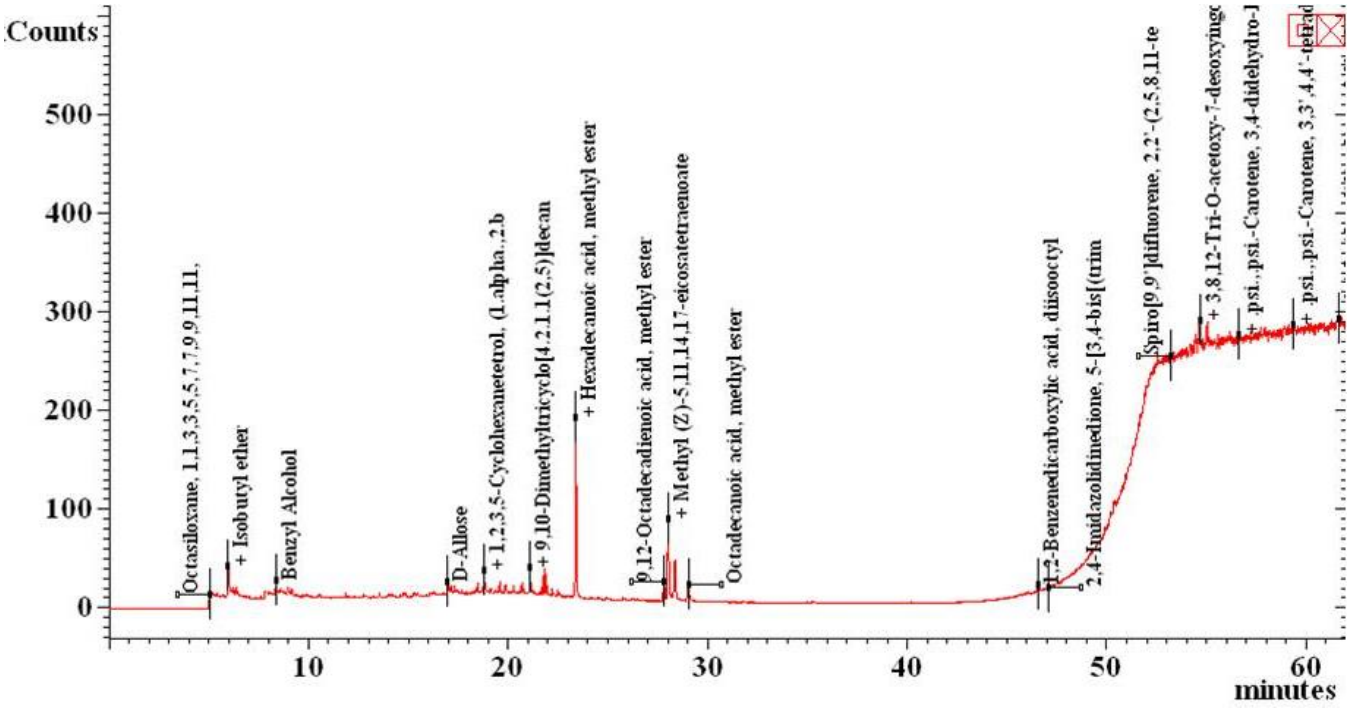

Figure 6. GC/MS chromatogram of MOLME.

Table 2. Chemical composition of MOLME analyzed by GC

SL.	Compounds	Retention time (min)	Area (%)
1	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	5.04	0.32
2	Isobutyl ether	5.98	1.96
3	2-Hexenal, (E)-	6.25	0.33
4	Ethanol, 2-butoxy-	6.39	0.51
5	Benzyl Alcohol	8.36	0.65
6	D-Allose	16.97	0.65
7	Hydroxylamine, O-decyl-	18.47	0.42
8	1,2,3,5-Cyclohexanetetrol, (1.alpha.,2.beta.,3.alpha.,5.beta.)-	18.79	1.68
9	Cycloheptasiloxane, tetradecamethyl-	19.58	0.32
10	3-Buten-2-one, 4-(6,6-dimethyl-1-cyclohexen-1-yl)-	20.69	0.33
11	9,10-Dimethyltricyclo[4.2.1.1(2,5)]decane-9,10-diol	21.09	1.26
12	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	21.80	4.46
13	2-Pentadecanone, 6,10,14-trimethyl-	21.92	1.02
14	Hexadecanoic acid, methyl ester	23.39	9.99
15	9,12-Octadecadienoic acid, methyl ester	27.82	1.41
16	Methyl (Z)-5,11,14,17-eicosatetraenoate	28.03	7.23
17	Octadecanoic acid, methyl ester	29.03	1.78
18	1,2-Benzenedicarboxylic acid, diisooctyl ester	46.54	0.53
19	2,4-Imidazolidinedione,5-[3,4-bis[(trimethylsilyl)oxy]phenyl]-3-methyl-5-phenyl-1-(trimethylsilyl)-	47.11	**13.73**
20	Spiro[9,9']difluorene, 2,2'-(2,5,8,11-tetraoxadodecane-1,12-diyl)-	53.23	2.56
21	3,8,12-Tri-O-acetoxy-7-desoxyingol-7-one	54.04	3.85
22	Cephalotaxine, 11-(acetyloxy)-, acetate (ester), (11.alpha.)-	54.19	5.86
23	Norcodeine di-TMS derivative	54.27	0.29
24	.psi.,.psi.-Carotene, 3,3',4,4'-tetradehydro-1,1',2,2'-tetrahydro-1-hydroxy-1'-methoxy-	54.38	**9.2**
25	Acetamide, N-[5-(diethylamino)-2-[(2,4-dinitrophenyl)azo]-4-methoxyphenyl]-	54.47	1.65
26	3,9-Epoxypregnan-14-ol-20-one, 3,11,18-triacetoxy-	54.90	0.37
27	Isoxazole, 5-[3,3-dicyano-1-cyclohexylidene-2-morpholino-prop-2-enyl)]-3-p-methoxyphenyl-	54.96	0.75
28	Gamma-Lumicolchicine	55.14	0.16
29	Lycoxanthin	55.21	0.77
30	4-(2-Methyl-1-cyclohexenyl)-trans-3-buten-2-one 2,4-dinitrophenylhydrazone	55.32	1.25
31	10-(Methoxycarbonyl)-N-acetylcolchinol	55.41	0.83
32	Butorphanol TMS derivative	55.45	0.78
33	Canthaxanthin	55.61	**5.51**
34	3,9.beta.;14,15-Diepoxypregn-16-en-20-one, 3,11.beta.,18-triacetoxy-	55.78	2.28
35	9-Desoxo-9-x-acetoxy-3-desoxy-7.8.12-tri-O-acetylingol-3-one	55.98	0.75
36	3,8,12-Tri-O-acetylingol 7-phenylacetate	56.07	1.99
37	2-Butenoic acid, 2-methyl-, 2-(acetyloxy)-1,1a,2,3,4,6,7,10,11,11a-decahydro-7,10-dihydroxy-1,1,3,6,9-pentamethyl-4a,7a-epoxy-5H-cyclopenta[a]cyclopropa[f]cycloundecen-11-yl ester, [1aR-[1aR*,2R*,3S*,4aR*,6S*,7S*,7aS*,8E,10R*,11R*(E),11aS*]]-	56.16	1.83
38	Alpha-Lumicolchicine	56.40	0.65
39	9-Desoxo-9-xi-hydroxy-3,7,8,9,12-pentaacetate ingol	56.83	0.61
40	Pregn-4-ene-3,11-dione, 17,20,21-tris[(trimethylsilyl)oxy]-, 3-(O-methyloxime), (20S)-	56.93	2.16
41	6,6'-Diacetyl-7,7'-dihydroxy-2,2',4,4',5,5'-hexamethoxy-1,1'-binaphthalene	57.01	1.65
42	Acetic acid, 1,1',4'-triacetoxy-5,5'-diisopropyl-6,7,6',7'-tetramethoxy-3,3'-dimethyl-[2,2']binaphthalenyl-4-yl ester	57.81	1.10
43	3,9-Epoxypregn-16-en-14-ol-20-one, 11,18-diacetoxy-3-methoxy-	57.91	0.82
44	Thymolphthalein	58.56	0.42
45	Silane, [[(3.beta.,5.alpha.,11.beta.,20S)-pregnane-3,11,17,20,21-pentayl]pentakis(oxy)]pentakis[trimethyl-	60.27	0.32
46	2,2-Bis[4-[(4,6-dichloro-1,3,5-triazin-2-yl)oxy]phenyl]-1,1,1,3,3,3-hexafluoropropane	60.59	0.42

Total area=96.74

DISCUSSION

Cancer is one of the deadliest diseases all over the world with towering rate of mortality, even if the success of its therapeutic option is not satisfactory [21]. Thus, it is a major concern for the scientists to discover potential safer treatment options in more effective manner. Interestingly, cell growth inhibition, inhibition in tumor weight and increase in survival time suggest MOLME as a potential source of anticancer agents.

Generally, prolongation of lifespan of the treated animal at the same time decreasing WBC count of blood is considered as the reliable criteria for evaluating an anticancer drug [22]. Our results showed an increased lifespan and reduction of WBC count in MOLME treated mice. Studies on induction of apoptosis also suggest that MOLME can inhibit EAC cell growth. Lacking apoptosis, results in abnormal accumulation of cells which eventually causes cancer [23]. In Figure 3, EAC cells from the MOLME treated mice showed cell growth inhibition and some morphological changes like membrane blebbing, cell shrinkage, chromosomal condensation and nuclear fragmentation under fluorescence and optical microscope which are the features of apoptosis. Molecular studies demonstrated that apoptosis is controlled by some regulatory proteins such as Bcl-2, Bcl-X, Bax, Bak, p53, Caspase-3,8,9, etc. [17,24]. It has been reported that, downregulation of Bcl-2 and upregulation of Bax and p53 mRNA illustrate mitochondria damage mediated apoptosis [17]. Therefore, in this study we investigated the expressions of p53, Bcl-2 and Bax mRNA of MOLME treated mice along with control. In support of morphological appearance of MOLME treated EAC cells, upregulation of p53, Bax and downregulation of Bcl-2 were found, indicating the mitochondria mediated apoptosis. The results of cell growth inhibition, morphological appearance along with apoptotic gene expression analysis of MOLME treated mice suggest MOLME induced anti-proliferation of EAC cells.

It is believed that ROS acts as a double-edged sword in cancer progression and prevention. In one hand, moderate levels of ROS maintain cancer cell survival, such as proliferation and angiogenesis, on the other hand high levels of ROS lead to destructive effects on cancer cells through pathways such as apoptosis and autophagy [25-27]. In this study, we revealed that treatment with MOLME increased ROS production in EAC cells significantly. Moreover, ROS are elevated in apoptotic cancer cells whereas NF-κB activation is inhibited [28,29]. Therefore, we intended to evaluate whether elevated level of ROS production is associated with NF-κB activity in MOLME treated EAC cells (Figure 4). Not surprisingly, reduced expression of NF-κB was found in MOLME treated EAC cells than untreated control. It was suggested that many chemical compounds exhibiting anti-carcinogenic activity can reduce NF-κB activity and elevate ROS level in cancer cells. A report showed that, in acute myelogenous leukemia stem cells niclosamide (an anti-carcinogenic compound) reduces NF-κB activity and at the same time it increases ROS concentration [30]. Evidently, the loss of NF-κB causes the accumulation of ROS because NF-κB activity might suppress the expression of some antioxidant genes [31-33]. The elevated ROS decreases the fluidity of the biological membranes and increases membrane permeability, as a result depolarizes the cell membrane, subsequently increasing cellular death.

GC/MS analysis suggests significant bioactive compounds in MOLME. Many of the identified compounds are known to possess several pharmacological activities [34, 35]. Hexadecanoic acid, methyl ester is one of the bioactive compounds found in *Pleurotus ferulae* ethanol extract. *Pleurotus ferulae* extract reported to inhibit the proliferation of murine melanoma B16 cells, human esophageal cancer Eca-109 cells, and human gastric cancer BGC823 cells through induction of apoptosis [34]. Canthaxanthin, another prominent bioactive compound of MOLME, appeared to prevent cancer initiation. It was suggested that dietary supplementation of Canthaxanthin resulted in 65 % reduction of mammary cancer in rats induced by dimethylbenzanthracene [35]. Carotene, another compound found in MOLME, has shown growth inhibitory activity against breast cancer cell line (MCF-7) [36]. It was also suggested that carotenes stimulate the immune system and regulate cell cycle and apoptosis [37]. Lycoxanthin, a carotenoid present in MOLME, has been attributed with various beneficial biological activities such as antioxidant, anticancer, anti-inflammatory, anti-obesity, and neuroprotective activities [38]. Therefore, observed activities of MOLME might be due to synergistic activity of these compounds.

In conclusion, it can be concluded that MOLME shows significant anti-proliferative effect through ROS induced apoptosis by inactivation of NF-κB activity. Therefore, it could be considered as a promising

resource of cancer chemotherapy with better host safety. However, before assuming that, it is important to carry out more research on this plant at advanced level, using other cell lines and higher animal models.

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AUTHOR CONTRIBUTIONS

PKD was involved in conception and design and perform the experiments. SYS and MAS analyzed data. MAR contributed to drafting the article. ARMT and AS experimented GC-Mass and analyzed data. FI and JAK contributed to revising it critically for important intellectual content. MAR made the final approval of the version to be published.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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