Cytotoxic and apoptotic potential of *Myristica fragrans* Houtt. (mace) extract on human oral epidermal carcinoma KB cell lines

Gayathri Rengasamy[1,3], Anuradha Venkataraman[2], Vishnu Priya Veeraraghavan[3], Mallika Jainu[4*]

[1]*Department Of Biochemistry, Research and Development Centre, Bharathiyar University, Coimbatore-641106, Tamil Nadu, India,* [2]*Department of Biochemistry, Mohammed Sathak College of Arts and Science, Sholinganallur, Chennai-600119, Tamil Nadu, India,* [3]*Department of Biochemistry, Saveetha Dental College and Hospital, Saveetha University, Velappanchavadi, Chennai-600 077, Tamil Nadu, India,* [4]*Department of Biochemistry, Biogen Care Research Centre, Chennai-600014, Tamil Nadu, India*

Several studies have revealed that certain naturally occurring medicinal plants inhibit the growth of various cancers. The present study was conducted to evaluate cytotoxicity and apoptotic induction potential of *Myristica fragrans* Houtt mace extract. The cytotoxic activity of the *Myristica fragrans* Houtt mace acetone extract was assayed by MTT assay on human oral epidermal carcinoma KB cell lines. KB cells were incubated with different concentration of mace extract ranging from 25 to 125 µg/mL for 24hrs. The apoptotic induction potential was also studied by the analysis of Bcl-2 protein and gene expression in mace extract incubated KB cell lines using western blotting technique and real-time polymerase chain reaction. The mace extract exhibited cytotoxicity and anticancer effect against KB cell lines and it also suppressed the growth of cancer cells, therefore growth inhibitory effect was noted in extract treated cell lines. The apoptotic potential of mace extract was accompanied by reduced gene expression of Bcl-2 compared to the untreated KB cells. The mace extract shows the cytotoxic activity and induced the apoptosis through the modulation of its target genes Bcl-2 in the KB cell lines, suggesting the potential of mace as a candidate for oral cancer chemoprevention. This can be further investigated *in vivo* for its anticancer potential.

Keywords: *Myristica fragrans* Houtt. (mace)/extract. Apoptosis. Cytotoxicity. KB cell lines. MTT assay. Bcl-2 gene.

INTRODUCTION

Oral cancer is one of the most common malignancies in the world. It is characterized by a high degree of local invasiveness and a high rate of metastasis to cervical lymph nodes. The migration of oral squamous cell carcinoma in to maxillary and mandibular bones is a common clinical problem (Sumitra, Nagani, 2013). The defective apoptosis (programmed cell death) which results in enhanced growth describes most cancer cells. Several proteins control the timing of the events in the cell cycle, which is tightly regulated to ensure that the cells divide only when necessary. The loss of this regulation is the hallmark of cancer (Rezafekrazad, Mehrad, Hamzeh, 2017). The increase in interest in apoptosis, or programmed cell

death has had a major impact on many fields with in the biological sciences, including Oncology. The delineation of discrete apoptotic pathway has affected not only our basic concepts of the development of cancer, but also our approaches towards the prevention and therapy of the disease (Maryam, Nadareh, Hossein, 2013). It is now evident that the balance between cellular proliferation and death plays a vital role in the maintenance of normal tissue homeostasis and the derangement of either of these processes can lead to the dysregulated clonal expansion that is characteristic of all neoplastic disease (Valter, Lombardi, Ramon, 2017).

Two different pathways, extrinsic and intrinsic, mainly play a role in the mechanisms of apoptosis. Also, in another pathway, the proteins Bax and Bcl-2, which both belong to the Bcl-2 family, play adverse roles in apoptosis with their ratio being an important determinant of the susceptibility to apoptosis by regulating the proapoptotic and antiapoptotic proteins. Bcl-2 family proteins govern

*Correspondence: M.Jainu. Department of Biochemistry, Biogen Care Research Centre, Chennai-600014, Tamil Nadu, India. E-mail: mallikajain@gmail.com

Braz. J. Pharm. Sci. 2018;54(3):e18028

Page 1 / 7

the mitochondrial outer membrane permeabilization (Ozgur *et al.,* 2014; Thazin *et al.,* 2017). Apoptosis results from a collapse of the cellular infrastructure through internal proteolytic digestion by some enzymes named caspases, leads to cytoskeletal disintegration, metabolic derangement and genomic fragmentation. The lack of effective diagnostic tool and the chemotherapeutics are the major constraints for controlling this type of cancer. Chemotherapy of oral cancer is largely ineffective and the clinical efficacy of the standard treatment with 5-fluorouracil (5-FU) is low. Furthermore, the resistance of oral cancer to chemotherapy and radiotherapy is a major problem (Jemal *et al.,* 2011).

The natural products, especially plants have been used in the treatment of various diseases for thousands of years (Tajuddin *et al.,* 2003). Mace is the aril part on the kernel of of *Myristica fragrans* Houtt (family: Myristicaceae), an evergreen aromatic tree cultivated in many tropical countries and is widely used as a spice and also to flavour many kinds of baked foods and vegetables (Kim *et al.,* 2016). *Myristica fragrans* Houtt exhibited a maximum number of plant constituents. The main constituents of *Myristica fragrans* Houtt have been found to be alkyl benzene derivatives (myristicin, elemicin, safrole, etc.), terpenes, alpha-pinene, beta-pinene, myristic acid, trimyristin, neolignan (myrislignan), and macelignan (Kim *et al.*, 2016).

The chemical constituents of *Myristica fragrans* Houtt have been investigated by the scientists from various disciplines for hypolipidemic and hypocholesterolemic effects, antimicrobial, antidepressant, aphrodisiac, memory-enhancing, antioxidant, and hepatoprotective properties (Ulyana *et al.,* 2016). Mace is widely used as a flavouring agent, a hair dye, and a folk medicine. It also possesses antipaillomagenic, anticarcinogenic, chemopreventive and anti-inflammatory activities (Kim *et al.,* 2016; Jannu, Hussain, Rao, 1991). In dentistry application, mace lignan, an active compound from seed had strong anticarcinogenic activity and antibacterial effect (Ulyana *et al.,* 2016). Since then there is no study that has been published to indicate the potential apoptotic activities of mace extract on oral carcinoma cell lines. Hence the aim of the study was to evaluate the cytotoxic and apoptotic potential of acetone mace extract on human oral epidermoid carcinoma KB cells.

MATERIAL AND METHODS

Chemicals and their sources

The authenticated sample of dried mace of *Myristica fragrans* Houtt was procured from the Herbal Care and Cure Centre (Chennai, India) and the sample was identified with the help of a botanist at the Central Drug Research Institute, Chennai before the study was carried out. All other chemicals used for this study were of analytical grade purchased from Sigma Aldrich Company, USA.

Extraction

The dried powder sample of mace (80g) was extracted three times by hot percolation method with 1:5 ratio volume of acetone at room temperature for 72 hours. The filtrates were concentrated under reduced pressure at 40ºC and stored at the room temperature for use in subsequent experiments (Gayathri, Anuradha, 2015).

Cell lines

KB cell lines were procured from the National Centre for Cell Sciences, Pune. The cells were maintained in Minimal Essential Medium enhanced with 10% FBS, streptomycin (100 μg/mL) and penicillin (100 U/mL), in a humidified atmosphere of 50 μg/mL CO_2 at 37 °C.

Maintenance of cell lines

The vial containing the KB cell lines acquired from ATCC (CCL -17) was removed from liquid nitrogen freezer and immediately placed in a 37 °C water bath. It was agitated continuously until the medium thawed. Then it was centrifuged for 10 min at 150 to 200 × g, room temperature. Supernatant was discarded and cells were washed with fresh medium to remove residual DMSO.

MTT assay for cytotoxicity

The MTT assay (Mossman, 1983) is based on the ability of live but not the dead cells to reduce a yellow tetrazolium dye to a purple formazan product. The cells were maintained in DMEM medium, supplemented with 10% Fetal Bovine Serum, at 37 ºC in humidified atmosphere with 5% CO_2. The KB cells were plated in 96 well flat bottom tissue culture plates at a density of approximately 1.2X 10^4 cells/well and allowed to attach overnight at 37 ºC. The medium was then discarded and cells were incubated with the different concentrations of the samples (25, 50, 75,100 and 125 μg/mL) for 24 h. After the incubation, medium was discarded and 100 μL fresh medium was added with 10 μL of MTT (5 mg/mL). After 4 h, the medium was discarded and 100 μL of DMSO was added to dissolve the formazan crystals. Then, the absorbance was read at 570 nm in a microtitre plate reader. Cyclophosphamide was used as a positive control.

Page 2 / 7

Braz. J. Pharm. Sci. 2018;54(3):e18028

The cell survival was calculated by the following formula:

$$\text{Viability \%} = (\text{Test OD/ Control OD}) \times 100$$
$$\text{Cytotoxicity \%} = 100 - \text{Viability\%}$$

Study of cellular morphology

KB cells were seeded in 6-well plates at 2×10^5 cells per well in 5 mL of complete growth medium, incubated with extract at concentrations 100 µg/mL. The control cells treated with 0.1% DMSO alone were also included. The morphological changes were observed under a phase contrast light microscope after 24 hours and 48 hours. The images were captured at a magnification of x200.

Annexin/PI Assay

The cells (2 mL) at a density of 5×10^4 were grown in 40-mm petri dishes and allowed to attach for 24 h after which cells were treated with the 100 µg/mL extract for 24 h and 48 h. After the various treatment periods, cells were harvested and centrifuged at 335 g for 10 min. The supernatants were washed in 1% PBS and re-suspended in Annexin V binding buffer. The cells were centrifuged at 335 g for 10 min and the supernatants were discarded. The cell extracts were suspended in 100 µL Annexin V binding buffer and 5 µL Annexin V Alexa Fluor 488 was added and allowed to incubate in the dark for 15 minutes. PI (4 µL) diluted in 1x Annexin V binding buffer (1 : 10) was added and allowed to incubate for 15 minutes in the dark at room temperature. Annexin V binding buffer (500 µL) was added to wash the Annexin/PI stained cells. Annexin/PI was evaluated on a Becton Dickinson FACScan instrument fitted with a 488 nm argon laser. A minimum of 10,000 cells per sample were acquired and analyzed using CellQuest Pro software.

Western blot analysis

The cell pellet was dissolved in a 1% cell lysate buffer and centrifuged for 10 min at 12,000 rpm. The total cellular protein (50 µg) was separated by using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked with 5% calf serum albumin. Afterwards the membrane was incubated for 2 hours with primary mouse anti-human Bcl-2 monoclonal antibody. The membrane was then probed for 1 hour at room temperature with horseradish peroxidase-labelled secondary antibody. The signal was revealed using 3,3-diaminobenzidine tetrahydrochloride (DAB) as the HRP substrate. The Quantification of the western blots was determined by scanning the blots with Adobe Photoshop and performing densitometry with Bio-1D Image.

Bcl-2 gene expression by RT-PCR

The total RNA from cell lines were isolated using the ONE STEP-RNA Reagent (Biobasic Inc.). After the RNA isolation, the RNA was immediately reverse transcribed with Easy Script Plus™ Reverse Transcriptase. It was a novel recombinant reverse transcriptase that exhibits much higher efficiency in the synthesis of first strand cDNA from RNA templates. It has been engineered for RNA templates with complicated secondary structure and high GC contents. For RT PCR reaction, 1-2 µg of RNA was used corresponding to 1-10 µL of the total RNA isolate.

cDNA preparation

RNA isolated from the fresh tissue samples was reverse transcribed where oligo-dT was used as a primer. Into a 1.5 mL Eppendorf PCR tube, 1-2 µg of RNA (at least 1 µL at most 10 µL), 2 µL of oligo-dT (stock was 10 µM) was added and the total volume was completed up to 12.5 µL with DEPC treated water. The tube was incubated at 65ºC for 5 min (Water Bath) and chilled on ice. Then, 4 µL of 5X reverse transcriptase buffer (final concentration 1X), 2 µL of 25 mM dNTP mix (final concentration 2.5 mM), and 0.5 µL of RNase inhibitor (40 U/µL) were added in the indicated order. After incubating at 42 ºC for 5 minutes, 1 µL of Easy Script reverse transcriptase (200 U/µL) was added. The reaction was carried out at 42 ºC for 50 min. Finally, the tube was heated up to 70 ºC for 10 min and chilled on ice. The samples were stored at -20 ºC until further use.

The polymerase chain reaction (PCR)

The cDNA obtained was amplified by PCR (Chomczynski, Sacchi, 1987). The Gene Specific PCR was used to amplify BCl_2 gene. A constitutively expressed gene, namely GAPDH, was chosen in order to assess the quality of PCR (Table I).

The primer sequences used for the PCR amplification were as follows:

Genes	Primer right	Primer left
BCl_2	5'-GGATTCATGACCAGGGAGTTC-3'	5'-GCGGTCTCCACTGAGAATAAT-3'
GAPDH	5'-GGCCAACCGTGAAAAGATG-3'	5'-GGATCTTCATGAGGTAGTCTC-3'

Braz. J. Pharm. Sci. 2018;54(3):e18028

Page 3 / 7

Statistical analysis

The data are given as the mean ± standard deviation (SD). The statistical analysis was performed by using one-way analysis of variance (ANOVA), followed by a Dunnett's multiple-comparison test with 95% confidence intervals. A P-value < 0.01 was considered to indicate a statistically significant difference.

RESULTS

The effect of anticancer from mace extract on KB cell lines was evaluated through the micro-culture tetrazolium assay (MTT). The multiple concentrations from the mace extract were used and the effective doses were calculated from the dose-response curve. The results of the cytotoxicity evaluation against KB cell line of the mace extract are shown in Figure 1. From the above figures, we can easily determine the percentage of cell viability for five different concentration of 25, 50, 75, 100 and 125 µg/mL mace extract. The shape of the curve shows significant inhibition of cell proliferation in KB cell line (Figure 2) in a dose-dependent manner after 24 hours of treatment. The cell viability of mace extract was 41.40, 46.99, 51.52, 55.61 and 58.51% respectively as shown in (Figure 1). Hence, the half maximal inhibitory concentration of mace extract was at the concentration of 75 µg/mL (Figure 2).
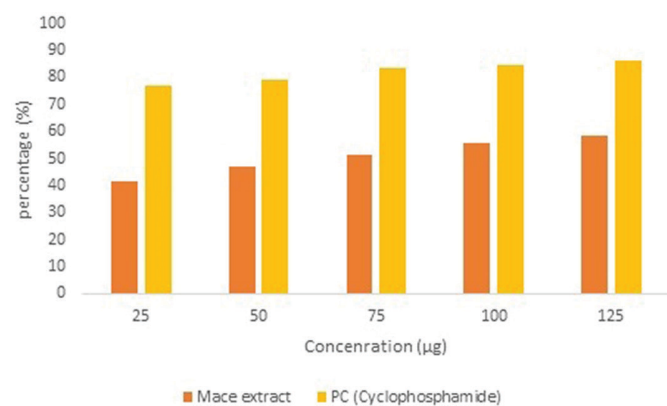

FIGURE 1 - Effect of mace extract and positive control cyclophosphamide cytotoxicity action on KB cells, 24h after exposure. The effect was measured by MTT cell viability assay. The data are mean ± SD of three independent experiments.

After 48 hours of treatment, the higher concentrations (100 µg/mL) killed more than 50% of cells. The apoptosis-inducing potential of the extract was further tested in the cancer cells using the Annexin-FITC/propidium iodide double staining flow cytometric assay (Table I). The
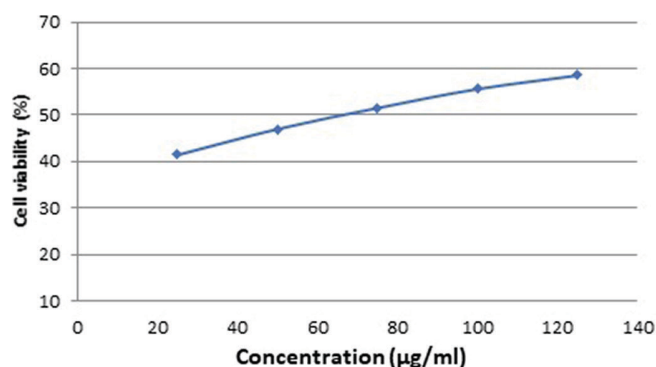

FIGURE 2 - Effects of IC_{50} concentration of acetone extract of mace extract.

decrease in the cell population was seen with the increase in the extract incubation. As can be seen in Figure 3, the cells with 100 µg/mL doses of extract revealed cellular shrinkage and overall morphological alterations.
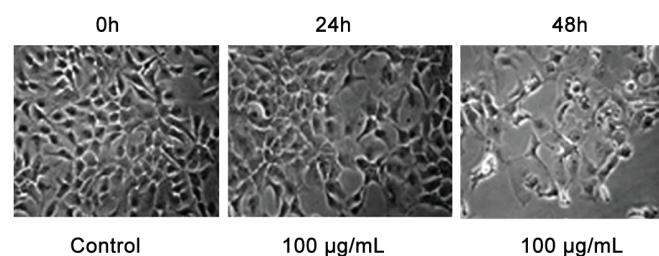

FIGURE 3 - The effect of Mace extract on morphology of KB cells. Cells were treated with vehicle (DMSO) or various doses of mace acetone extract (100 µg/mL for KB cells) for 24 h and 48 h. Cell viability was determined by photographs captured by phase contrast microscope at magnification, ×200.

TABLE I - The percentage of cell population in different stages (live, apoptotic and necrotic) following the extract treatment and evaluated by double staining I Annexin V-FITC/PI using flow cytometric assay

	24 h	48 h
Live (%)	72.15	20.16
Early apoptosis (%)	33.17	30.45
Late apoptosis (%)	20.54	40.12
Dead (%)	0.12	51.36

As observed in the Figure 4 and 5, Bcl-2 protein expression reduced after treatment with the mace extract. This advocates a key role for the mitochondria in mace extract -induced apoptosis. Levels of Bcl-2 genes expression in KB cells treated with acetone extract results are represented in Figure 6. The Bcl-2 gene expression was found to be decreased in the extract treated cells in

Page 4 / 7

Braz. J. Pharm. Sci. 2018;54(3):e18028

comparison to the untreated cells, whereas GAPDH gene expression remained the same in both sample treated and untreated cells. The quantitative measurements of Bcl-2 expression was done and it is shown in the Figure 7.
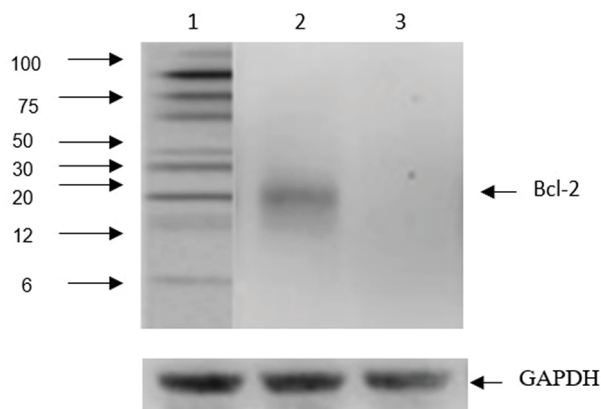

FIGURE 4 - Effect of mace extract treatment on Bcl-2 protein expression in KB cells. Lane 1 -Marker Lane; Lane 2- Bcl-2 protein expression in control KB cells; Lane 3 – Bcl-2 protein expression in extract treated KB cells.

FIGURE 6 - Effect of mace extract treatment on BCl_2 gene expression in KB cells. Lane 1 - 1 1KB DNA Ladder; Lane 2 - GAPDH expression in control KB cells; Lane 3 - GAPDH expression in mace extract treated KB cells; Lane 4 - BCl_2 gene expression in control KB cells; Lane 5 - BCl_2 gene expression in extract treated KB.
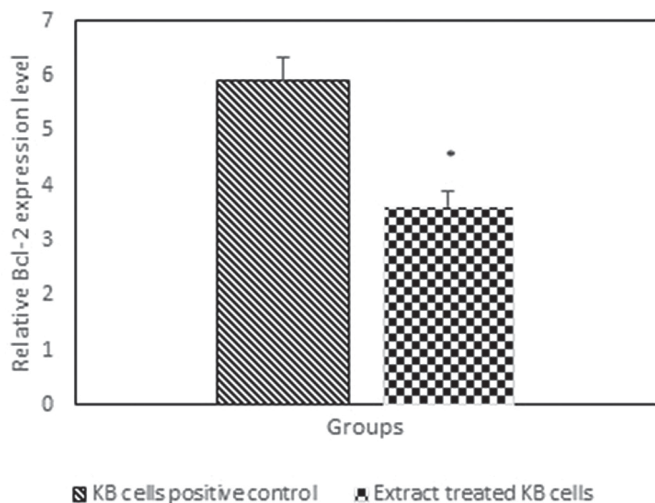

FIGURE 5 - Relative intensity of Bcl-2 protein levels were expressed as means in bar graphs. Significant differences from positive control were noted as $^*P<0.01$.

FIGURE 7 - The m-RNA expression levels were analyzed by densitometry, and the data is presented as mean intensity in relative arbitrary densitometric units (ADU) $\pm$ SEM with n = 3. $^*P < 0.01$ are statistically significant compared to KB untreated cells.

DISCUSSION

The discovery and development of chemotherapeutics that are effective for the treatment and control of oral cancer is urgently needed. The natural products are still important sources to discover new anticancer drugs. Several studies have revealed that certain naturally occurring medicinal plants inhibit the growth of various cancers (Eun-Sun *et al.,* 2012). The methanol extract of the bark of *M. fragrans* Houtt
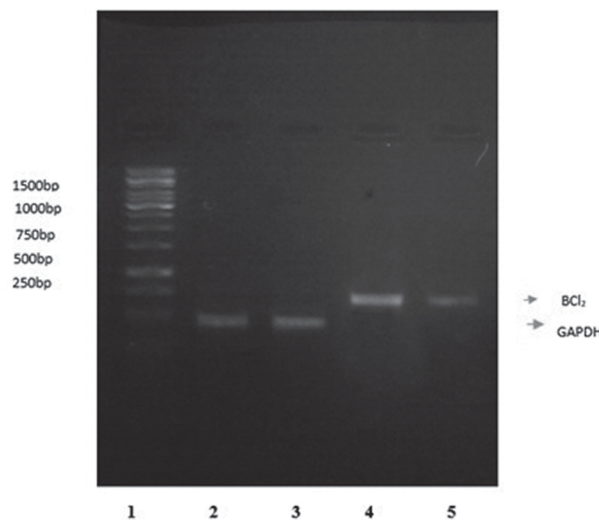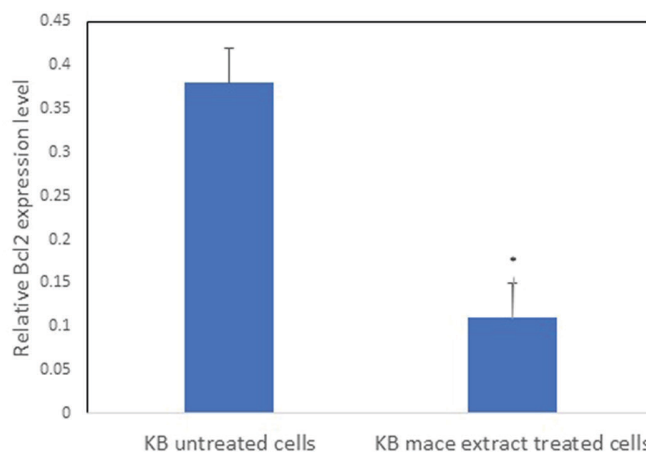
inhibited jurkat T Leukemia cell line proliferation through apoptosis induction and induced SIRT1m RNA down regulation (Chirathaworn *et al.,* 2007).

The mace extract was screened for its cytotoxicity against KB cell lines at different concentrations to determine the IC_{50} (50% growth inhibition) by MTT assay. The exposure of KB cell lines with varying concentration of mace extract resulted in a concentration dependent inhibition of cell proliferation, suggesting that the acetone

mace extract inhibited the proliferation of KB cells, cyclophosphamide was used as a positive control.

The Annexin/PI assay also confirmed the ability of the extract to induce early and late apoptosis. Cancer cells, however, exhibit resistance to apoptosis in order to sustain their uncontrolled proliferation and, therefore, any apoptosis modulating compound is desirable as a plausible chemotherapeutic agent against cancer. KB cells were treated with 100 μg/mL concentration of mace extract for 24 and 48 hours. The extract induced both early and late apoptosis as compared to the untreated control.

The mitotic inhibitory effect of *M. fragrans* Houtt. was cytotoxic because this activity of the extract was induced in a concentration-dependent manner. Our results are in accordance with the previous report on the cytotoxic activity of *M. fragrans* Houtt. (Chirathaworn *et al.,* 2007). Lignans, which are important class of plant-derived compounds, possess a variety of biological activities such as antitumor, antimitotic, antiviral and antiatherosclerotic activities. The antitumor activity of lignans can also justify the observed cytotoxic effect of *M. fragrans* Houtt. (Essam, Aseel, Massody*,* 2012). The previous results suggest mitodepressive and antimutagenic potentials of water extract of the leaves of *M. fragrans* Houtt. as desirable properties of a promising anticancer agent (Akeem *et al.,* 2011). Some constituents present in mace extract possess potent antioxidant and anti-inflammatory activities and some of them exhibit cancer preventive activity in experimental carcinogenesis (Plengsuriyakarn *et al.,* 2012). These previous findings support the current study.

The Bcl-2 oncoprotein suppresses or delays the induction of apoptosis in prostate, skin, lymphoid tissues, and mammary gland. The apoptotic pathway includes the extrinsic (cytoplasmic) and intrinsic (mitochondrial) pathways. Permeabilization of the mitochondrial outer membrane is importantly associated with Bcl2 family proteins that regulate the integrity of the mitochondria (In-Hyoung *et al.,* 2015).

The acetone mace extract treated and cultured cells were harvested and further analysed for the expression of BCl_2 gene, and the results showed a decrease in the expression. This is in agreement with the previously reported potential antitumour activity of the mace extract (Datta, Kojima, Yoshida*,* 1997). Thus, acetone mace extract exerts its antitumour role by lowering the expression of BCl_2 gene in KB cells, which points to the mechanism of action related to activation of apoptosis. The activities of this plant may be due to the presence of mace lignans compound. Their biological effects include antibacterial, anti-thrombotic and vasodilatory, antimutagenic,

lipoxygenase and cycloxygenase inhibition scavenging of reactive oxygen species, and antitumourigenic (Essam, Aseel, Massody*,* 2012). In conclusion, the results of the present study suggest that the acetone mace extract may contain bioactive compound myristicin, that induces apoptosis of KB cells. The additional studies are required to characterize the bioactive compounds responsible for the observed activities of mace plant as a novel resource for new anticancer drugs.

CONCLUSION

In conclusion, **the** mace extract functions as a broad-spectrum anticancer agent in human cancer cells by blocking cell cycle and further triggering apoptosis via the intrinsic pathway. In addition, **the** mace extract has less cytotoxic effect on normal cells, and is selective at inducing apoptosis between cancer and normal cells. Therefore, mace is a potential candidate, as a powerful chemotherapeutic agent.

CONFLICTS OF INTEREST STATEMENT

There is no conflict of interest.

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