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CYTOGENETIC STUDY OF THE PURE MYRISTICIN FROM NUTMEG (*MYRISTICA FRAGRANS*) ON RHABDOMYOSARRCOMA CELL LINE (*IN VITRO*)

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ABSTRACT
This study was conducted with the aim to extract and purify apolyphenolic compound "Myristicin" from the dried seeds of nutmeg *Myristica fragrans* available in Iraqi markets. The partial purified myristicin is obtained after column chromatography application which has been detected in the essential oil by TLC and vanillin-H_2SO_4 reagent. The cytotoxic effect of pure and partial purified myristicin extract concentrations ranging (31.25– 500) µg/ml are used to treat the Rhabdomyosarrcoma (RD) cell line for 24, 48 and 72 hours intervals. The highest percentage of inhibition appears in 500, 250 and 125 µg/ml at 48 h (82.3, 82.1 and 75.9 %) respectively on RD cell line when treated with pure myristicin , While when treated with partial pure myristicin at the same times and with the same concentrations this percentage decreases without any significant difference.

KEY WORDS: Cell line**,** Cytogenetic, Inhibitor rate %, myristicin, *Myristica fragrans*, Rhabdomyosarrcoma,

INTRODUCTION

Medicinal plants have been used for disease relief and health maintenance for a long period of time (Al-Amiry, 2010). The term complementary and alternative medicine (CAM) is customary in describing various alternative approaches to augment the health of mind, body and spirit in order to enhance traditional medical approach to disease treatment (Mazzio and Soliman, 2009). Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years, and have served humans well as valuable components of seasoning, beverages, cosmetics, dyes and medicine. Most of this therapy observed by Ibrahim and Aqel (2010). The essential oil is an active ingredient of several pharmaceutical products and cosmetics (Marsik *et al.,* 2005). The medicinal value of nutmeg in the treatment of several ailments ranging from nervous to digestive disorders (Gable, 2006) has been recognized worldwide since ancient times and it was regarded as a cure for plague. Recent studies relating to its anti-bacterial (Khalid and Hung,2010), anti-viral (Goncalves *et al*., 2005), anti-cancer (Mahady *et al*., 2005), anti-proliferative (Lee *et al*., 2005), anti-oxidant (Sen *et al.,* 2010), hepatoprotective (Morita *et al*., 2003) and neuroprotective (Ban *et al*., 2004) anti-obesity(Yuliana *et al.*,2011) effects reveal a wide scope for its application in the health sector.

In vitro studies indicate herbs, spices, and their bioactive components can inhibit, and sometimes induce, pathways that regulate cell division, cell proliferation, and detoxification, in addition to the inflammatory and immune response (Shishodia *et al*.,2003). *Myristica fragrans* extract has been shown to contain antibacterial activity against different genera of bacteria and antiviral activity against rotavirus (Dorman and Deans, 2000; Goncalves *et al.*,2005). For its role as anticancer agent, myristicin, found in *M. fragrans Houtt* has cytotoxic and apoptotic effects in human cell line (Lee *et al* ., 2005).The use of *in vitro* assay system for screening has been a common practice since the beginning of cancer chemotherapy in 1946, following the discovery of antineoplastic activity of nitrogen mustard. Some phytochemicals have been shown to exhibit cytotoxic effects against cancer cell through cell cycle modulation (Rana *et al*., 2002).In the present study, were extraction and Purification of myristicin by using column chromatography. The cytotoxicity of a partial and pure myristicin on RD cell line *in vitro* were determinate i.e. inhibitor rate %.

MATERIALS AND METHODS

Myristica fragrans was obtained from local market in Baghdad. The covers of the seeds were removed by hand and the dried kernel have been crushed to small pieces by mortar and pestle then grinded by a coffee grinder to a fine powder and stored in a closely tight container in freeze until used. Thirty grams of the powder plant material has been placed in a thumb of Soxhelt apparatus according to Narasimhan and Dhake (2006) with some modification. Then (200) ml of chloroform was added for a period of 3 hours, the solution have been filtered through a filter paper and distilled under reduce pressure to yield a semisolid residue. The residue dissolved in ethanol followed by cooling .The filtrate was reserved for the separating of myristicin. The trimyristin residue was recrystallized from ethanol. The filtered solution remaining after separation of trimyristin was distilled to remove ethanol. The oily product that remained after distillation was followed by using patch way method by putting silica gel M.F.C about 100-200 in flask and was washed it many times by petroleum ether then it was added the filtered solution and was put in a water bath and was moved every 10 min. for half an hour then the upper layer was collected and added to the flask petroleum ether .The method was repeated again ,finally benzene was added and collected the sample .All samples had been preceded using glass column (3.5× 6) cm filled with activated alumina special for column chromatography .The oily product had been dissolved in 1-2 ml petroleum ether and chromatographed on the activated alumina column using the elution solvent of petroleum ether to separate the pure myristicin.

This stage involved defatting of nutmeg essential oil by using Soxhelt apparatus according to Ozaki *et al*.,(1989) with some modification. So (50)gram of the nutmeg seed powder was put in a thumble and refluxed with 350ml of 70% methanol (1:7) for 8hours time when over , the solution have been filtered through a filter paper and evaporated to dryness under vacuum at 40°C,the dried extract have been weighed and stored at 4°C (Al-Amiry, 2010).

Thin layer chromatography: According to Mirzaie *et al*., (2007), 1 ml of the oil was taken to be examined and dissolved in toluene. A suspension was formed; this suspension can be loaded on thin layer chromatography (TLC) which is a layer of aluminum coated with (silica gel) with (20x20) cm dimensions. The layer was (0.2) mm thickness (Fluka) with a fluorescent substance when exposed to UV light, the sample spotted below a plate from its ending border using capillary tubes. Therefore, two different solvent separation systems have been used as follows: Ethyl acetate : toluene (7:93) : Benzene. These solutions have been placed in a tank separately, then a spotted plate, was placed inside the tank and was left there, when the solvent system moved about 20 cm from the spots, the plate was directly pulled out, dried in the air and sprayed with vanillin reagent, after that the plate has been heated in the oven at 100- 105 °C for 10 minutes. The R*f* value was calculated when examined in day light.

Cytotoxic effects of Myristicin on cell lines *in vitro:* Human rhabdomyosarcoma (RD) provided by the Iraqi Center for Cancer and Medical Genetics Research (ICCMGR), Baghdad, Iraq and were maintained at 37°C. Cell lines were grown on RPMI-1640 medium with helps buffer and L-glutamin, supported by ICCMGR with fetal calf serum 100ml, Benzyl penicillin 0.5ml, Nystatin 0.25ml, Streptomycin 0.5ml.

Cytotoxicity assay: Approximately 7.5×10^3 cells were cultured in complete medium growing in 96-well plates and incubated at 37 °C under a 5% CO_2 atmosphere. The cells were allowed to grow for 24 h and then exposed to different concentrations of myristicin (dissolved in DMSO, not exceeding 0.4%), ranging from 31.25 to 500 µg/ml, for 24-h and 48-h and 72-h periods. DMSO at 0.4% (v/v) was added to the wells without chemical (control cultures). After incubation period was finished, the cells in the wells were gently washed by the addition of sterile PBS twice, finally 50 µl of Neutral red stain was added to the wells and the plates were incubated for 30 minutes at 37°C, then the plates were washed gently with distilled water and left to dry.
The plates of different cell culture at the end of the assay were examined by ELISA reader at 492 nm transmitting wave length. Only viable cells were able to take the stain, the dead cells were not. The inhibitors rate was measured according to (Chumchalova and Smarda, 2003) and as follows:

$$\text{Inhibitor rate \%} = \frac{\text{Abs. at 492 nm of control} - \text{Abs. at 492 nm of test}}{\text{Abs. at 492 nm of control}} \times 100$$

Abs = Absorbance
The –Ve results referred to the inhibition rate %
While the +Ve results referred to proliferation rate %
All values were analyzed statically

RESULTS AND DISSUCION

Myristicine was isolated from *M. fragrans* . The resultant myristicin is detected by TLC silica gel 60 F_{254} plate of 0.75 mm thickness using the mobile phase benzen which gave a big spot of brown to purple in color of R*f* 0.5(Narasimhan and Dhake ,2006;Al-amiry, 2010) . The TLC chromatogram in for the myristicin and the standard has given one brown spot of R*f* 0.9 in toluene: ethylacetate (7:93) as mentioned by Harborn (1998). According to the results demonstrated in Fig (1), significant differences were observed for 24 h within all concentrations used, for RD cell line, the highest percentage of inhibition rate appeared within 250µg/ml and reached to 81.32%for partial pure Myristicin. So, apparent significant differences were observed within this concentration between the pure and partial pure extract. Both the partial and pure myristicin effects on human rhabdomyosarcoma (RD) cells *in vitro.* RD inhibition by both the pure and methanolic extracts partial pure of *M. fragrans was* higher. This reflected the sensitivity of RD cells compared with other cell lines. Al- Hilli (2004) showed in his study on the effect of crude extracts of *Cyperus rotundus* L. that RD cells were more sensitive to each of the hexane, aqueous, and ethanolic extracts compared with HEp-2 cells. Highest inhibition was recorded after 48 hrs of treatment with any of the extracts. According to the data in Figure 2, the effect of pure myristicin is apparent with no significant differences within all concentrations at 48 h. of exposure, except within 125 µg/ml, the inhibitory effect of pure Myristicin on RD cell line which started within 31.25 µg/ml with a percentage of 27.1% with a significant difference and this effect continued in its elevation when within 62.5µg/ml reached to 56.5 % and increased within 125µg/ml until reaching to 75.9 % with a significant difference. No significant differences were observed at this concentration between the pure and partial pure Myristicin. The highest inhibitory rate was reported within 500 µg/ml when reached to 82.3% for pure myristicin. So, apparent significant differences were observed within this concentration between the pure and partial pure extract.

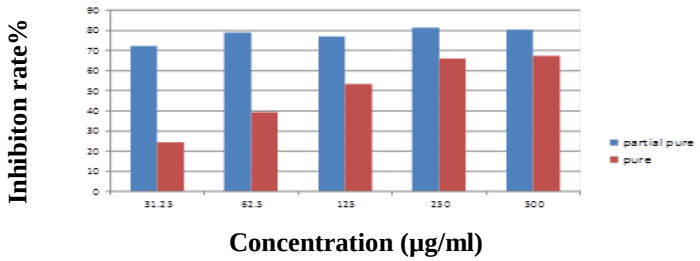

Figure 1. Inhibition rate (%IR) histogram for different concentrations of pure and partial pure myristicin on RD cell line at 24 hours.

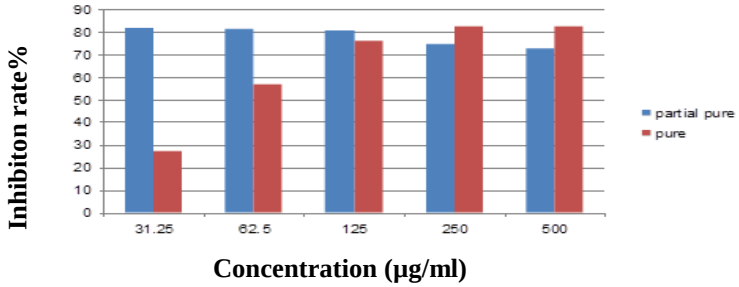

Figure 2. Inhibition rate (%IR) histogram for different concentrations of pure and partial pure myristicin on RD cell line at 48 hours.

At 72h. according to the result in Fig (3) there is apparent high significant differences which were observed between the pure and partial pure Myristicin at two concentrations. The highest inhibitory rate was reported within 62.5 µg/ml for pure and partial pure extract when reached to 55.4%, 56.8% .Among two types of treatment (pure and partial pure) at all concentrations and for different intervals of exposure (24, 48 and 72 hours); the extracted purified myristicin had the best efficiency in inhibiting RD cell growth within (500µg/ml) at 48 hours. The pure myristicin can inhibit the growth of AA8 and EM9 cells were analyzed for cell viability, after treatment with a wide range of concentrations of myristicin, from 50 to 2000 µM for 24 h, using the MTT assay protocol. Both compounds displayed similar dose dependent decreases in viability .For a 24 h exposure, the cell viability was reduced below 50% when cells were treated with concentrations higher than 500 µM or 1000 µM, for AA8 and EM9 cells, respectively. (Martins *et.al*, 2011).
The methanol extract of the *myristica fragrans* seeds demonstrated potent inhibitions on the proliferations of the cultured human tumor cells such as A549 (non-small cell lung), SK-OV-3 (ovary), SK-MEL-2 (melanoma), XF498 (central nerve system), and HCT-15 (colon) (Lee *et al*., 2005).

CONCLUSIONS

Extraction method can yield both Myristicin and myristic acid.Myristicin has cytotoxic effect against RD cancer cell lines.

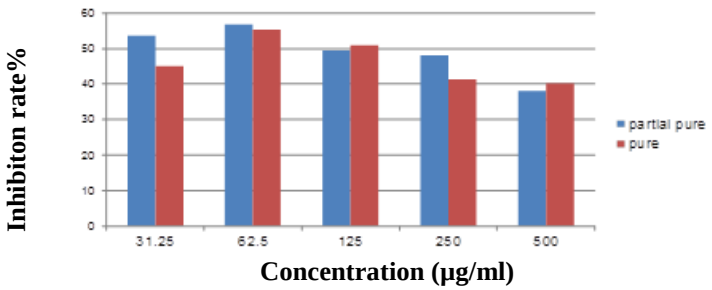

Figure (3): Inhibition rate (%IR) histogram for different concentrations of pure and partial pure myristicin on RD cell line at 72 hours.

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