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Research Article

CHEMISTRY

Cytotoxic effect of citrus fruits osthole in lung cancer

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Abstract: Osthole, 7-methoxy-8-(3-methyl-2-butenyl) - 2H-1-Benzopyran-2-one, is an active constituent of Cnidium monnieri genus and apiaceae family. The anticancer effect of osthole has been reported due to osthole may inhibit the growth, invasion and metastasis of cancer cells **Aim**: Preparation of osthole from different citrus fruit plant sources and evaluates its cytotoxic effect on lung cancer. **Methods**: osthole from different cultivar of citrus fruits was extracted in reflux by using petroleum ether and acetone, the solvent was evaporated and then precipitates and dried then crystallized. Osthole structure was characterized by IR and GC-MS. Lung cancer cells A549was treated with different concentration of osthole, cell proliferation was measured by MTT assay. **Result**s: Osthole was extracted from lemon (*citrus limon*), grapefruit (*Citrus paradise)* and orange (*Citrus sinensis*) plant sources The highest concentration of osthole exist in grapefruit The IR spectrum grapefruit osthole with the peaks at 1747 cm^{-1} (coumarinic carbonyl), 1048cm^{-1} (C-O streaching) and 1439 cm^{-1} (aromatic C=C) confirms the skeleton of osthole. The grapefruit osthole showed well separated peaks with retention times16.56 identical with the osthole standard solution. The IC_{50} value of grapefruit osthole was 46 µg/ ml against lung cancer cell line after 48 hrs treatment. **Conclusion**: our findings suggest the osthole exist in citrus fruit as lemon, grape fruit, orange practically free of osthole. Osthole has therapeutic application in human lung cancer.

Key words: *Osthole, Lung cancer, Cnidium monnieri, Citrus fruits*

Introduction:

Lung cancer is one of the most commonly diagnosed cancers and the leading cause of death in the world, non-small cell lung cancer accounts for approximately 85% of all cases, small cell lung cancer accounts about 15% **(Parkin *et al*., 2002; Jemal *et al*., 2008)**.Despite advances in diagnostic and therapeutic, the survival rate in many countries is generally less than 15% **(Erridge *et al*., 2007)**. In order to improve the survival rate, intensive efforts have been made to find new anticancer agents, and many attentions have been drawn to herbal medicines owing to their wide range of biological activities, low toxicity and side effects **(Carter *et al*., 2008; Hsu *et al*., 2009)**.

Osthole, 7-methoxy-8-(3-methyl-2-butenyl) - 2H-1-Benzopyran-2-one (figure 1), is a bioactive simple coumarin derivative extracted from many medicinal plants such as Cnidium monnieri (L.) Cusson, has long been used in traditional Chinese medicine for the treatment of eczema, cutaneous pruritus, trichomonas vaginalis infection, and sexual dysfunction **(Dien *et al*., 2012)**. osthole has many medicinal application including anti-inflammatory, anti-oxidant, anti-osteoporotic **(Du *et al*., 2015)**, anti-bacterial, and anti-allergic effects **(Cai *et al*., 1991; Hideaki *et al*., 2002; Zimecki *et al*., 2009; Ming *et al*., 2011).** Furthermore, accumulating evidence indicates that osthole possesses anti-tumor effects by inhibiting tumor cell growth and inducing apoptosis **(Yang *et al*., 2003; Riviere *et al*., 2006 Chou *et al*., 2007; Xu *et al*., 2011**).It has been reported that osthole was able to inhibit the migration and invasion of breast cancer cells **(Yang *et al*., 2010)**. However, the effects of osthole on hepatocellular carcinoma (HCC) remain unknown. The molecular mechanism of osthole's anti-tumor effect was not yet clearly known. It has been shown that osthole-induced G2/M arrest and apoptosis in lung cancer A549 cells were associated with the inhibition of Cyclin B1, p-Cdc2 and p-Akt expressions and upregulation of the Bax/Bcl-2 ratios **(Xu *et al*., 2011)**.Osthole could also downregulate fatty acid synthase (FASN) expression. Furthermore, osthole has been shown to effectively inhibit (matrix metalloproteinase-2 promoter) MMP-2 promoter and enzyme activity, which might be one of the causes that lead to the inhibition of migration and invasion of breast cancer cells by osthole **(Yang *et al*., 2010; Duan *et al*., 2016)**. More studies are needed to fully address the molecular mechanisms of the anti-tumor effects of osthole. In this study we evaluate the prescence of osthole in some citrus fruits and detect its cytotoxic effect on lung cancer.

Material and methods:

1. Plant materials:

Grapefruit (*Citrus paradise)*, lemon (*citrus limon*) and orange (*Citrus sinensis*) were purchased from tanta market (Egypt).

2. Chemicals:

(3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide)) (MTT), Dimethyl Sulfoxide (DMSO) and Standard osthole were purchased from Sigma Chemical Co. (St. Louis, Mo, U. S. A.).All other chemicals used were of high grade.

3. Extraction and isolation of osthole:

Extraction of osthole from citrus fruit according to **(YU *et al*., 2002)** method with some modifications, Dried citrus fruits were ground and dafatted twice by petroleum ether. the defatted samples were extracted by acetone in heated reflux,extracted liquid was filterated and evaporated to obtain yellow residue.this residue dried and recrystallized in diluted ethanol, a light yellow acicular crystals were obtained.

4. FTIR analysis:

light yellow acicular crystals were blended with potassium bromide. A JNS-CO Spectrum System 4100 LE FTIR spectrometer (Japan) was used for the analysis in the range between 4000 cm^{-1} and 400 cm^{-1} at a resolution of 4 cm^{-1} **(Liu *et al*., 2010)**.

5. GC-MS analysis and conditions:

Gas chromatography combined with mass spectrometry (GC-MS) was used for identification of the components. The analysis was performed on Focus GC, DSQII (thermo Scientific, USA) a Thermo-TR.5MS column, 30m x 250µm x 0.25µm. Inlet temperature was 250 °C. Oven temperature was initially held at 50°C for 1 min, then increased to 150 °C at 30°C /min for 2 min, then increased to 280°C at 10°C /min and held for 5 min. The injection volume was 1µl (splitless). Detection was operated under full-scan and selected ion monitoring (SIM) mode (**Sajjadi *et al.,* 2009; Mahier *et al.,* 2010**).

6. Determination of cytotoxic effect of grapefruit osthole:

6.1 Cell line and culture conditions:

The human lung cancer cell line A549 was obtained from the institution Serum and Vaccine, (Cairo) and maintained in DEMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 **(Xu *et al*., 2011)**.

6.2 MTT Assay:

Cell proliferation was measured using the MTT assay. A549 cells were plated at a density of 1×10^4cells per well in 96-well plates overnight and then treated with different concentrations of Osthole (0, 10, 20, 40, 60, 80and 100µg/ml). After 48 h treatment, 20 µl of MTT solution (2 mg/ml in PBS) were added to each well and the cells were cultured for another 4 h at 37°C. Then the medium was totally removed and 150 µl DMSO was added to solubilize MTT formazan crystals. Finally, the plates were shaken and the optical density was determined at 570 nm (OD570) using a ELISA plate reader **(Yang *et al*., 2010)**.

Results:

Grapefruit, orange and lemon osthole were extracted by acetone in reflux to give light yellow crystals. The light yellow crystals isolated from Grapefruit and lemon with melting point of 83-85 °C, with the peaks at 1747 cm^{-1} (coumarinic carbonyl), 1048cm^{-1} (C-O stretching) and 1600 cm^{-1} (aromatic C=C) confirms the skeleton of osthole but orange extract not contain on C=O bond so orange free of osthole **Fig. (2).** The grapefruit extract showed well separated peaks with retention times16.56 identical with the osthole standard solution with molecular weight 244 **Fig. (3).** After analyzing the light yellow crystals by IR and GC-MS the structure of osthole were confirmed in Grapefruit and lemon whereas orange was practically free of this compound.

In addition, the results showed that grapefruit osthole inhibited the proliferation of lung cancer cells in vitro when the cells were treated with different concentrations of grapefruit osthole for 48 h then the inhibition rate was evaluated by MTT assay. The IC_{50} value was 46 µg/ ml as shown in **Fig. (4).**

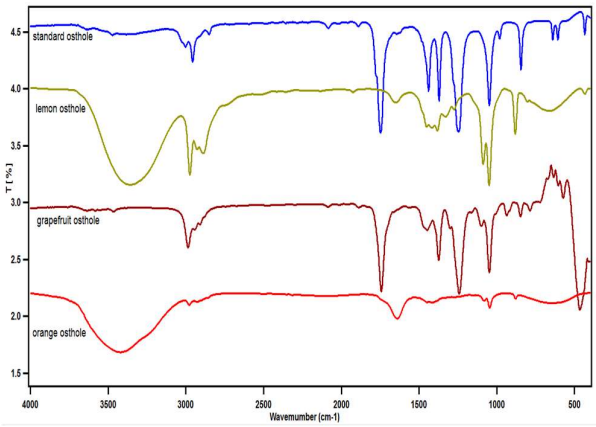

Fig (2): FTIR of extracted osthole from different citrus fruits compared to standard

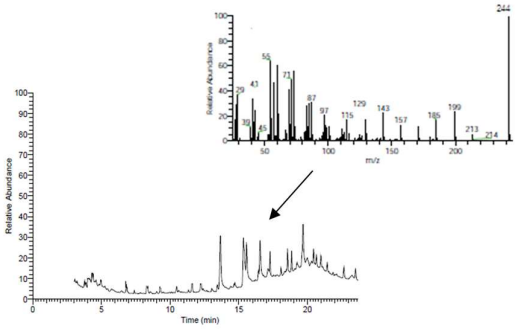

Fig (3): GC-MS chromatogram of grapefruit extract. The arrow shows the peak of osthole

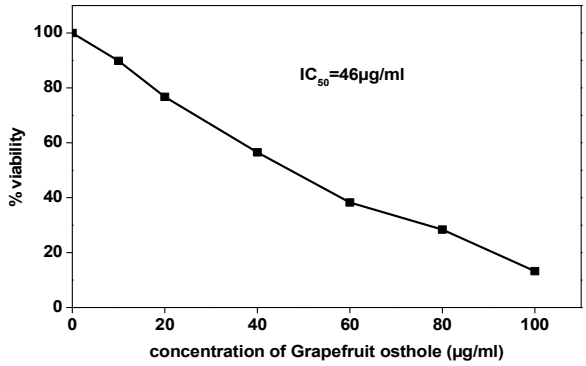

Fig (4): Effect of osthole on human cancer A549 cells growth. ***P*< 0.001 versus control group.***

Discussion:

Osthole, is an active constituent of Cnidium monnieri (L.) Cusson, extracted from many medicinal plants and herbs such as Cnidium monnieri and Angelica pubescens **(YU *et al*., 2002)**. Our study indicated that osthole exist in some citrus fruits and confirmed by IR and GC-MS. IR the peaks at 1747 cm^{-1} (coumarinic carbonyl), 1048cm^{-1} (C-O stretching) and 1439 cm^{-1} (aromatic C=C) confirms the skeleton of osthole. Osthole has been shown to have comprehensive and wider applications as anti-hepatitis, anti-oxidation, anti-inflammatory, anti-microbacterial, and antiallergic effects **(Dien *et al*., 2012)**. Furthermore, the anticancer effect of Osthole has been reported in a few papers. Both in vitro and in vivo studies showed that Osthole possessed an anticancer effect by inhibiting human cancer cells growth and inducing apoptosis **(Okamoto *et al*., 2005)**. It is reported that Osthole is able to inhibit the migration and invasion of breast cancer cells **(Yang *et al*., 2010)**. In our study we showed that osthole has anti proliferating effect on human cancer cells. The induction of cell cycle arrest and apoptosis are common mechanisms proposed for the cytotoxic effects of anticancer-drug extracted from herbal medicine **(Xavier *et al*., 2009)**.Cell cycle arrest can trigger proliferation inhibition and apoptosis in cancer cells **(Chao *et al*., 2004)**.During cell cycle, the G2/M checkpoint is a potential target for cancer therapy. It prevents DNA-damaged cells from entering mitosis and allows for the repair of DNA that was damaged in late S or G 2 phases prior to mitosis **(Wang *et al*., 2009)**.The G2/M checkpoint is controlled by Cdc2 and Cyclin B1 **(Dash *and* El-Deiry, 2005)**, and some anticancer-drugs could induce G2/M arrest through down-regulating the expressions of Cyclin B1 and Cdc2 **(Yang *et al*., 2009)**.

This study showed that citrus fruits as grapefruit and lemon contain osthole whereas orange free of osthole, and show the effect of osthole extracted from grapefruit on proliferation of human lung cancer A549 cell. The results indicated that osthole has anticancer effect so osthole may be a good compound for developing anticancer drugs.

Conclusion:

Our study demonstrated that osthole present in grapefruit and lemon. Osthole inhibited the growth of human lung cancer A549 cells our findings suggest that Osthole may have a therapeutic application in the treatment of human lung cancer.

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التأثير السمى لاثول الموالح على سرطان الرئه

حامد عادل ابراهيم ابوشرف

قسم الكيمياء- كلية العلوم – جامعة طنطا

توجد مادة الاوثول فى العائلة الخيميه والاسم العلمى لها **7-ميزوكسى-8-(3-ميثيل-2-بيوتيل-2هيدرو-1-بينزوبيوران-2-أون)** وهذه الماده كانت مستخدمه كعلاج صينى قديم فاستخدمت فى علاج الامراض الجلديه مثل الاكزيما وعلاج الامراض الجنسيه وفى بعض الابحاث القليله ثبت ان الاوثول يعالج الامراض السرطانيه. تهدف هذه الدراسه الى عزل الاثول من مصادر نباتيه مختلفه (الموالح) وتقييم النشاط الساملاوثول الموجود فى اعلى مصدر. فى هذه الدراسه تم استخلاص الاثول من الموالح مثل الجريب فروت والليمون والبرتقال باستخدام البتروليم ايثر والاسيتون. الاثول المستخلص من الجريب فروت تم التاكد من وجوده باستخدام تقنيه FTIR و GS- MS وHPLC . بالاضافه الى ذلك تم تقييم التاثيرالسام للاثول المستخلص من الجريب فروت على خلايا سرطان الرئه البشريه باستخدام تحليل MTT. لقد اوضحت نتائج تحليل IR الى وجود رابطه بين C=O عند 1747سم$^{-1}$وبين C=C عند 1439سم$^{-1}$وبين C-Oعند طول 1048سم$^{-1}$ وذلك فى الجريب فروت والليمون ولكن البرتقال خالى من الاثول. بالاضافه الى ذلك لقد اوضحت النتائج ان حضانه خلايا سرطان الرئه البشريه مع تركيزات مختلفه من اثول الجريب فروت لمده 48 ساعه ادى الى تقليل حيويه هذه الخلايا حيث ان تركيز اثول الجريب فروت على تثبيط نمو 50% من هذه الخلايا هو 46 ميكروجرام لكل مل. ويمكن من هذه الدراسه ان نستنتج ان اثول الجريب فروت يثبط نمو خلايا سرطان الرئه لذلك من الممكن ان يكون له تطبيقات علاجيه فى المستقبل.