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CHEMICAL COMPOSITION, ANTIMICROBIAL ACTIVITY ,CHEMOPREVENTIVE POTENTIAL OF THE PEEL ESSENTIAL OIL OF *CITRUS DELICIOSA* TEN. GROWING IN EGYPT

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ABSTRACT

The chemical composition of the peel essential oil (PEO) of *Citrus deliciosa* Ten. growing in Egypt was investigated using gas chromatography-mass spectrometry(GC-MS) analysis .As a result, sixteen components were identified representing 98.79% of the total oil yield(0.55 %v/w).The major components belong to monoterpene -type viz., *d*-limonene (46%),*p*-cymene(40%) and γ -terpinene (9%). Consequently , PEO of *C. deliciosa* Ten . belonged to *d*-limonene/p-cymene chemotype. The PEO was screened for the antimicrobial activity and it showed observed inhibitory activity against Gram-positive bacteria (*Bacillus subtilis* ,*Staphylococcus aureus*) and marked inhibition against fungi (*Aspergillus flavus* ,*A . niger and Candida albicans*) but no activity was detected against Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). The induction of the phase II detoxifying enzymes like glutathione-S- transferase (GST) and glutathione peroxidase (GPx) was investigated in a way to evaluate the chemoprevention and/or chemotherapeutic potential of the PEO -alone and in combination with vitamin E on rat alimentary tract. An increase of 2.72 and 2.12-folds in GST activity in liver and intestinal mucosa in addition to 50.4% increase in large intestinal mucosa was observed with PEO gavaging .On the other hand ,PEO alone had no significant effect on GPx enzyme activity in all the tested tissues except liver. A combination of vitamin E with each of GST and GPx led to potentiation of activity of both enzymes. In such a way ,the peels of *C. deliciosa* Ten. which is normally considered as environmental waste is turned into promising antimicrobial, chemopreventive and/or chemotherapeutic nutraceutical or pharmaceutical product with an extra added economic value .

INTRODUCTION

Essential oils and their components impressively demonstrate potential activities in different medical products, in food industry, cosmetics, perfumery and aromatherapy [1, 3].

Citrus fruits stand out among the most common source of essential oil and most common dietary sources. The genus *Citrus* (family Rutaceae) encompasses around 16 species which are mainly distributed in subtropical regions[4] . Mandarins are among the major genus fruits cultivated in Mediterranean region[5]. Among the popular mandarins: *Citrus deliciosa* Ten. (Mediterranean mandarin) and *Citrus reticulata* Blanco (common mandarin)[6, 7].

Citrus Essential oils occupies the largest sector of the world production of essential oils [4] and have been identified in leaves, flowers and fruits, being more abundant in the fruit peel (flavedo) [8]. These *Citrus* by-products are relatively abundant ,non-expensive ,consisting mainly of terpene hydrocarbons viz., α-,β-pinene, ,myrcene, γ - terpinene, *d*-limonene, *p*-cymene, myrcene, α -phellandrene and others. Other compounds include oxygenated hydrocarbons like terpinen- 4-ol and α-terpineol while sesquiterpene hydrocarbons are found in traces [9-12]

Citrus plants contribute actively in traditional medicine allover the world. In the Chinese traditional medicine, *Citrus* peels are used as tonic, carminative, in treating dyspepsia, to reduce phlegm, in treating cough, cold, anorexia, malignant breast sores, prolepses of uterus and anus, diarrhea, blood in faeces, hernia and also in cancer[1]. *d*-Limonene which is the most widely distributed component in *Citrus* essential oils has been shown to inhibit several induced tumors like forstomach, colonic, pulmonary and skin tumors in laboratory animals [13,14] It is well documented that ,an inverse relation has been established between initiation of these malignancies and fruit as well as vegetables intake. Several natural products elicit an increased activity of phase II detoxifying enzymes like glutathione -s-transferase (GST) or glutathione peroxidase (GPx) in the presence of glutathione (GSH) in laboratory assays. This provoked enhancement of enzyme activity is one of the mechanism behind the inhibitory activity of certain compounds against carcinogenesis [15,16] .

Previous reported studies [17-19] covered the phytochemical and botanical investigation of *C. deliciosa* Ten. growing in Egypt and provided a chemotaxonomic marker for identification of this plant. Reviewing the available literature, neither the composition of PEO of *C. deliciosa* Ten. growing in Egypt nor its antimicrobial activity or its chemopreventive potential has been explored .

So, based on the potentialities of *Citrus* peels in both traditional and modern medicine, it was deemed of interest to investigate the chemical composition to evaluate the biological activity of the oil in an attempt to find a natural antimicrobial agent and chemopreventive product with activity against alimentary tract carcinogenesis since colon cancer is the third most malignant neoplasm in the world and the second cause of death in the USA[20]. Achieving this goal could add to the medicinal or pharmaceutical or nutritional value in addition to favorite environmental impact by converting what is usually taken as an eco-burden into a product with an added economical value.

EXPERIMENTAL

Plant material:

The ripe fruits of *Citrus deliciosa* Ten. were collected in April 2005 from local farms and Agrarian reform farms in El-Sharkia Governorate, Egypt. Samples were kindly authenticated by Prof. Abdalla M.A. Mohsen; Professor of Horticulture, Faculty of Agriculture, Zagazig University. A voucher specimen is deposited at the Pharmacognosy Department, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt.

Preparation of the essential oil:

The PEO of *C. deliciosa* Ten. was isolated by cold pressing following the reported methods [21,22].

Oil analysis and identification:

GC-MS analysis was carried out on a Hewlett Packard 5890A gas chromatograph (HP, Santa Clara, CA, USA) equipped with a fused silica analytical column (DB5, 30 m x 0.32 mm x 0.25 µm) (J&W Scientific, Waldbronn, Germany). The capillary column was directly coupled to a sector field mass spectrometer (GC Mate, Jeol, Tokyo, Japan). The conditions applied were: split injection (ratio; 1:10); injector and transfer line were set at 280°C; Oven temperature: 45°C (3 minutes) to 160°C (0 min.) at 4°C min^{-1} then 10°C min^{-1} and the EI-MS spectra were recorded at 70 eV. The components were identified based on combination of Retention Index (RI) [23] and mass fragmentation pattern compared to library data of the GC/MS system and to those documented by published data [24] . Retention indices of the oil components were calculated relative to co-chromatographed standard n-alkanes

Antimicrobial activity:

The PEO of *C. deliciosa* was evaluated for its antimicrobial and antifungal activities by applying the agar disc diffusion method [25]. All the tested microorganisms were laboratory isolates of the Department of Microbiology, Faculty of Pharmacy, Zagazig University. Two strains of Gram-positive bacteria viz., *Bacillus subtilis*, *Staphylococcus aureus* and a couple of Gram-negative bacteria(*Escherichia coli* , *Pseudomonas aeruginosa*) and three fungi *(Aspergillus niger*, *A. falvus* and *Candida albicans*) were tested.

Bacteria and fungi were cultured on nutrient agar and Sabouraud dextrose agar (Difco,USA), respectively. Paper discs (each was 6 mm in diameter) were impregnated with 25 µl of the PEO and applied onto the surface of the inoculated plates.

Standard antimicrobial and antifungal discs (Oxoid ,UK) were used as references. Plates with impregnated discs and references were incubated at 35°C (24 hr.) and 25°C (48 hr.) for bacteria and fungi, respectively. The diameters of the zones of inhibition were measured and expressed in millimeters.

Animal, diet and treatment:

Twenty four male Sprague-Dawley rats (180 ± 5 gm) were housed in stainless cages (four per cage). Rats were maintained under controlled conditions of 50% relative humidity, temperature of 20-25°C and 12 hour light/12 hour dark cycles.

Rats were fed on semipurified fresh - daily diet which was similar in composition to that of American Institute of Nutrition Reference Diet (Ain-76A)[13] with modification of source of carbohydrate and removal of antioxidant. In this current study, sucrose was replaced by a mixture of starch and glucose and water was given *ad libitum*.

After one week of starting the diet, rats were divided into three groups (eight rats per group) as follows:

Group I (control group) was given only 0.3 ml cotton seed oil by gavaging.

Group II was given 1,000 mg/kg/day *C. deliciosa* essential oil in 0.3 ml cotton seed oil according to Hamada *et al.*[26].

Group III was given citrus oil in combination with vitamin E (PHAR^{CO} Pharmaceuticals ;100 mg/kg/day) in 0.3 ml cotton seed oil as reported [27,28].

The duration of treatment of all groups was eight consecutive days and rats were fed fresh diet daily.

Tissue preparation:

Twenty-four hours following the last administration, rats were sacrificed by cervical dislocation. Esophagus, stomach, small intestine, colon as well as liver were isolated instantly for enzyme preparation. The mucosal surfaces of the stomach and intestine were collected by scraping with a scalp and the tissue was homogenized in cold 1.15% KCl solution (pH 7.4). The homogenate was centrifuged at 9000 x g for 20 minutes. The supernatant was centrifuged at 100,000 x g at 4°C for one hour. Aliquots of the cytosol fraction were frozen in liquid nitrogen and stored at -80°C until used following a previous report[27].

Assay of glutathione- s-transferase (GST):

The activity of cytosolic was determined according to Habig *et al.*[29] using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay was carried out at 25°C in the presence of 0.1 M phosphate buffer (pH 6.5) in the presence of 5 mM GSH, 1 m M CDNB, and 20 µl of cytosol. Protein concentration of these samples was determined according to the method of Lowry *et al.*[30] using bovine serum albumin as a standard.

Glutathione peroxidase (GPx) assay:

Glutathione peroxidase activity was measured using both hydrogen peroxide and t-butyl hydroperoxide as substrates basically as described by Howie *et al.*[31].

Statistical analysis:

The data were checked, entered, and analyzed by using SPSS version 11; data were expressed as means ± SEM. ANOVA tests were used for comparison and the Least Significant Difference (LSD) was calculated and $p < 0.05$ was considered significant.

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RESULTS AND DISCUSSION

A total of 16 compounds were identified representing 98.79 % of the peel oil. The oil exhibited a strong aroma characteristic of the fruit. As shown in table (1) , the oil was rich in monoterpene hydrocarbons, which constituted about 97.89 of the total oil with *d*-limonene, *p*-cymene and γ-terpinene as major component (46, 40 and 9 % respectively). The oxygenated monoterpenes represented about 0.54% of the total oil with α-terpineol (0.24%) terpinen-4-ol (0.2%) while trans-carveol appeared in 0.1% in addition to traces of thymol . Sesquiterpenes viz., α-phellandrene, α-farnesene and β-caryophyllene were only present as traces .

Unlike the dominance of *d*-limonene in peel essential oil (46%) in the current study , the previous investigation of oil of the leaf [19] showed that methyl-N-methyl anthranilate was the major constituent of the foliage oil by 41.4%. The latter compound is represented in the current study by only 0.3%. Moreover, γ-terpine, *d*-limonene, *p*-cymene accounted for 24.2, 0, 6 and 5.3% of total foliage essential oil . It is noteworthy to say that myrcene, α-phellandrene, α-terpineol, α-farnesene and β-caryophyllene were not reported in the foliage essential oil. Thus, PEO of *C. deliciosa* Ten is a *d*-limonene / p-cymene chemotype. The results also, showed that there were qualitative and quantitative variations in the chemical composition between foliage and the peel essential oil of *C. deliciosa* Ten. growing in Egypt. This finding could be utilized to determine the authenticity of the *C. deliciosa* peel essential oil which was found -unlike the foliage oil- to be rich in *d*-limonene. *d*-Limonene plays several important biological roles as it will be demonstrated here- in-after.

Concerning the antimicrobial activity, essential oils offer a wide array of potential antimicrobials against bacterial pathogens and fungi [32] According to the results shown in table(2) , the PEO of *Citrus deliciosa* Ten. demonstrated an interesting activity against all the tested microorganisms except Gram-negative bacteria (*Escherichia coli and Pseudomonas aeruginosa*). The pronounced activity against the Gram-positive bacteria like *Bacillus subtilis , Staphylo coccus aureus* and fungi (*Aspergillus flavus, A. niger and Candida albicans*) could be attributed partly to the monoterpenes viz. *d* - limonene , and α-, β - pinene as reported [33,34] .However, It should be emphasized that, one should not nullify the interaction of other oil components .

Unlike the Gram-positive bacteria, Gram-negative bacteria possess an outer membrane with a distinct barrier of hydrophilic polysaccharide chain that hampers the diffusion of the PEO thus, making them resistant to several antimicrobials in conformity with reported data[35]. Thus, the oil could be listed among the promising natural antimicrobials not only in the pharmaceutical field but also in the area of food safety. In this connection, the PEO could be used to prevent the spoilage of foodstuffs via contamination with toxin producing fungi e.g. *A. flavus* which produces the mycotoxin (aflatoxin) leading to serious health hazards [36-38].

As shown in table 3, PEO of *C. deliciosa* Ten. elicited an increase GST activity by 2.72 and 2.12-folds higher than those reported for controls in liver tissue and intestinal mucosa, respectively. Moreover, an increase of 50.4% in the GST activity in large intestinal mucosa was observed with oil gavaging. However, EO showed no significant effect in the stomach tissues (group II).

On the other hand, a combination of vitamin E with the oil (group III) demonstrated a synergistic effect on the GST activity. As shown in table 3, there was 3.32- and 2.61-folds increase higher than that of control concerning enzyme activity in liver and small intestinal mucosa of the rats.

As far as total glutathione peroxidase (GPx) activities are concerned, the highest activities were detected in the liver and stomach while the lowest levels were demonstrated in small and large intestinal mucosa. PEO alone had no significant effect on GPx enzyme activity (group II) except in liver. Combining EO with vitamin E showed significant increase in GPx activities in all examined tissues fractions (Table 4).

It is well documented that the phase II enzymes viz., GST and GPx utilize GSH as a substrate for detoxifying several carcinogens but in two different mechanisms . GST catalyses the conjugation of GSH to a wide array of electrophiles including carcinogens that ends up in the elimination of such reactive and , noxious moieties. On the other hand, the major role of GPx in cellular defense mechanism is to catalyze the conversion of H_2O_2 into H_2O or fatty acyl hydroperoxides to the corresponding fatty alcohol in the presence of reduced GHS. [14,16,39, 40] . Vitamin E is a well-known antioxidant, which scavenges harmful radicals in a multicomponent system involving GPx [41].

Upon tracking the published data , it was found that the presence of *d*-limonene in the oil could provide a plausible explanation to the obtained results of the current study. These results were in harmony with several investigations reporting the role of *d*-limonene in induction of GST enzyme activity [42,43] . *d*-Limonene has been also reported to inhibit polycyclic aromatic hydrocarbons and nitrosamine induced carcinogenesis in laboratory animals [44]

Table (1): Identified constituents, their relative indices, fragmentation pattern and relative percentage of the peel essential oil of *Citrus deliciosa* Ten.

Component	RI	M^+	BP	Main fragments	Relative %
α-Thujene	913	136	93	121, 105, 91, 77, 65, 53	0.25
α-Pinene	918	136	93	121, 105, 91, 77, 67, 53	0.80
Sabinene	942	136	93	121, 107, 91, 77, 69	traces
β-Pinene	966	136	93	121, 107, 91, 79, 67	0.70
β-Myrcene	989	136	93	121, 107, 91, 97, 69	1.00
α-Phellandrene	1008	136	93	121, 105, 91, 77, 65	traces
α-Terpinene	1014	136	93	121, 105, 91, 77, 65	0.20
P-cymene	1022	134	119	117, 103, 91, 77, 65	40.00
d-Limonene	1027	136	67	121, 107, 93, 79, 67	46.00
γ-Terpinene	1056	136	93	121, 105, 93, 77, 65	9.00
Terpinen-4-ol	1174	154	71	136, 121, 111, 93, 86, 77, 71, 67	0.20
α-Terpineol	1188	UI	59	136, 121, 107, 93, 81, 71, 67	0.24
Trans-carveol	1217	152	109	136, 134, 119, 109, 91, 84	0.10
Thymol	1298	150	135	115, 107, 91, 77	traces
Methyl-N-methyl anthranilate	1403	165	165	150, 132, 116, 105, 91, 77, 66	0.3
β-Caryophyllene	1414	204	91	189, 161, 147, 133, 119, 105, 79, 67	traces
α-Farnesene *	1512	UI	93	135, 123, 119, 107, 91, 97, 69	traces

UI: un-identified

* Tentative

Table (2) : The antimicrobial activity of the peel essential oil of *Citrus deliciosa* Ten

Microorganism		Diameter of inhibition zone (mm)					
		The tested oil	The standard antimicrobial agent				
			AK 30 µg/disc	AMx 25 µg/disc	GM 10 µg/disc	TE 30 µg/disc	NS 100 µg/disc
Bacillus subtilis	(Gram- positive)	16	26	16	20	10	-
Staphylococcus aureus	(Gram- positive)	12	13	20	17	22	-
Escherichia coli	(Gram- negative)	-	18	24	18	14	-
Pseudomonas aeruginosa	(Gram- negative)	-	15	-	20	-	-
Aspergillus niger	(fungi)	19	-	-	-	-	-
Aspergillus flavus	(fungi)	18	-	-	-	-	14
Candida albicans	(fungi)	13	-	-	-	-	16

AK: Amikacin; AMx: Amoxycillin; GM: Gentamicin; TE: Tetracycline; NS: Nystatin -: no detected activity

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Table (3): Effect of peel essential oil of *Citrus deliciosa* Ten. alone and in combination with vitamin E on rat alimentary tract glutathione -s-transferase (GST) (nmol /minute-mg protein) (mean ± SEM)

Group	Stomach	Small intestinal mucosa	Large intestinal mucosa	Liver
Control: cotton seed oil 0.3 ml	420 ± 56	421 ± 55	160 ± 16	770 ± 48
PEO (1000 mg. kg/d) in 0.3 ml cotton seed oil	493 ± 30*	990 ± 75*	239 ± 32	2290 ± 106*
PEO + vit. E (100 mg/kg/d) in 0.3 ml cotton seed oil	594 ± 24*	1055 ± 54*	277 ± 42	2505 ± 160*

*$p < 0.05$ when group II or III was compared with control (group I)

Table (4): Effect of peel essential oil of *Citrus deliciosa* and alone and in combination with vitamin E on rat alimentary tract glutathione peroxidase (GPx) (nmol/minute-mg protein) (mean ± SEM)

Group	Stomach	Small intestinal mucosa	Large intestinal mucosa	Liver
Control: cotton seed oil 0.3 ml	878 ± 50	122 ± 8	107 ± 9	1107 ± 99
PEO (1000 mg .kg/ d) in 0.3 ml cotton seed oil	773 ± 30	119 ± 13	124 ± 12	1508 ± 209
PEO+ vit. E (100mg/kg/d) in 0.3 ml cotton seed oil	1068 ± 64*	146 ± 8*	130 ± 9*	1650 ± 158*

*$p < 0.05$ when group II or III was compared with control (group I)

CONCLUSION

The current study reports the chemical composition of the oil of the peel of *Citrus deliciosa* Ten. which revealed a major share of monoterpenes and the dominance of *d*-limonene followed by *p*-cymene i.e. *d*-limonene / *p*-cymene chemotype. Thus in addition to flavor and aroma, the PEO *Citrus deliciosa* Ten. offers an ideal proposed natural alternative to chemical-based antimicrobials against selected gram positive bacteria and fungi .

The present work also demonstrated through a preliminary screening of the induction of two detoxifying enzymes (GST and GPx) that the PEO possessed a promising chemopreventive and / or chemotherapeutic potential on the rat alimentary tissues. This finding however, awaits further clinical studies before being considered in human application.

To the sum up, the current study offers a low cost, orally bioavailable, less toxic product for pharmaceutical, food, flavor and cosmetic industries out of what is normally regarded as an eco-burden.

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المحتوى الكيميائي والأثر المثبط للميكروبات و الواقي من المسرطنات للزيت الطيار لقشر يوسفي البحر الأبيض المتوسط (سترس دلسيوز/ تينور- العائله السذابيه) الذي ينمو في مصر

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قسم العقاقير والنباتات الطبيه- كليه الصيدله و قسم الكيمياء الحيويه بمستشفيات جامعه الزقازيق•- جامعه الزقازيق- الزقازيق-مصر

في هذا البحث تمت دراسة مكونات الزيت الطيار لقشر يوسفي البحر الأبيض المتوسط (سترس دلسيوزا تينور- العائله السذابيه) باستخدام كروماتوجرافيا الغاز المتصل بمطياف الكتلة.وقد أمكن التعرف الكمي والكيفي على ستة عشرة مكونا تمثل 98.79% من المكونات الكلية للزيت الذي غلبت عليه التربينات الأحادية والتي تمثلت نسبتها الأعلى في دليمونين 46% والبارسيمين 40 % والجاما تربنين 9% فيما بلغت نسبة المركبات الأكسجينية 0.54% ووجدت أثار للسيسكيتربينات وعليه فيعتبر الزيت من النوع: د- ليمونين/ باراسيمين وذو قيمه طبية لمحتواه من الليمونين ذو الفاعلية المختبرة والموثقه.

وقد تم استجلاء الفعالية ضد الميكروبات الموجبة و السالبة الجرام والفطريات فثبت أن للزيت المختبر تأثير مثبط على النوعين الأول والأخير كما وجدت للزيت الطيار فعالية للوقاية من مسرطنات القناة الهضمية منفرداً ومع فيتامين هـ في الاستقصاء المختار لتحفيز انزيم جلوتاثيون- اس- ترانسفيريز منها فيما لم يحفز الزيت منفردا انزيم الجلوتاثيون بر أوكسيديز الا في أنسجه الكبد. هذا ويذكر أن للانزيمين المختبرين علاقه موثقه بكبح المسرطنات الكيميائيه والتخلص الحيوي منها.

وبناء على ماتقدم يعد الزيت المذكور مرشحا مأمولا لتجارب متقدمه في مجال الوقاية والعلاج من سرطان القناة الهضمية إضافة لثبوت كونه مضادا لفطر أسبرجيلس فلافس الضائر صحيا لما يفرزه من ميكوتوكسينات كالأفلاتوكسين ذات التأثير الخطير على صحة الإنسان وبهذا تضاف لقشور النبات المذكور- والتي تعتبر عاده عبء بيئيا- قيمه اقتصاديه في مجالي الدواء والغذاء.