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Screening and Detection of Egyptian *Citrus* Essential Oils Extracted from Food and Juice Industrial Waste Products as Natural Preservatives and Antioxidants in Food Industries.

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Industrial waste products, essential oils, preservatives.

ABSTRACT

Background: Chemical preservatives cause mutagenicity and carcinogenicity and a lot of other side effects in food industries. **The aim of the study:**Screening and detection of Egyptian Citrus essential oils extracted from food and juice industrial waste products as natural preservatives in food industries. **Methodology:** In our screening experimental study, we could extract the essential oils of the waste products of the food and juice industries. They were extracted by the hydrodistillation method, separated and analyzed by gas chromatography and mass spectrometry. We assessed their chemical composition and antioxidant and antimicrobial activities of them with their preservative activity against *Listeria monocytogenes* inoculated in minced beef meat at 4 ^{0}C. **Results:**They showed antimicrobial and preservative effects through the disc diffusion method for screening and assay of their antimicrobial effects in comparison with standard antimicrobial drugs. They were excellent natural preservatives because they are devoid of carcinogenicity. They also, showed moderate antiviral activity against *Herpes viruses* as indicated by the inhibition of the cytopathic effect and viral replication of these viruses. They also showed antioxidant activities and inhibited the development of cancers. **Conclusion:**In our study, we could discover new natural antioxidants, and antimicrobial and preservative compounds obtained from essential oils of *Citrus* industrial waste products.

INTRODUCTION

Micro-organism resistance against current antibiotics represents a seriously irresistible juncture globally (Parveen Kumar, 2017). This necessitates exploring new origins of antibacterial to get over this natural event (Caroline S, Zeind Michael G, 2018). The global difficulty of antibacterial drug resistance makes the demand for antimicrobial berth apparent (Trevor Anthony, Katzung Bertram, Kruidering-Hall Marieke, 2021). The revelation of antibiotics is prodigious of the outstanding advances in medicinal drugs and their usage has substantially diminished mortality and morbidity globally (Olson James, 2020).

Unfortunately, with far-flung antibiotic usage, we have uttered the egress-ion of multi-drug resistant infectious agents and reduced efficacy of numerous of our most potent antibacterials(Bardal Stan, Waechter Jason, Martin Douglas, 2020). In step-up, we have as well acknowledged many adverse effects of antibiotics, to the highest degree notably the ascending rates of *Clostridium difficile* inflammatory bowel disease (Levinson Warren, 2021). Mechanism of bacterial resistance: Bacterial resistance to drugs is mediated by four major mechanisms. (i) The antibiotic is inactivated by enzymes produced by bacteria (cephalosporins and penicillins can be inactivated by beta-lactamases via clearing the beta-lactam ring of the antibiotic (Swanson Larry N, Souney Paul F, Muntnick Alan H, Shargel Leon, 2019). (ii) Modified targets are synthesized by bacteria against which the antibiotic possesses a decreased effect such as the resistance to streptomycin can result from a mutant protein in the 30S ribosomal subunit, as well as, the resistance to erythromycin can result from a methylated 23S ribosomal RNA (Fisher Bruce, Champe pamela, Harvey Richard, 2021). (iii) The permeability to an antibiotic can be decreased by bacteria such that an effective drug intracellular concentration is not reached such as the amount of penicillin entering the bacterial cells is decreased by alterations in porins (Dipro Cecily, Schwinghammer Terry, Dipro Joseph, Well Barbara, 2021). (iv) The antibiotics are actively exported by bacteria using a multi-drug resistance efflux pump. Protons are imported by a multidrug resistance pump (MDR) and a variety of diverse molecules including certain antibiotics such as tetracyclines are exported, in an exchange-type reaction (Golderg Stephen, 2020). A genetic change in bacteria either the acquisition of a plasmid or transposon or a chromosomal mutation causes most antibiotic resistance (Wilson Golder N, 2019).

Disinfectant drugs provide the chief cornerstone of the medical care of microorganism infections (Metting Patricia J, 2019).

Since the disclosure of them and their uses as chemotherapeutic factors There was an impression in the medical assemblage that this would ground to the ultimate obliteration of contagious diseases. Nevertheless, over-exploitation of macrobiotic medicates has become the leading cause for the egress-ion and dispersion of multi-drug resistant strains of various groups of microorganisms (Neelima Mahato *et al.,* 2019). Chemical preservatives used in juice, food and pharmaceutical industries evoked carcinogenicity and mutagenicity (Eman A. Mahmoud, 2017). Earthy sources of preservatives are destitute of mutagenicity and carcinogenicity. *Citrus* fruits are the leading origins of essential oils used in food and medicinal purposes. Plants of *Citrus* are flowing plants that belong to the *Rutaceae* family (Anis Ben Hsouna *et al.,* 2017). In our study, we aimed to overcome this challenge by utilizing the essential oils extracted from Citrus from food industries waste products as natural antimicrobial agents and preservatives.

MATERIALS AND METHODS

Chemicals:

All chemical materials used were of analytical reagent grade. All reagents were purchased from Algomhoria pharmaceutical company in Cairo, Egypt and Alnasr pharmaceutical company in Qalyobia, Egypt.

Place and the Date of The Study:

The present study was carried out in faculty of pharmacy, at Cairo university between February 2021 and March 2022.

Type of Study:

Screening pharmaceutical experimental study.

Collection of the Samples:

Collection of 100 kg of waste products of Citrus food industries from different food and juice industries in Qaha, Qalyobia, Egypt by hand at the beginning of 2021.

Extraction of the Essential Oils:

The oil extraction was obtained by steam distillation for 3 hrs using Cleveger-

type apparatus. we extracted the aqueous phase with dichloromethane and then dried it with anhydrous sodium sulfate. Following the filtration, we eliminated the solvent by pressure distillation than the pure oils were stored at 4 C in obscurity.

Oil(W%/V) = observed volume of oil(ml)/weight of sample(g)*100.

The *Citrus* essential oils were solubilised in n-Hexane for gas chromatography and mass spectrometry analysis.

Equipment:

Table 1. List of instruments.

Instrument	Model and manufacturer
Autoclaves	Tomy, Japan
Aerobic incubator	Sanyo, Japan
Digital balance	Mettler Toledo, Switzerland
Oven	Binder, Germany
Deep freezer -80	Artiko
Refrigerator 5	whirlpool
PH meter electrode	Mettler-toledo, UK
Deep freezer -20	whirlpool
Gyratory shaker	Corning gyratory shaker, Japan
190-1100nm Ultraviolet-visible spectrophotometer	UV1600PC, China
Light(optical) microscope	Amscope 120X-1200X,China

Methods:

Estimation of the Antimicrobial Activity Of Citrus Essential Oils:

In our study we screened and bio-assessed the antimicrobial activity of Citrus herbal drugs on microbes (5 bacteria including *Salmonella typhi, Escherichia coli, Staphylococcus aurus, Listeria monocytogenes and Pseudomonas aeuroginosa* and 2 fungi *Candida Albicans and Aspergillus niger*). Also, we analyzed the chemical composition of herbal Citrus essential oils using gas chromatography/mass spectrometry. Then we Detected and bio-assessed bioactive components responsible for the antimicrobial effects of herbal drugs after their separation by gas chromatography/mass spectrometry through the disc diffusion method. We compared the antibacterial herbal bioactive drugs with the effect of cephalosporin(cefotaxime) and quinolone (ciprofloxacin) antibiotics on the previous bacteria. Also, we added a mixture of the bioactive antimicrobial components of the essential oils with cefotaxime and ciprofloxacin antibiotics and observed the antibacterial activity of combination (synergism, antagonism, or addition) on the previous bacteria. The minimum fungicidal concentrations (MFCs), as well as the minimum inhibitory concentrations (MICs), were determined. The in-situ effect of Citrus essential oils was evaluated through physicochemical parameters (PH and thiobarbituric acid reactive substance (TBARS), and also against *Listeria monocytogenes* in the minced beef meat model.

Estimation of the Antiviral Activity Via the Determination of Viral Nucleic Acids in Food and Juice Samples:

Nucleic acids of viruses, either the viral mRNA or viral genome, could be noticed in the juice and food samples with complementary RNA or DNA (cRNA or cDNA) like a probe.

Estimation of the Antioxidant Activity of *Citrus* Essential Oils:

This was determined according to the beta-carotene bleaching assay and by 2.2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity.

Antioxidant Testing Assays:

DPPH Radical Scavenging Activity:

Using DPPH radical as a reagent, the Radical scavenging activity of the different fractions was determined. Briefly, 600 µl of sample solutions (different concentrations) was blended with 2 ml of a 5% (w/v) DPPH radical solution in ethyl alcohol. For 30 minutes in the dark at room temperature, the inter-mixture was incubated. Scavenging capacity was interpreted using UV spectrophotometer by observing the drop-off of the optical density at 517 NM. Higher free radical scavenging activity was indicated by Lower absorbance of the mixture of reaction. Ascorbic acid was utilized as a standardized antioxidant. According to the formula: DPPH radical scavenging activity (%) = $[(OD_{blank} - OD_{sample})/ OD_{blank}] \times 100$, DPPH radical scavenging action was measured. OD_{blank} was the optical density of the control reaction bearing all reagents. OD_{sample} was the optical density of the tested compound. Extract

concentration rendering fifty percent inhibition (IC50) was measured from the graph plotting extract concentration against inhibition percentage. Tests were performed in triplicates.

β-Carotene Bleaching Assay:

The antioxidant action was dictated accordant to the β-carotene bleaching assay. We prepared a stock solution of linoleic/ β-carotene acid inter-mixture as postdate: 0.6 mg of β-carotene was liquefied in 1.1 ml of chloroform and 190 mg of Tween-20 with 26 µl of linoleic acid. Chloroform was wholly evaporated, utilizing an evaporator of vacuum. And so, we saturated 100 ml of distilled water with oxygen and the acquired solution was smartly agitated. 5 ml of that reaction inter-mixture was distributed into test tubes and 199 µl of each sample, processed at various concentrations, was added. The emulsion system was incubated for 1 h at 45 °C. We repeated the identical procedure with a blank as a negative control and Butylated hydoxytoluene (BHT) as a positive control. Later this incubation period, the optical density of each mixture was calculated at 480 nm. The activity of antioxidants in β-carotene bleaching form in percentage (A %) was measured with the succeeding equation: A % = 1 − (A0 – At/A' 0−A' t) × 100, where A0 and A'0 were optical densities of the blank and the sample, respectively, calculated at zero time, and A t and A't were optical densities of the sample and the blank, respectively, measured after 1 h. Every test was performed in triplicates.

Gas Chromatography/Mass Spectrometry (GC-MS):

GC-MS was used to determine and identify the major constituents of the extracted *Citrus* essential oils.

Formulation of the Essential Oils of *Citrus* as Topical Oil In Water(O/W) Emulsions:

Medication Order: Essential oils of *Citrus* 20 ml; Acacia q.s.;Distilled water, q.s. a.d. 90 ml and Sig: 1 tablespoon q.d.

Manufacturing Procedure: An initial emulsion (primary emulsion) was settled with the dry gum method, utilizing one part (4.5 g) of powdered acacia, two parts (7 ml) of water, and four parts (20 ml) of oils. In a Wedgwood mortar, the acacia was emulsified with mineral oil.

The e7 ml of water was added all at one time and, with fast broiement, settled the primary emulsion, which for about 5 mins was triturated. The leftover water was merged in small amounts with broiement. To a 90-mL prescription bottle, the emulsion was transferred and to the container, a "shake well" label was affiliated.

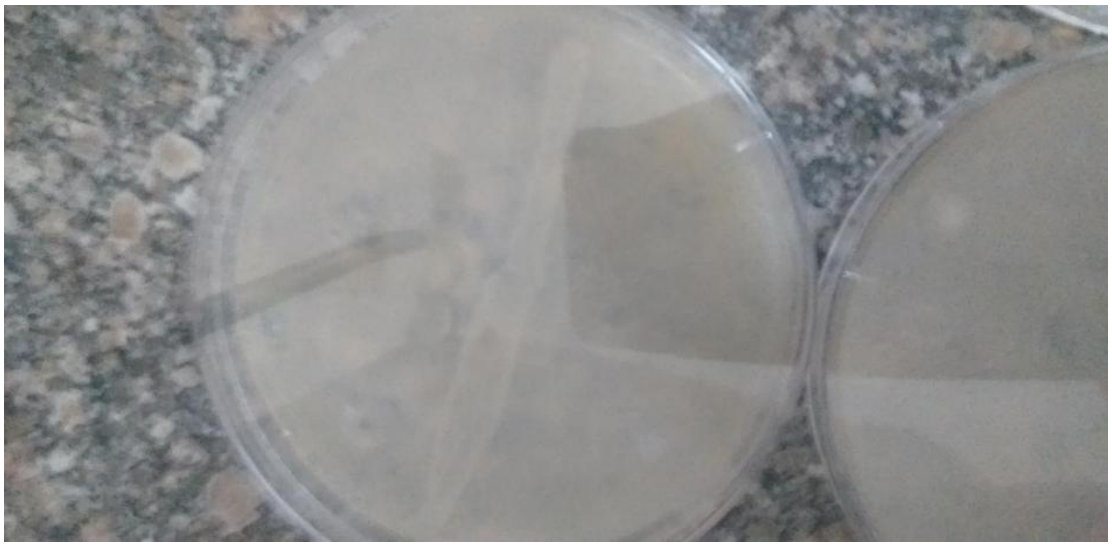
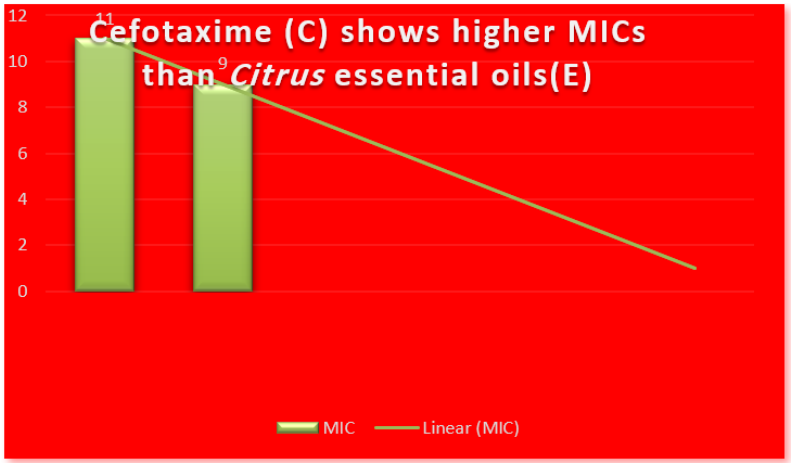


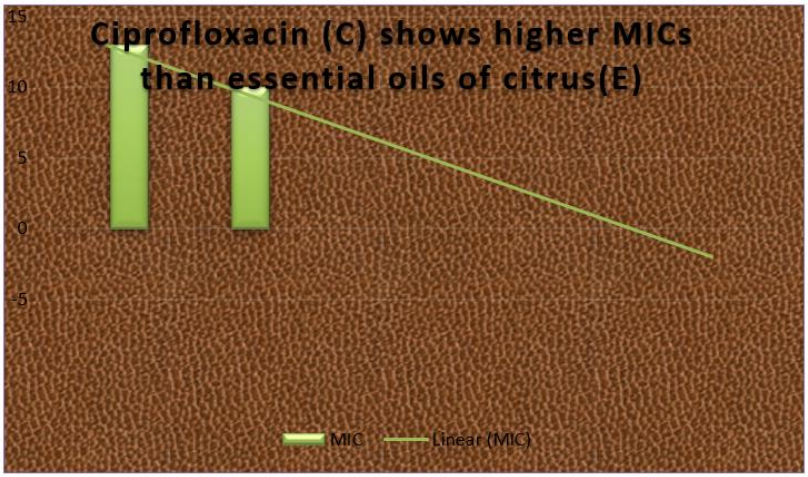
Fig. 1. It shows the antimicrobial effect of Egyptian Citrus essential oils against *Eschrechia coli*.

Statistical Analysis:

All cultures were conducted in triplets. Their presentation was by means and standard deviation. One way analysis of variance (p value≤.05) was used as means for performing statistical analysis and also, statistical analysis based on excel-spreadsheet-software. F-test was utilized in this study.



Graph 1. It represents MIC of cefotaxime(C) versus essential oils of Citrus(E) against Listeria monocytogenes.



Graph 2. It represents MIC of ciprofloxacin(C) versus *Citrus* essential oils(E) against *Listeria monocytogenes*.

RESULTS

Thirty-four components were identified in the Citrus essential oils and the two dominant components were limonene (29.68%) and beta-pinene (21%). These compounds displayed an excellent scavenging DPPH ability with an extract concentration providing 50% inhibition (IC50) of 19.073 microgram/milliliter and a strong beta-carotene bleaching inhibition after 94 minutes of incubation with an IC50 of 32.89 microgram/milliliter. Zones of inhibition ranged from 21.4 to 24.6 mm in diameter. The minimum inhibitory concentrations varied from 0.051 to 1.42 mg/ml for Gram-positive and Gram-negative bacteria and fungi. The meat-preserving potential of Citrus essential oils was investigated against

Listeria monocytogenes.They successfully inhibited the arising of *Listeria monocytogenes* in minced beef meat at concentrations 0.045 and 0.299 mg/g when stored at 4 C.Additionally during the storage

period, physicochemical values (PH and TBARS) were higher in control meat than treated meat with *Citrus* essential oils suggesting an efficient antioxidant activity of Citrus essential oils. They showed no carcinogenicity or mutagenicity while chemical preservatives showed mutagenicity and carcinogenicity.They showed antiviral activity by the inhibition of the cytopathic effect of *Herpes simplex* viruses. Figure 1 represents antimicrobial activity against *E.coli.* Graphs 1 and 2 represents MIC of essential oils of Citrus compared to quinolone and cephalosporin antibiotics respectively.

Tables [2 and 3] represent MICs of cefotaxime and ciprofloxacin versus essential oils of *Citrus*(E) against *Listeria monocytogenes*.

Table 2. It represents MIC of cefotaxime(C) versus essential oils of *Citrus*(E) against *Listeria monocytogenes*.

	C	E
MIC	11	9

Table 3. It represents MIC of ciprofloxacin(C) versus *Citrus* essential oils(E) against *Listeria monocytogenes***.**

	C	E
MIC	13	10

DISCUSSION

Anti-microbial Activity of Egyptian *Citrus* Essential Oils Extracted from Food and Juice Industries:

Anti-microbial activity o was due to limonene, alpha and beta-pinene derivatives with limonene being the main essential oil responsible for antimicrobial activity against various pathogens. Essential oils were aromatic compounds.

Antibacterial and Anti-Fungal Activities Determination:

This was done by determining MIC against the test bacterial and fungal pathogens by the measurements of zones of inhibition which ranged from 21.4 to 24.6 mm in diameter.

Synergism and Antagonism:

The bioactive components showed synergism in antibacterial activity with quinolone and cephalosporin antibiotics.

Antiviral Activity Determination:

The bio-active components showed moderate antiviral activity against different viruses such as *Herpes* viruses. This was revealed by inhibition of the cytopathic effect and inhibition of viral replication. They were ineffective as antiviral agents against SARS-COV2 and *influenza* viruses.

Determination of Preservative Activity:

The essential oils of C*itrus* waste products extracted from food and juice industries were excellent natural preservatives without inducing mutagenicity or carcinogenicity. This was compared with standard chemical preservative drugs such as benzoic acid. Chemical preservatives induced mutagenicity and carcinogenicity effects because their sources were not natural but the Citrus essential oils were derived from natural sources.

Determination of the Anti-Oxidant Activity:

They showed superior antioxidant activity to standard chemical preservatives and also prevented the development of different types of cancers.

Formulation of the Main Antimicrobial Bio-Active Components of The Essential Oils of Citrus as Topical Oil in Water(O/W) Emulsions:

They were able to show excellent antimicrobial activity as compared to standard topical antimicrobial emulsions against burns, wounds, different topical fungal infections such as *Candida albicans* and topical viral infections such as herpes simplex virus type 1 and 2 infections.

Comparison with the Previous Study:

In a comparison with a previous study (Karoui and Marzouk,2013) conducted in Tunisia. The previous study declared that the essential oils of *Citrus* had fewer antioxidant and antimicrobial actions than standard antioxidants and antimicrobial drugs, while our present study displayed higher antioxidant

and antimicrobial activities than standard ones.

Conclusion:

Citrus waste products collected from different food industries in Egypt showed natural preservatives without inducing carcinogenicity or mutagenicity and antimicrobial activities and synergism with other antimicrobial agents. In our study, we could overcome the multi-drug resistance due to mutant strains of pathogens. we recommend other studies to determine the optimal doses and formulations of bio-active components of Citrus essential oils as natural preservatives and antimicrobial agents.

Conflict of interest: There is no conflict of interest.

Fund: This study was carried out in a research project number 46362/2021 funded by STDF.

Data availability: Raw data were generated at faculty of pharmacy, Cairo university,Egypt. Derived data supporting the findings of this study are available from the corresponding author Dr. Mohammed Kassab up on request.

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ARABIC SUMMARY

فحص وكشف الزيوت العطرية من الحمضيات المصرية المستخلصة من منتجات المخلفات الصناعية للأغذية والعصائر كمواد حافظة طبيعية ومضادات للأكسدة في الصناعات الغذائية

محمد كساب
قسم علوم المايكروبيولوجي والمناعة كلية الصيدلة جامعة القاهرة

خلفية:تتسبب المواد الحافظة الكيميائية في حدوث الطفرات الجينية والسرطنة والعديد من الآثار الجانبية الأخرى في الصناعات الغذائية.
الهدف من الدراسة:فحص وكشف الزيوت العطرية من الحمضيات المصرية المستخرجة من منتجات المخلفات الصناعية للأغذية والعصائر كمواد حافظة طبيعية في الصناعات الغذائية.
المنهجية:في دراستنا التجريبية للفحص ، يمكننا استخراج الزيوت الأساسية من نفايات صناعات الأغذية والعصائر. تم استخلاصها بطريقة التقطير المائي ، وتم فصلها وتحليلها بواسطة كروماتوجرافيا الغاز وقياس الطيف الكتلي. قمنا بتقييم تركيبها الكيميائي وأنشطتها المضادة للأكسدة والميكروبات مع نشاطها الوقائي ضد ليستريا مونوسيتوجينز الملقحة في لحم البقر المفروم عند 4 درجة مئوية.
نتائج :أظهروا تأثيرات مضادات الميكروبات والمواد الحافظة من خلال طريقة انتشار القرص لفحص ومعايرة تأثيراتهم المضادة للميكروبات مقارنة بالأدوية القياسية المضادة للميكروبات. كانت مواد حافظة طبيعية ممتازة لأنها خالية من المواد المسببة للسرطان. كما أظهروا نشاطًا معتدلًا مضادًا للفيروسات ضد فيروسات الهربس كما يتضح من تثبيط تأثير الاعتلال الخلوي والتكاثر الفيروسي لهذه الفيروسات. كما أظهروا أنشطة مضادة للأكسدة وأعاقوا تطور السرطانات.
استنتاج:في دراستنا ، تمكنا من اكتشاف مضادات أكسدة طبيعية جديدة ومركبات مضادة للميكروبات والمواد الحافظة تم الحصول عليها من الزيوت الأساسية لمنتجات النفايات الصناعية من الحمضيات.