Egypt. J. Chem. **Vol. 66**, No. SI: 13 pp. 21 - 37 (2023)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Phytochemical Study, Antioxidant Potential and Preparation of a Clove Nanoemulsion Loaded with Pomegranate Peel Extract

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Abstract

The aim of this study was to investigate the phytochemical composition and antioxidant activity of pomegranate peel and chicory leaf extracts as well as clove and cinnamon essential oils (EOs). This is the first report for the preparation and characterization of nanoemulsion of clove EO loaded with pomegranate peel extract. The chemical composition of clove and cinnamon EOs was identified by GC-MS analysis, while phenolic and flavonoid compounds in pomegranate peel and chicory leaf extracts were evaluated by HPLC. The antioxidant activity test was determined using the DPPH and ABTS methods. A clove oil nanoemulsion (O/W) was prepared and characterized and used for the development of a clove oil-based nanoemulsion loaded with pomegranate extract. According to GC-MS analysis, most of the cinnamon EO contains of (E)-cinnamaldehyde (62.4%), benzyl alcohol (25.29%) and methoxyacetic acid, benzyl ester (10.71%), while most of the clove-EO contains of eugenol (79.26%), triacetin (11.72%) and benzyl alcohol (6.76%). As shown by the HPLC results, the pomegranate peel extract contained ten phenolic components, while the Cichorium leaf extract contained a total of fifteen phenolic compounds in varying amounts. The total phenolic content of pomegranate peel extract, chicory leaf extract, and clove oil and cinnamon oil was 34.4, 17.8, 36.6 and 22.5 mg per 100 g dry weight (DW) or 100 g oil, respectively. The total content of flavonoids is 161.4, 64.5, 15.4 and 11.1 mg per 100 g DW or oil, respectively, expressed in units of quercetin equivalents (QE). Results showed that pomegranate peel extract and clove oil had the highest levels of phenols and flavonoids and high DPPH and ABTS inhibition and scavenging activity (IC50, g/mL) 298.6, and 327.2 µg/ml of DPPH and 97.1 and 74.7 µg/ml of ABTS, respectively. In this study, we prepared a clove oil nanoemulsion using a self-emulsification method with Tween 80 as a surfactant. We have optimized the oil to surfactant ratio and the sonication time to obtain the best nanoemulsion with a droplet size of 155.2 nm and good physicochemical properties. These results suggest that nanoemulsions based on pomegranate peel extract and clove oil are good sources of natural antioxidants and can be used to protect humans from the toxicity of xenobiotics such as pesticides and heavy metals.

Keywords: antioxidant, DPPH, ABTS, phytochemical, GC-MS, HPLC, nanoemulsion, clove oil, pomegranate

1. Introduction

Free radicals (FRs) are unstable molecules with an unpaired electron, short lived and highly reactive. They are extremely reactive and can damage tissues and cells. They can be formed naturally by the body through metabolism or generated externally through exposure to pollutants, radiation and cigarette smoke[1]. FRs can damage various cellular components such as DNA, proteins and lipids by stealing electrons from them and disrupting cellular cell membranes, leading to cell death [2], [3]. The mechanism by which free radicals produced disease and toxicity involved several steps. Free radicals first cause a cascade of lipid peroxidation in cell membranes that alters the fluidity and permeability of the membranes [4]. Second, free radicals alter gene

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EJCHEM use only: Received date 28 May 2023; revised date 10 July 2023; accepted date 16 July 2023
DOI: 10.21608/EJCHEM.2023.213866.8034

expression and damage DNA in the mitochondria and nucleus, leading to mutations. Third, free radicals affect cellular signaling and metabolism by altering the structure and functionof proteins, enzymes, and receptors. Fourth, free radicals cause apoptosis or necrosis by disrupting the balance of calcium ions in cells [1], [5].

There are a number of treatments that can scavenge free radicals and reduce their risk and damage. One way is to eat a diet rich in antioxidants, such as plants like fruits, vegetables and whole grains that can neutralize free radicals. Over recent decades, it has been widely accepted that plants have positive medical benefits [6], [7]. The pharmaceutical industry uses around 20% of all known plants, which has a good influence on health care by helping to treat hazardous diseases like cancer [8], [9]. Plants can produce a broad spectrum of bioactive components that act as antioxidants and can protect against free radical damage and pesticide-induced oxidative damage [8], [10]. In contrast to synthetic compounds, there has recently been an increase in interest in natural plant metabolites compounds for industrial and pharmacological usage [11][12][13].

The pomegranate peel (*Punicagranatum* L.) is a rich source of bioactive metabolites that have antioxidant and anticancer properties [14], [15]. The peel contains various phytochemicals, such as ellagic acid, gallic acid, anthocyanins, and tannins, that can modulate cellular signaling pathways and induce apoptosis in cancer cells [16][17]. Another plant with high therapeutic potential is chicory (*Cichoriumintybus* L.), which has a wide range of bioactive substances, such as alkaloids, flavonoids, saponins, and tannins [18], [19]. Chicory is mainly composed of inulin, a prebiotic fiber that can improve gut health and lower blood glucose levels. Fresh chicory has 68% inulin, 14% sucrose, 5% cellulose, 6% protein, 4% ash, and 3% other compounds, whereas dry chicory has 98% inulin and 2% other compounds [20].

Essential oils (EOs) are concentrated plant extracts that have various health benefits. They can be used in aromatherapy or applied to the skin with a carrier oil. Some of the most studied and effective EOs include clove, cinnamon, and lavender [21][22]. Clove EO is one of the most potent antioxidant and free radical scavengers of all EOs. It contains high levels of polyphenolic compounds like eugenol, which may protect the liver from oxidative damage and inflammation. Clove oil also has anti-inflammatory, antibacterial, antiviral, and antifungal properties that can help with infections and wounds [21]–[26]. Cinnamon (*Cinnamomumzeylanicum*) EO is another powerful EO that has multiple biological effects. It can inhibit the growth of bacteria and fungi such as *Escherichia coli* and *Candida albicans* and modulate the immune system. Cinnamon oil also has an antioxidant and antidiabetic effect as it can lower blood sugar and cholesterol levels. In addition, cinnamon oil has been shown to have nematicidal, insecticidal, anticancer and anti-inflammatory effects thanks to its main component, trans-cinnamaldehyde and other bioactive substances [27]–[31].

One of the major challenges in using EOs and plant bioactive compounds for therapeutic purposes is their low aqueous solubility, stability, bioavailability, and high susceptibility to first-pass metabolism. These factors reduce their therapeutic potential and limit their clinical application. Nanoemulsions using is a promising strategy to overcome these limitations and improve the delivery and absorption of bioactive components. Nanoemulsions can improve various pharmacokinetic properties and increase the therapeutic value of phytochemicals, as previous studies have shown [32]–[34]. Nano-phyto-ingredients have been shown to be more potent than native phyto-ingredients in terms of bioavailability and therapeutic efficacy [35]. Therefore, nanoemulsions can be a useful approach to increase the bioavailability and absorption of beneficial compounds [36], [37].

The objective of this study was to investigate the phytochemical composition and antioxidant potential of pomegranate peel and chicory leaves extracts and Clove and Cinnamon essential oils that have been reported to have health benefits. Furthermore, a novel pomegranate peel extract-loaded clove nanoemulsion was prepared and characterized for its physicochemical properties and antioxidant activity.

2. Materials and methods

1.1. Chemicals and reagents

Tween 80, also known as Polysorbate 80, was purchased from VWR International, located at 201 Rue Carnot in Fontenay/Bois, France. The ascorbic acid was supplied by Sigma-Aldrich Chemie, Steinheim, Germany. DPPH (2,2′-

diphenylpicrylhydrazyl) and ABTS (2,2 azinobis-(3-ethylbenzthiazoline-6-sulphoric acid)) were purchased from Sigma, St. Louis, Missouri, UAS. Other chemicals were purchased from local scientific suppliers in Egypt and Sigma-Aldrich.

1.2. Essential oils and plant extractions

Clove and Cinnamon essential oils were extracted from the NRC oil extraction facility in Dokki, Cairo, Egypt. Both dried ground pomegranate peel (*Punicagranatum*) and dried ground chicory leaves (*Cichoriumintybus*) were obtained from the local market in Cairo, Egypt. Plant materials were peeled, crushed and hydrodistilled using a Clevenger apparatus to obtain the EOs. The EOs were separated, dried over anhydrous sodium sulfate (Na_2SO_4) solution, placed in an amber glass flask and stored undercooled until use. To obtain plant extracts, dried, ground plant material was soaked in ethanol (70%) in a weight ratio of 1:3 for two hours, filtered and the residue extracted twice more under the same circumstances. Combine and condense the obtained extracts using a rotary evaporator at 40 °C

1.3. GC-MS analysis of EOs

Gas chromatography-mass spectrometry (GC-MS) analysis was used to identify the volatile components of clove and cinnamon EOs. The gas chromatograph THERMO TRACE 2000 and the mass spectrometer FINNIGAN SSQ 7000 (GC-MS) were used. A quartz glass capillary column (DB-5MS; 5% phenyl, 95% methylpolysiloxane) with 30 m×0.25 mm×0.25 m internal diameter was installed in the GC (internal diameter). The carrier gas helium was used at a rate of 1 ml/min. injector volume, 1 l; injector temperature, 220; Column temperature, isotherm at 40 °C for 3 minutes, then programmed to ramp to 250 °C at 5 °C/minute; held at this temperature for 3 min; and ion source temperature, 250 . The GC column effluent was introduced directly into the source of the MS. The MS was used under the following conditions: ionization voltage of 70 eV, 0.5 second scan period, and 40-400 m/z range. By comparing the fragmentation pattern of the spectrum to the patterns found in the Wiley Mass Spectral Library data, several oil compounds were identified.

1.4. HPLC analysis of phenolic contents

High performance liquid chromatography (HPLC) analysis was performed using an Agilent 1260 series instrument. An Eclipse C18 column with an internal diameter of 4.6 mm x 250 mm and a length of 5 m was used for the separation. Water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) were the constituents of the mobile phase, which had a flow rate of 1 mL/min. The following linear gradient was used to program the mobile phase in the order: 0 min (82% A), 0–5 min (80% A), 5–8 min (60% A), 8– 12 min (60% A).), 12-15 minutes (85% A) and 15-16 minutes (82% A). At 280 nm the multi-wavelength detector could be seen. A 10 L injection volume was used for each sample solution. The column was maintained at a constant temperature of 35°C. Caffeic acid, syringe acid, catechol, rutin, ellagic acid, coumaric acid, vanillin, ferulic acid, naringenin, taxifolin, cinnamic acid, and kaempferol have been used as standards for phenolic compounds.

1.5. Phytochemical study

2.5.1 Total phenolic content

Total phenolic content (TPC) was determined using the method cited in previous studies[38], [39]. 1 mL of freshly prepared (1:10 v/v) Folin-Ciocalteu reagent was combined with 200 µL of each extract or EO (3 mg/mL in water or DMSO) and shaken for 30 seconds before incubating at room temperature in the dark for 5 minutes. Then 0.8 ml of sodium carbonate (7.5% w/v) was added to the mixture, shaken for 30 seconds and allowed to stand at room temperature for 15 minutes. A spectrophotometer at 765 nm (Shimadzu UV-VIS Recording 2401 PC, Japan) was used to measure the absorbance. A standard curve for gallic acid was constructed using regression solution concentrations of 2, 4, 8 and 12 g/mL in distilled water. The TPC was expressed in mg gallic acid equivalent/100 g DW extract.

2.5.2 Total flavonoids content

Total flavonoid (TF) content was determined using the method cited in previous studies [38], [39]. TF was determined by mixing 0.5 mL of plant extracts or EOs (3 mg/mL in water or DMSO) with 0.5 mL of 2% AlCl3 (w/v) and incubating in the dark for 15 min. The absorbance at 415 nm was then measured as a blank value in comparison with water

using a spectrophotometer. Using a standard calibration curve of quercetin, results were expressed as quercetin equivalents (QE)/g DW.

1.6. In vitro antioxidant activity study

1.6.1. DPPH radical scavenging activity

The scavenging activity of selected extracts and EOs was determined using DPPH scavenging ability using the method cited in previous studies [38], [39]. Plant extracts, EOs or standards (vitamin C and BHT) at concentrations ranging from 5 g/mL to 1 mg/mL were combined with 0.5 mL of 0.1 mM DPPH in methanol, mixed well and left at room temperature for 30 minutes in the dark, then the absorbance was determined at 520 nm. As a control, methanol and DPPH radical absorption were measured with no sample. Ascorbic acid was chosen as the standard for polar plant extracts, while butylatedhydroxytoluene (BHT) served as the standard for non-polar essential oils.

The following equation was used to calculate the scavenging ability of the samples, presented as percent inhibition of DPPH:

$$I\ (\%) = [(A_c\text{-}A_s)/A_c] \times 100$$

Where Ac and As represent the sample's and the control's absorbance's.

1.6.2. ABTS radical scavenging activity

The radical scavenging capacity of the selected plant extracts and essential oils was determined using ABTS [39], [40]. 2.45 mM potassium persulfate was prepared by dissolving 0.0066 g in 10 ml distilled water and 0.0384 g ABTS was used to prepare the ABTS cation (7 mM). Combine the two solutions in equal amounts, then leave in the dark at room temperature for 12 to 16 hours. When stored in the dark, the radical is stable for two days. ABTS cation (7 mM) was prepared by dissolving 0.0384 g ABTS in 10 ml distilled water and 2.45 mM potassium persulphate by dissolving 0.0066 g in 10 ml distilled water. Mix equal amounts of the two solutions and keep in the dark at room temperature for 12-16 hours. The radical in this case was stable for two days when kept in the dark. The working solution of the ABTS cation (at an absorbance of 0.700 0.02 at 734 nm) was prepared with distilled water. 1 mL of ABTS solution was added to 1 mL of various plant extracts, EOs, ascorbic acid and BHT concentrations ranging from 5 g/mL to 1 mg/mL and the mixture was left to stand in the dark at room temperature for 30 minutes before measuring at 734 nm. ABTS without sample was used as a control. The following equation was used to calculate the antioxidant scavenging activity of plant extracts, EOs, and the standard as a percentage of inhibition:

ABTS scavenging activity (%) = $([A_{control} - A_{sample})/A_{control}] \times 100$

Where $A_{control}$ is absorbance of the control and A_{sample} is the absorbance of the sample.

Due to their powerful antioxidant effects, pomegranate peel extract and clove essential oil were selected for further studies to develop a nano-formulation.

1.7. Preparation of clove oil nanoemulsion

Clove oil was formulated with deionized water and the nonionic surfactant polysorbate 80 (Tween 80) at a concentration of 10% into an oil-in-water nanoemulsion (O/W). (D.W.)[41], [42]. The aqueous phase was stirred at 400 rpm for 30 minutes while the organic phase (oil and surfactant) was added. The resulting emulsion was subjected to ultrasonic treatment (Sonics & Materials, INC. 53 Church Hill Rd. Newtown, CT, USA) with a probe diameter of 13 mm at a high frequency of 20 kHz and 750 W for different periods of time (2.5 minutes). , 5 mins and 10 mins). The organic phase was prepared with clove oil and Tween 80 in different weight ratios (1:1, 1:2 and 2:1). The energy was delivered with a sonicator probe and reduced with ice.

1.8. Nanoemulsion physicochemical and stability study

Clove nanoemulsion stability and physico-chemical experiments were performed according to standard protocols [42]–[44]. The stability of the clove nanoemulsions of samples A1 to C3 was investigated using different stresses including centrifugation, heating, cooling and freezing cycles. Eventually, phase separation or creaming was observed when using the stable nanoemulsion formulation. Two nanoemulsions, samples A3 and B3, were found to be stable according to the physico-chemical test of the study, therefore these formulations were subjected to a droplet size determination (samples A3 and B3).

1.9. Characterization of nanoemulsion

1.9.1. Droplet size and zeta potential

Using a dynamic light scattering instrument (DLS) (PSS, Santa Barbara, CA, USA) at 23 °C, the droplet size was determined at the 632.8 nm line of a He-Ne laser projected as incident light with an angle of incidence of 90 acted. The zeta potential was examined at an external angle of 13.9.

1.9.2. Transmission electron microscopy (TEM)

Transmission electron microscopy was used to examine the morphology of the clove nanoemulsion (model JEM-1230, Jeol, Tokyo, Japan). A drop of carnation nanoemulsion (Sample B3) was diluted with deionized water before being placed on a carbon-covered copper grid and stained with a phosphotungstic acid solution (2%, pH = 6.7) for one minute. The replica was lifted to dry at 27°C and the image was viewed with a TEM at an accelerating voltage of 80 kV.

2. Results and discussion

Plants are rich sources of natural antioxidants that can protect humans from oxidative stress and chronic diseases. Among the bioactive compounds found in plants, phenols are of particular interest as they possess a hydroxyl group that confers on them antioxidant activity, making them versatile molecules for therapeutic and preventive applications [45]–[47]. In this study, we aimed to evaluate the phytochemical composition and antioxidant potential of pomegranate peel and chicory leaf extracts, as well as clove and cinnamon essential oils, which have been shown to have health benefits. In addition, we prepared and characterized a novel nanoemulsion containing pomegranate peel extract and clove essential oil and evaluated its physicochemical properties and antioxidant activity.

The chemical composition of clove and cinnamon oil was identified by GC-MS analysis (Figure 1).Table 1 shows that the main components of cinnamon EO are (E)-cinnamaldehyde (62.4%), benzyl alcohol (25.29%), methoxyacetic acid, benzyl ester (10.71%), (Z)-3-phenylacrylaldehyde (0 .99%), and phenol, 2-methoxy-4-(2-propenyl)-acetate (0.24%). These results agree with the previous study by Behbahani*et al.*[48], who reported that the main components of cinnamon essential oil (*Cinnamomumzeylanicum*) are (E)-cinnamaldehyde (71.50%), linalool (7.00%), -caryophyllene (6.40%)), eucalyptol (5.40% and eugenol (4.60%). In addition, several other studies have confirmed that cinnamaldehyde is the main chemical component of essential oils [49], [50]. The GC-MS analysis of clove oil revealed that it contained eugenol (79.26%), triacetin (11.72%), benzyl alcohol (6.76%), 1-propanol, 3,3'-oxybis-(0.78 %), 1-propanol contains , 2-(2-hydroxypropoxy)- (1.3%) and 7-octen-2-ol, 2,6-dimethyl (0.17%) (Table 2). This is stated in the Consistent with the results of other studies that have also reported that eugenol is the main component of clove EO [51], [52].

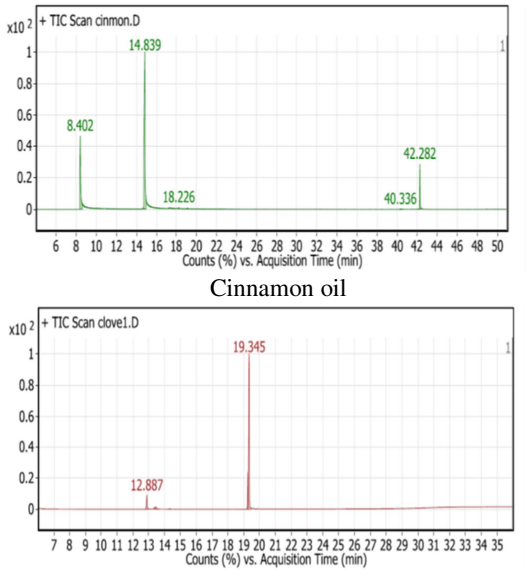


Figure 1: GC-MS chromatogram of cinnamon and clove oils

The phenolic compounds in Cichorium leaf and pomegranate peel extracts were analyzed by HPLC and compared to 16 reference phenolic compounds. The reference phenolic compounds were gallic acid, chlorogenic acid, catechin, methyl gallate, caffeic acid, syringe acid, catechol, rutin, ellagic acid, coumaric acid, vanillin, ferulic acid, naringenin, taxifolin, cinnamic acid and kaempferol. They were detected at a wavelength of 280 nm (Figure 2). The HPLC results showed that pomegranate peel extract contained ten phenolic compounds (Table 3). The most abundant phenolic compounds were gallic acid (59.534 mg/g), catechin (83.312 mg/g), naringenin (7.254 mg/g), syringic acid (1.316 mg/g) and methyl gallate (1.133 mg/g). Other phenolic compounds present at lower concentrations were caffeic acid (852.59 g/g), ellagic acid (34.302 mg/g), vanillin

(487.19 g/g), taxifolin (182.53 g/g) and cinnamic acid (38, 78g/g). These results are consistent with previous studies that found gallic acid to be the main phenolic compound in pomegranate peel [53]. In addition, pomegranate peel contains significant amounts of organic and phenolic acids, the flavonoids anthocyanins, catechins and other complexed flavonoids, as well as punicalin, pedunculagin, punicalagin, gallic and ellagic acids. [54], [55]. The cichorium extract contained fifteen phenolic compounds with different concentrations. The most abundant ones were gallic acid (1.026 mg/g), chlorogenic acid (2.758 mg/g), pyro catechol (1.427 mg/g), rutin (4.770 mg/g), ellagic acid (1.247 mg/g), and naringenin (1.478 mg/g). Other phenolic compounds present in lower amounts were catechin (103.23 µg/g), methyl gallate (12.83 µg/g), coffeic acid (259.92 µg/g), coumaric acid (800.57 µg/g), vanillin (3.62 µg/g), ferulic acid (115.99 µg/g), taxifolin (137.00 µg/g), cinnamic acid (36.54 µg/g) and kaempferol (7.62 µg/g) as shown in Table 4. According to previous study, chicory leaf extract is rich in phenolic compounds, which are bioactive substances with antioxidant and anti-inflammatory properties [56]–[58]. However, the phenolic content and composition of plants can vary widely depending on various factors, such as cultivar, soil type, environmental conditions, harvest timing and post-harvest processing methods [59]. Therefore, it is important to consider these factors when evaluating the phenolic profile and potential health benefits of chicory leaves.

Table 1

GC-MS analysis of cinnamon essential oil

Peak	RT	Name	Formula	Area (%)
1	8.402	Benzyl alcohol	C_7H_8O	25.29
2	14.839	(E)-cinnamaldehyde	C_9H_8O	62.4
3	17.276	(Z)-3-Phenylacrylaldehyde	C_9H_8O	0.99
4	18.226	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	$C_{12}H_{14}O_3$	0.24
5	19.079	2-Propenoic acid, 3-phenyl-, 1-ethenyl-1,5-dimethyl-4-hexenyl ester	$C_{19}H_{24}O_2$	0.21
6	40.336	Benzenepropanenitrile	C_9H_9N	0.17
7	42.282	Methoxyacetic acid, benzyl ester	$C_{10}H_{12}O_3$	10.71

RT: retinal time

Table 2

GC-MS analysis of clove oil

Peak	RT	Name	Formula	Area (%)
1	12.9	Benzyl alcohol	C_7H_8O	6.76
2	13.4	1-Propanol, 3,3'-oxybis-	$C_6H_{14}O_3$	0.78
3	13.5	1-Propanol, 2-(2-hydroxypropoxy)-	$C_6H_{14}O_3$	1.3
4	14.3	7-Octen-2-ol, 2,6-dimethyl-	$C_{10}H_{20}O$	0.17
5	19.3	Triacetin	$C_9H_{14}O_6$	11.72
6	19.3	Eugenol	$C_{10}H_{12}O_2$	79.26

RT: retinal time

Table 3

HPLC analysis of phenolic compounds in pomegranate extract

No.	Retention Time (min)	Compound	Concentration (µg/g)
1	3.108	Gallic acid	59534.03
2	4.265	Catechin	83312.11
3	4.829	Methyl gallate	1133.39
4	5.424	Coffeic acid	852.59
5	5.831	Syringic acid	1316.74
6	7.625	Ellagic acid	34302.91
7	8.806	Vanillin	487.19
8	9.941	Naringenin	7254.26
9	12.122	Taxifolin	182.53
10	13.264	Cinnamic acid	38.78

Table 4

HPLC analysis of phenolic compounds in the cichorium

No.	Retention Time (min)	Compound	Concentration (µg/g)
1	3.108	Gallic acid	1026.20
2	3.797	Chlorogenic acid	2758.27
3	4.265	Catechin	103.23
4	4.829	Methyl gallate	12.83
5	5.424	Coffeic acid	259.92
6	6.174	Pyro catechol	1427.32
7	7.126	Rutin	4770.66
8	7.625	Ellagic acid	1247.47
9	8.103	Coumaric acid	800.57
10	8.806	Vanillin	3.62
11	9.465	Ferulic acid	115.99
12	9.941	Naringenin	1478.48
13	12.122	Taxifolin	137.00
14	13.264	Cinnamic acid	36.54
15	14.055	Kaempferol	7.62

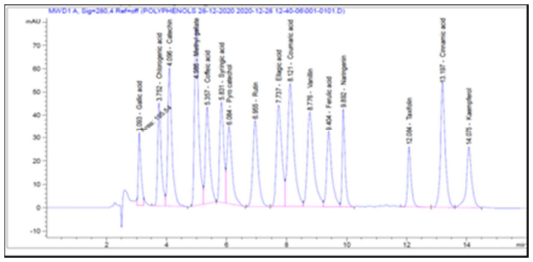
Table 5

Total phenolic and flavonoids contents of pomegranate, cichorium extracts, clove and cinnamon oils

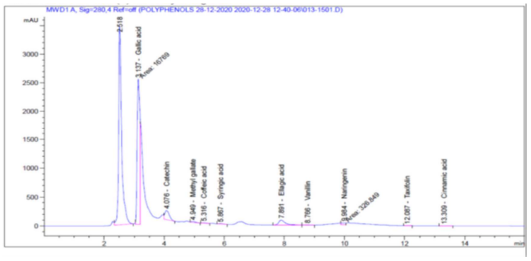
Plant extract or oil	TPC (mg Gallic acid equivalent/100 g DW extract or oil weight)	TFC (mg Quercetin equivalents (QE)/g DW or oil weight)
Pomegranate extract	34.4±3.3	161.4±1.6
Cichorium extract	17.8±0.1	64.5±1.9
Clove oil	36.6±2.3	15.4±1.2
Cinnamon oil	22.5±0.1	11.1±7.0

TPC, Total Phenolic Content; TFC, Total Flavonoid Content; DW, dry weight
Values are means ± SD

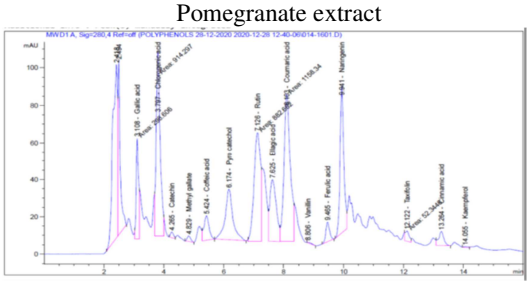
As far as we know, plants defend themselves against biotic and abiotic stress by producing secondary metabolites, so-called phenolic compounds. These compounds include a large and diverse group of chemicals, such as flavonoids and phenolic acids, which have diverse biological activities. Among these activities, the ability of phenolic compounds to act as antioxidants has attracted much attention in the field of traditional medicine. Many studies have reported that phenolic compounds can modulate oxidative stress and inflammation, thus having potential therapeutic effects in various chronic diseases [60], [61]. In this study, we measured the total phenolic and flavonoid content of these four extracts and oils using spectrophotometric methods. Results showed that pomegranate extract and clove oil had the highest total levels of phenols and flavonoids among the samples tested (Table 5 and Figure 3). The total phenolic content of pomegranate peel extract, chicory leaf extract, and clove oil and cinnamon oil was 34.4, 17.8, 36.6 and 22.5 mg gallic acid equivalent (GAE) per 100 g dry weight (DW) or per 100 g oil, respectively. The total flavonoid content is 161.4, 64.5, 15.4 and 11.1 mg quercetin equivalent (QE) per 100 g DW or per 100 g oil, respectively. This finding reveled that both pomegranate peel extract and clove oil have higher phenolic and flavonoid content compared to chicory leaf extract and cinnamon oil. These results are consistent with previous studies reporting the high phenolic and flavonoid content of pomegranate extract and clove oil [62], [63]. Therefore, pomegranate extract and clove oil can be considered effective sources of natural antioxidants for food and pharmaceutical applications.



Calibration of standard phenolic compounds

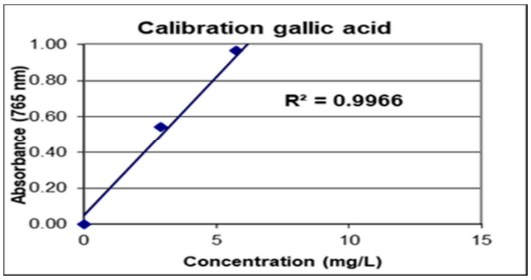


Pomegranate extract

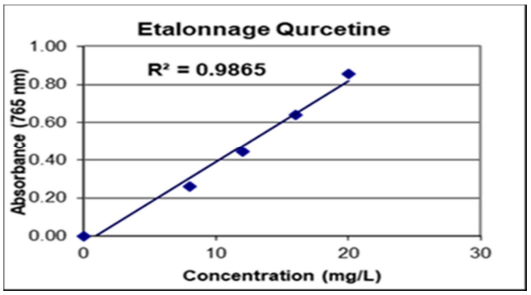


Cichorium extract

Figure 2: HPLC chromatogram of calibration of standard phenolic compounds, pomegranate and cichorium extracts at wavelengths of 280 nm



Gallic acid



Quercetin

Figure 3: Calibration line of standard phenolic "gallic acid" and standard flavonoid "quercetin"

The ABTS and DPPH tests are widely used methods to assess the antioxidant capacity of natural products. Both are spectrophotometric techniques based on the quenching of stable colored radicals (ABTS+ or DPPH) and demonstrate the scavenging ability of antioxidants even when present in complex biological mixtures such as plant or food extracts. The principle of these tests is that antioxidants can donate hydrogen atoms or electrons to the radicals and reduce them to their stable forms. The degree of radical reduction is measured by the decrease in absorbance at a specific wavelength [64], [65]. The antioxidant capacity is then expressed as BHT Equivalent Antioxidant Capacity (BHTEAC) or Vitamin C Equivalent Antioxidant Capacity (VCEAC), depending on the standard used for calibration (Figure 4). In this study, the DPPH and ABTS methods were used to evaluate the antioxidant activity of pomegranate peel, chicory leaf extracts, clove oil, and cinnamon oil. The results showed that crude extracts and EOs had a significant inhibitory effect compared to VC or BHT (Table 6). The DPPH scavenging activity (IC50, g/mL) of pomegranate peel extract, chicory leaf extract, vitamin C, clove oil, cinnamon oil and butylatedhydroxytoluene (BHT) was 298.6, 1392.1, 37.2, 1.6, 327.4, 1392, 1 and 2.5 g/ml, while the ABTS scavenging activity (IC_{50}, g/ml) was 97.1, 947.5, 1.3, 74.7, 206.0 and 2.2 g/ml, respectively. The highest levels of DPPH and ABTS inhibition were observed for pomegranate peel extract and clove oil (Figures 5 and 6). This indicates that they have the highest levels of antioxidants that can quickly scavenge DPPH and ABTS radicals. Previous studies have also reported high antioxidant activity of pomegranate peel extract [66], [67] and clove oil [68], [69]. The results also showed that among the four samples tested, pomegranate peel extract and clove oil had the highest levels of phenols and flavonoids, as well as higher levels of DPPH and ABTS inhibition. Chicory leaf extract and cinnamon oil had lower phenolic and flavonoid content than pomegranate peel extract and clove oil. This finding indicates that pomegranate peel extract and clove oil may have more antioxidant properties than chicory leaf extract and cinnamon oil. Therefore, they were chosen to develop a nano formulation loaded with plant extracts.

One of the most promising methods is the use of

nanoemulsions for formulation based on plant extracts enriched to increase stability and oral bioavailability [70]–[72]. In this study, a nanoemulsion (O/W) was formulated with 10% clove oil using deionized water (DW) and polysorbate 80 (Tween 80) as nonionic surfactant. The nanoemulsion was sonicated for 2.5, 5, and 10 minutes and then subjected to stability tests such as centrifugation, heat-cool cycling, and freeze-thaw cycling. The results of the physico-chemical and stability studies are presented in Table 7 and Table 8. We have optimized the oil to surfactant ratio and the sonication time to obtain the best nanoemulsion with a droplet size of 155.2 nm and good physicochemical properties. Nanoemulsion samples A3 and B3 showed the best stability with droplet sizes of 276.8 and 155.2 nm, respectively, as shown in Figure 7. The morphology and dispersion of the clove oil-based nanoemulsion sample B3 were confirmed by transmission electron microscopy (TEM) in Figure 8. The TEM images showed that the nanoemulsion droplets had a spherical shape and high dispersion. Nanoemulsions are colloidal systems consisting of oil droplets dispersed in an aqueous phase and stabilized by surfactants and co-surfactants. They have several advantages over conventional formulations, such as improved solubilization of lipophilic compounds, protection against degradation, improved absorption and bioavailability and reduced side effects [73], [74]. However, their poor stability, and poor bioavailability limit the therapeutic potential of plant extracts. By loading nanoemulsions with plant extracts, these disadvantages can be overcome and their effectiveness increased. Several studies have reported the successful preparation and characterization of nanoemulsions containing plant extracts for various applications such as wound healing, skin care, oral health and diabetes management [75]–[77]. However, this study is also designed to explore the potential of pomegranate extract as a natural antioxidant in clove oil nanoemulsions. Pomegranate extract contains a high proportion of polyphenols, which have a high antioxidant effect. However, polyphenols are subject to degradation by environmental factors such as light, oxygen, and pH. Therefore, a nanoemulsion containing pomegranate extract in clove oil was proposed to protect the bioactive compounds and improve their stability and potency. Clove oil is a rich source of eugenol, a phenolic compound with antioxidant, antimicrobial, and anti-inflammatory properties. The clove oil nanoemulsion was prepared by the spontaneous emulsification method using Tween 80 as a surfactant. The solubility of pomegranate extract in clove oil and its nanoemulsion at different concentrations was studied. Pomegranate extract was incorporated into clove oil nanoemulsion to obtain pomegranate clove nanoemulsion. The concentration of the extract in the nanoemulsion (25 mg/ml) was determined based on the experimental design for animal studies.

Table 6
DPPH and ABTS free radical scavenging of pomegranate, cichorium extracts and clove and cinnamon oils

Plant extract or oil	Scavenging activity (IC_{50},µg/ml)	
	DPPH	ABTS
Pomegranate extract	298.6 ± 1.5	97.1± 1.6
Cichorium extract	1392.1 ± 37.2	947.5± 6.3
Vitamin C (V.C.)	1.6± 0.001	1.3± 0.001
Clove oil	327.4 ±6.30	74.7±0.80
Cinnamon oil	1392.1 ±37.20	206.0±2.40
Butyl hydroxytoluene (BHT)	2.5±0.01	2.2 ±0.00

Values are means ± S.D.

Table 7
Cod samples and formulation of a nanoemulsion (O/W) based on clove oil.

Sample cod	Sonication time (mints)	Formulation		
		Organic phase		Aqueous phase
		Clove oil	Tween 80	DW
A1	2.5	10	10	80
A2	5	10	10	80
A3	10	10	10	80
B1	2.5	10	20	70
B2	5	10	20	70
B3	10	10	20	70
C1	2.5	20	10	70
C2	5	20	10	70
C3	10	20	10	70

Deionized water (DW)

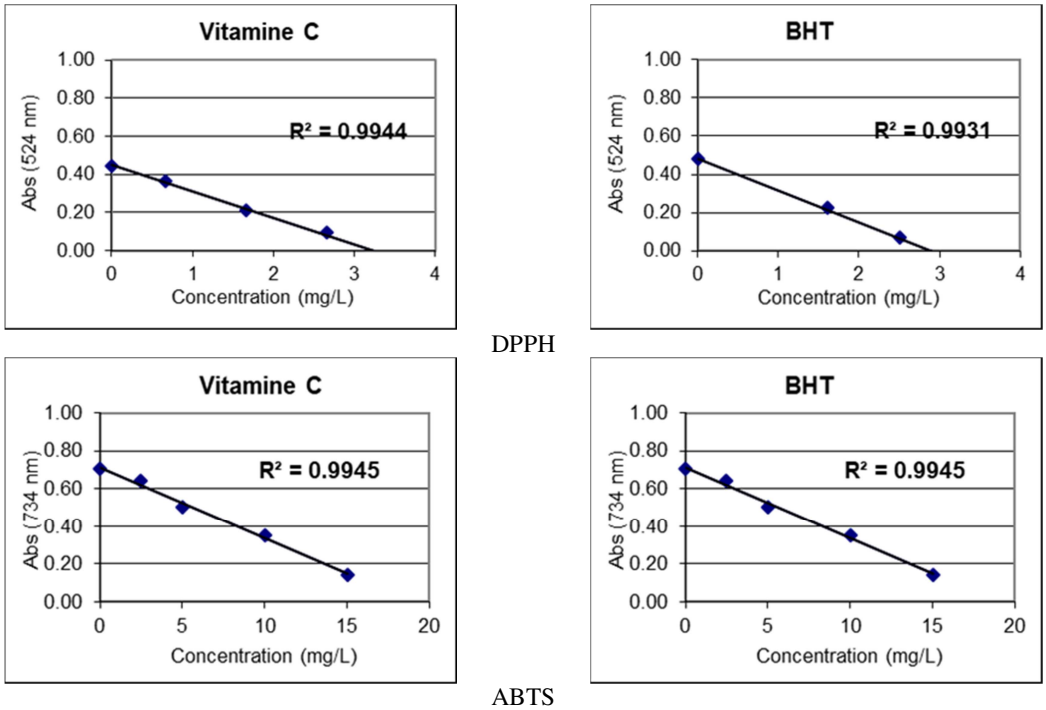


Figure 4: Calibration line of ascorbic acid (V.C.) and butyl hydroxytoluene (BHT) by DPPH and ABTS

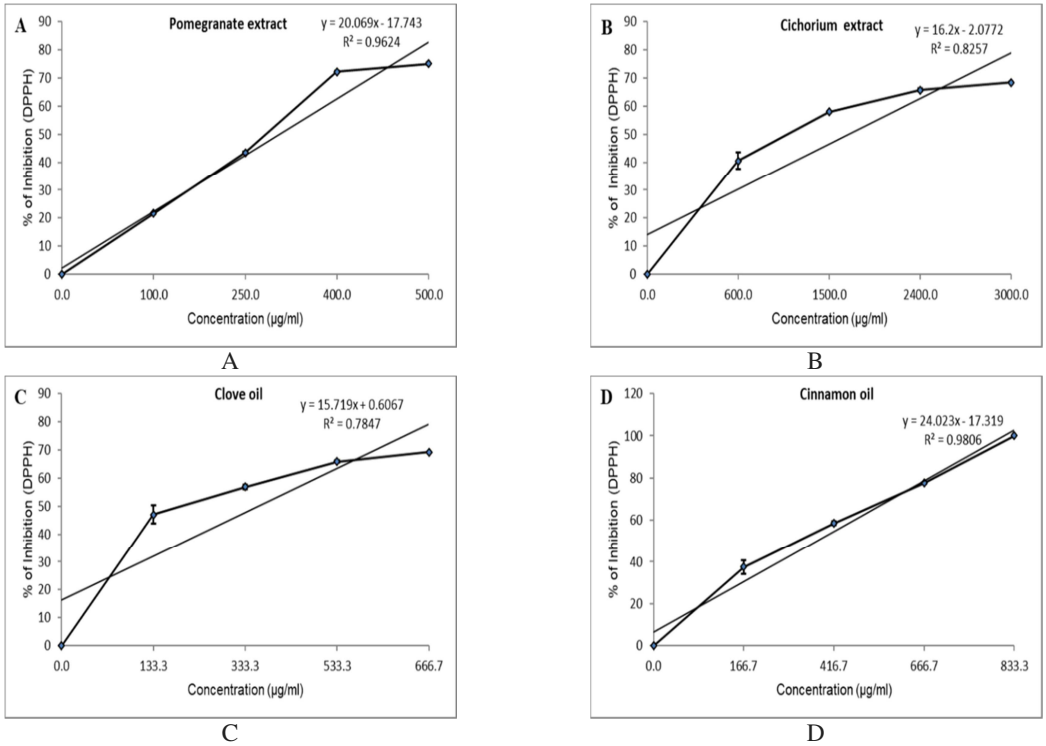


Figure 5: DPPH free radical scavenging (% inhibition vs concentration graph) of pomegranate (A), cichorium (B) extracts and clove (C) and cinnamon (D) oils

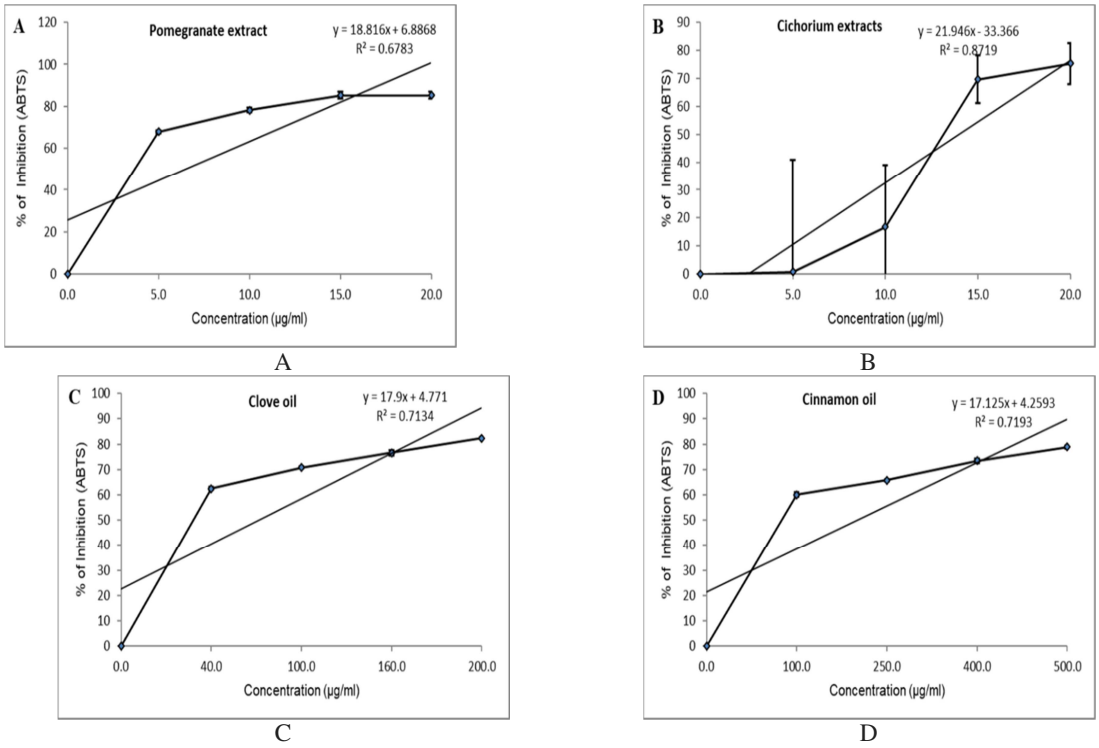


Figure 6: ABTS free radical scavenging (% inhibition vs concentration graph) of pomegranate (A), cichorium (B) extracts and clove (C) and cinnamon (D) oils

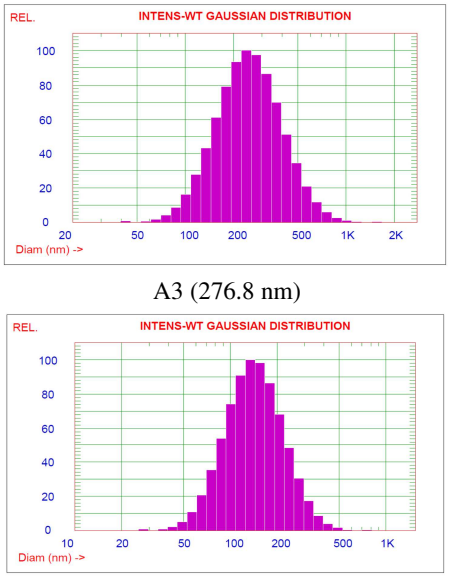


Figure 7: Droplets size disruption of clove-oil based nanoemulsion A3 (276.8 nm) and B3 (155.2 nm).

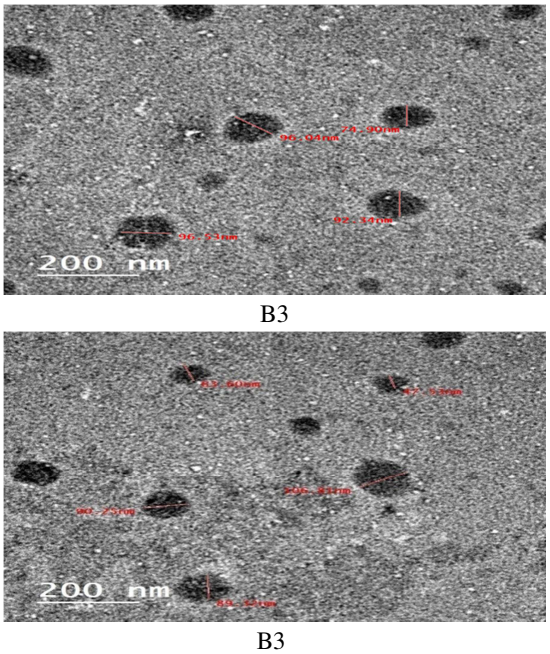


Figure 8: Transmission electron microscopy (TEM) of clove-oil-based nanoemulsions sample B3

Table 8
Evaluation of the stability and droplet size of clove oil-based nanoemulsions

Sample Cod	Ration of Oil: tween 80	Sonication time (min)	Stability test			Stability Result	Droplet size (nm)
			Centrifugation	Freeze–Thaw cycle	Heating–Cooling cycle		
A1	1:1	2.5	-	-	+	x	-
A2	1:1	5	-	-	+	x	-
A3	1:1	10	+	+	+	√	*276.8*
B1	1:2	2.5	-	-	+	x	-
B2	1:2	5	-	-	+	x	-
B3	1:2	10	+	+	+	√	155.2
C1	2:1	2.5	-	-	+	x	-
C2	2:1	5	-	+	+	x	-
C3	2:1	10	+	+	+	x	-

X = failed; √ = passed

3. Conclusion

According to GC-MS analysis, most of the cinnamon EO contains of (E)-cinnamaldehyde (62.4%), benzyl alcohol (25.29%) and methoxyacetic acid, benzyl ester (10.71%), while most of the clove-EO contains of eugenol (79.26%), triacetin (11.72%) and benzyl alcohol (6.76%). As shown by the HPLC results, the pomegranate peel extract contained ten phenolic components, while the Cichorium leaf extract contained a total of fifteen phenolic compounds in varying amounts. The total phenolic content of pomegranate peel extract, chicory leaf extract, and clove oil and cinnamon oil was 34.4, 17.8, 36.6 and 22.5 mg per 100 g dry weight (DW) or 100 g oil, respectively. The total content of flavonoids is 161.4, 64.5, 15.4 and 11.1 mg per 100 g DW or oil, respectively, expressed in units of quercetin equivalents (QE). Results showed that pomegranate peel extract and clove oil had the highest levels of phenols and flavonoids. The results also showed that pomegranate peel extract and clove oil had high DPPH and ABTS inhibition and scavenging activity (IC50, g/mL) 298.6, and 37.2 µg/ml of DPPH and 97.1 and 74.7 µg/ml of ABTS, respectively. In this study, we prepared a clove oil nanoemulsion using a self-emulsification method with Tween 80 as a surfactant. We have optimized the oil to surfactant ratio and the sonication time to obtain the best nanoemulsion with a droplet size of 155.2 nm and good physicochemical properties. These results suggest that nanoemulsions based on pomegranate peel extract and clove oil are good sources of natural antioxidants and can be used to protect humans from the toxicity of xenobiotics such as pesticides and heavy metals.

4. Acknowledgment

The authors extend their appreciation to the National Research Centre, Cairo, Egypt for funding this work through grant thesis no. 1/4/9.

5. Conflict of interests

The author(s) declare(s) that there is no conflict of interests regarding the publication of this article."

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