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Phytoconstituents, LC-ESI-MS Profile, Antioxidant and Antimicrobial Activities of *Citrus* x *limon* L. Burm. f. Cultivar Variegated Pink Lemon

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Abstract

Variegated pink lemon is a valuable citrus tree, which didn't receive enough phytochemical and biological studies. *n*-Hexane, dichloromethane and ethyl acetate fractions of hydro alcoholic extracts of stem, leaf and fruit rind were subjected to chromatographic investigation. Thirteen compounds were isolated and identified as limettin, limonin, chrysoeriol, *p*-coumaric acid, scoparin, vitexin, chrysoeriol-7-*O*-glucoside, and hesperidin in addition to friedlin, lupeol, behenic acid, *β*-sitosterol and stigmasterol mixture and *β*-sitosterol-*O*-glucoside. The isolated compounds were identified by UV, EI-MS, ^{1}H-NMR, ^{13}C-NMR, COSY, HSQC, DEPT-135, APT and HMBC. HPLC-PDA-ESI-MS/MS analysis of dichloromethane and ethyl acetate fractions afforded the identification of 90 compounds including organic acids and their glycosides, flavonoids and their HMG derivatives, coumarins and limonoids. Total phenolics and flavonoids contents were quantified by Folin-Ciocalteu and aluminum chloride - potassium acetate colorimetric methods, respectively. Ethyl acetate fraction and total alcoholic extract of stem showed the highest phenolics (113.01 ± 0.23 mg GAE/g extract) and flavonoids (557.10 ± 0.82 mg quercetin and 625.22 ± 0.91 mg rutin equivalent/g extract) concentrations, respectively. Antioxidant activity was evaluated using DPPH radical scavenging capacity where leaf ethyl acetate fraction showed the highest activity (SC_{50}=19.01µg/mL) compared with ascorbic acid (SC_{50}=13.4 µg/mL). Antimicrobial activity was assayed by agar well diffusion and MIC for different fractions where rind and leaf ethyl acetate fractions exceeded activity of gentamycin against *Klebsiella pneumoniae*. Additionally, Juice and rind ethyl acetate showed the best activity against *Bacillus subtilis* (MIC=0.49 µg/mL) and *Saccharomyces cerevisiae* (MIC=0.98 µg/mL). Moreover, *Enterococcus faecalis* was highly sensitive to juice ethyl acetate (MIC=0.49 µg/mL).

Keywords: *Citrus*, variegated pink lemon, LC-ESI-MS, phenolics, flavonoids, antioxidant, antimicrobial.

1. INTRODUCTION

Genus *Citrus* (Rutaceae) is considered as a treasure trove for different classes of bioactive secondary metabolites such as flavonoids, volatile oils, limonoids, coumarins, alkaloids, sterols and carotenoids in addition to vitamins (specially vitamin C), minerals and dietary fibers which make citrus a health-benefit promoting fruit. So, daily consumption of fresh citrus fruits is beneficial for prevention and treatment of chronic diseases [1-3]. Citrus flavonoids exhibited anti-oxidant activities via free radical scavenging capacity [4]. Additionally, they possess anti-inflammatory activity through inhibition of protein kinase C, phosphodiesterase, phospholipase, lipoxygenase, and cyclooxygenase enzymes leading to inhibition the formation of biological mediators responsible for the activation of especial endothelial cells involved in inflammation [5]. Moreover, flavonoids prevent atherosclerosis by inhibiting the formation of atheroma [6]. Polymethoxylated flavones significantly reduced serum total and very low-density lipoprotein [7]. Hesperetin exhibited a moderate antimicrobial activity against *Salmonella. typhi* and *S. typhimurium* [8]. Hesperidin and hesperetin exhibited protective effects against different microbes and toxins including human intestinal microbes in addition to their antiparasitic and cytotoxic activities as reviewed [9].

Citrus limonoids especially limonin exert anticancer effects *via* selective cytotoxicity, antiproliferative actions and apoptosis [10, 11]. It was previously reported that some isolated compounds from genus *Citrus* showed antimicrobial activity as limonin, limonol and nomilinic acid had antifungal activity against *Puccinia arachidis* [12]. Variegated pink lemon tree is quite ornamental due to its showy flowers and variegated green - white leaves. The rind has green and cream stripes with rough surface. At full stage of maturity the fruit interior is light pink, low-seeded with acidic taste.

In course of the chemical investigation of *Citrus* species growing in Egypt, chemical composition, and biological activities of the essential oil of pink lemon were reported [13]. The current study involves detection, isolation and identification of different groups of secondary metabolites of pink lemon in addition to its total flavonoids and phenolic contents, anti-oxidant and antimicrobial activities.

2. MATERIALS AND METHODS

2.1. Plant materials

The fresh ripe fruits, stems and leaves of *Citrus* x *limon* L Burm. f. cultivar variegated pink lemon, (also called the variegated Eureka lemon or pink-fleshed Eureka lemon) were collected from the garden of Faculty of Agriculture, Moshtohor, Benha University, Egypt in March 2012. The identity of the plant was confirmed by Prof. B. Houlyel, Faculty of Agriculture, Benha University, Egypt. A voucher specimen (P-78, 79**)** was deposited in Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt.

2.2. Apparatus

Shimadzu UV-1700 (Japan) was used to measure the UV absorption. Mass spectrometry was carried out using Quadropole mass analyzer in Thermo Scientific GCMS model ISQ LT (USA) using Thermo X-Calibur software. ^{1}H- and ^{13}C-NMR spectra were obtained using Bruker (Switzerland), 400 and 100 MHz, respectively.

2.3. Extraction and fractionation

Dried stem, fruit rind and leaf powders (1.3, 0.5 and 1.3 kg, respectively) were extracted with 80% ethanol at room temperature till exhaustion, filtered and evaporated under reduced pressure to yield 161, 236.8 and 460 g of residues, respectively. Each total extract was suspended in MeOH: H_2O (1:9) and partitioned against *n*-hexane, dichloromethane and ethyl acetate, successively, dried over sodium sulphate anhydrous and concentrated. The yielded fractions for the stem were 8.7, 2.5 and 8 g while the rind fractions were 6.7, 4.8 and 25.2 g and the leaf fractions were 25, 8 and 22 g for *n*- hexane, dichloromethane and ethyl acetate, respectively. The juice (2L) was extracted by ethyl acetate till exhaustion to yield 50 g total extract and fractionated similarly to afford 0.5, 5 and 8 g for *n*- hexane, dichloromethane and ethyl acetate, respectively.

2.4. Chromatographic investigations

All fractions were investigated by TLC using light petroleum: dichloromethane: methanol (15: 15: 1, S1), dichloromethane: methanol (9.5:0.5, S2) and ethyl acetate: methanol: water (6:1:0.6, S3). TLC plates were visualized by UV, anisaldehyde sulphuric acid, ammonia and ferric chloride. Promising fractions of major spots were subjected to chromatographic investigations.

The *n*-hexane soluble fraction of stem (SH, 8 g), rind (RH, 4.5 g) and rind dichloromethane soluble fraction (RC, 4.5 g) were separately chromatographed on silica gel column packed with *n*-hexane and the polarity was increased by dichloromethane then methanol. Similar fractions were collected according to TLC profile using S1 and S2, concentrated and crystallized to afford separation of compounds 1, 2, 5 and 9 from SH while 3, 4 and 5 were isolated from RH. Additionally, compounds 6, and 8 were isolated from RC.

The ethyl acetate soluble fraction of the leaf (LE, 20 g), stem (SE, 6 g) and rind (RE, 20 g) were separately subjected to silica gel column packed with dichloromethane and the polarity was increased gradually using methanol for LE and by ethyl acetate then methanol for SE and RE. Fractions were examined by TLC using S2 and S3 and similar fractions were combined and crystallized to afford separation of compounds 7, 11, 12 and 13 from LE while compounds 10, 11 and 13 were isolated from SE in addition to compounds 10 and 13 from RE.

2.5. High performance liquid chromatography-Mass spectrometry (HPLC-PDA-ESI-MS/MS)

Dichloromethane and ethyl acetate fractions of leaf, stem, rind and juice were subjected to HPLC-PDA-ESI-MS/MS analysis. Thermofinigan HPLC (Thermo electron Corporation, USA) coupled with an LCQ-Duo ion trap mass spectrometer with an ESI source (ThermoQuest) was used. The separation was achieved using a C18 reversed-phase column (Zorbax Eclipse XDB-C18, rapid resolution, 4.6 × 150 mm, 3.5 µm, Agilent, USA). A gradient of water and acetonitrile (ACN) (0.1 % formic acid each) was applied from 5% to 30% ACN in 60 min and then was increased to 90% ACN in the next 60 min with flow rate 1 ml/min with a 1:1 split before the ESI source. The samples were injected automatically using autosampler surveyor ThermoQuest. The instrument was controlled by Xcalibur software (Xcalibur^{TM}2.0.7, Thermo Scientific). The MS operated in the negative mode with a capillary voltage of − 10 V, a source temperature of 200°C, and high purity nitrogen as a sheath and auxiliary gas at a flow rate of 80 and 40 (arbitrary units), respectively. Collision energy of 35% was used in MS/MS fragmentation. The ions were detected in a full scan mode and mass range of 50 - 2000 *m/z*.

2.6. Quantitative estimation of the total phenolics and flavonoids contents

Spectrophotometric determination of the total phenolic content was carried out using the Folin-Ciocalteu colorimetric method [14]. The crude ethanolic extracts and ethyl acetate fractions of different plant organs were dissolved in methanol at a concentration of 4 mg/ml. Four replicates were carried out and total phenolics were expressed as mg of gallic acid equivalents (mg GAE)/g extract deduced from calibration curve of standard gallic acid (40-300 µg/ml).

Total flavonoids contents were carried out using the aluminum chloride-potassium acetate spectrophotometric assay [15]. Crude extracts and their fractions of *n*-hexane, dichloromethane and ethyl acetate of different organs were dissolved in 95% ethanol (2 mg/ml). Four replicates were carried out and total flavonoids were expressed as mg of quercetin equivalents/g extract and as mg of rutin equivalents/g extract by using calibration curve of for quercetin and rutin (12.5-1200 µg/ml) in 95% ethanol.

2.7. Antioxidant Activity

Stem and leaf total alcoholic extracts in addition to the ethyl acetate fractions of stem, leaf and rind as well as the leaf dichloromethane fraction were evaluated for their antioxidant capacity using 2, 2-diphenyl-1-picrylhydrazyl (DPPH**).** Freshly prepared (0.1 mM) solution of DPPH and different fractions prepared at 5, 10, 20, 40, 80, 160 and

320 µg/ml in methanol were used [16]. The absorbance of the DPPH radical without antioxidant (control) and the reference compound ascorbic acid prepared in the same concentrations as tested fractions were also measured. All the determinations were performed in three replicates and averaged. The percentage inhibition of DPPH radical was calculated according to the formula:

% DPPH radical-scavenging = (AC-AS)/AC × 100

Where AC is the absorbance of the control solution and AS is the absorbance of sample in DPPH solution. The percentage of DPPH radical-scavenging was plotted against each fraction concentrations and ascorbic acid (µg/ml) to determine SC_{50}.

2.8. Antimicrobial activity

Different fractions of pink lemon were evaluated for their antibacterial and antifungal activities by using well diffusion method [17] against three Gram-positive bacteria (*Staphylococcus aureus* RCMB 010027, *Enterococcus faecalis* RCMB 010063 and *Bacillus subtilis* RCMB 010067); three Gram-negative bacteria (*Enterobacter cloacae* RCMB 010072, *Klebsiella pneumoniae* RCMB 010093 and *Escherichia. coli* RCMB 010052) and three fungi (*Aspragillus fumigatus* RCMB 02568, *Candida albicans* RCMB 05036 and *Saccharomyces cerevisiae* RCMB 05177) obtained from the regional center for mycology and biotechnology, Egypt.

The assay was performed on nutrient agar medium for bacterial strains and Saboroud dextrose agar for fungi. The tested fractions were dissolved in dimethyl sulfoxide (DMSO) at concentration of 500 µg/ml. Ampicillin and gentamicin (100 µg/ml) were used as positive control for bacteria, while amphotericin B (100 µg/ml) was used for fungi. The wells were filled with 100 µl from stock solution of each sample, the standards and DMSO as a negative control. Cultures were incubated at 37ºC for 24 hours for bacteria and for 2-7 days for fungi.

All the assays were done in triplicate. Results were expressed in mean zone of inhibition diameter in mm ± standard deviation (SD) and the percentage inhibition of diameter growth was calculated as reported before [18].

$$\%\ \text{of inhibition} = \frac{\text{Inhibition zone diameter of sample} - \text{inhibition zone diameter of solvent}}{\text{Inhibition zone diameter of standard} - \text{inhibition zone diameter of solvent}} \times 100$$

2.9. Determination of minimum inhibitory concentration (MIC)

MIC values were determined using the agar plate dilution method and all MIC ranges were determined [19, 20]. Fractions which showed the biggest inhibition zone diameters against the tested organisms were evaluated for MIC. The tested fractions concentrations ranged from 5 to 250 µg/ml [21]. Inocula were obtained from a suspension containing approximately $1\text{-}2\times10^{8}$ colony-forming unit (cfu/ml). The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity comparable to that of the 0.5 McFarland standards.

2.10. Statistical analysis

All experiments were repeated at least three times. Results are reported as means±SD.

3. RESULTS AND DISCUSSION

3.1. Characterization of the isolated compounds

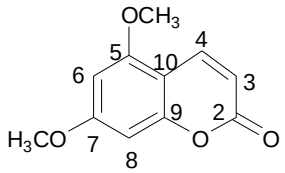
Chromatographic investigation of different fractions afforded the isolation of thirteen compounds (**1-13**). The structural elucidation was carried out using UV, EI-MS, ^{1}H-NMR, ^{13}C-NMR, HSQC, HMBC, DEPT- 135 and APT. Friedlin **(1)**, lupeol **(2)**, behenic acid (docasanoic acid) **(3)**, *β*-sitosterol and stigmasterol mixture **(5)** and *β*-sitosterol-*O*-glucoside **(9)** were identified by comparison of spectroscopic data with the reported literature [22-26] and Co-TLC with available authentic.

Compounds **4, 6-8** and **10-13** (Figure 1) were characterized as following

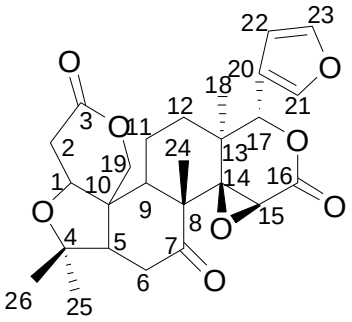
Compound 4: Yellowish-white powder (50 mg) with R_f value 0.64 (S1). UV**:** λ_{max} (MeOH) nm: 247 (sh), 251 (sh) and 324 nm. EI-MS, *m/z* (relative abundance %): 206 (M^+, 100), 178 (84.7), 163 (50.3), 149 (6.1), 148 (2.8), 147 (2.4), 135 (26.8), 121 (4.6), 120 (7.0), 103 (1.6), 91 (4.8) and 43 (0.8).^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm 6.18 (1H, *d*, J=10, H-3), 7.99 (1H, *d*, J=10, H-4), 6.31 (1H, *d*, J=2.4, H-6), 6.44 (1H, *d*, J=2.4, H-8), 3.88 (3H, *s*, O-CH_3) and 3.92 (3H, *s*, O-CH_3).^{13}C-NMR (100 MHz, $CDCl_3$): δ ppm 161.44 (C-2), 111 (C-3), 138.66 (C-4), 157.01 (C-5), 94.85 (C-6), 163.73 (C-7), 92.88 (C-8),156.87 (C-9), 104.05 (C-10), 55.93 (O-CH_3) and 55.78 (O-CH_3).These spectral data are matching with the reported for limettin [27, 28].

Compound 6: White needles (28 mg) with R_f value 0.2 (S1). EI-MS, *m/z* (relative abundance %): 413 (M^+-57, 1.82), 347 (100), 329 (8.16), 287 (6.22), 241 (3.83), 201 (4.91), 187 (5.58), 147 (6.46), 136 (9.10), 135 (19.08), 108 (19.94), 95 (39.22), 69 (18.33) and 43 (27.15). ^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm 4.04 (1H, *br. s*, H-1), 2.23 (1H, *dd*, J=3.2, 16, H-2a), 2.68 (1H, *dd*, J=2, 16.8 H-2b), 2.46 (1H, *dd*, J=3.2, 14.4, H-5), 2.98 (1H, *dd*, J=3.6, 16.8, H-6a), 2.85 (1H, *t*, J=15.2, H-6b), 2.55 (1H, *dd*, J=2.8, 12.6, H-9), 1.85 (1H, *m*, H-11a), 1.91 (1H, *m*, H-11b), 1.50 (1H, *m*, H-12a), 1.77 (1H, *m*, H-12b), 4.04 (1-H, *br. s*, H-15), 5.47 (1-H, *s*, H-17), 1.18 (3H, *s*, H-18), 4.46 (1H, *d*, J=12.8, H-19a), 4.75 (1H, *d*, J=13.2, H-19b), 7.41 (1H, *br. s*, H-21), 6.34 (1H, *br. s*, H-22), 7.40 (1H, *br. s*, H-23), 1.07 (3H, *s*, H-24), 1.29 (3H, *s*, H-25) and 1.17 (3H, *s*, H-26).^{13}C-NMR (100 MHz, $CDCl_3$): δ ppm 79.3 (C-1), 35.8 (C-2), 169.23 (C-3), 80.46 (C-4), 61.71 (C-5), 36.54 (C-6), 206.23 (C-7), 51.48 (C-8), 48.27 (C-9), 46.09 (C-10), 19.07 (C-11), 30.31 (C-12), 38.09 (C-13), 65. 81 (C-14), 53.99 (C-15), 166.75 (C-16), 77.94 (C-17), 17.77 (C-18), 65.50 (C-19), 120.12 (C-20), 143.39 (C-21), 109.82 (C-22), 141.26 (C-23), 20.86 (C-24), 31.00 (C-25) and 21.53 (C-26). Upon comparison of the spectral data with the available literature, compound 6 was confirmed to be limonin [29, 30].

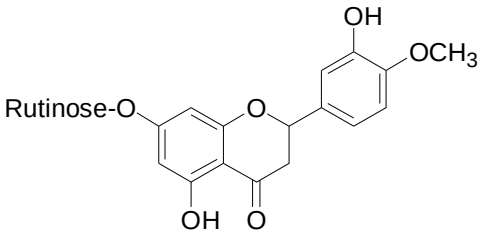
Compound 7: Yellow amorphous powder (20 mg) with R_f 0.6 (S2). UV: λ_{max} (MeOH) nm: 241 (sh), 268, 346; (+ NaOMe): 265, 332 (sh), 407; (+ $AlCl_3$): 261 (sh), 275 (sh), 360, 386; (+ $AlCl_3$ + HCl): 260 (sh), 277 (sh), 352, 382; (+ NaOAc): 279, 318 (sh), 395; (+ NaOAc + Boric acid): 269, 318 (sh), 347. EI-MS: *m/z* (relative abundance %): 300 (M^+, 0.8), 240 (12), 225 (8.6), 136 (0.8), 121 (3.2), 119 (7.2), 107 (7.5), 106 (100), 105 (1.7), 91 (10.9), 79 (9.1), 78 (86.6), 77 (9.8) , 64 (8.7), 63 (8.5), 57 (3), 51 (33) and 43 (21.6). ^{1}H-NMR (400 MHz, DMSO-d_6): δ ppm 6.89 (1H, *s*,



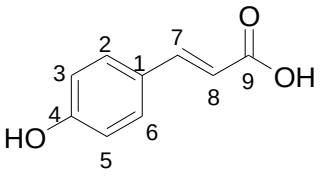
Limettin **(4)**



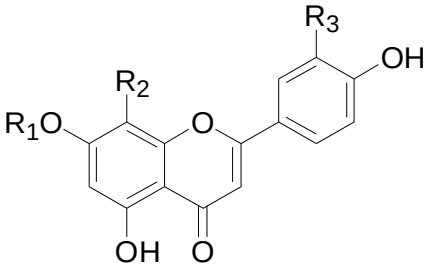
Limonin **(6)**



Hesperidin **(13)**



p-Coumaric acid **(8)**



Compound	R_1	R_2	R_3
Vitexin **(11)**	H	Glucose	H
Scoparin **(10)**	H	Glucose	O-CH_3
Chrysoeriol **(7)**	H	H	O-CH_3
Chrysoeriol-7-*O*-glucoside **(12)**	Glucose	H	O-CH_3

Figure 1: Chemical structures of compounds isolated from the different organs of pink lemon.

H-3), 6.2 (1H, *s*, H-6), 6.51 (1H, *s*, H-8), 7.58 (1H, *d*, J=2, H-2`), 6.94 (1H, *d*, J=8.4, H-5`), 7.56 (1H, *dd*, J=2, 8.4, H-6`), 3.9 (3H, *s*, O-$CH_3$) and 13 (1H, *s*, OH at C-5). $^{13}$C-NMR-APT (100 MHz, DMSO-*d6*): δ ppm 164.12 (C-2), 103.66 (C-3), 182.25 (C-4), 161.89 (C-5), 99.33 (C-6), 164.78 (C-7), 94.55 (C-8), 157.82 (C-9), 104.11 (C-10), 121.97 (C-1`), 110.66 (C-2`), 148.51 (C-3`), 151.23 (C-4`), 116.25 (C-5`), 121.12 (C-6`), and 56.43 (O-CH_3). Spectral data of UV, EI-MS and NMR of this compound were found to be identical to chrysoeriol [31-34].

Compound 8: Colorless needles (30 mg) with R_f 0.22 (S2). UV: λ_{max} (MeOH) nm: 284. EI-MS: *m/z* (relative abundance %): 164 (M^+, 12.9), 163 (6.0), 147 (9), 136 (2.2), 120 (100), 119 (32.5), 118 (7.2), 107 (11.2), 91 (75.6), 89 (12.4), 65 (28.6), 63 (15.4), 60 (31.1) and 51 (10).^{1}H-NMR (400 MHz, CD_3OD-*d4*): δ ppm 6.83 (2H, *d,* J=8, H-2 and H-6), 7.46 (2H, *d,* J= *8*, H-3 and H-5), 7.61(1H, *d*, J=16, H-7), 6.3 (1H, *d*, J=16, H-8). ^{13}C-NMR (100 MHz, CD_3OD-*d4*)**:** 161.14 (C-1), 116.83 (C-2, C-6), 130.92 (C-3, C-5),127.32 (C-4), 146.56 (C-7), 115.78 (C-8), 171.04 (C-9).When spectral data of compound 8 were compared with the available literature, it is clear that this compound is *p*-coumaric acid [35, 36]

Compound 10: Yellow amorphous powder (30 mg) with R_f value 0.7 (S3)**.** UV: λ_{max} (MeOH) nm: 254 (sh), 270 (sh), 343; (+ NaOMe): 272, 386; (+ $AlCl_3$): 275, 294 (sh), 360, 388; (+ $AlCl_3$ + HCl): 278, 294 (sh), 353, 381; (+ NaOAc): 278, 324 (sh), 361; (+ NaOAc + Boric acid): 254 (sh), 271, 344. EI-MS *m/z* (relative abundance %): 444 (M^+-18, 1.9), 426 (1.0), 408 (2.6), 372 (3.8), 354 (4.1), 342 (6.8), 314 (19.7), 313 (100), 312 (5.7), 300 (12.6) 165 (62.9), 152 (3.0), 151 (12.4) and 148 (13.5).^{1}H-NMR (400 MHz, DMSO-*d6*): δ ppm 6.74 (1H, *s*, H-3), 6.27 (1H, *s*, H-6), 7.52 (1H, *s*, H-2'), 7.07 (1H, *d*, J=8, H-5'), 7.67 (1H, *dd*, J=2, 8, H6'), 4.70 (1H, *d*, J=9.6, H-1"), 3.18- 3.87 (4H, *m*, H-2"-H-5", sugar protons), 3.58 (1H, *m*, H-6"), 3.82 (1H, *m*, H-6"), 3.89 (3H, *s*, O-CH_3), 4.97 (2H, *s*, OH-glu) and 13.14 (1H, *s*, 5-OH).^{13}C-NMR (100 MHz, DMSO-*d6*): δ ppm 163.68 (C-2), 103.08 (C-3), 182.04 (C-4), 160.38 (C-5), 98.17 (C-6), 162.65 (C-7), 111.77 (C-8), 156.01 (C-9), 104.07 (C-10), 123.46 (C-1`), 113.57 (C-2`), 146.78 (C-3`), 151.09 (C-4`), 112.25 (C-5`), 119.13 (C-6`), 73.37 (C-1"), 70.76 (C-2"), 78.75 (C-3"), 70.67 (C-4"), 82.06 (C-5"), 61.68 (C-6") and 55.82 (O-CH_3). Comparison of the above mentioned spectroscopic data with the available literature proved that compound 10 is chrysoriol-8-C-glucoside known as scoparin [37].

Compound 11: Yellow amorphous powder (50 mg) with R_f 0.69 (S3). UV: λ_{max} (MeOH) nm: 270, 335; (+ NaOMe): 279, 330 (sh), 395; (+ $AlCl_3$): 276, 303 (sh), 346, 381; (+ $AlCl_3$ + HCl): 277, 301 (sh), 340, 378; (+ NaOAc): 279, 378; (+ NaOAc + Boric acid): 271, 335. EI-MS, *m/z*

(relative abundance %): 342 (M^+-90, 6.1), 336 (2.6), 324 (4.9), 312 (10.6), 284 (20.8), 283 (100), 282 (4.7), 270 (3.7), 165 (67.2), 152 (2.2), 121 (12.4), 118 (14.4), 69 (17.4), 61 (23.3), 60 (20.2), 55 (10.9) and 43 (29.7).^{1}H-NMR (400 MHz, DMSO-*d6*): δ ppm 6.78 (1H, *s*, H-3), 6.27 (1H, *s*, H-6), 8.02 (2H, *d*, J=6.8, H-2`, 6`), 6.89 (2H, *d*, J=7.2, H-3`, 5`), 4.69 (1H, *d*, J=9.6, H-1"), 3.81-4.69 (4H, *m*, glucose protons), 3.52 (1H, *m*, H-6"), 3.75 (H, *m*, H-6"), 4.98 (glucose OH), 13.16 (1H, *s*, 5-OH), and 10.36 (1H, *br.s*, 4`-OH).$^{13}$C-NMR (100 MHz, DMSO-*d6*): δ ppm 163.91 (C-2), 102.42 (C-3), 182.06 (C-4), 160.37 (C-5), 98.15 (C-6), 162.63 (C-7), 104.59 (C-8), 155.98 (C-9), 103.98 (C-10), 121.59 (C-1`), 128.94 (C-2`, C-6`), 115.79 (C-3`, C-5`), 161.12 (C-4`), 73.37 (C-1"), 70.83 (C-2"), 78.64 (C-3"), 70.51 (C-4"), 81.83 (C-5") and 61.27 (C-6"). Compound 11 data are matched with vitexin [38-41].

Compound 12: Yellow amorphous powder (50 mg) with R_f 0.67 (S3). λ_{max} (MeOH) nm: 251 (sh), 268 (sh), 346; (+ NaOMe): 245 (sh), 262 (sh), 391; (+ $AlCl_3$): 274, 357 (sh), 388; (+ $AlCl_3$ + HCl): 275, 354 (sh), 385; (+ NaOAc): 256, 268 (sh), 349, 408; (+ NaOAc + Boric acid): 251 (sh), 268, 347. EI-MS, *m/z* (relative abundance %): 300 (M^+-glucose, 100), 285 (2.2), 257 (19.4), 228 (1.2), 152 (7.9), 151 (4.6), 148 (24.6), 137 (2.0), 136 (17.5), 133 (23.67), 124 (13.6), 123 (10.1), 116 (2.1), 95 (3.4), 90 (1.4), 73 (18.7), 69 (24.1), 60 (23.1), 57 (15.5), 55 (11.8), 44 (24.5) and 40 (98.1). ^{1}H-NMR (400 MHz, DMSO-*d6*): δ ppm 6.99 (1H, *s*, H-3), 6.45 (1H, *s*, H-6), 6.87 (1H, *s*, H-8), 7.59 (2H, *d*, *dd*, J=2.8, 8.4, H-2`, H-6`), 6.95 (1H, *d*, J=8.4, H-5`), 5.06 (1H,*d*, J=8, H-1``), 3.18-3.44 (4H, *m*, H-2``-H-5``, sugar protons), 3.46 (1H, *m*, H-6``), 3.71 (1H, *m*, H-6``), 3.89 (3H, *s*, O-CH_3), 12.97 (1H, *s*, OH at C-5) and 10 (1H, *br. s,* OH at C-4`).$^{13}$C-NMR (100 MHz, DMSO-*d6*): δ ppm 164.14 (C-2), 103.42 (C-3), 182.04 (C-4), 161.08 (C-5), 99.48 (C-6), 162.96 (C-7), 95.01 (C-8), 156.9 (C-9), 105.33 (C-10), 121.3 (C-1`), 110.28 (C-2`), 148.05 (C-3`), 150.95 (C-4`), 115.76 (C-5`), 120.49 (C-6`), 99.98 (C-1"), 73.1 (C-2"), 76.44 (C-3"), 69.57 (C-4"), 77.23 (C-5"), 60.59 (C-6") and 55.95 (O-CH_3). Compound 12 was characterized as chrysoeriol 7-*O*-glucoside [31, 33, 42, 43].

Compound 13: Buff amorphous powder (466 mg) with R_f 0.5 (S3). λ_{max} (MeOH) nm: 283, 324 (sh.); (+ NaOMe): 242, 287, 361; (+ $AlCl_3$): 306, 380 (sh); (+ $AlCl_3$+ HCl): 305, 381; (+ NaOAc): 283, 325 (sh); (+ NaOAc + Boric acid): 283, 325 (sh). EI-MS: *m/z* (relative abundance %): 302 [M^+ - 308] (28.48), 301 (12.93), 286 (10.41), 285 (8.95), 271 (4.89), 259 (3.66), 179 (32.23), 165 (10.65), 152 (13.76), 150 (64.78), 137 (100), 135 (64.61), 129 (15.72), 124 (24.51), 111 (10.85), 107 (20.83), 84 (37.13), 78 (10.31), 77 (21.76), 73 (32.65), 71 (39.32), 69 (41.87), 60 (42.58), 57 (32.52) and 43 (40.68).^{1}H-NMR (400 MHz, DMSO-*d6*): δ ppm 5.50 (1H, *dd*, J=4, 12.4, H-2), 3.27 (1H, *m*, H-3a), 2.78 (1H, *dd*, J= 2.6, 17.2, H-3b), 6.12 (1H, *d*, J=1.2, H-6), 6.14 (1H, *d*, J=1.2, H-8), 6.94 (1H, *d*, J=1.6, H-2'), 6.96 (1H, *d*, J=8, H-5'), 6.92 (1H, *dd*, J=1.6, 8, H-6'), 4.97 (1H, *d*, J=6.8, H-1"), 4.52 (1H, *br.s*, H-1'"), 3.14- 3.63 (10 H, *m*, sugar protons), 1.08 (3H, *d*, J=5.6, CH_3), 12.02 (1H, *s*, 5-OH), 9.1 (1H, s, 3`-OH) and 3.77 (3H, *s*, O-$CH_3$). $^{13}$C-NMR (100 MHz, DMSO-*d6*): δ ppm 78.38 (C-2), 42.02 (C-3), 197.04 (C-4), 163.04 (C-5), 96.38 (C-6), 165.14 (C-7), 95.55 (C-8), 162.50 (C-9), 103.32 (C-10), 130.90 (C-1`), 114.15 (C-2`), 146.46 (C-3`), 147.97 (C-4`), 112.03 (C-5`), 117.97 (C-6`), 99.45 (C-1"), 70.27 (C-2"), 75.52 (C-3"), 68.32 (C-4"), 76.27 (C-5"), 66.02 (C-6"), 100.60 (C-1'"), 69.60 (C-2'"), 70.27 (C-3'"), 72.99 (C-4'"), 75.52 (C-5'") 17.84 (CH_3) and 55.69 (O-CH_3).Compound 13 was identified as hesperidin by comparison of the spectral data with the available literature and Co-TLC with authentic sample [31, 44].

3.2. HPLC-PDA-ESI-MS/MS

Dichloromethane and ethyl acetate fractions of stem, leaf, rind and juice were analyzed by HPLC-PDA-ESI-MS/MS negative mode. Totally, 90 secondary metabolites were identified based on their UV spectrum, MS/MS information given by mass of the precursor ion and their fragments, together with neutral mass loss and known fragmentation patterns for the given classes of compounds as well as comparison with the available literature [45-75]. The compounds were arranged according to relative retention time (RRt) to chrysoeriol in case of dichloromethane fractions and hesperidin in case of ethyl acetate fractions (Table 1).

Free organic acids

Free organic acids as malic (1) [45, 46], isocitric (2) and citric (3) [47, 48], ascorbic acid (5) [50, 51], 3,5-dihydroxybenzoic (12) [55], *p*-hydroxybenzoic (11), protocatechuic (13), feruloyl quinic (22), vanillic (9) and its isomers (23 and 35), syringic (25), ferulic (18), *p*-coumaric (28) [52, 57], *o*-coumaric (29) [53], sinapic (31) [52] and dihydroferulic acid (32) [57] were identified as previously published.

Phenolic acid derivatives

Phenolic acid derivatives are mostly glycosides, their first fragmentation stage is the cleavage of the glycosidic linkage to yield the *m/z* of the phenolic acid and the corresponding neutral mass loss of sugar molecules, then neutral mass losses of hydroxyl, methyl or carboxylic groups were helpful in identification of the specific phenolic acid. Caffeic acid hexoside-*O*-pentoside (4) [49], protocatechuic acid hexoside (6), *p*-coumaric acid hexoside (16), ferulic acid hexoside (65) [52], dihydro-feruloyl-*O*-glucoside (19) [48, 57], *p*-coumaroyl quinic (7) and its isomer (20) [53], dihydroferulic acid-HMG-glucoside (26) [48], dihydro-caffeoyl-*O*-glucoside (24), sinapoyl-*O*-glucoside (33), dihydrosinapoyl-*O*-glucoside (34) [57], caffeoylglucaric acid (58) [49], sinapaldehyde (79) [54] and ferulic acid derivative (66) [68] were identified.

Additionally, two glucosylated abscisic acid derivatives were observed. The molecular ion signal $[M-H]^-$ of 8`-hydroxy-abscisic acid glucoside (30) was detected at *m/z* 441 and MS/MS fragments at *m/z* 397, 330, 161 were identical to the reported structural data of this compound [57]. The other derivative was abscisic acid-*O*-glc-HMG (27) which consisted of an additional HMG (hydroxyl-methylglutaryl) substitution. The MS/MS spectra of (27) with $[M-H]^-$ at *m/z* 585 showed the neutral loss cleavage of *m/z* 144 for a HMG unit to produce the fragment ion *m/z*

441 (abscisic acid -*O*-glc). The mass fragment at m/z 330 corresponding to isophorone glucoside deduced from the cleavage of side chain $C_6H_7O_2$ [57].

Coumarin derivatives

Five coumarin derivatives were found in dichloromethane or ethyl acetate fractions of the juice. They gave same fragmentation patterns with common fragment ions at *m/z* 173, 143, 111 and UV spectra characteristic for substituted coumarins [76]. Limettin (5, 7- dimethoxy coumarin) (8) shows deprotonated molecular ion peak at *m/z* 205 and was identified as previously reported [51]. MS data indicated the presence of one ethoxyl and methoxyl groups in compound (10) and its isomer (14) ([M-H] $^-$ at m/z 219). Propoxyl and methoxyl groups in (15) and its isomer (21) ([M -H] $^-$ at m/z 233) but did not allow their position to be established. In any case, 10 and 14 were tentatively identified as ethoxy-methoxy coumarin while (15) and (21) were identified as propoxy-methoxy coumarin.

Flavone C-glucosides

The UV spectrum of compounds (43), (47), (48), (50), (54) and (62) are characteristic of flavone structures. The presence of ([M-H]$^-$ -90), and ([M-H]$^-$ -120) in negative ionization mode confirmed that these compounds are mono -C-hexosylated flavones. Investigation of MS/MS spectrum indicated that the sugar lies on the position 8 due to the absence of the fragment peak at *m/z* ([M-H]$^-$-18) as in compounds (47), (50) and (54) which were identified as vitexin, chrysoeriol-8-C-glucoside (scoparin) and orientin-4'-methyl ether (diosmetin-8-C-glucoside), respectively [63]. In contrast, the loss of water and presence of the mass fragment m/z ([M-H]$^-$ -18) confirmed the presence of C-6 glucoside as in isoorientin (43) [48], isovitexin (48) and isoorientin-4'-methyl ether (diosmetin-6-C-glucoside) (62) [63]

In compound (37), position 6 and 8 are substituted where characteristic fragment at *m/z* 383 corresponding to ([M-H]$^-$ -120- 120) in MS-MS that confirm the two C-glucosides substitution. The compound was identified as lucenin-2 4'-methyl ether [60, 77].

Rutinoside and neohesperidoside flavonoids

Elution of rutinosides earlier than neohesperidosides was evidenced [44]. The rutinosides [Glc (6→1) Rha] favour mostly the complete loss of the disaccharide unit ([M -H]$^-$ -308) giving this respective aglycone peak with high abundance [57]. Three compounds with pesudomlecular ion at m/z 609 were detected. Hesperidin (hesperetin-7-*O*-rutinoside) (59), homoeriodictyol 7-*O*- rutinoside (60) and its 7-*O*-neohesperidoside isomer (neohesperidin) (61) were identified with MS/MS fragment ion at m/z 301 for a hesperitin aglycone [66]. Subsequently, identification of isorhamentin-3-rutinoside (57) [63] and its neohesperidoside isomer (72), eriocitrin (46) and neoeriocitrin (49) [57] were deduced. In addition, the MS spectra of all rutinosides eriocitrin (46), luteolin-7-*O*-rutinoside (51), didymin (52), kaempferol-3-*O*-rutinoside (53), narirutin (56) and hesperidin (59) showed an aglycone pseudomolecular ion peak in addition to the corresponding pseudomolecular ion, while the neohesperidosides neoeriocitrin (49) and neohesperidin (61) showed only pseudomolecular ion [44]. The signal at *m/z* 607 was assigned as diosmin (77) with an aglycone mass of *m/z* 299 [64].

Flavonol-O-glycosides

The favoured positions of glycosidation in flavonols are C-3 and/or C-7. The signal at *m/z* 593 was assigned as kaempferol-3-*O*-rutinoside (53) with an aglycone mass of *m/z* 285. Isorhamnetin-3-*O*-rutinoside (57) and its neohesperidoside isomer (72) were characterized by a [M-H]$^-$ at *m/z* 623 with a product ion at *m/z* 315 (aglycone ion derived from the loss off a rutinosyl or neohesperidosyl moiety ([M-H]$^-$ -308) [57, 63]

Flavonol-O-glucosides- with 3-hydroxy-3-methylglutaric acid (HMG) substitution

Citrus species are rich with 3-hydroxy-3-methylglutaric acid (HMG) containing flavonoids, and the respective glucosides [57]. They showed natural statin and hypoglycemic activities in type-2 diabetic animals *via* decreasing hepatic 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase and acyl CoA: cholesterol acyl transferase (ACAT) activities and increased fecal cholesterol [78].

Isorhamentin-3-*O*-[6``-HMG] glucoside (42) with [M-H]$^-$at *m/z* 621 showed an ion fragment at *m/z* 477 attributed to the loss of one unit of HMG, and the loss of glucose unit was deduced by the presence of ion fragment at m/z 315 [62].

The limocitrin (85) (8-methoxy isorhamnetin) showed a pseudomolecular ion peak at *m*/z 345. HMG-substitutions in compounds (41) and (45) were deduced by the neutral loss cleavages at m/z 62, 102 and 144 to the respective fragment ions *m/z* 589, 549 and 507, respectively. The neutral loss of *m/z* 162 to give a fragment at *m/z* 345 indicated the cleavage of a glucose moiety to the aglycone limocitrin. The pseudomolecular ion peak [M-H]$^-$at *m/z* 651 of limocitrin-*O*-glc-HMG (41), and its positional isomer (45) and the daughter ion fragments at *m/z* 345 and 330 for the aglycone moiety with one HMG unit were detected.

Limocitrin-*O*-glc. HMG-HMG (36) was structurally related to the limocitrin derivatives (41) and (45) with [M-H]$^-$ at m/z 795. Two indicative neutral loss cleavages revealed an additional HMG- unit [57].

Limocitrol aglycone (82) is a C-6 methoxy-limocitrin which produced pseudomolecular ion peak at *m/z* 375 and daughter ions at *m/z* 330 and 345 in the ESI–MS/MS analysis. Limocitrol-*O*-glucoside (55) showed [M-H]$^-$ at *m/z* 537 and it was recognized by the neutral loss cleavage of *m/z* 162 to produce a fragment at *m/z* 375. All detected limocitrol glucosides showed two indicative fragment ions at *m/z* 360 and 375 [57, 62].

Limocitrol-*O*-glc-HMG (40) and its isomer (44) were detected by pseudomolecular ion peak at *m/z* 681. The loss of HMG was confirmed by daughter fragment ions at *m/z* 619 ([M-H]$^-$-62), 579 ([M-H]$^-$ -102), 537 ([M-H]$^-$ -HMG). Further loss of glucose

moiety was deduced from the fragment ion at *m/z* 375 ([M-H]$^-$-HMG-glc.).
Limocitrol-*O*-glc-HMG-HMG (38) and its isomer (39) with [M-H]$^-$at *m/z* 825, showed identical MS/MS fragmentation pattern. Fragment ions at *m/z* 681 ([M-H]$^-$-HMG), 537 ([M-H]$^-$-HMG-HMG) and 375 ([M-H]$^-$-HMG-HMG-glc.) indicated that these compounds (38 and 39) have two HMG-units and one glucosyl moiety [57].

Flavonoid aglycones
The UV spectrum of compounds (73), (74), (75), (81), (83) and (84) are characteristic of flavone structures. Eriodictyol (73) was recognized by comparing its MS/MS fragmentation pattern with the previously reported data [72, 73].
Compound (75) was deduced to be luteolin while compound (83) gave a [M-H]$^-$ ion at *m/z* 299, was assigned as chrysoeriol and compound (81) with [M-H]$^-$ ion at m/z 269 as apigenin [53]. Tetramethoxy flavone (74) [74] and trihydroxymethoxy flavone (84) were tentatively identified as well.
The UV spectrum of compounds (86), (87) and (90) are characteristic of flavonol structures. Quercetin dimethyl ether (86) and jaceidin (87) were identified as previously reported [64] while myricetin tetramethyl ether (90) was tentatively identified.

Limonoids
Six limonoids aglycones were identified from the dichloromethane fractions based upon comparison with literature data as following: Calamin (67), cyclocalamin (68), isocyclocalamin (69), limonexic acid (70), limonexic acid isomer (71) [51], limonin (89) [75], and an unknown limonoid derivative (80).

Miscellaneous compounds
Chrysoeriol-7-*O*-glucoside (63) was recognized by comparing its MS/MS fragmentation pattern with the reported data [51].
Compound (64) showed a deprotonated molecular ion at *m/z* 579 and an intense fragment at *m/z* 417, attributed to the loss of hexoside moiety. Further, MS analysis produced identical product ions tentatively identified as syringaresinol lignin, so compound (64) can be identified as syringaresinol hexoside 1 [54].

Table 1: Metabolites identified in pink lemon stem (S), leaf (L), rind (R) and juice (J) dichloromethane and ethyl acetate fractions using HPLC-PDA-ESI-MS in negative ionization mode

No	Compound name	RRt*	RRt** (min)	UV λmax (nm)	[M-H]$^-$ (m/z)	MS^2 fragments (m/z)	Ref.	DCM				EtOAc			
								S	L	R	J	S	L	R	J
1	Malic acid		0.035	213	133	115, 71	45, 46	-	-	-	+	-	-	-	+
2	Isocitric acid		0.045	213, 285	191	173, 111, 85	47, 48	-	-	-	+	-	-	-	+
3	Citric acid		0.061	209, 295	191	173, 111, 87	47, 48	-	-	-	+	-	-	-	+
4	Caffeic acid hexoside-*O*-pentoside	0.027		210	473	341 ([M-H]$^-$ -132), 312 ([M-H]$^-$ -162)	49	+	-	-	-	-	-	-	-
5	Ascorbic acid	0.038		289	175	143, 113, 101, 99	50, 51	+	-	+	-	-	-	-	-
6	Protocatechuic acid hexoside		0.061	251	315	153 ([M-H]$^-$ -glc.)	52	-	-	-	-	-	+	-	-
7	*p*-Coumaroyl quininc acid	0.053		292	337	191, 173 ([M-H]$^-$ -Coumaroyl), 155, 111	53	-	-	+	-	-	-	-	-
8	Limettin (Citropten)		0.096	209, 295	205	187, 173, 143, 131, 111, 87	51	-	-	-	-	-	-	-	+
9	Vanillic acid	0.087		251, 283	167	152, 123 ([M-H]$^-$ -CO_2)	52, 54	-	-	+	-	-	-	-	-
10	Ethoxy- methoxy coumarin †		0.141	278	219	173, 143, 111		-	-	-	-	-	-	-	+
11	*p*-Hydroxybenzoic acid	0.087	0.143	285 sh	137	93 ([M-H]$^-$ - CO_2)	52	+	-	-	-	-	-	-	-
12	3,5-Dihydroxybenzoic acid		0.148	254	153	109	55	-	-	-	-	-	+	-	-
13	Protocatechuic acid		0.15	254, 286	153	109	52	-	-	-	-	-	+	-	-
14	Ethoxy- methoxy coumarin isomer †		0.169	268	219	173, 143, 111		-	-	-	-	-	-	-	+
15	Propoxy-methoxy coumarin †		0.208	255	233	215, 173, 157, 143, 111		-	-	-	-	-	-	-	+
16	*p*- Coumaric acid hexoside		0.209	266, 296	325	163	52	-	-	-	-	-	+	-	-

No	Compound name	RRt*	RRt** (min)	UV λmax (nm)	$[M-H]^-$ (m/z)	MS^2 fragments (m/z)	Ref.	DCM				EtOAc			
								S	L	R	J	S	L	R	J
17	Meranzin hydrate	0.124		286	277	259, 189	51, 56	-	-	+	-	-	-	-	-
18	Ferulic acid	0.227		283, 306 sh	193	178, 149, 134	52	+	-	+	-	-	-	-	-
19	Dihydro-feruloyl-*O*-glucoside	0.234	0.214	277	357	195 ($[M-H]^-$ -glc.), 151 ($[M-H]^-$ -glc.- CO_2), 136	48, 57	+	-	+	-	+	-	+	-
20	*p* -Coumaroyl quininc acid isomer		0.232	251	337	191, 173 ($[M-H]^-$ -Coumaroyl), 155, 111	53	-	-	-	-	-	+	-	-
21	Propoxy- methoxy coumarin isomer †		0.245	278	233	201, 187, 157, 143		-	-	-	-	-	-	-	+
22	Feruloyl quinic acid		0.254	253, 284	367	193, 173	52, 58	-	-	-	-	-	+	-	-
23	Vanillic acid isomer		0.26	254, 284	167	152, 123 ($[M-H]^-$ -CO_2), 108 ($[M-H]^-$ -CO_2- CH_3)	52, 54	-	-	-	-	-	+	-	-
24	Dihydro-caffeoyl-*O*-glucoside	0.247	0.261	274	343	181 ($[M-H]^-$ -glc.), 163, 137 ($[M-H]^-$ -glc.- CO_2)	57	+	-	+	-	-	+	+	-
25	Syringic acid		0.304	285	197	182, 153	52	-	-	-	-	-	+	-	-
26	Dihydroferulic acid–HMG - glucoside		0.341	-------	501	399, 357 ($[M-H]^-$ -HMG, 195($[M-H]^-$ -HMG- glc.), 151	48	-	-		-	-	-	+	-
27	Abscisic acid -*O*-glucoside-HMG		0.363	-------	585	483 ($[M-H]^-$ -102), 441 ($[M-H]^-$ -HMG), 330 ($[M-H]^-$ - HMG-$C_6H_7O_2$) (isophorone glucoside)	57	-	-	-	-	-	-	+	-
28	*p*-Coumaric acid	0.256	0.371	269	163	119	52 , 53,55	+	-	+	-	-	+	-	-
29	*o*-Coumaric acid	0.258	0.411	277	163	119	53	+	-	-	-	-	+	-	-
30	8`-Hydroxy-abscisic acid glucoside		0.417	-------	441	397, 330, 161	57	-	-	-	-	-	-	+	-
31	Sinapic acid	0.28		307	223	208, 179, 164	59	+	-	+	-	-	-	-	-
32	Dihydroferulic acid	0.304		276	195	177, 151, 136	57	+	-	+	-	-	-	-	-
33	Sinapoyl-*O*-glucoside		0.452	210, 290	385	265, 223, 179	57	-	-	-	-	-	+	-	-
34	Dihydrosinapoyl-*O*-glucoside	0.337		278 sh	387	225, 181	57	-	-	+	-	-	-	-	-
35	Vanillic acid isomer	0.416		251, 283	167	152, 123 ($[M-H]^-$ - CO_2), 108	57	+	+	-	-	-	-	-	-
36	Limocitrin-*O*-glc. HMG-HMG	0.44	0.541	285 sh.	795	693 ($[M-H]^-$ - 102), 651 ($[M-H]^-$ -HMG), 589, 549, 507 ($[M-H]^-$ -HMG-HMG), 345 ($[M-H]^-$ -HMG-HMG-glc.)	57	-	-	+	-	-	-	+	-
37	Lucenin-2 4'-methyl ether	0.451		280 sh, 318	623	605 ($[M-H]^-$ -18), 533 ($[M-H]^-$ -90), 503 ($[M-H]^-$ -120), 413 ($[M-H]^-$ -90-120), 383 (($[M-H]^-$ -120-120)	60	+	-	-	-	-	-	-	-
38	Limocitrol - *O*- glc. HMG-HMG		0.552	277	825	723 ($[M-H]^-$ -102), 681($[M-H]^-$ - HMG), 619, 579, 537($[M-H]^-$ - HMG-HMG), 375 ($[M-H]^-$ - HMG-HMG-glc.)	57	-	-	-	-	-	-	+	-
39	Limocitrol –*O*- glc. HMG-HMG isomer		0.556	278	825	723 ($[M-H]^-$ - 102), 681($[M-H]^-$ - HMG), 619, 579, 537($[M-H]^-$ - HMG-HMG), 375 ($[M-H]^-$ - HMG-HMG-glc.)	57	-	-	-	-	-	-	+	-
40	Limocitrol-*O*-glc. HMG	0.478	0.653	282, 379	681	619 (($[M-H]^-$ -62), 579 ($[M-H]^-$ -102), 537 ($[M-H]^-$ -HMG), 375 ($[M-H]^-$ -HMG-glc.)	57, 61	-	-	+	-	+	-	+	-

No	Compound name	RRt*	RRt** (min)	UV λmax (nm)	[M-H]$^-$ (m/z)	MS^2 fragments (m/z)	Ref.	DCM				EtOAc			
								S	L	R	J	S	L	R	J
41	Limocitrin-*O*-glc. HMG	0.48	0.658	282, 379	651	589 (([M-H]$^-$ -62), 549 ([M-H]$^-$ -102), 507 ([M-H]$^-$ -HMG), 345 ([M-H]- -HMG-glc.)	57, 61	-	-	+	-	+	-	+	-
42	Isorhamnetin-3-*O*-[6"-HMG]-glucoside		0.737	278, 323, 379	621	559 ([M-H]$^-$ - 62), 519 ([M-H]$^-$ - 102), 477 ([M-H]$^-$ -HMG), 315 ([M-H]$^-$ -HMG-glc.)	62	-	-	-	-	+	-	-	-
43	Isoorientin		0.776	281, 310, 379	447	429 ([M-H]$^-$ -18), 357 ([M-H]$^-$ -90) , 327 ([M-H]$^-$ - 120), 297	48	-	-	-	-	+	-	-	-
44	Limocitrol -*O*- glc. HMG isomer	0.482	0.784	277, 331, 357	681	619, 579, 537, 375, 360	57, 61	-	-	+	-	+	-	+	-
45	Limocitrin -*O*- glc. HMG isomer	0.484	0.79	278, 327	651	589 (([M-H]$^-$ -62), 549 ([M-H]$^-$ -102), 507 ([M-H]$^-$ -HMG), 345 ([M-H]- -HMG-glc.), 330	57, 61	-	-	+	-	+	-	-	-
46	Eriocitrin	0.558	0.812	281, 379	595	287	57	+	+	-	-	+	+	+	+
47	Vitexin		0.838	273, 343	431	341 ([M-H]$^-$ -90), 311([M-H]$^-$ -120)	63, 64	-	-	-	-	+	+	-	-
48	Isovitexin		0.849	279, 342	431	413 ([M-H]$^-$ -18) , 341([M-H]$^-$ -90), 311([M-H]$^-$ -120)	63, 64	-	-	-	-	+	-	+	-
49	Neoeriocitrin	0.56	0.849	280, 324	595	287	57, 65	-	+	-	-	+	+	+	+
50	Chrysoeriol 8-C-glucoside (Scoparin)		0.879	273, 351	461	371 ([M-H]$^-$ -90), 341([M-H]$^-$ -120)	57, 63	-	-	-	-	+	-	+	+
51	Luteolin-7-*O*-rutinoside		0.882	274, 329	593	285 ([M-H]$^-$ -Rut.)	53	-	-	-	-	+	-	-	-
52	Didymin (Neoponcirin)		0.884	271, 338	593	285 ([M-H]$^-$ -Rut.), 241	57	-	-	-	-	+	-	-	-
53	Kaempferol-3-*O*-rutinoside		0.885	270, 340, 380	593	285 ([M-H]$^-$ -Rut.)	57	-	-	-	-	+	-	-	-
54	Orientin-4`-methyl ether (Diosmetin-8-C- glucoside)		0.911	278, 325	461	371 ([M-H]$^-$ -90), 341 ([M-H]$^-$ -120)	63	-	-	-	-	+	-	+	+
55	Limocitrol-*O*-glucoside		0.958	280, 318	537	522, 375, 359	57	-	-	-	-	+	+	+	+
56	Naringenin -7-*O* -rutinoside (Narirutin)		0.962	280, 321	579	271 ([M-H]$^-$ -Rut.)	57	-	-	-	-	+	-	-	-
57	Isorhamnetin-3-rutinoside		0.97	278, 323	623	315 ([M-H]$^-$ - Rut.)	63	-	-	-	-	+	-	-	-
58	Caffeoylglucaric acid		0.983	276, 327	371	209 ([M-H]$^-$ -Caf.)	49	-	-	-	-	+	-	+	-
59	Hesperidin		1	282, 326 sh	609	459, 325, 301([M-H]$^-$ -Rut.)	66	-	-	-	-	+	+	+	+
60	Homoeriodictyol-7-*O*-rutinoside		1.017	282, 327 sh	609	301 ([M-H]$^-$ -Rut.)	67	-	-	-	-	+	-	+	-
61	Neohesperidin		1.02	282, 331, 380	609	343, 301	66	-	-	-	-	+	-	-	- -

No	Compound name	RRt*	RRt** (min)	UV λmax (nm)	$[M-H]^-$ (m/z)	MS^2 fragments (m/z)	Ref.	DCM				EtOAc			
								S	L	R	J	S	L	R	J
62	Isoorientin -4'-methyl ether (Diosmetin- 6-C-glucoside)		1.056	270, 316	461	443 ($[M-H]^-$ - 18), 371($[M-H]^-$ - 90), 341($[M-H]^-$ -120)	63	-	-	-	-	+	+	-	-
63	Chrysoeriol-7-*O*-glucoside		1.056	252, 268, 346	461	446 ($[M-H]^-$ -CH_3), 445, 371, 357, 341, 299 ($[M-H]^-$ -glc.), 283, 269, 257	51	-	-	-	-	+	+	-	-
64	Syringaresinol hexoside 1	0.648		281 sh	579	417, 402, 387	54	-	+	-	-	-	-	-	-
65	Ferulic acid hexoside	0.653		285, 324	355	193, 179, 149, 134	52	-	+	-	-	-	-	-	-
66	Ferulic acid derivative†	0.656		327	-	193, 178, 149, 134	68	-	+	-	-	-	-	-	-
67	Calamin	0.677		282	519	487, 475, 459, 415, 371	51	-	-	+	-	-	-	-	-
68	Cyclocalamin	0.703		300	501	457, 413	51, 69	+	+	+	+	-	-	-	-
69	Isocyclocalamin	0.71		310	501	501, 457, 425, 371	51, 70	+	-	+	-	-	-	-	-
70	Limonexic acid (Limonexin)	0.727		305	501	457, 439, 413	51, 69	+	-	+	-	-	-	-	-
71	Limonexic acid isomer (Isolimonexic acid)	0.729		260	501	457, 413, 372	51, 71	+	-	+	-	-	-	-	-
72	Isorhamnetin neohesperidoside		1.201	281, 320, 379	623	315 ($[M-H]^-$ - Neoh.)	57	-	-	-	-	+	-	-	-
73	Eriodictyol		1.215	287, 324	287	151, 135, 125, 107	72, 73	-	-	-	-	-	-	+	-
74	Tetramethoxy flavone	0.758		278	341	326, 311 , 285	74	-	+	-	-	-	-	-	-
75	Luteolin		1.298	276, 326	285	285, 267, 257, 243, 241, 217, 213, 199, 197, 175, 151, 133	53, 72	-	-	-	-	-	-	+	-
76	Trihydroxy octadecadienoic acid	0.802		279	327	229, 211, 171	64	+	+	+	-	-	-	-	-
77	Diosmin	0.807		224 sh, 310	607	299	64	-	+	-	-	-	-	-	-
78	Trihydroxy octadecadienoic acid derivative †	0.869		222, 269, 314	565	429, 391		+	-	-	-	-	-	-	-
79	Sinapaldehyde	0.869		324	207	207, 192	54	-	+	-	-	-	-	-	-
80	Limonoids derivative †	0.928		------	545	501 ($[M-H]^-$ -44), 457, 397		-	-	+	+	-	-	-	-
81	Apigenin		1.562	209, 270, 324	269	269, 251, 225, 197, 149	53	-	-	-	-	-	+	+	-
82	Limocitrol		1.587	280, 324 sh, 390 sh	375	360, 345	57	-	-	-	-	-	-	+	+
83	Chrysoeriol	1	1.601	222, 247 sh, 321	299	299, 284	53	+	+	+	-	+	+	+	-

No	Compound name	RRt*	RRt** (min)	UV λmax (nm)	$[M-H]^-$ (m/z)	MS^2 fragments (m/z)	Ref.	DCM				EtOAc			
								S	L	R	J	S	L	R	J
84	Trihydroxy methoxy flavone†		1.62	270, 331	299	299, 284	-	-	-	-	-	-	+	-	-
85	Limocitrin	1.013	1.634	280, 323 sh, 379	345	345, 330	57	-	-	+	-	-	+	+	+
86	Quercetin dimethyl ether		1.674	474	329	314, 299	64	-	-	+	-	-	-	-	+
87	Jaceidin		1.772	277, 379	359	344, 329	64	-	-	-	-	+	+	-	+
88	Coumaroyl derivative†	1.157	1.89	280	379	163, 119		-	-	+	-	+	+	-	-
89	Limonin	1.219		277 sh	469	455, 411, 367, 191	75	-	-	+	-	-	-	-	-
90	Myricetin tetramethyl ether†		1.943	277, 379	373	358, 343		-	-	-	-	-	-	+	+

†= Tentative identified; RRt* = Relative retention time to chrysoeriol; RRt**= Relative retention time to hesperidin; glc. = glucose; Rut.= Rutinosyl; Neoh.= Neohesperidosyl; Caf.= Caffeoyl; HMG= 3- hydroxyl-3-methyl glutaryl; DCM= Dichloromethane; EtOAc= Ethyl acetate.

3.4. Quantitative estimation of the total phenolics and flavonoids contents

Phenolic compounds exhibited antioxidant activity and played a role in prevention of heart diseases and cancer [79]. Total phenolic contents of leaf, stem, juice and rind, as determined by Folin-Ciocalteu method, are expressed as gallic acid equivalents (GAE) by reference to standard calibration curve ($y = 0.0043 x + 0.0583$, $r^2=0.9916$) and shown in Table (2).

Table 2: The total phenolic and flavonoids contents of the total alcoholic extract and different fractions of pink lemon leaf, stem, juice and rind

Plant organ	Plant extract / fraction	Total phenolics mg GAE/g extract	Total flavonoids	
			mg quercetin equivalent /g extract	mg rutin equivalent /g extract
Leaf	*n*-Hexane	ND	485.48 ± 0.50	545.64 ± 0.56
	Dichloromethane	ND	11.35 ± 2.22	19.53 ± 2.46
	Ethyl acetate	29.72 ± 0.13	354.60 ± 0.82	400.22 ± 0.91
	Total alcoholic extract	30.11 ± 0.12	136.85 ± 1.29	158.28 ± 1.43
Stem	*n*-Hexane	ND	365.98 ± 1.26	412.86 ± 1.40
	Dichloromethane	ND	10.98 ± 0.50	18.42 ± 0.56
	Ethyl acetate	113.01 ± 0.23	79.35 ± 0.58	94.39 ± 0.64
	Total alcoholic extract	12.96 ± 0.23	557.10 ± 0.82	625.22 ± 0.91
Juice	*n*-Hexane	ND	7.35 ± 0.58	14.39 ± 0.64
	Dichloromethane	ND	0.134 ± 0.06	0.262 ± 0.02
	Ethyl acetate	4.4 ± 0.13	2.806 ± 0.19	4.93 ± 0.06
	Total alcoholic extract	6.6 ± 0.36	2.753 ± 0.50	4.83 ± 0.18
Rind	*n*-Hexane	ND	7.98 ± 0.22	15.08 ± 0.56
	Dichloromethane	ND	1.26 ± 0.58	7.63 ± 0.32
	Ethyl acetate	91.19 ± 0.23	130.85 ± 0.50	151.61 ± 0.64
	Total alcoholic extract	26.03 ± 0.59	56.73 ± 1.71	69.25 ± 0.56

ND: Not determined

The results showed that the total phenolic contents ranged from 4.4 ± 0.13 to 113.01 ± 0.23 mg GAE/g in the tested extracts where ethyl acetate fraction of stem contains the highest amount of phenolics.Total flavonoids contents of different fractions were determined by aluminum chloride-potassium acetate spectrophotometric method, and expressed as quercetin and rutin equivalents by reference to calibration curves where y = 0.001 x - 0.0128 and r^2 = 0.996 in case of quercetin as standard while y = 0.0009 x + 0.0019 and r^2 = 0.9966 in case of rutin as standard. The results showed that total alcoholic extract of the stem is the most abundant fraction with flavonoids followed by the *n*-hexane fraction of leaf (Table 2).

Comparison between the results of this study and other citrus plants showed that total phenolic contents of different fraction of *C. sinensis* peel ranged from 3-115 mg/g extract and ethyl acetate fraction had the highest concentration, [80] which is in agreement with our results. On the other hand, *C. microcarpa* phenolic contents is less than the ethyl acetate fraction of pink lemon stem while *C. hystrix* had the highest total phenolic content (490.74 ± 1.75) significantly higher than other tested *Citrus* species [81]. The variation in total phenolics levels may be due to varieties, geographic origin, growing seasons, agricultural practices, and differences in methods of analysis [82].

3.5. Antioxidant activity

It is well known that the plant flavonoids and phenols in general, are highly effective free radical scavengers and antioxidants. Thus, they are used for the prevention and cure of various diseases which are mainly associated with free radicals. Fractions which showed high total flavonoids and/or phenolic contents (Table 2) were evaluated for their radical scavenging capacity. Series of concentrations were used ranged from 5 to 320 µg in methanol. The DPPH scavenging percentage of different fractions as well as ascorbic acid and SC_{50} values (the concentration required to scavenge DPPH by 50%) are shown in Figure 2 and 3, respectively. Stem and leaf ethyl acetate fractions showed the highest antioxidant activities as indicated by their high DPPH scavenging percentage (79.6 and 78.9%, respectively) at 320 µg and low SC_{50} values (30 and 19.01 µg, respectively) which approached the activity of the standard ascorbic acid. Their activity can be attributed to their contents of vitexin, scoparin and hesperidin as reported before [83, 84]. During this work, hesperidin was isolated from ethyl acetate fractions of stem, leaf and rind which was further supported by its detection in LC-MS analysis of ethyl acetate fraction of all organs. Its high antioxidant activity was evidenced and attributed to its chemical structure where 3'-hydroxy, 4'-*O*- methoxy system in the ring B, reciprocal configuration of the 5-hydroxyl group and the double bond C5-C6 of the A ring. It was reported that hesperidin at 6 mg/L showed DPPH scavenging activity 83% [85]. In a previous study, apigenin, kaempferol, neohesperidin and neoeriocitrin (detected in LC-MS of different organs extracts of pink lemon), showed significant antioxidant activity at concentration of 10 µM [86]

Phenolic compounds can act as antioxidants by many potential pathways such as free radical scavenging, oxygen radical absorbance, peroxide decomposition, suppression of singlet oxygen, chelating of metal ions and enzymatic inhibition and increasing the levels of endogenous defenses [87, 88]. So, the higher the total polyphenolic content, the greater the antioxidant activity [89] as there is a direct correlation between radical scavenging activity, vitamin C concentration and total phenolic contents [79]. It was reported that antioxidant activity showed a higher significant correlation with total phenolics than total flavonoids which indicated that phenolic compounds may be responsible for the antioxidant activity on a large proportion [90]. In a similar study, *C. pyriformis* (Ponderosa lemon) showed lower antioxidant activity as indicated by SC_{50} values (13.3-625.9 µg/ml) [91].

Correlation between SC_{50} values, radical-scavenging ability and total phenolic and flavonoids contents of all fractions tested in this study in addition to the identified phenolic acids in LC-MS analysis is significant as fractions with higher phenolics and/or flavonoids contents showed lower SC_{50} value and higher antioxidant activity.

Flavonoids which have a chromanol ring system, had stronger antioxidant activity as compared to limonoids and bergapten, which lack the hydroxyl groups [92]. Limonin aglycone possessed a relatively stronger antioxidant capacity than the limonin glucoside [86].

Gorinstein et al. [89] stated that lemons peel showed the highest antioxidant potential among different studied citrus fruits and are preferred for dietary prevention of cardiovascular and other diseases. Citrus fruits' peels were proven to be rich in dietary fibres and phenolic compounds as ferulic, sinapic, *p*-coumaric and caffeic acids and suitable for industrial processing.

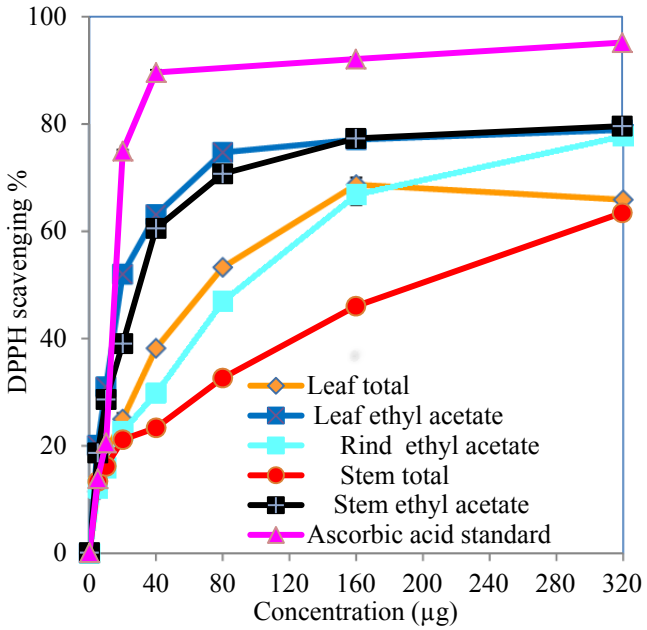


Figure 2: DPPH scavenging capacity of pink lemon fractions**.**

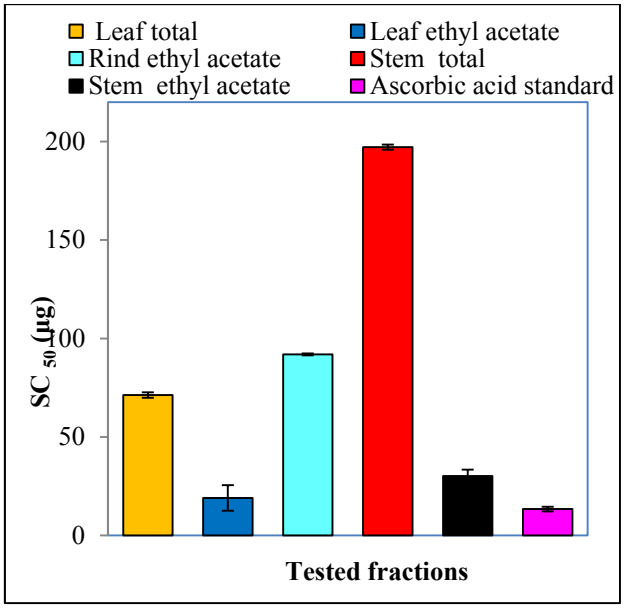


Figure 3: SC_{50} of pink lemon fractions and ascorbic acid.

3.6. Antimicrobial activity

Results of antibacterial and antifungal activity of total alcoholic extract and different fractions against different microorganisms by well diffusion technique were expressed as diameter of inhibition zone and percentage of activity (Table 3). Juice and stem *n*-hexane, and rind dichloromethane fractions didn't inhibit the growth of the all tested bacteria and fungi. These results also indicated that ethyl acetate fraction of juice (JE) showed MIC values 0.49 µg/ml that is equal to MIC of ampicillin against both *E. faecalis* and *B. subtilis*. The potential use of ethyl acetate fractions of leaf (LE) and rind (RE) in management of bacterial diseases caused by *K. pneumonia* as it showed strong antibacterial activities with MIC = 1.95 µg/ml which exceeded the activity of gentamicin with MIC = 3.9 µg/ml (Figure 4). Only the total extract of leaf could exhibit antifungal activity against *C. albicans,* reached to 92.69% activity of amphotericin B with MIC = 0.98 µg/ml. The best antifungal activity was observed for ethyl acetate of juice and rind against *S. cerevisiae* (MIC = 0.98 µg/ml compared to amphotericin (MIC = 0.24 µg/ml).

In a previous report, methanolic extract of *C. limetta* (sweet lime) peel was more effective compared to other extracts as ethyl acetate, chloroform and water in their anti-microbial activity against the pathogenic *E. coli, Pseudomonas sp., Klebsiella sp*. and methicillin resistant *S. aureus* due to the presence of flavones and phenolic contents [93]. Moreover**,** *C. medica* L. peel extract and the juice of the ripen and unripe fruit of *C .limon* possessed significant antimicrobial activity against *S. aureus*, *Klebsiella sp., E. coli, P. aeruginosa* and *C. albicans* [94, 95].

Table 3: Results of the antimicrobial screening of the different total alcoholic extract and fractions of pink lemon

Tested material	Inhibition zone diameter (mm ±S.D) (% of inhibition)								
	G-ve			G+ve			Fungi		
	E. cloacae	*K. pneumoniae*	*E. coli*	*S. aureus*	*E. faecalis*	*B. subtilis*	*A. fumigatus*	*C. albicans*	*S. cerevisiae*
Ampicillin	-	-	-	28.9 ± 0.14 (100)	25.3 ± 0.58 (100)	26.4 ± 0.34 (100)	-	-	-
Gentamicin	23.8 ± 0.63 (100)	20.2 ± 0.12 (100)	27.3 ± 0.44 (100)	-	-	-	-	-	-
Amphotricin B	-	-	-	-	-	-	23.7 ± 0.10 (100)	21.9 ± 0.12 (100)	27.8 ± 0.58 (100)
JC	17.8± 0.63 (74.79)	16.4 ± 0.37 (81.19)	18.5 ± 0.58 (67.77)	16.9 ± 0.58 (58.48)	18.3 ± 0.44 (72.33)	20.4 ± 0.44 (77.27)	16.2 ± 0.58 (68.35)	NA	18.4 ± 0.58 (66.19)
JE	21.7 ± 0.36 (91.18)	18.9 ± 0.25 (93.56)	20.4 ± 0.63 (74.73)	21.9 ± 0.44 (75.78)	23.7 ± 0.37 (93.68)	24.2 ± 0.25 (91.67)	19.8 ± 0.25 (83.54)	NA	21.7 ± 0.25 (78.06)
JT	22.4 ± 0.25 (94.12)	20.3 ± 0.37 (100.50)	19.8 ± 0.63 (72.53)	21.4 ± 0.58 (74.05)	22.2 ± 0.44 (87.75)	23.8 ± 0.58 (90.15)	20.6 ± 0.58 (86.92)	NA	21.3 ± 0.63 (76.62)
SC	16.2 ± 0.44 (68.07)	15.8 ± 0.12 (78.22)	18.2 ± 0.44 (66.67)	16.2 ± 0.37 (56.06)	17.4 ± 0.44 (68.77)	18.3 ± 0.37 (69.32)	18.2 ± 0.25 (76.79)	NA	18.9 ± 1.2 (67.99)
SE	16.8 ± 0.58 (70.59)	14.2 ± 0.25 (70.30)	17.6 ± 0.63 (64.47)	17.4 ± 0.44 (60.21)	19.3 ± 0.37 (76.28)	20.1 ± 0.25 (76.14)	16.7 ± 0.25 (70.46)	NA	20.3 ± 0.25 (73.02)
ST	21.3 ± 0.25 (89.50)	18.7 ± 0.44 (92.57)	20.3 ± 0.63 (74.36)	18.2 ± 0.44 (62.98)	19.8 ± 0.58 (78.26)	21.2 ± 0.37 (80.30)	17.3 ± 0.58 (73.00)	NA	19.2 ± 0.58 (69.06)
RH	18.3 ± 0.58 (76.89)	16.8 ± .37 (83.17)	17.3 ± 0.58 (63.37)	16.9 ± 0.58 (58.48)	16.5 ± 0.44 (65.22)	18.4 ± 0.44 (69.7)	14.2 ± 0.58 (59.92)	NA	14.2 ± 0.58 (51.08)
RE	22.4 ± 0.44 (94.12)	19.9 ± 0.25 (98.51)	21.3 ± 0.44 (78.02)	19.3 ± 0.58 (66.78)	21.4 ± 0.63 (84.58)	23.2 ± 0.58 (87.88)	21.3 ± 0.44 (89.87)	NA	22.4 ± 0.44 (80.58)
RT	19.7 ± 0.48 (82.77)	16.5 ± 0.37 (81.68)	17.6 ± 0.25 (64.47)	17.4 ± 0.25 (60.21)	17.9 ± 0.58 (70.75)	19.2 ± 0.44 (72.73)	19.1 ± 0.63 (80.59)	NA	21.2 ± 0.58 (76.26)
LH	NA	NA	NA	13.7 ± 0.58 (47.40)	14.1 ± 0.58 (55.73)	16.3 ± 0.32 (61.74)	NA	NA	NA
LC	20.1 ± 0.63 (84.45)	17.8 ± 0.63 (88.12)	19.2 ± 0.72 (70.33)	19.3 ± 0.44 (66.78)	19.8 ± 0.44 (78.26)	20.4 ± 0.36 (77.27)	20.4 ± 0.58 (86.08)	NA	21.2 ± 0.58 (76.26)
LE	22.4 ± 0.58 (94.12)	20.8 ± 0.19 (102.97)	21.3 ± 0.58 (78.02)	20.9 ± 0.44 (72.32)	18.6 ± 0.58 (73.52)	21.9 ± 0.36 (82.95)	20.4 ± 0.58 (86.08)	NA	20.4 ± 0.58 (73.38)
LT	17.1 ± 0.63 (71.85)	18.6 ± 0.24 (92.08)	20.5 ± 0.58 (75.09)	20.3 ± 0.58 (70.24)	20.9 ± 0.58 (82.61)	21.2 ± 0.32 (80.30)	17.2 ± 0.32 (72.57)	20.3 ± 0.58 (92.69)	21.4 ± 0.32 (76.98)

JC: Juice dichloromethane fraction, JE: Juice ethyl acetate fraction, JT: Juice total extract, SC: Stem dichloromethane fraction, SE: Stem ethyl acetate fraction, ST: Stem total extract, RH : Rind *n*-hexane fraction, RE: Rind ethyl acetate fraction, RT: Rind total fraction, LH : Leaf *n*-hexane fraction, LC: Leaf dichloromethane fraction, LE: Leaf ethyl acetate fraction , LT: Leaf total fraction.NA: No activity

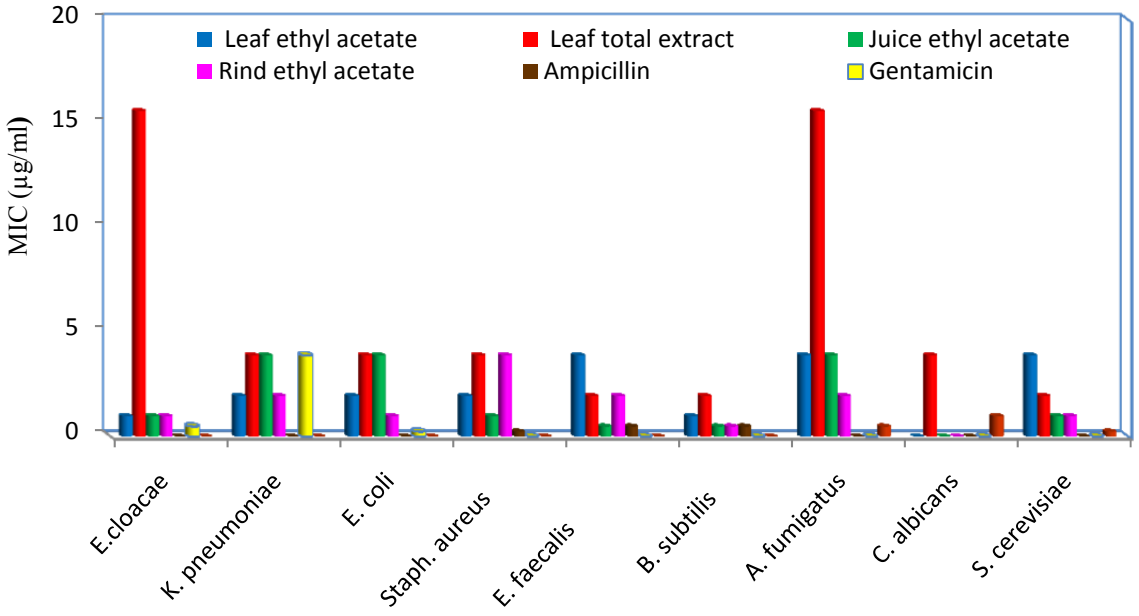


Figure 4: Minimum inhibitory concentration (MIC) of pink lemon fractions, antibiotics and antifungal.

Fruit juice of *C. limon* and *C. aurantium* showed high antimicrobial activity against *E. coli*. Their activity is probably due to the presence of flavonoids and steroids that are able to complex with extracellular and soluble proteins and to complex with bacterial cell wall [96]. Additionally, methanolic extract of *C. aurantifolia* leaves showed high potency against *E. coli* and *S. aureus* using erythromycin as positive control

The antibacterial activity may be attributed to the presence of flavonoids, tannins and steroidal alkaloids [97]. Moreover, ethyl acetate extract of lemon seeds (*C. limon*) showed its maximum activity against *E. coli* while hot water extract of lemon peel was found to be maximum against *S. aureus* [98]

In a previous report, chrysoeriol showed a strong inhibitory effect on *K. pneumonia* and *B. subtilis* and to less extent against *E. cleacae*, *E. coli*, *S. aureus* and *C. albicans* [99]. LC-MS analysis indicated the presence of apigenin in LE and vitexin in stem ethyl acetate (SE) and RE. Basile et al. [100] confirmed that apigenin and vitexin exhibited antimicrobial activities against *E. coli, K. pneumoniae*, and *E cloacae*, which is in agreement with our results.

β-Sitosterol and oleic acid extracted from flavedo and albedo of *C. grandis* Osbeck showed higher antimicrobial activity than limonin against *E. coli*, *B. subtilis* and *S. aureus* which may be attributed to the synergistic effects of *β*- sitosterol and oleic acid [101].

C. grandis carotenoids extract was suggested as a natural alternative for chemicals in food preservation as *β*- carotene could lead to the accumulation of lysozyme, an antibacterial immune enzyme that digests bacterial cell walls [102].

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CONCLUSION

Thirteen compounds were isolated and characterized for the first time in variegated pink-fleshed eureka lemon cultivated in Egypt. Organic acids and their glycosides, flavonoids and their HMG derivatives, coumarins and limonoids were identified in different organs using HPLC-PDA-ESI-MS/MS analysis. Ethyl acetate fraction and total alcoholic extract of the stem showed the highest concentration of phenolics and flavonoids, respectively while leaf ethyl acetate fraction showed the highest *in vitro* antioxidant activity compared with other tested fractions. Juice and rind ethyl acetate fractions exhibited the best antimicrobial activities. This study recommended the consumption of variegated pink lemon and its use as food additive of natural origin or pharmaceutical supplement products.

CONFLICT OF INTEREST

The authors declared no conflict of interest

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