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Research article

Unveiling the bioactive potential of Egyptian loquat leaves: Seasonal and cultivar-based variation in phenolic profiles, antimicrobial, anticancer, antioxidant, and xanthine oxidase inhibitory activities

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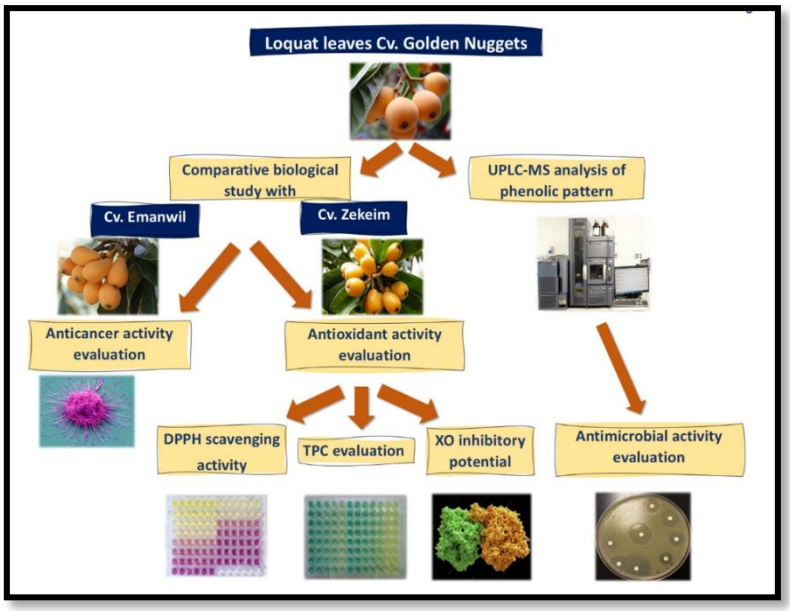
Abstract:

In this study, UPLC-MS/MS analysis was applied to unveil the phenolic pattern of loquat leaves cultivar Golden Nuggets. The leaves extracts were further assessed for their antimicrobial activity. Additionally, the cytotoxic potential, antioxidant capacity, total polyphenolic content (TPC), and xanthine oxidase (XO) enzyme inhibitory activity of leaves extracts together with the other two cultivars, Emanwil and Zekeim were evaluated at different growing seasons. A total of **28** compounds belonging to different metabolite classes were identified in cultivar Golden Nuggets, among which phenolic compounds, especially flavonoids, were the most predominant class. The methylene chloride fraction of cultivar Golden Nuggets significantly inhibited the growth of Gram-positive *Staphylococcus aureus*. The total ethanolic extracts of the tested cultivars effectively suppressed the proliferation of A549 lung cancer cell line, showing IC_{50} values between 175 and 345 µg/mL, with Golden Nuggets being the most active.



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Among Golden Nuggets fractions, the residual aqueous extract showed the most potent anti-proliferative activity (IC_{50} = 20 µg/mL) followed by the methylene chloride fraction (IC_{50} = 65 µg/mL). The tested cultivars showed significant antioxidant activities with remarkable TPC. The highest antioxidant activity and TPC were observed for Emanwil cultivar, followed by Zekeim, then Golden Nuggets, with the flowering stage recording the highest values for the three cultivars. Moreover, the examined cultivars significantly inhibited XO enzyme, showing varying efficacy through different seasons. The highest activities were observed for ethyl acetate, then methylene chloride fractions. A total of five ethyl acetate fractions showed promising inhibition rates (>95%) at a concentration of 2.5 mg/mL, comparable to that of allopurinol. This study shed light on Egyptian loquat leaves as a potential source of bioactive constituents that could serve as leads to produce effective and less toxic anticancer, antioxidant, and hypouricemic agents.

Keywords: *Eriobotrya japonica*; Phenolic profile; Xanthine oxidase; DPPH; Cytotoxic.

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1. Introduction

Eriobotrya japonica (Thunb.) Lindl., also known as "loquat", is a rosaceous evergreen shrub native to south-eastern China. The plant later became naturalized in several countries, including Japan, Korea, Brazil, Egypt, Spain, Turkey, India, and many other countries. Loquat is considered a plant with high medicinal value, and almost all organs of the plant have been used traditionally as folk medicines for many years [1,2]. Among different organs, loquat leaves attract considerable interest by researchers and have been widely used as traditional medicine for treating respiratory, gastrointestinal, and metabolic disorders, e.g., coughs, phlegm, high fever, nausea, vomiting and diabetes [3–6]. Many of these ethno-medical claims have been supported by various studies and indeed, *E. japonica* leaves have displayed high degree of efficacy in chronic bronchitis, inflammatory diseases, diabetes and against certain tumors [3,7–10]. A number of pharmacologically active constituents have been identified in loquat leaves, for instance, triterpenes, flavonoids, phenolic acids, megastigmane glycosides, and sesquiterpene glycosides [3,11–13]. Many of these compounds were correlated to hypoglycemic, anti-inflammatory, hypolipidemic, antiviral, hepatoprotective, antioxidant, and antitumorigenic activities [7,14–16]. Numerous cultivars of *Eriobotrya japonica* have been developed. Two groups are distinguished based on origin; the Chinese group with large, pyriform, deep orange fruit, which can be kept for 1-2 weeks, and the Japanese group with small, slender, light- colored fruit, maturing earlier and having a shorter life span [17,18]. A list of world-important cultivars is: "Advance", "Tanaka", "Thales" (syn. "Golden Nuggets"), "Algerie", "Akko 13", "Champagne", "Magdall", "Premier", and "Saint Michel". Cultivars grown in Egypt include "Golden Nuggets", "Emanwil", "Zekeim", "ElSukary", "Advance", "Premiere", and "Late" [19,20].

Several studies have demonstrated that seasonal changes, including variations in temperature, light, and humidity, significantly influence the accumulation of primary and secondary metabolites in plants [21–23]. The overall phytochemical composition of a plant is highly adaptive to its needs at any given time. The synchronized control of primary and secondary metabolism ensures that phytochemicals are in harmony with environmental demands [24,25]. These season-based phytochemical fluctuations, in turn, greatly impact the plant's nutritional value and overall medicinal potential [21]. The favorable actions of different natural medicines seem to be derived from their antioxidant potential. Interest in plant phytochemicals has greatly focused on phenolic constituents, which, for their

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ubiquity and diversity, represent the most beneficial antioxidant constituents in plants. As a consequence, appreciable efforts have been devoted to characterization and quantification of the phenolic constituents of the most common species [26,27]. Species of *Eriobotrya* were described among the species with the highest reducing powers, and may be candidates for the development of new natural antioxidants [28]. Nonetheless, data available about the polyphenolic content of *E. japonica* leaves is restricted to total polyphenolics, and only a few reports have pinpointed the complete phenolic pattern [29,30]. Further, no data have been reported to date about the potential health-relevant bioactivities of *E. japonica* leaves grown in Egypt. Hence, the study of the functional potential of Egyptian loquat leaves would give critical scientific information for further studies about medicinal applications, likely promoting loquat production in Egypt on a long-term basis as a high medicinal value plant.

The attempted work in this study represents a comprehensive study of the phenolic pattern of *E. japonica* leaves cultivar Golden Nuggets cultivated in Egypt using the powerful hyphenated chromatographic tool, UPLC-MS/MS. Screening of the antimicrobial activity of various extracts of the plant was performed. Additionally, the cytotoxic potential, the antioxidant capacity, the total polyphenolic content and xanthine oxidase inhibitory activity of leaves extracts of cultivar Golden Nuggets along with other two cultivars (Emanwil and Zekeim) were further examined at three different growing seasons aiming to investigate the influence of seasonal changes and inter-cultivar variations on the plant biological activities.

2. Materials and methods

2.1. Reagents, cell lines and apparatus

The reference standards gallic acid, allopurinol, (1, 1-diphenyl-2-picrylhydrazyl) DPPH free radical, Folin Ciocalteu, xanthine oxidase (XO), (Grade I, from bovine milk, approximately 10.4 units ml^{-1}), xanthine substrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) culture medium, antibiotics solution, and fetal bovine serum (FBS) were purchased from Invitogen, Life Sciences, USA. Petroleum ether (40-60ºc), chloroform, methylene chloride, ethyl acetate, *n*-butanol, ethanol, methanol, dimethyl sulfoxide (DMSO), Ultra- pure water of analytical grade and methanol of UPLC grade, Potassium phosphate buffer with 1% NaOH in distilled water and 1M HCl were procured from Fisher Scientific, UK. The Microbial strains used; *Staphylococcus aureus* (code: ATCC6538P), *Bacillus subtilis* (code: ATCC19659), *Escherichia coli* (code: ATCC8739), *Pseudomonas aeruginosa* (code: ATCC9027) and *Candida albicans* (code: ATCC2091) are local isolates provided from the Department of Pharmaceutical microbiology, Faculty of Pharmacy, Alexandria University. Lung carcinoma cell line (A549), a cell line isolated from a male nude mouse with lung carcinoma, was obtained from the American Type Culture Collection (ATCC). UPLC XEVO QqQ triple quadruple instrument, Waters Corporation, Milford, MA01757 U.S.A., Rotary evaporator; Buchi Rotavapor R-200, Switzerland, Microplate reader, BioTek, 800 TS Absorbance Reader, Micropipettes (Gilson, France) and Microtiter plates (Falcon, NJ, USA) were utilized.

2.2. Collection of loquat cultivars and sample preparation

Nine samples of *E. japonica* leaves cultivars: Golden Nuggets, Zekeim and Emanwil cultivated in Egypt were collected, one sample of each at three different growing seasons (flowering, fruiting and post- fruiting stages) as depicted in **Table S.1**. Samples

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were identified at Plant Taxonomy Department, Faculty of Science, Alexandria University, Egypt. Voucher samples (EJ101, EJ102 and EJ103) were kept at Pharmacognosy Department, Faculty of Pharmacy, Alexandria University, Egypt. Powdered air-dried *E. japonica* leaves of each cultivar collected at different growing stages were extracted using 70% ethanol and the obtained extracts were filtered, concentrated under reduced pressure using a rotary evaporator. Each extract was then subjected to solvent partitioning starting with petroleum ether, methylene chloride, ethyl acetate, and finally butanol. All fractions, in addition to the residual water extract, were concentrated under reduced pressure using a rotary evaporator.

2.3. UPLC–ESI–MS/MS analysis

Refer to the supplementary material.

2.4. Antimicrobial activity

The agar diffusion method was used for assessing the antimicrobial activity of the tested loquat samples. One milliliter of 24 hr broth culture of each of the tested organisms was separately inoculated into 100 mL of sterile molten nutrient agar maintained at 45°C. Each of the inoculated media was mixed well and poured into sterile 10 cm diameter petri dishes. After setting, ten cups, each 8 mm in diameter, were cut in the agar medium (Oxoid). Accurately weighed 25 mg of the total extract, fractions and the residual extract after fractionation were dissolved in 1 mL DMSO (9%), and the solutions were inserted in the cups and then incubated at 37°C for 24 hours. Triplicate plates were prepared for each treatment. DMSO (9%) was used as a negative control. The antimicrobial potency of the test samples was measured by determining the diameter of the zone of inhibition (measured in mm, including disc size). The antibacterial potential of extracts was compared with standard antibiotic ciprofloxacin, and the antifungal potential was compared with standard antifungal clotrimazole. The results were expressed as averages of three determinations.

2.5. Anticancer activity

Plant extracts and fractions were prepared by serial two-fold dilutions (100, 200, 300 & 400 g/mL). Lung carcinoma cell line (A549) was cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 0.2% sodium bicarbonate and 1% penicillin-streptomycin. Cells were grown in 5% CO_2 at 37 °C in high high-humidity atmosphere [31,32]. Cells were seeded in 96-well flat-bottomed microtiter plates at a cell concentration of $1x10^4$ cells per well in 100 µL of growth medium. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO_2. After incubation of the cells for 24 hr, fresh medium containing various concentrations of plant extract dissolved in DMSO was added using a multichannel pipette. The DMSO concentrations in the cell culture medium did not exceed 0.2% (*v*/*v*). Three wells were used for each concentration of the test sample. Control cells were incubated with DMSO. After adding the sample plant extract, incubation continued for 48 hours. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/mL of stock in FBS) was added to each well (10 µl/well in 100 µl of cell suspension). After incubating the cells for another 4 hr, supernatants were discarded, and the resulting MTT formazan product was dissolved by the addition of 200 µl of DMSO. The amount of formazan was determined by measuring the absorbance at 595 nm in a microplate reader. All experiments were carried out in triplicate. Treated samples were compared with the control cells. All results were corrected for background absorbance detected in wells without added MTT. The % viability of cells was determined using: % inhibition = control-sample/control [31,33].

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2.6. Antioxidant activity

Please refer to the supplementary material

2.6.1. DPPH radical scavenging assay

Please refer to the supplementary material

2.6.2. Total polyphenolic content (TPC)

Please refer to the supplementary material.

2.6.3. Xanthine oxidase inhibitory activity

Accurate weights (100 mg) from each fraction were separately transferred into a 10-mL volumetric flask, dissolved in 10% dimethyl sulfoxide (DMSO), and the volume was adjusted to 10 mL with the same solvent. Hence, 10 mg/mL stock solution for samples was prepared, and then the stock solution was diluted to the required concentrations with distilled water to prepare 2.5 mg/mL solution for ethyl acetate and methylene chloride fractions and 5 mg/mL solutions for butanol and petroleum ether fractions exploited for *in vitro* xanthine oxidase inhibition testing. The final concentrations were prepared to contain less than 1% DMSO (*v/v*), which did not affect the enzyme assay. The XO inhibitory activity was examined spectrophotometrically under aerobic conditions by following the decrease in uric acid production at 295 nm using xanthine as substrate according to the procedure developed by F. Liu *et al.*, 2017, and Nessa *et al.*, 2010 [34,35] with slight modifications. Briefly, the assay mixture was prepared by adding 1 mL of the test inhibitor solution, 1.9 mL of potassium phosphate buffer (50 mM, pH 7.6) and 0.1 mL of XO enzyme solution (0.24 units/mL in potassium phosphate buffer, pH 7.6), which had been prepared immediately before use. After pre-incubation at 37 °C for 15 min, the reaction was started by the addition of 1 mL of substrate solution (0.4 mM). The assay mixture was incubated at 37 °C for 30 min, and further reaction was stopped by adding 1 mL of hydrochloric acid (1M). The concentration of uric acid was determined by measuring the absorbance value at 295 nm using UV/vis spectrophotometer. A blank was prepared similarly, but by adding 1M HCl to the assay mixture before adding the substrate. Allopurinol: a well-known XO inhibitor, was set up as a positive control (5.2 µM, i.e., concentration that yields 100% inhibition) for the inhibition test. All determinations were performed in triplicate, thus, % inhibition was expressed as the mean of three observations. XO inhibitory activity was expressed as % inhibition of the enzyme in the above assay mixture system, calculated by the following equation:

[1- (Abs $_{\text{Test inhibitor}}$- Abs $_{\text{positive control}}$) / (Abs $_{\text{negative control}}$ - Abs $_{\text{positive control}}$)] x 100 Where Abs $_{\text{negative control}}$ is the absorbance of the control solution without the tested samples, Abs $_{\text{Test inhibitor}}$ is the absorbance of the tested samples solution and Abs $_{\text{positive control}}$ is the absorbance of Allopurinol solution that yield the maximal inhibitory effect.

3. Results and Discussion

3.1. UPLC–ESI–MS/MS analysis of phenolic pattern of loquat leaves cultivar Golden Nuggets at fruiting stage.

UPLC–ESI–MS/MS technique was employed to determine the maximum number of antioxidant phytochemicals, especially polyphenols, in leaves extracts from *E. japonica* cultivar Golden Nuggets cultivated in Egypt. The ethyl acetate and butanol fractions were chosen for the present study as preliminary screening demonstrated their abundance with polyphenols (Preliminary TLC screening). Leaves extracts were analyzed by gradient reversed phase UPLC–ESI–MS/MS using a QqQ–MS analyzer. The chromatographic conditions were optimized so that most of its chemical compositions could be separated in less than 30 min. The MS acquisition was performed in negative ionization mode as a large number of flavonoids exhibit higher sensitivity and much cleaner mass spectral background compared to the positive ion mode [36], making it most suitable to determine the molecular mass of the separated flavonoids.

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The addition of 1% formic acid in the mobile phase has not only improved chromatographic peaks and enhanced resolution, but also triggered the formation of adduct ions of $[M - H + HCOOH]^-$, $[2M - H]^-$ which were helpful for further confirmation of the molecular ion $[M - H]^-$, especially in phenolic and glycosylated compounds [37,38].

Our established method of analysis enabled the putative identification of **28** compounds belonging to different metabolite classes, including phenolic acids, flavonoids, megastigmane glycosides, sesquiterpene glycosides, and triterpene acids, with flavonoids being the most predominant class. The abundance of such class was also evident in the base peak ion (BPI) chromatogram, which was extremely rich in the elution range (t_R 7.23–19.16 min). Phenolic acids appeared first in the chromatogram, followed by flavanol monomers, flavonoidal *C*-glycosides, flavonoidal *O*-glycosides, cinchonain derivatives, procyanidin derivatives, megastigmane glycosides, sesquiterpene glycosides, and finally triterpene acids. However, a certain degree of overlapping was observed between the individual members of different classes due to the structural diversity of the compounds. It is noteworthy that the identified compounds were tentatively characterized for the first time in *E. japonica* leaves cultivar Golden Nuggets cultivated in Egypt, and the obtained results coincided with other earlier studies that dealt with the chemical composition of *E. japonica* leaves [4,39]. The typical MS base peak ion (BPI) chromatogram of *E. japonica* leaves extracts traced with numbered peaks were illustrated in **Fig. 1**. The identified compounds were presented in **Table 1** along with their retention times (t_R), deprotonated molecular ions $[M - H]^-$, molecular formula, molecular weight and the MS/MS ions used for the identification of each compound. Compounds were numbered according to their elution order. The interpretation of the mass spectra, while taking into consideration the data provided by the literature and databases, was the main tool for putative identification of compounds.

Table 1. Compounds identified in the ethyl acetate and butanol fractions of *E. japonica* leaves cultivar Golden Nuggets by UPLC-ESI- MS/MS

ID	Name	t_R (min)	Ion type	Class	Mwt	Formula	MS^n Fragments	Fraction
1	Caffeoylquinic acid	6.53	353 $[M - H]^-$	Phenolic acid	354	$C_{16}H_{18}O_9$	191, 179, 173, 161, 135	Ethyl acetate & butanol
2	Schaftoside	7.38	563 $[M - H]^-$	Flavonoid	564	$C_{26}H_{28}O_{14}$	545, 503, 473, 443, 383, 353	Butanol
3	Epicatechin	7.57	289 $[M - H]^-$ & 335 $[M - H+ HCOOH]^-$	Flavonoid	290	$C_{15}H_{14}O_6$	245, 205, 179, 135	Ethyl acetate
4	*p*-Coumaroylquininc acid	7.61	337 $[M - H]^-$	Phenolic acid	338	$C_{16}H_{18}O_8$	191, 163, 119	Butanol
5	Caffeoylshikimic acid	7.79	335 $[M - H]^-$	Phenolic acid	336	$C_{16}H_{16}O_8$	179, 173, 161, 135	Ethyl acetate
6	Methyl chlorogenic acid	7.99	367 $[M - H]^-$	Phenolic acid	368	$C_{17}H_{20}O_9$	191, 179, 161, 135	Butanol
7	Naringenin-6,8-di-*C*-glucoside	8.38	595 $[M - H]^-$	Flavonoid	596	$C_{27}H_{32}O_{15}$	577, 505, 475, 385, 355	Ethyl acetate & butanol
8	Feruloylquinic acid	8.48	367 $[M - H]^-$	Phenolic acid	368	$C_{17}H_{20}O_9$	193, 191, 134	Ethyl acetate
9	Eugenyl-rutinoside	8.59	471 $[M - H]^-$	Phenylpropene derivative	472	$C_{22}H_{32}O_{11}$	163, 148	Butanol

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10	Naringenin-6-*C*-glucoside	8.73	433 [M – H] $^-$	Flavonoid	434	$C_{21}H_{22}O_{10}$	343, 313, 415	Ethyl acetate
11	Naringenin 8-*C*-rhamnopyranosyl -(1"-2')-glucoside	8.82	579 [M – H] $^-$	Flavonoid	580	$C_{27}H_{29}O_{15}$	415, 459, 343, 313	Ethyl acetate & butanol
12	Cinchonain Ia	8.94	451 [M – H] $^-$	Flavonoid	452	$C_{24}H_{20}O_9$	287, 107	Ethyl acetate
13	3-oxo-ionyl-9-*O*-xylopyranosyl-glucoside (Eriojaposide A)	9.19	501 [M – H] $^-$& 547 [M – H+ HCOOH] $^-$	Megastigmane glycoside	502	$C_{24}H_{38}O_{11}$	369, 207	Ethyl acetate & butanol
14	Eriojaposide A isomer	9.46	501 [M – H] $^-$& 547 [M – H+ HCOOH] $^-$	Megastigmane glycoside	502	$C_{24}H_{38}O_{11}$	369, 207	Butanol
15	Kaempherol-3-*O*-glucoside	9.49	447 [M – H] $^-$	Flavonoid	448	$C_{21}H_{20}O_{11}$	285, 284, 246, 179, 151	Ethyl acetate
16	Naringenin-6-*C*-(2"-*O*-acetyl)-glucoside	9.80	475 [M – H] $^-$	Flavonoid	476	$C_{23}H_{24}O_{11}$	433, 415, 385, 355, 343, 313	Ethyl acetate
17	Vomifoliol-9-*O*-pentosyl-glucoside	9.88	517 [M – H] $^-$	Sesquiterpene glycoside	518	$C_{24}H_{38}O_{12}$	385, 223, 205	Butanol
18	Kaempherol-3-*O*-rhamnoside	10.12	431 [M – H] $^-$	Flavonoid	432	$C_{21}H_{20}O_{10}$	285, 284, 246, 179, 151	Ethyl acetate
19	Catechin 3-*O*-gallate	10.29	441 [M – H] $^-$& 883 [2M – H] $^-$	Flavonoid	442	$C_{22}H_{18}O_{10}$	289, 169, 125, 245, 205, 137	Ethyl acetate & butanol
20	Cinchonain Ib	10.42	451 [M – H] $^-$	Flavonoid	452	$C_{24}H_{20}O_9$	287, 107	Ethyl acetate
21	Epicatechin 3-*O*-gallate	10.88	441 [M – H] $^-$& 883 [2M – H] $^-$	Flavonoid	442	$C_{22}H_{18}O_{10}$	289, 169, 125, 245, 205, 137	Ethyl acetate & butanol
22	Nerolidol 3-*O*-[rhamnopyranosyl-rhamnopyranosyl-rhamnopyranosyl]-glucoside (Loquatifolin A)	12.58	821 [M – H] $^-$& 867 [M – H+ HCOOH] $^-$	Sesquiterpene glycoside	822	$C_{39}H_{66}O_{18}$	675, 659, 383, 221	Ethyl acetate & butanol
23	Procyanidin B-2	13.11	577 [M – H] $^-$	Flavonoid	578	$C_{30}H_{26}O_{12}$	451, 425, 407, 289	Ethyl acetate
24	Nerolidol 3-*O*-[rhamnopyranosyl-rhamnopyranosyl]-glucoside	13.53	675 [M – H] $^-$& 721 [M – H+ HCOOH] $^-$	Sesquiterpene glycoside	676	$C_{33}H_{56}O_{14}$	513, 529, 383, 221	Ethyl acetate
25	Nerolidol 3-*O*-[rhamnopyranosyl-rhamnopyranosyl]-glucoside	13.88	675 [M – H] $^-$& 721 [M – H+ HCOOH] $^-$	Sesquiterpene glycoside	676	$C_{33}H_{56}O_{14}$	513, 529, 383, 221	Ethyl acetate & butanol
26	Nerolidol 3-*O*-{rhamnopyranosyl-rhamnopyranosyl]-(feruloyl)-rhamnopyranosyl}-glucoside	14.92	997 [M – H] $^-$	Sesquiterpene glycoside	998	$C_{49}H_{74}O_{21}$	821, 675, 659, 383, 221	Ethyl acetate & butanol
27	Corsolic acid	24.49	471 [M – H] $^-$	Triterpene acid	472	$C_{30}H_{48}O_4$	423, 405, 393	Ethyl acetate

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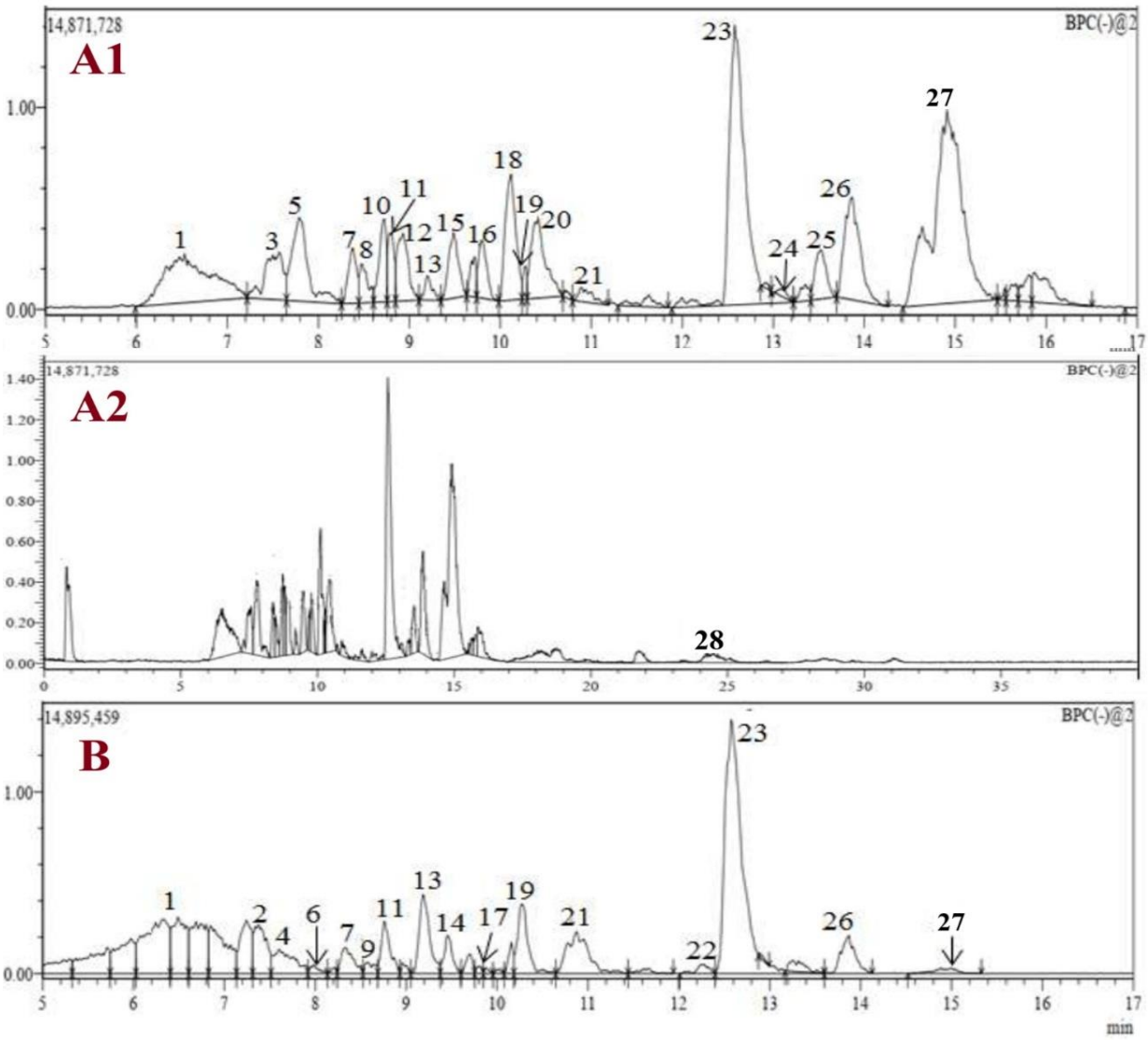

Fig. 1. Base peak ion chromatograms of *E. japonica* leaves extracts, (A1: Time scale from 5 to 60 minutes, A2: Time scale from 0- 35 minutes) ethyl acetate fraction and (B) butanol fraction.

3.1.1. Characterization of phenolic acids

Compound **1** exhibited base peak $[M - H]^-$ ion at 353 Da. Fragment ions were detected at 179 and 191 Da, consonant with deprotonated caffeic and quinic acids, respectively. Other characteristic product ions at 173, 135, 161 Da were also noticed, consistent with [quinic acid – H $–H_2O]^-$, [caffeic acid – H – $CO_2]^-$, and [caffeic acid – H – $H_2O]^-$, respectively. Hence, compound **1** was assigned as caffeoylquinic acid [40,41]. In the same respect, compounds **6 & 8** displayed the same deprotonated molecular ion at 367 Da, yet they exhibited different CID product ions (**Table 1**).

The precursor ion peaks of these compounds were 14 Da higher than that of compound **1,** suggesting an additional methyl group. Compound **6** gave rise to daughter ions like those observed for compound 1, appearing at 179, 191, 161, and 135 Da, corresponding to deprotonated caffeic acid and quinic acids, [caffeic acid – H_2O – H] $^-$ and [caffeic acid – H – $CO_2]^-$, respectively. Accordingly,

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compound **6** was suggested to be a caffeoylquinic acid derivative and was tentatively assigned as methyl chlorogenic acid [42]. On the other hand, compound **8** was annotated as feruloylquinic acid, relying on the main fragment ions at 193 and 191 Da, assigned to deprotonated ferulic acid and quinic acid, respectively. Moreover, the intense ion noticed at 134 Da, representing deprotonated ferulic acid after subsequent loss of CO_2 and methyl radical, further confirmed the identity of the compound [43]. In the same manner**,** compound **5** with a precursor ion peak appearing at 335 Da and t_R of 7.79 min, was proposed to be caffeoylshikimic acid. Fragmentation of the precursor ion yielded two main daughter ions at 179 and 173 Da **(Table 1),** consistent with deprotonated caffeic acid and shikimic acid, respectively, along with another peak at 135 Da assignable to deprotonated caffeic acid after loss of CO_2, which further confirmed the proposed speculation [40,44].

3.1.2. Characterization of flavonoids

In our study, flavonoids represented the major class of phenolic compounds identified. Fourteen different flavonoids belonging to various classes were characterized, and most of them were glycosylated derivatives, either *O*- or *C*-glycosides**.** The identified compounds included one flavone *C*-glycoside **2**, four flavanone *C*-glycosides (**7, 10, 11 & 16**), two flavonol *O*-glycosides (**15 & 18),** and seven flavan-3-ol derivatives **(3, 12, 19, 20, 21, 22 & 24**).

3.1.2.1. Characterization of *C*-glycosides

The spectra of *C*-glycosides displayed losses of 90 and 120 Da, resulting from cross-ring cleavages of the sugar unit [45]. Compound **2** presented a deprotonated molecular ion at 563 Da releasing fragments at; 473 Da $[M - H - 90]^-$, 503 Da $[M - H - 60]^-$, 443 Da $[M - H - 120]^-$ and 545 Da $[M - H - 18]^-$ suggesting the presence of di-C-hexosyl/pentosyl flavone. This assumption was corroborated by the characteristic fragment ions noticed at 353 Da (aglycone + 83) and 383 Da (aglycone + 113), which characterize apigenin aglycone. The higher intensity of the ion at 443 Da $[M - H - 120]^-$ compared with the ion at 473 Da $[M - H - 90]^-$ was indicative of the presence 6-*C*-hexosyl-8-*C*-pentosyl linkage. Hence, compound **2** was tentatively characterized as apigenin 6-*C*-pentosyl-8-*C*-hexoside (schaftoside) [46]**.** Similarly, compound **7** showed a pseudo-molecular ion peak at 595 Da and was putatively assigned as naringenin 6,8-di-*C*-glucoside. The MS/MS product ion scan of this compound showed characteristic neutral losses of 90 Da (*m/z* 505) and 120 Da (*m/z* 475) from the parent ion corresponding to cross-ring cleavages in the sugar unit together with a minor fragment ion at 577 Da $[M - H - 18]^-$ corresponding to neutral loss of a water molecule. In addition, the ions detected at 385 Da [aglycone + 113] $^-$ and 355 Da [aglycone + 83] $^-$ (**Table 1**) further confirmed the identity of the compound [45]. In the same regard, the precursor ion peak of compound **10** was detected at 433 Da with t_R of 8.73 **(Table 1)**. The MS/MS product ions showed fragmentation characteristic of mono-*C*-hexosyl flavanone, with losses of 90 and 120 and 18 Da giving rise to fragment ions at 343, 313, and 415 Da coincident with [aglycone + 71], [aglycone + 41] and $[M - H - H_2O]^-$, respectively. This information allowed the characterization of the aglycone as naringenin. The intense abundance of the peaks at 343 Da $[M - H - 90]^-$ and 415 Da $[M - H - H_2O]^-$ confirmed the substitution in C-6. Therefore, the tentative assignment of the compound was naringenin 6-*C*-glucoside [29,45,47]. In the same sense, compound **11** gave a molecular ion peak at 579 Da. A fragment ion was detected at 459 Da $[M - H - 120]^-$ with another characteristic one at 415 Da $[(M - H) - (146+18)]^-$ indicating the loss of a rhamnosyl moiety and a water molecule suggesting a 2"-*O*-glycosylated *C*-glycoside

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with rhamnose as the *O*-glycosidic residue. A naringenin aglycon was suggested from the ions noticed at 343 Da [aglycone + 71] $^-$ and 313 Da [aglycone + 41] $^-$. The absence of the fragment ion [M − H − 90] $^-$ assigned the *C*-glycosylation site to C-8. Hence, compound **11** was characterized as naringenin 8-*C*-rhamnopyranosyl-(1-2")- glucopyranoside in agreement with literature [29,47]. Similarly, compound **16** exhibited a molecular ion peak at 475 Da, showing a fragmentation pattern typical of a 2-*O*-acetylated *C*-mono-glycoside [45]. Characteristic fragment ions were observed at 415 Da [M − H − acetyl − 18] $^-$ and 433 Da [M − H − acetyl] $^-$ a long with ions at 385 Da [M − H − 90] $^-$, 355 Da [M − H− 120] $^-$ arising from cross-ring cleavage of the sugar moiety and at 343 Da [aglycone + 71] $^-$, 313 Da [aglycone + 41] $^-$ indicative of the agylcone structure **(Table 1)**. The higher relative abundance of the fragment ion at 415 Da relative to the ion at 433 Da, along with the high intensity of the fragment ion at 385 Da, assigned glycosylation site to C6 rather than C8. Accordingly, compound **16** was identified as naringenin 8-*C*-rhamnopyranosyl -(1"-2')-glucoside [45,48].

3.1.2.2. Characterization of *O*-glycosides

Two flavonol -*O*-glycosides were detected in *E. japonica* leaves extracts (compounds **15 & 18**), both were kaempferol glycoconjugates. Compound **15** exhibited a deprotonated molecular ion at *m/z* 447 Da and was characterized as kaempferol-*O*- glucoside based on the characteristic fragment ions noticed at 284 Da [kaempferol − 2H] $^-$ (base peak) and 285 Da [kaempferol − H] $^-$ **(Table 1)** as a result of the neutral loss of a hexosyl moiety together with the characteristic ions detected at 151 and 179 Da consistent with $[^{1,3}A]^-$ and $[^{1,2}A]^-$ ions resulting from Retro-Diels–Alder (RDA) cleavage of ring C [46,49]. Similarly, compound **18** gave an intense peak at 431 Da. MS/MS fragmentation of the molecular ion peak yielded the characteristic fragment ions at 285 and 284 Da inferred to the kaempferol aglycone after neutral loss of a rhamnosyl moiety [M − H − 146] $^-$. The kaempefol aglycone was also affirmed from RDA ions noticed at 151 Da $[^{1,3}A]^-$ and 179 Da $[^{1,2}A]^-$. Based on these features, compound **18** was assigned as kaempferol 3-*O*-rhamnoside [39].

3.1.2.3. Characterization of flavanol derivatives

3.1.2.3.1. Monomeric derivatives

Five monomeric flavanol derivatives (compounds **3, 12, 19, 20 & 21**) were identified in our study. Compound **3** showed quasi-molecular ion peaks at 289 Da [M − H] $^-$ and 335 Da [M − H − HCOOH] $^-$. The MS/MS spectra were characterized by the product ion at 245 Da assignable to the deprotonated molecular ion peak after the loss of CO_2 [M − H − 44] $^-$, as described by Beltrame *et al.*, 2006 [50] **(Table 1)**. A fragment ion very characteristic of flavanol was also observed at 137 Da, corresponding to the ion $[^{1,3}A]^-$ generated by RDA fragmentation. Other fragment ions were observed at 205 Da corresponding to the loss of two ketene groups (C_2H_2O) from ring A simultaneously, and at 179 Da representing a loss of CO from ring A. Hence, compound **3** was identified as epicatechin in agreement with previous studies [44,51]. On the other hand, compounds **12 & 20**, eluting at t_R of 8.94 and 10.42 min, respectively, exhibited molecular ion peaks at the same *m/z* value 451. Similar fragment ions were observed for both compounds, in agreement with the published data [50]. Two main fragment ions were detected at 287 and 107 Da ascribed to fragments1c/2c (obtained by the loss of a caffeiloxy moiety incorporated in the pyranone ring connected to ring A) and 1d/2d (corresponding to a catechol moiety). Thus, compounds **12 & 20** were tentatively characterized as the flavalignan isomers; cinchonain Ia and cinchonain Ib (phenylpropanoid-substituted epicatechins)

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[44,52]. In the same sense, compounds **19** & **21** exhibited precursor ion peaks at the same *m/z* values 883 $[2M - H]^-$ & 441 $[M - H]^-$ (**Table 1**), suggesting the possibility of being an isomeric pair. MS/MS fragmentation of the precursor ion yielded similar daughter ions with a major peak at 289 Da ascribable to catechin or epicatechin, 169 and 125 attributed to deprotonated gallic acid and deprotonated gallic acid after loss of CO_2, respectively. Additionally, the fragment ions at 245, 205 and 135 Da characterizing catechin and its isomer epicatechin were also observed [44]. Accordingly, compounds **19 & 21** were proposed as tetrahydroxyflavanol 3-*O*-gallate isomers. The earlier eluted isomer (compound **19**) was tentatively identified as catechin 3-*O*-gallate and the later eluted one (compound **21**) was assigned as epicatechin 3-*O*-gallate in accordance with previous studies [53].

3.1.2.3.2. Oligomeric derivatives

Two procyanidins (**22 & 24**) were identified in our study, both belonged to B-type procyanidins in which the epicatechin units are linked by a single bond [54]. Compound **22** exhibited a quasi-molecular peak $[M - H]^-$ at 865 Da and was suggested to be a trimer. On the other hand, compound **24** demonstrated a quasi-molecular ion peak $[M - H]^-$ at 577 Da and was suggested to be a dimer. A similar fragmentation pattern was observed for both suggested compounds, in agreement with the previous studies [55,56]. The major fragments were generated after the neutral loss of 126 Da ($C_6H_6O_3$, phloroglucinol) from ring A of an epicatechin unit, 152 Da ($C_8H_8O_3$) from RDA cleavage of the heterocyclic C ring, 170 Da (RDA + water loss) and sequentially 288 Da ($C_{15}H_{12}O_6$, epicatechin − 2H) by cleavages at the interflavanoid linkages [57]. Accordingly, compounds **22 & 24** were annotated as procyanidins C1 and B2, respectively.

3.1.3. Characterization of phenylpropene derivatives

One phenylpropene derivative **(9)** was detected, displaying a precursor ion peak at 471 Da and was annotated as eugenyl - rutinoside in accordance with previous data. The precursor ion gave fragments at 163 and 148 Da, coincident with the subsequent losses of rutinosyl moiety (eugenol) and CH_3 from the methoxy group attached to the benzene ring of eugenol, confirming its assignment [58].

3.1.4. Characterization of megastigmane glycosides

Three megastigmane glycosides were characterized in our study. Two of them were 3-oxo-- ionol derivatives (**13 & 14**), while the other was vomifoliol derivative [17] (**Table 1**). Compounds **13 & 14** exhibited the same quasi-molecular ions at 547 Da $[M - H + HCOOH]^-$ and 501 Da $[M - H]^-$. CID of the precursor ions yielded a fragment ion at 369 Da attributed to neutral loss of a pentosyl moiety, together with another fragment ion at *m/z* 207 ascribed to 3-oxo-ionol aglycone after the neutral loss of pentosyl and hexosyl moieties. Consequently, compounds **13 & 14** were tentatively assigned as eriojaposide A and its isomer [11,59]. Similarly, compound **17** gave deprotonated ion at 517 Da $[M - H]^-$. Two major fragment ions were observed at 385 and 223 Da, indicating sequential loss of pentosyl and hexosyl moieties, generating vomifoliol aglycone. Another fragment ion was noticed at 205 Da [M-H- 294-18] corresponding to neutral loss of H_2O from vomifoliol aglycone. Based on these features, compound **17** was annotated as vomifoliol-9-*O*-pentosyl-*O*-glucopyranoside [11].

3.1.5. Characterization of sesquiterpene glycosides

In our study, four sesquiterpene glycosides (**23, 25, 26 & 27**) were detected; all of them were derivatives of nerolidol, the typical sesquiterpenol found in loquat leaves [60]. The $[M - H]^-$ ions of these compounds appeared

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at 821, 675 and 997 Da and many of them formed adducts with formic acid [M − H + HCOOH] $^{-}$ as reported previously [58]. Compound **23** gave an intense peak at 867 Da [M − H + HCOOH] $^{-}$. Fragmentation of the precursor ion yielded a product ion at 659 Da corresponding to neutral loss of a glucosyl moiety along with other fragment ions at 675 $^{-}$, 529 and 383 Da consistent with subsequent losses of three rhamnosyl moieties. Another intense peak was detected at 221 Da inferred to nerolidol aglycone ($C_{15}H_{26}O$). Hence, compound **23** was determined to have the sesquiterpenol nerolidol as aglycone with a branched sugar chain linked at C-3 formed from one glucose, three rhamnose. Thus, compound was putatively identified as (loquatifolin), the major sesquiterpene glycoside in *E. japonica* leaves [61]. In the same regard, compound **27** displayed the [M − H] $^{-}$ ion at 997 Da. The molecular ion peak was 176 mass units higher than that of compound **23,** consonant with an additional methoxyl-C6-C3 ester moiety. A similar fragmentation pattern to compound **23** was observed**,** showing product ions at 821*,* 659, 383 & 221 Da. Accordingly, compound **27** was assigned a feruloyl derivative of loquatifolin [59,62].

3.1.6. Characterization of triterpene acids

In our study, only one triterpene acid (compound **28**) was detected in the ethyl acetate fraction at 471 Da [M − H] $^{-}$ (**Table 1**). The base peak of this compound appeared at the end of the chromatographic run, displaying the longest retention time. The fragmentation pattern of the compound seemed consistent with corsolic acid, relying on the main fragment ions detected at 423 Da [M − H − 48] $^{-}$, attributed to the loss of H_2O and HCHO, and at 405 Da [M − H − 96] $^{-}$. Furthermore, a characteristic fragment ion was observed at 393 Da [M − H − 78] $^{-}$ assignable to the loss of H_2O, COOH, and CH_3, which further corroborated the proposed assumption [63].

3.2. Evaluation of antimicrobial activity of *E. japonica* leaves extracts cultivar Golden Nuggets at fruiting stage.

In this study, the total ethanolic extract and various fractions of loquat leaves cultivar Golden Nuggets were screened for their antibacterial activity against Gram-positive *S. aureus* and *B. subtilis*, Gram-negative *P. aeruginosa* and *E. coli* and their antifungal activity against the fungus *C. albicans* **(Table 2)**. The results obtained revealed that the tested samples displayed varying degrees of efficacy against different tested microbes. The methylene chloride fraction significantly inhibited the growth of Gram-positive *S. aureus*. Regarding the ethyl acetate and butanol fractions, they showed moderate antibacterial activity against Gram-positive *B. subtilis*. The fungus candida *C. albicans* and the other tested Gram-negative bacterial strains showed little or no sensitivity to the tested extracts. Generally, Gram-positive bacteria were more sensitive to the extracts followed by Gram-negative bacteria while the fungus *C. albicans* was the least sensitive. Variations in the activity among the tested extracts can be partly attributed to qualitative and quantitative disparities in the secondary metabolites existing in these extracts. Previous phytochemical analyses of *E. japonica* leaves revealed the presence of triterpenoids, flavonoids and sesquiterpenoids [13,64,65]. According to a previous study [66], triterpenoids exhibited considerable antimicrobial activities against vast array of microbes.

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Table 2. Results of the antibacterial and antifungal screening of the total extract and fractions of *E. japonica* leaves cultivar Golden Nuggets

Tested sample	Inhibition zone (IZ) in mm[a]									
	Bacteria								Fungi	
	Gram-positive				Gram-negative					
	S. aureus		*B. subtilis*		*P. aeruginosa*		*E. coli*		*C. albicans*	
	C	S	C	S	C	S	C	S	C	S
Total extract	9	9	9	9	9	9	9	9	9	9
Petroleum ether	9	9	9	9	9	9	9	9	9	10
Methylene chloride	9	15	9	9	9	9	9	9	9	9
Ethyl acetate	9	9	9	12	9	9	9	9	9	9
Butanol	9	9	9	12	9	10	9	9	9	10
Residual extract	9	9	9	9	9	10	9	11	9	10
Ciprofloxacin	9	30	10	30	9	30	9	30	-	-
Clotrimazole	-	-	-	-	-	-	-	-	9	18

[a]values expressed are averages of three replicates
*DMSO (9%) was used as a control, S: sample, C: control.

This activity was ascribed to their effect on the non-mevalonate pathway essential for the synthesis of cell membrane components and as a secondary source of carbon in microbes [67]. Triterpenes of *E. japonica* leaves were reported to be concentrated in low polarity fractions suggesting the possibility of being responsible for the significant antimicrobial activity observed for methylene chloride fraction [12,68]. Furthermore, flavonoids have been reported to possess antimicrobial properties probably due to its capability of complexing extracellular and soluble proteins of the bacterial cell walls [69]. Flavonoids had been reported to be more abundant in polar fractions (ethyl acetate and butanol) of *E. japonica* leaves [70] and this was also corroborated by our study of the UPLC-MS profile of these fractions (**Section 3.1**). The greater susceptibility of Gram-positive test organisms to the tested fractions could be explained by the disparity in the cell wall composition between Gram-positive and Gram-negative bacteria. Gram-positive bacteria lack lipopolysaccharide coat (LPS) and hydrolytic enzymes of the Gram-negative bacteria, which are responsible for selective hindering of infiltrating substances and devastating potentially threatening foreign substances, respectively [71,72].

3.3. Evaluation of anticancer activity of leaves extracts of *E. japonica* cultivars Golden Nuggets, Emanwil and Zekeim at fruiting stage

The ethanolic extracts of the leaves of the three *E. japonica* cultivars cultivated in Egypt, (Golden Nuggets, Emanwil and Zekeim collected at fruiting stage) in addition to fractions of Golden Nuggets cultivar were evaluated for their *in-vitro* growth inhibitory activities on lung carcinoma cell line A549. Cytotoxicity of the tested samples was evaluated using viability assay. Data were arranged with inhibition on the Y- axis and concentration on the X- axis and IC_{50} values were calculated for each sample. The results obtained revealed that the tested extracts and fractions were effectively able to suppress the proliferation of A549 lung cancer cell lines in a dose dependent manner showing varying degrees of potency (**Fig. 2**). The total ethanolic extracts of the leaves of the three *E. japonica* cultivars showed IC_{50} values between 175 µg/mL and 345 µg/mL. A549

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cell line was most sensitive to ethanolic extracts of Golden Nuggets and Zekeim with IC_{50} values of 175 µg/mL and 180 µg/mL, respectively while the ethanolic extract of cultivar Emanwil was less active showing IC_{50} value of 345 µg/mL (**Fig. 2**). Among all tested Golden Nuggets fractions, the residual aqueous extract showed the most potent anti-proliferative activity against A549 cell line (IC_{50} = 20 µg/mL) followed by methylene chloride fraction (IC_{50} = 65 µg/mL), then petroleum ether fraction (IC_{50} = 175 µg/mL). While the ethyl acetate and butanol fractions exhibited the lowest cytotoxic activity against A549 cell line with IC_{50} values > 400 µg/mL (**Fig. 2**). It is worth mentioning that the residual aqueous extract exerted a highly potent cytotoxic activity with a very low IC_{50} value (20 µg/mL). According to the American National Cancer Institute, the accepted IC_{50} value to consider an extract for further studies should be lower than 30 µg/mL [(73,74)]. Relying on these criteria, the residual aqueous extract of cultivar Golden Nuggets could be considered a promising candidate for further analysis. The anticancer activities observed for the various extracts and fractions could be attributed to the activity of the secondary metabolites present in these extracts, including triterpenoids, megastigmane glycosides, and high molecular weight polyphenolics. The high cytotoxic activity observed for the residual aqueous extract could be attributed to its high content of highly polar polymeric procyanidins (flavan-3-ol polymers) especially the procyanidin oligomer as reported in literature [(70)] The high cytotoxic activity of these compounds was ascribed to their prooxidant capacity and free radical generation when exist in high concentrations, besides induction of DNA fragmentation [(70)]. The considerable cytotoxic activity exerted by the low polarity fractions (methylene chloride and petroleum ether) could be ascribed to their content of triterpene acids [(12,68)]. Regarding the more polar fractions (ethyl acetate and butanol fractions), the low cytotoxic activity observed may be owing to their abundance with simple phenolics such as phenolic acids and flavonoids [(12,29)], which may increase cell viability by acting as free radical scavengers. The abundance of such fractions with flavonoids and phenolic acids was also evident in their UPLC- MS profile as discussed before (**Section 3.1**). Based on these findings, Egyptian loquat leaves could be established as a potential source of cytotoxic phytochemicals that could play a pivotal role in the treatment of selected cancers by working in concert with conventional chemotherapeutic agents, thereby improving their efficacy or reducing their toxicity.

3.4. Evaluation of antioxidant activity of leaves extracts of *E. japonica* cultivars Golden Nuggets, Emanwil and Zekeim at three different growing stages

In our study, thirty-six extracts prepared from *E. japonica* leaves of cultivars Golden Nuggets, Zekeim and Emanwil were screened for their DPPH radical scavenging assay, total polyphenolic content and xanthine oxidase inhibitory potential at different growing seasons. The extracts were prepared with four different solvents: petroleum ether, methylene chloride, ethyl acetate and *n*-butanol.

3.4.1. DPPH radical scavenging assay

Five concentrations (0.025, 0.05, 0.15, 0.25, 0.3 mg/mL) of standard gallic acid were used to construct the calibration curve **(Fig. S1).** The relationship between % inhibition and the concentration showed linearity over the range 0.025-0.3 mg/mL. The good linearity was proven by the high value of the correlation coefficient (r = 0.9926). The results obtained **(Fig. 3, Table S2)** revealed that all three tested *E. japonica* cultivars showed significant DPPH radical scavenging activities with varying degrees of efficacy. Emanwil cultivar exerted the most potent

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DPPH radical scavenging activity among the three cultivars, followed by Zekeim cultivar, then Golden Nuggets cultivar. Of the three examined seasons, the flowering stage was the richest season with antioxidant phytochemicals and exhibited the highest DPPH radical scavenging activity in all cultivars followed by fruiting stage, whereas the lowest activity was observed in the post-fruiting stage. The fractions with the highest DPPH radical scavenging of Emanwil cultivar were butanol and methylene chloride fractions in both flowering and fruiting stages, while petroleum ether and methylene chloride were the most active fractions in the post-fruiting stage. Regarding Zekeim cultivar, the highest DPPH radical scavenging activity was exerted by butanol and methylene chloride fractions in the three examined seasons. On the other hand, the butanol and ethyl acetate fractions of Golden Nuggets cultivar were the most active in both flowering and post-fruiting stages, while in the fruiting stage, the methylene chloride and petroleum ether fractions displayed the highest activity.

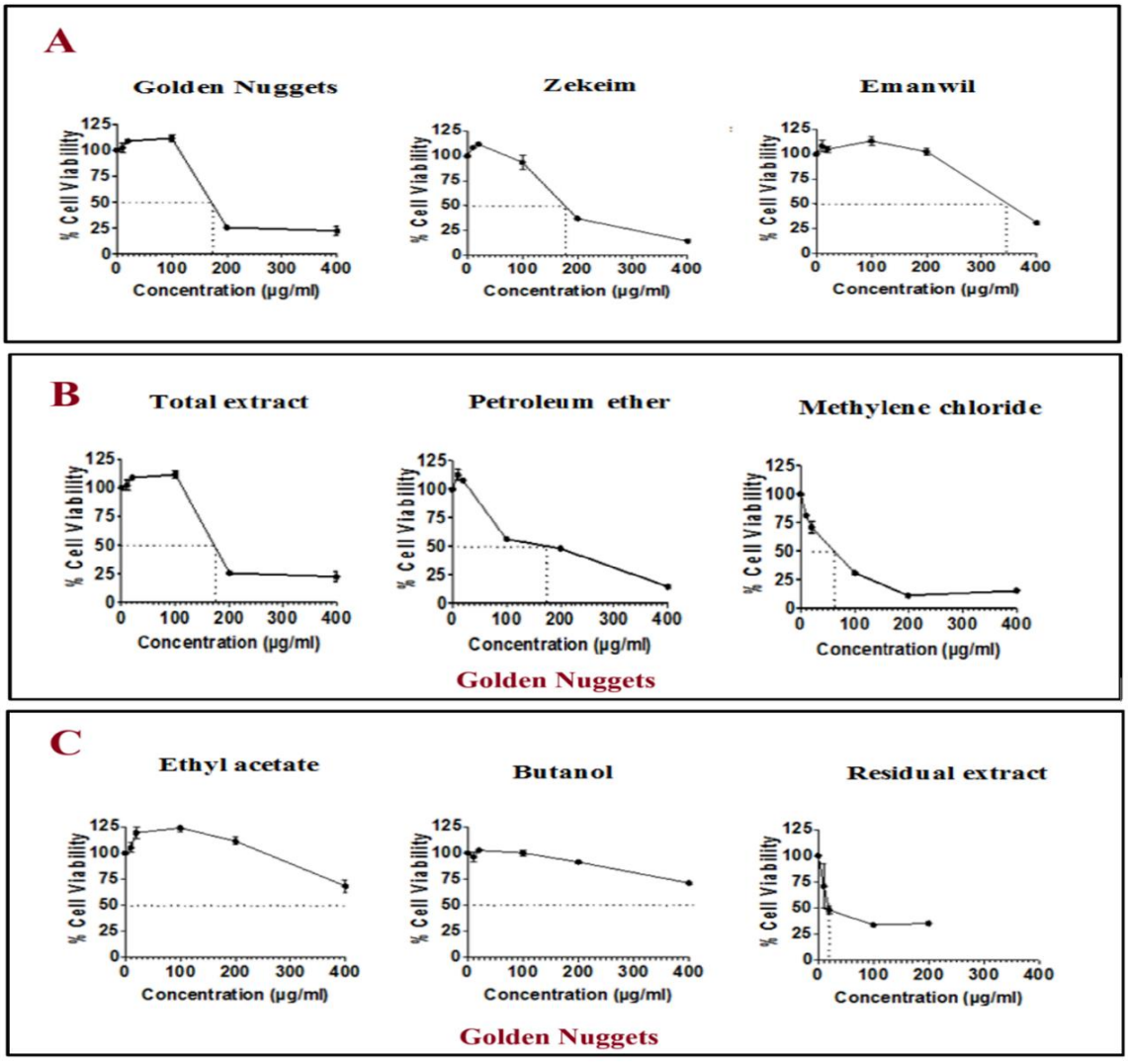

Fig. 2. Dose-Response curves of *in vitro* preliminary antitumor screening of *E. japonica* leaves extracts against lung carcinoma cells, A: total ethanolic extracts of the three examined cultivars, B & C: total ethanolic extract, fractions and residual aqueous extract of cultivar Golden Nuggets.

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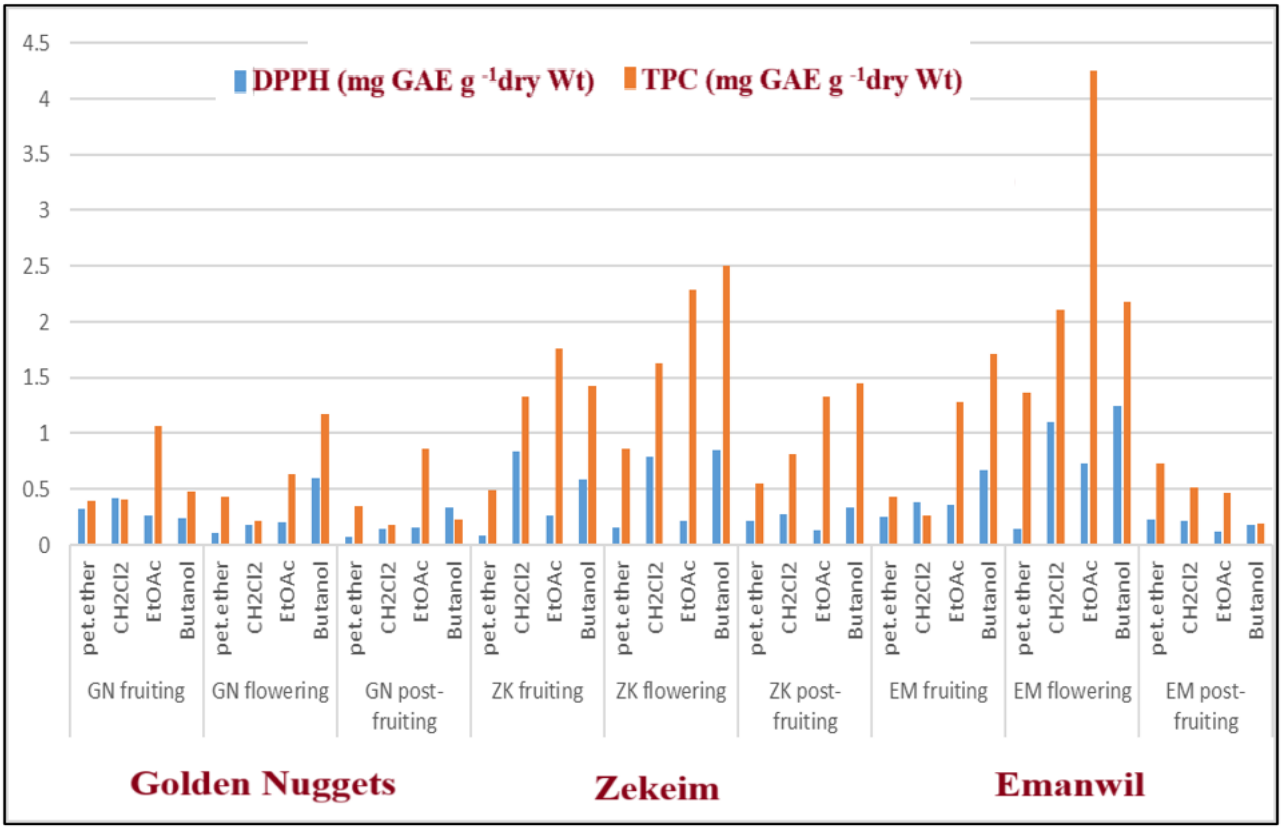

Figure 3. Bar chart demonstrating the DPPH inhibitory activity and total polyphenolic content as (mg GAE g $^{-1}$) of various leaves extracts of the three examined *E. japonica* cultivars at different growing seasons, GN: Golden Nuggets, ZK: Zekeim & EM: Emanwil.

3.4.2. Total polyphenolic content (TPC)

Five concentrations (0.025, 0.05, 0.15, 0.25, 0.3 mg/mL) of standard gallic acid were used to construct the calibration curve (**Fig. S2**). The relationship between absorbance difference and concentration showed linearity over range 0.1-0.4 mg/mL. The good linearity was proven by the high value of the correlation coefficient (r = 0.9977). The obtained results **(Fig. 3, Table S3)** disclosed that TPC of the tested extracts varied widely among the three examined cultivars and extracts of the same cultivar at different seasons. Emanwil cultivar showed the highest TPC among the three examined cultivars followed by Zekeim cultivar then Golden Nuggets cultivar. Of the three examined seasons, the flowering stage exhibited the highest TPC in all cultivars followed by fruiting stage, whereas the least TPC was observed in the post-fruiting stage. Concerning Emanwil cultivar, the fractions with the highest TPC were butanol and ethyl acetate in both flowering and fruiting seasons. On the other hand, the methylene chloride and petroleum ether fractions showed the highest TPC in post post-fruiting stage. However, in the case of Zekeim cultivar, the butanol and ethyl acetate fractions displayed the highest TPC in the three examined seasons. Regarding the Golden Nuggets cultivar, the butanol and ethyl acetate fractions demonstrated the highest TPC in both the flowering and fruiting stages. Whereas, in the post-fruiting stage, the highest TPC was observed for the ethyl acetate and petroleum ether fractions.

The results showed that the three examined loquat cultivars showed great variability in terms of their antioxidant capacity and TPC

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(Fig. 3, Table S2 & S3). TPC content and DPPH radical scavenging activity of the three tested cultivars varied significantly with regard to seasonal changes. This indicates that the chemical composition of the leaves is highly affected by growing season. The variation in the active constituents could be attributed to the seasonal changes in humidity, temperature and, also to different stages of plant metabolism. It was evident that there was a good correlation between TPC and DPPH radical scavenging activity of the tested samples; Emanwil cultivar showed the highest TPC and DPPH radical scavenging activity among the tested cultivars followed by Zekeim cultivar, then Golden Nuggets cultivar. Additionally, the highest TPC and DPPH radical scavenging activity of the three cultivars was observed in the flowering stage, followed by fruiting stage, while the post-fruiting stage showed the least TPC and DPPH radical scavenging activity. However, minor variations were observed between the TPC and the DPPH radical scavenging activity of the fractions of each cultivar in the same season. This indicates the presence of other phytochemicals in *E. japonica* leaves besides polyphenolics that can directly scavenge free radicals. Examples of these compounds are triterpene acids that were abundant in *E. japonica* leaves and were reported by various studies to have significant DPPH radical scavenging activity [75,76]. Also, these minor variations could be attributed to the presence of different classes of phenolic compounds in *E. japonica* leaves, for instance, flavonoids, cinchonain derivatives, procyanidin derivatives and phenolic acids. These phenolic compounds have different chemical structures and may exhibit different behavior in different antioxidant assays based on the nature of hydroxyl groups, molecular weight and number of aromatic rings [77]. Besides, the Folin- Ciocalteu reagent is reported to react with other constituents in plants rather than polyphenolics such as ascorbic acid and minerals. Thus, it may overestimate TPC of plant extracts [78,79]. The obtained results revealed that the leaves of the three investigated *E. japonica* cultivars are rich sources of antioxidant phytochemicals that could play a substantial role in protection against oxidative damage associated with various diseases, thus in turn supporting the traditional medicinal use of the leaves. The observed antioxidant activity could be attributed to various phytochemicals, of which phenolic compounds take the largest part, as manifested by the positive correlation between the DPPH radical scavenging activity and TPC.

3.4.3. Xanthine oxidase (XO) inhibitory activity

In our study, the ethyl acetate and methylene chloride fractions of the three cultivars were tested at a concentration of 2.5 mg/mL (**Tables S4, Fig. 4)**, as preliminary screening indicated significant inhibitory activity at this level. In contrast, the petroleum ether and butanol fractions exhibited weak inhibition at this concentration, so their concentration was doubled to 5 mg/mL for testing (**Tables S5, Fig. 4).** Based on the obtained results, it was evident that all the tested fractions of the three tested cultivars inhibited XO enzyme with varying degrees of potency through different seasons. Of all the tested fractions, a total of 13 ethyl acetate and methylene chloride fractions demonstrated substantial XO inhibitory activity with % inhibition (>40%) at 2.5 mg/mL. On the other hand, a total of 7 petroleum ether and butanol fractions exerted XO inhibitory activity with the same potency at a concentration of 5 mg/mL. The ethyl acetate fraction exhibited the highest xanthine oxidase inhibitory activity in the three tested cultivars in most cases where a total of five fractions possessed an inhibition rate >95%, comparable to that of the standard, allopurinol. Among the three cultivars, the ethyl acetate fraction of Zekeim

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cultivar showed the highest efficacy in all seasons with % inhibition reaching 99.18% and 97.48% at fruiting and flowering stages, respectively. The highest XO inhibitory action of the three cultivars was observed in the fruiting stage where the ethyl acetate fraction of all of them showed inhibition rate > 97% **(Table S4, Fig. 4)**. Regarding the methylene chloride fraction, the highest inhibition was observed for the methylene chloride fraction of cultivar Golden Nuggets at fruiting stage with % inhibition of 96.89% comparable to that of the ethyl acetate fraction (97.15%) **(Table S4, Fig. 4)**.

At a concentration of 5 mg/mL, the petroleum ether and butanol fractions demonstrated pronounced XO inhibitory effects in only few cases **(Table S5, Fig. 4)**. As concerns the petroleum ether fraction, Emanwil cultivar showed potent XO activity with % inhibition reaching 99.43% and 83.36% in flowering and fruiting stages, respectively. Regarding the butanol fraction, Emanwil cultivar displayed the highest potency in the flowering stage with % inhibition reaching 83.36%. Whereas the butanol fraction of Golden Nuggets cultivar exerted the highest XO inhibitory activity in the fruiting stage with % inhibition of 82.12%. **(Table S5, Fig. 4)**. The variable efficacies displayed by the tested fractions through different seasons confirmed that the chemical profile of the leaves was highly affected by seasonal variations. Furthermore, it was evident that XO inhibitory potential was strongly correlated with the antioxidant capacity since the process of uric acid formation by XO is accompanied by the formation of ROS. Accordingly, the radical scavenging activity of the plants suggested the XO inhibitory potency of these plants and their constituents [80]. In this respect, many of the tested leaves fractions of the three examined *E. japonica* cultivars, especially the ethyl acetate fractions showed powerful free radical scavenging capacity and high TPC (**Sections 3.4.1 & 3.4.2**). The abundance of the ethyl acetate fraction of cultivar Golden Nuggets with phenolic compounds was also evident in our study of its UPLC-MS profile (**Section 3.1**).

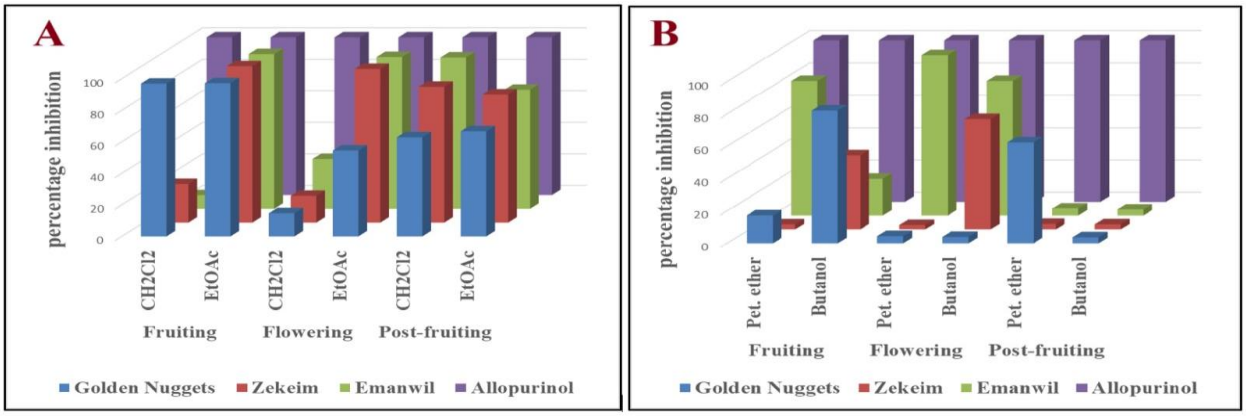

Fig. 4. Bar chart demonstrating the XO inhibitory activity as % inhibition of the leaves extracts of the three examined *E. japonica* cultivars at different growing seasons, A: ethyl acetate and methylene chloride fractions at a concentration of 2.5 mg/mL, B: petroleum ether and butanol fractions at concentration of 5 mg/mL.

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Moreover, *E. japonica* leaves were reported to be rich in triterpene acids with ursolic acid being the most abundant [8,81]. Ursolic acid was reported to significantly decrease the serum uric acid levels *in vivo* by 79.9% and exerted potent xanthine oxidase inhibitory activity *in vitro* with IC_{50} value = 10.3 µg/mL [82]. The present work reported for the first time a systematic comprehensive study on the XO inhibitory activity of various leaves fractions of loquat cultivars; Golden Nuggets, Zekeim and Emanwil cultivated in Egypt at different growing seasons. The leaves of the tested cultivars could serve as promising candidates for the development of new natural anti-hyperuricaemic agents with better safety profile for treatment of gout.

4. Conclusion

In this study, UPLC - MS technique was successfully applied to unveil the phenolic profile of *E. japonica* leaves cultivar Golden Nuggets cultivated in Egypt. The leaves were proven to be a rich source of structurally diverse classes of phenolic compounds, along with many other biologically active constituents with high medicinal values. Moreover, our study provided preliminary insights on the potential health functionalities of the leaves of three *E. japonica* cultivars; Golden Nuggets, Zekeim and Emanwil cultivated in Egypt. The leaves of the three examined cultivars were shown to exert promising antitumor potential against lung cancer. Moreover, they exerted significant antioxidant activity and proved to be highly effective in the inhibition of XO enzyme. Additionally, the obtained results presented further proof for the outstanding impact of growing season on the chemical profile of the cultivars examined which was reflected on their biological activities. Collectively, the findings of our study suggested that Egyptian *E. japonica* leaves might be a potential candidate for the development of new cytotoxic, antioxidants, and hypourecaemic agents for gout.

Authors Contributions:

Ali M. Metwally, Samah M. El Sohafy and Rasha M. Abu El-Khair introduced the concepts, Mariam M. Elattar wrote the draft and gathered the data, Ali M. Metwally, Samah M. El Sohafy and Rasha M. Abu El-Khair: reviewing, editing and making the final approval for publication.

Conflict of interest:

The authors declare no conflict of interest.

Highlights:

- UPLC-MS/MS analysis was applied to analyze the phenolic pattern of loquat leaves cultivar Golden Nuggets resulted in identification of **28** compounds.
- The leaves extracts of three loquat cultivars were assessed for different biological activities at different growing seasons.
- The leaves of the three examined cultivars exerted promising cytotoxic activity.

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