Antimutagenic and Cytotoxic Potential of Punica Granatum L. And Opuntia Ficus-indica L. Peels

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Abstract

Introduction: This study pointed to validate the phytochemistry, antioxidant and antimutagenic activities of two common plant peels; *Punica granatum* L. and *Opuntia ficus-indica* L.

Material and methods: HPLC analysis was performed for identification of phenolics and flavonoids, beside to isolation of some triterpenes and phenolics from both plant peels.

Results: *P. granatum* peels composed of 16 flavonoids and 18 phenolics, while *O. ficus-indica* comprised of 18 flavonoids and 10 phenolics. α-Amyrin acetate **(1)**, friedelin **(2),** lup-20(29)-en-3β-ol **(3),** quercetin-3,4'-dimethyl ether-7-*O*-α-Larabinofuranosyl β-D-glucopyranoside **(4),** punicaflavanol **(5),** and two hydrolyzabl tannins **(6&7)** were isolated from *P. granatum* peels, while friedelin **(8),** 24-Methylene-ergosta-5-en-3β-ol **(9),** apigenin-7-*O*-glucoside **(10),** isorhamnetin 3-*O*-β-D-glucopyranoside **(11),** and betanin pigment **(12)** were isolated from *O. ficus-indica* peels. Compounds (**1-5** and **8, 10, 11, 12**) were isolated for the first time from both plant peels. *P. granatum* and *O. ficus-indica* peel extracts have relatively significant antioxidant, cytotoxic and antimutagenic effects.

Discussion and conclusion: The tested plant peel extracts could be a reliable source as natural antioxidant, antimutagenic and cytotoxic agents with a high level of safety. The novelty of this study is the comparison of such activities of the peels under study.

Introduction

The reactive species are highly toxic and mutagenic. They can promote DNA damage and initiate auto oxidation reactions causing cancer, and other degenerative diseases, therefore the intake of natural antioxidants is crucial for free radicals scavenging by different mechanisms, such as active species prohibition or by seizing metal ions, leading to repairing damage [1]. Consequently, many evidences of mutation-related carcinogenesis were established resulting in much detailed studied on mutagenesis [2].

Mechanism of mutagenesis mainly includes the generation of reactive oxygen species. Therefore, the current study focusing on edible plant peels which are rich in flavonoids, terpenes, phenolic compounds, and natural pigments which are reported as anticancer, antioxidant and antimutagenic agents [3].

Pomegranate (*Punica granatum* L.), family Punicaceae, is native to the Mediterranean region and the previous phytochemistry studies of pomegranate fruits revealed the presence of polyphenolic compounds that include flavonoids and hydrolyzable tannins beside other main classes [4]. According to literature, many bioactivities of *P. granatum* L. have been reported such as antioxidant, anti-inflammatory, antimicrobial and others **[5; 6].** Although there are a fair number of scientific studies on *P. granatum* fruits and peel extracts regarding *in vitro* and *in vivo* anticancer activities, insufficient data are available on the antimutagenic effects of *P. granatum* peels **[7; 8].**

Prickly pear or cactus pear, *Opuntia ficus-indica* L. belongs to family Cactaceae. The fruits have been widely used in folk medicine to treat many diseases. The majority of studies had focused on cactus fruit as a source of bioactive compounds [9]. *Opuntia* peels are usually discarded after fruit consumption. However,

there are no adequate studies on the peels, although they contain a diversity of significant amounts of bioactive constituents with various biological activities [10]. A recent study investigated the prophylactic effect *O. ficus-indica* petroleum ether peel extract against irradiation-induced colitis in rats. The fruit peel extract proved to possess a potential antioxidant and anti-inflammatory activity as well as limiting the colonic complications caused by irradiation. The activities were attributed to the richness of peel extract in fatty acid methyl ester content, terpenes and sterols [11].

Chemical Investigation

Plant material

Punica granatum L. and *O. ficus-indica* L. (Pink red cultivar) fresh fruits were purchased in January 2019 from the local market, Giza, Egypt. The specimens of fruits were identified by Prof. Dr. Gamal Farag professor, Horticulture Research Center, Ministry of Agriculture, Giza, Egypt. The fruits were washed. The peels were collected separately, dried under shade and grinded into powder. Crude ethanolic extracts were prepared by maceration of powdered samples in 80% ethanol. It was then kept at room temperature, shaken every 2 h for one day. Thereafter, it was filtered by using Whatmann filter paper No. 1. and evaporated by rotary evaporator at 50° C till dryness. The crude extracts were kept in the refrigerator for the phytochemical and biological analyses.

Instruments

UV spectra were obtained by UV–Visible Spectrophotometer double beam UVD– 3500 spectrophotometer, Labomed, Inc. Color detection was monitored by visible spectrophotometer, Shimadzu UV 240 (PIN 204-58000) (Japane). MS chromatograms were carried out using Bruker Avance 400 spectrometer (9.4 T, Karlsruhe, Germany). FT-IR spectra were obtained with infra-red spectrophotometer,

Perkin-Elmer 283 (Germany), spectrophotometer. The NMR spectra were performed on BRUKER spectroscopy; (^{1}H, 400MHz; ^{13}C, 125 MHz). Melting point measurement was done using Koffler's heating stage microscope.

Phytochemical screening

The powdered plant peels were subjected to the phytochemical screening for carbohydrates, sterols and / triterpenes, tannins, flavonoids, alkaloids and nitrogenous compounds as mentioned in **[12].**

HPLC analysis

HPLC analysis were carried out according to **Matilla [13]** using Agilent Technologies 1100 series liquid chromatography equipped with an auto sampler and a diode-array detector. The analytical column is Eclipse XDB-C18 (150 X 4.6 µm; 5 µm) with a C18 guard column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 0.8 mL/min for a total run time of 70 min and the gradient program was as follows: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min and 0% B to 100% B in 5 min. The injection volume

was 50 µL and peaks were monitored simultaneously at 280 and 320 nm for the benzoic acid and cinnamic acid derivatives, respectively. All samples were filtered through a 0.45 µm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectra and compared with those of the standards.

Determination of total phenolic and flavonoid contents

The total phenolic content was determined according to the Folin-Ciocalteu procedure. The total phenolic content was determined by means of a calibration curve prepared with gallic acid, and expressed as milligrams of gallic acid equivalent (mg GAE) per gram of sample. While the total flavonoid content was expressed as mg of catechin equivalent (CE) per g of sample according to **[14].**

Isolation of the main compounds from *P. granatum* and *O. ficus-indica* peels

The total extract of both plant peels was concentrated and partitioned with chloroform targeting triterpene isolation, the residue was fractioned with ethyl acetate for isolation of phenolics in both plant peels. While further fractionation step with butanol was performed for *P. granatum* peels for isolation of hydrolysable tannins. The chloroform soluble fraction was concentrated and loaded on a silica gel column, eluted with various ratios of petroleum ether and chloroform. The fractions were tested for the presence of triterpenes using Liebermann–Burchard reagent, then they were spotted on TLC plate and sprayed with 10%H_2SO_4 reagent **[15].** The positively tested fractions were purified on TLC silica gel plates using benzene:ethyl acetate solvent system (19:1 & 8:2 v/v).

Furthermore, the ethyl acetate fraction was concentrated and purified on TLC silica gel chromatography using $CHCl_3$: CH_3OH (9:1 v/v). The obtained bands were examined under UV at 254 and 366 nm. The presence of flavonoid constituents was confirmed by spraying with 1% ethanolic solution of $AlCl_3$ **[16]**. The isolated compounds were further purified several times with preparative TLC and their structure elucidation were confirmed by different spectral analyses.

On the other hand, the butanol fraction of *P. granatum* was subjected to silica column chromatography using acetone: water solvent system at different concentrations with increasing order of polarity, similar fractions was collected purified on sephadex column chromatography aiming for isolation of tannins.

Based on the spectral analyses (FT-IR, ^{1}H-NMR, ^{13}C-NMR and Mass spectroscopy) of the isolated compounds, the structures of the compounds were interpreted.

Isolation and characterization of betanin pigment from *O. ficus-indica* peels

Fresh peels of *O. ficus-indica* were carefully chopped and extracted with ethanol: water (80%), under continuous mechanical stirring for 1 h in the dark. The pH of the extraction solvent was adjusted to 4.5 for more stability of the extracted pigment. The extract was centrifuged at 15000 g for 30 minutes at 4˚C. Then it was subjected to silica gel column chromatography for purification of betanin with the elution solvent system of methanol: water: glacial acetic acid (9:0.3:0.7). Purification was performed using TLC silica gel plates and developed using methanol : 5% aqueous acetic acid (1:1) v/v, visualized under short and long UV

wavelengths (254, 366 nm), then sprayed with vanillin reagent mixture. Similar bluish red bands with the same R_f values were collected and concentrated under vacuum using the rotatory evaporator and kept in freezer till further spectral investigation. The confirmation and structure elucidation of betanin was done by UV-visible, FT-IR, and ^{1}HNMR spectroscopy **[19]**.

Biological investigations

Median lethal dose (LD_{50})

Adult albino mice, of Sprauge Dawely strain 25-30 g (6 animals/ group) were obtained from the animal house colony of National Research Centre, Cairo, Egypt. They were kept under the same hygienic conditions and well-balanced diet and water. Doses of the two extracts were calculated according to **[20]**, and were administered orally. The median lethal doses (LD_{50}) of the ethanolic extracts of *P. granatum* and *O. ficus-indica* fruit peels were determined according to the method described by **[21].** The mice were observed for 24 hours, symptoms of toxicity and mortality rates in each group were recorded and LD_{50} was calculated for each extract.

In vitro antioxidant activity

Free radical scavenging capacities of the sample were determined on the stable 1,1-diphenyl-2-picryl-hydrazyl (DPPH) using Trolox as a standard**.** The final concentration was 200 µM for DPPH and the final reaction volume was 3 mL. The absorbance was measured at 517 nm against a blank of pure methanol after 60 min of incubation in a dark condition. Then the absorbance was taken at 517 nm using the spectrophotometer. The standard curve was prepared using Trolox. Results were expressed as mg Trolox equivalents (TE)/g sample **[14].**

In vitro cytotoxic activity

Potential cytotoxic activity of the two ethanolic extracts was tested using the method of **[22]** against HEPG2 (liver carcinoma cell line), PC3 (prostate Cancer), MCF7 (breast carcinoma cell line, HCT116 (colon carcinoma cell line), and A549 (lung cancer). The potency was compared with reference drug (Doxorubicin). Statistical analysis for cytogenetic analysis, the significance of the results from the negative control data and between plant extracts and CP group comparing to CP alone was calculated using t-test**.**

Antimutagenicity study (chromosome evaluation in somatic and germ cells)

Laboratory-bred strain Swiss albino male mice of 10-12 weeks old with an average weight of 25-30 g were housed in six groups (5 animals/ group) and maintained under standard conditions.

Group I was the non- treated group (negative control).

Group II was treated by injection of cyclophosphamide (CP) (Sigma Aldrich, St. Louis, MO, USA) 20 mg/kg body weight for 24 h (positive control).

Group III was treated by repeated oral administration with the dose of 150 mg/kg b.wt of each extract for 7 days.

Groups IV, V, VI was treated by repeated oral administration with the doses of 50,100 and 150 mg/kg b.wt of each extract for 7 days with i.p. injection of (CP) 20 mg/kg body weight for 24 h.

For somatic and germ cell preparations, animals from the different groups were injected i.p. with colchicine, 2-3 h before sacrifice.

Chromosome preparations from bone marrow (somatic cells) carried out according to the method of **[23]** methods for spermatocyte cells. One hundred well spread metaphases were analyzed per mouse. Metaphases with different abnormalities in somatic and germ cells were recorded with a 100X magnification light microscope (Olympus, Saitama, Japan).

Evaluation of the effect of the two plant extracts to inhibit DNA damage induced by CP was carried out according to **[24]** equation as follows:

Inhibitory index = [1- (plant extracts plus CP – negative control) / (CP - negative control)] X100.

Statistical analysis for cytogenetic analysis, the significance of the results from the negative control data and between the plant extracts plus CP comparing to CP alone was calculated using t-test.

Ethics

Anesthetic procedures and handling of animals were complied with the ethical guidelines of the Medical Ethical Committee of the National Research Centre in Egypt and performed ensuring that the animals do not suffer at any stage of the experiment under registration No.19288.

Results And Discussion

Chemical investigation

The air dried powder of the two investigated plants, were separately extracted by maceration with 80% ethanol until complete exhaustion. Percentage yield of the plants are illustrated in Table 1.

Table 1: Percentage yield of the crude extract of the collected plants:

Name of the plant	% (wt/wt)
Punica granatum	0.30
Opuntia ficus- indica	0.06

Preliminary phytochemical screening

The air-dried powders of all plants under study were subjected to the phytochemical tests and the results are combined in Table 2.

Preliminary phytochemical screening revealed that the air dried, powdered plant peels under study contain carbohydrates and glycosides, sterols and triterpenes, and flavonoids. It can be noticed also that anthraquinones, and saponins appeared to be absent from all tested plants, while proteins and tannins present in all tested plant material, while tannins present only in *P. granatum* peels.

Table 2: Results of preliminary phytochemical screening of the air dried powdered plant peels

Test	*P. granatum*	*O. ficus -indica*
Sterols &/or Triterpenes	+	++
Carbohydrates &/or glycosides	+	+
Anthraquinones	-	-
Flavonoids	+	+
Coumarins	-	-
Alkaloids&/or nitrogenous Compounds	-	-
Proteins	+	+
Tannins	+	-
Saponins	-	-

(++): Appreciably present

Determination of the total phenolics and flavonoids

The total amount of phenolics was calculated as gallic acid equivalent while total flavonoids were as expressed as mg of catechin equivalent (CE) per g. The results are summarized in Table 3. Total phenolic and flavonoid concentrations of *P. granatum* peels were much higher than that of *O.ficus-indica* (27.600 mg GAE/g and 1.195 mg CE/g), respectively.

Table 3: Identification of total phenolics and flavonoids in *P. granatum* and *O.ficus-indica* peel extracts

Sample	Total phenols (mg GAE/g)	Total flavonoids (mg CE/g)
P. granatum	27.600	1.195
O. ficus-indica	5.820	0.350

HPLC Identification and quantification of flavonoids and phenolics

The analysis revealed that *P. granatum* peels composed mainly of 16 flavonoidal and 18 phenolic compounds, while *O. ficus-indica* comprised of 18 flavonoids and 10 phenolics. The most predominant flavonoid was *hesperidin* (31885.85 mg/100g), while the major phenolic compounds were pyrogallol (116278.78 mg/100g), catechein (41864.38 mg/100g), and gallic acid (13396.79 mg/100g). On the other hand, HPLC analysis of *O. ficus-indica* ethanol extract revealed that the main compound was *kaempferol*-3,7-dirhamnoside (2919.31 mg/100g) followed by *isorhamnetin 3-O-rutinoside* (1738.24

mg/100g). Nevertheless, the main phenolic compounds were quinic and malic acids (4825.71and 3527.14 mg/100g), respectively (Tables 4and 5).

Table 4: Identification and quantification of flavonoids of the ethanolic extracts of *P.granatum* and *O. ficus-indica* by HPLC analysis

Flavonoids	Concentration (mg/100g)	
	P.granatum	*O. ficus-indica*
Apigenin -7- glucoside	287.87	295.23
Apigenin -6- arabinose-8- galactoside	1182.32	752.21
Apigenin -6- rhamnose-8- glucoside	936.16	452.32
Apigenin -7-*O*-neohespiroside	376.45	211.23
Naringin	2489.85	231.21
Hesperidin	31885.85	114.65
Isorhamnetin 3-O-rutinoside	----	1738.24
Isorhamnetin 3-O-galactoside	----	864.51
Quercetin 3-O-rutinoside	----	498.25
Quercetin 3-O-glucoside	----	291.35
Rutin	1504.15	425.35
Kaempferol-3,7-dirhamnoside	1981.26	2919.31
Quercetrin	1069.09	258.35
Acacetin-7- neo hesperside	78.54	238.25
Quercetin	124.73	----
Naringenin	25.11	61.25
Hespirtin	218.68	21.20
Kaempferol	13.13	----
Luteolin-7-O-glucoside	-----	152.21
Rhamentin	67.45	----
Apigenin	17.68	69.25

Table (5): Identification and quantification of phenolic compounds of the ethanolic extracts of *P. granatum* and *O. ficus-indica* by HPLC analysis

Phenolic compounds	Concentration (mg/100g)	
	P. granatum	*O. ficus-indica*
Gallic acid	13396.79	157.58
Pyrogallol	116278.78	----
4-Amino benzoic acid	618.40	----
Protocatchuic acid	3049	251.32
Catechein	41864.38	625.25
Chlorogenic acid	8787.60	----
Catechol	5656.81	452.23
Caffeine	1543.62	----
ρ-Hydroxy benzoic acid	12391.13	----
Caffeic acid	321.36	851.32
Quinic acid	----	4825.71
Malic acid	----	3527.14
Vanillic acid	1149.96	----
ρ-Coumaric acid	261.38	125.31
Ferulic acid	581.26	247.15
Iso-Ferulic acid	496.96	185.21
Ellagic acid	3418.32	----
Benzoic acid	5066.72	----
3,4,5- Methoxycinnamic acid	170.58	----
Cinnamic acid	49.29	----

Isolation of *P. granatum* compounds (Fig. 1)

Triterpenes from chloroform fraction

α-Amyrin acetate (Compound 1) was isolated from the column by petroleum ether: chloroform (60%:40% v/v), purification was performed on TLC plates by benzene-ethyl acetate (19:1 v/v) as a solvent system. It was in a form of white needles, melting point 226 °C. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$, (ppm) revealed signals at δ 0.89 to δ 1.12 (m, 18H, 6 xCH_3), δ 1.22 to δ 1.45 (m, 18H, 9 xCH_2). At δ 1.89 to δ 2.44 (8H, methine protons), δ 3.61 (1H, CHOH), δ 5.36 (1H, vinylic proton), δ 5.02 and δ 5.14) that's a differentiating from β-amyrin olefinic protons that exist at δ 5.16. Mass spectrum showed M^+ at *m/z* 468 for

molecular formula $C_{32}H_{52}O_2$ and 218 (100%) abundance. The other major fragments appeared at m/z: 408, 393, 365, 273, 249, 203, 189. These data are in accordance with **[5]** which previously isolated this compound from *P. granatum* leaves.

Friedelin (Friedelan-3-one) (Compound 2) was isolated from petroleum ether: chloroform (50%:50% v/v), then purified on TLC by benzene-ethyl acetate (8:2), the melting point of the white crystals was 263ºC. FT-IR: peak at 2965 and 2859 cm^{-1} for C-H stretching, 1710 cm^{-1} for C=O stretching, 1442 cm^{-1} for C-H bending. Mass spectrum showed *m/z* at 426 $[M]^+$ for molecular formula $C_{30}H_{50}O$, in addition to other major peaks at 411, 302, 273, 218, 205, 163, 44 (100).^{1}H NMR (400 MHz, $CDCl_3$) (δ ppm): 0.75 (3H, s, H-24), 0.89 (3H, s, H-25), 0.90 (3H, *d*, H-23), 0.98 (3H, s, H-30), 1.00 (3H, s, H-26), 1.03 (3H, s, H-27), 1.08 (3H, s, H-28), 1.21 (3H, s, H-29), 1.24 (3H, s, H-30), 1.99 (1H, m, H-1a), 2.29 (2H, m, H-2b, H-4), 2.42 (1H, m, H-2a), 1.27-1.79 (m, rest of the protons), These data are in accordance with that mentioned in **[25].** This compound was previously isolated from *P. granatum* leaves by **[26].**

Lup-20(29)-en-3β-ol(compound 3) was in a form of colorless amorphous powder, eluted with petroleum ether: chloroform (40%:60% v/v), ^{1}H NMR (400 MHz, $CDCl_3$) (δ ppm): 4.73 (brs, 1H, H2-29), 4.60 (brs,1H, H2-29), 3.18 (dd, 1H, H-3), 2.99 (brs, 1H, H-19), 1.68 (brs, 3H, Me-30), 1.36 (brs, 3H, Me-23), 1.25 (brs, 3H, Me-25), 0.92 (brs, 3H, Me-24), 0.89 (brs, 3H, Me-26), 0.84 (brs, 3H, Me-28), 0.75 (brs, 3H, Me-27). 26), 14.67 (C-27), 20.82 (C-28), 109.66 (C-29), 22.66 (C-30). Mass spectrum show *m/z* at 426 $[M]^+$ for molecular formula $C_{30}H_{50}O$, in addition to 411, 408, 396, 393, 381, 363, 286, 272, 258, 236, 222, 219(100), 207, 205, 191 (41.0), 189. By comparing these data with previous literature **[27]** and on the basis of spectral data analyses, this compound was identified as **lup-20(29)-en-3β-ol**.

Flavonoids from ethyl acetate fraction

Quercetin-3,4'-dimethyl ether-7-O-α-Larabinofuranosyl β-D-glucopyranoside (Compound 4) was isolated in the form of yellow powder, m.p. 176 °C**.** It gave a positive test for flavonoids. Mass spectrum m/z: 625 $[M + H]^+$, 493 $[M + H\text{-arabinosyl}]^+$, m/z 331 was attributed to the aglycone moiety, and two diagnostic peaks at *m/z* 153 and 149 generated through retro-Diels Alder fragmentation were consistent with the presence of two hydroxy groups in ring A and one hydroxy, one methoxy in ring B**.** An intense peak at m/z 287 (aglycone – $CH_3CO]^+$ provided evidence for aglycone moiety being a quercetin 3,4'dimethyl ether. ^{1}H NMR (400 MHz, CD_3OD, δ ppm) : 12.73 (5-OH), 7.60 (1H, d), 7.46 (1H, dd, 6'-H), 6.94 (1H, d, 5'-H), 6.75 (1H, d, 8-H), 6.42 (1H, d, 6-H), 5.06 (1H, d) was assigned to a glucosyl anomeric proton and suggested that the glycosidic bond had a **β**-linkage, 4.74 (1H, d, 1-H) was assigned to the arabinosyl anomeric proton with an α- linkage, The singlet 3.80 (3H, s, 3-OCH_3), 3.71 (3H, s, 4'-OCH_3) were indicative of a methoxyl group attached to the B and C rings. The above spectral data approved that this compound had both the diglycoside moiety and methoxy groups. This compound was previously isolated from the ethyl acetate fraction of *P. granatum* bark by **[28].**

Punicaflavanol named **5, 6, 7, 8, 2', 3', 5'- heptahydroxy -4'-methoxy flavanone** (compound 5) was obtained as pale yellow crystals from chloroform–methanol (9:1). It responded positively to the test of flavonoids. The mass spectrum exhibited a molecular ion peak at *m/z* 366, pointing to the molecular formula $C_{16}H_{14}O_{10}$.

The retro-Diels-Alder fragmentation of ring C yielded the diagnostic peaks at m/z 184 and 182, supporting the presence of four hydroxyl groups in ring A and three hydroxyl and one methoxyl groups in ring B, respectively. The generation of important ion fragments at m/z 140, 188, and 155, 211 also supported the substitution pattern. The ion fragments at m/z 338, 323, and 351 $[M - Me]^+$ arose from the removal of the carbonyl group and the methyl group from the molecular ion peak. The ^{1}H NMR spectrum (400 MHz, CD_3OD, δ ppm) showed a one-proton broad signal at δ 7.31 attributed to H-6 suggesting the 2,3,4,5-tetraoxygenated pattern of ring B. δ 5.32 (dd), 2.97 and δ 2.81 (mm) was characteristic of H-2β of the flavanone moiety. A three-proton broad signal at δ 3.62 was attributed to the methoxyl protons attached at C-4'. The ^{13}C-NMR (125 MHz, CD_3OD)spectrum displayed important signals for the C-4 carbonyl carbon (δ 192.06) and the flavanone carbon between δ 173.23–37.01. The signals at δ 52.03 confirmed the existence of one methoxyl group in the molecule. On the basis of spectral data analyses and comparing with literature, the structure has been elucidated as 5,6,7,8,2,3,5-heptahydroxy-4-methoxyflavanone and was previously isolated from *P. granatum* flower **[29].**

Hydrolysable tannins from butanol fraction

In the current study, two major hydrolysable tannins were isolated and identified from *P. granatum* butanol fraction.

Punicalin (compound 6) was isolated as yellow amorphous powder, m.p. 247°C in accordance to that mentioned in **[30].** Its R_f value was 0.35 in water: acetic acid (3:2)v/v solvent system and gave bluish-black color with 5% $FeCl_3$ spraying agent. The ^{1}H NMR spectrum (400 MHz, CD_3OD, δ ppm) showed the presence of signals at 4.6 (proton of anomer of glucose), 2.00–4.9 (protons of glucose moiety), 6.68–7.21 (protons of gallagyl moiety), 7.50–10.00 (protons of hydroxy group). ^{13}C-NMR (125 MHz, DMSO): 88.50 & 97.15 (α and β-anomeric carbon of glucose), 63.15–75.31 (other carbons of glucose), 168.21 & 169.54 (C=O associated with gallayl moiety), 158.00 & 158. 12 (carbon associated with lactone moiety), 109.84–148.62 (carbon associated with gallagyl moiety). FT-IR: Peak at 2 900 cm^{-1} for C-H stretching, 3 455 cm^{-1} indicated the presence of the O-H stretching, 1 720 cm^{-1} for C=O stretching, 1 355 cm^{-1} for C-H bending, 1170 cm^{-1} for C-O stretching), 1600 cm^{-1} represented C = C benzene ring.

punicalagin (compound 7) was in the form of yellow amorphous powder, melting point 251°C (247-250°C, **30**]. Its R_f value 0.43 in water: acetic acid (3:1)v/v solvent system. On the other hand, the H^1 NMR spectrum (400 MHz, CD_3OD, δ ppm) showed the presence of signals at δ 5.02 for the anomeric glucose proton, signals at 4.91, 5.11, 4.81, 3.21 & 4.18 (other protons of the glucose moiety), at 7.01 & 6.52 for gallagyl moiety protons, while signals values at 6.593 & 6.596 represented the hexa hydroxy diphenoyl moiety protons. Hydroxy group protons appeared at 8.094. The ^{13}C NMR (125 MHz, DMSO) specrtal data were as following: peaks at 90.12 & 93.90 assigned for α and β-anomeric carbon of glucose, 66.91–75.56 for other glucose carbons, while peaks at 169.12 & 168.01 represented the C=O associated with hexa hydroxy diphenoyl moiety. Gallagyl moiety C=O appeared at 168.62 & 169.25. The lactone carbon showed peak at 156.91 & 157.82. Finally peaks at 108.81-146.90 referred to the hexa hydroxy diphenoyl & gallagyl moiety carbon. FT-IR spectra: peak at 2930-2730 cm^{-1} represented the C-H stretching, while peak 3445 cm^{-1} assigned for O-H

stretching, 1732 cm^{-1} indicated the carbonyl group stretching, C- H bending represented by peak at 1350cm^{-1}, peaks at 1175–1 181 cm^{-1} associated with the C-O stretching, and the aromatic conjugation showed at 1620 cm^{-1}.

It is to be mentioned that compounds (1-5) were isolated and identified for the first time in this study from the fruit peel while, compounds 6 and 7 were previously isolated from *P. granatum* peels by **[30].**

Isolation of *O. ficus-indica* compounds (Fig. 2)

Triterpenes from chloroform fraction

Friedelin(Friedelan-3-one) (compound 8). This compound was previously isolated and characterized from the *O. dillenii* stems **[31].** The spectral data are as fore mentioned in compound 2.

24-Methylene-ergosta-5-en-3β-ol (compound 9) was isolated as white powder, EI-MS, *m/z* 398 [M^+] (100%) for the molecular formula $C_{46}H_{28}O$. Other main fragments were m/z 383 [M-CH_3]$^+$, 365 [M-CH_3-H_2O]$^+$, 314 [M-C_5H_9-CH_3]$^+$, 299 [M-C_7H_{13}-2H]$^+$, 281 [M-C_7H_{13}-H_2O-2H]$^+$, 271 [M-side chain-2H]$^+$, while ^{1}H-NMR (400 MHz; $CDCl_3$, d ppm) showed δ ppm 0.69 (*s*, H18), 0.98 (*d*, 6.6, H21), 1.00 (s, H19), 1.04, 1.06 (d, 6.8, H26, H27), 3.55 (m, H3), 4.67 (s, H28), 4.73 (s, H28), 5.36 (d, 4.9, H6). This compound was previously isolated from *O. ficus-indica* peels by **[11].**

Flavonoids from ethyl acetate fraction

Apigenin-7-*O*-glucoside (Compound 10) was in the form of yellow crystal, melting point 227 °C, ^{1} H NMR (400 MHz, CD_3OD, δ ppm) 7.81 (2H, d, H-2'/6'), 7.36 (2H, d, H-3'/5'), 6.65 (1H, s, H-3), 6.55 (1H, d, H-6), 6.88 (1H, d, H-8), 5.01 (1H, d). Mass spectrum showed peak at *m/z* M^+ 432 for molecular formula $C_{21}H_{20}O_{10}$, other observed fragments at 431 (100%), and 269 after the loss of an hexose moiety from the parent ion. By comparing the resultant data with the available literature **[16],** this compound was identified. It was previously isolated from *O. ficus-indica* fruits by **[17].**

Isorhamnetin 3-*O*-β-D-glucopyranoside (compound 11) was isolated as pale yellow amorphous powder, mass spectrum *m/z*: M^+ 478(100) for molecular formula $C_{22}H_{22}O_{12}$, 316 [M-Glc]$^+$, 285, 271. ^{1}H NMR (400 MHz, CD_3OD, δ ppm): 7.95 (1H, d, , H-2'), 7.42 (1H, dd, H- 6'), 6.85 (1H, d, H-5'), 6.41 (1H, d, H-8), 6.19 (1H, d, H-6), 5.48 (1H, d, H-1"), 3.80 (3H, s, O-CH3), 3.71 (1H, m, H-4"), 3.60 (1H, m, H-2"), 3.52 (1H, m, H-6"a), 3.41 (1H, m, H-5"), 3.38 (1H, m, H-3"), 3.36 (1H, m, H-6"b); ^{13}C NMR (125 MHz, DMSO, δ): 176.4 (C-4), 164.4 (C-7), 162.2 (C- 5), 155.1 (C-9), 157.4 (C-2), 149.5 (C-4'), 147.1 (C-3'), 134.1 (C-3), 123.6 (C-6'), 122.1 (C-1'), 114.9 (C-5'), 112.9 (C-2'), 103.8 (C-10), 102.3 (C-1"), 99.2 (C-6), 94.6 (C-8), 76.1 (C-3"), 72.9 (C-5"), 71.2 (C-2"), 68.3 (C-4"), 61.1 (C- 6"), 55.8 (O-CH_3). These data are in accordance with that previously illustrated in **[18].**

Characterization of the isolated betanin pigment (compound 12)

The lyophilized pigment exhibited an absorbance at UV spectroscopy with significant single peak at 538 nm which expressed to λmax of betanin as reported. The mass calculated by ESI-mass m/z for M^+ 551

(89%) for molecular formula $C_{24}H_{26}N_2O_{13}$. The spectrum also showed base peak at m/z 389 $[betanidin]^+$ aglycone that was produced by fragmentation of the parent ion of m/z of 551 assigned to glucose loss of betanin. Our results are in accordance with that reported by **[17].** FT-IR (KBr/cm^{-1}) showed peak at 2920 cm^{-1} for C-H stretching, 3420 cm^{-1} indicated the presence of the O-H stretching, the NH stretching appeared 3250 cm^{-1}, 1700 cm^{-1} for C=O stretching, 1250 cm^{-1} for C-H bending, 1162 cm^{-1} for C-O stretching), 1638 cm^{-1} represented C = C of benzene ring.

This compound was previously detected in *O. ficus-indica* peeled fruits using LC-MS analysis in a study performed by **[17]**. By comparing these spectral data with previous research studies **[19],** this compound could be identified as betanin (Fig. 2). Only a few fruits and vegetables contain betalains and the best known is beetroot (*Beta vulgaris*), an important food colorant and *Opuntia spp*. fruits (prickly pear). Several investigations have also reported beneficial impact of betanin as a significant antioxidant and anti-inflammatory factor, cancer cells suppressor, lipid peroxidation and in heme disintegration **[32; 17; 33].**

It should be noted that compounds (8, 10, 11, 12) are isolated from the first time from the fruit peels in the current study whereas, compound 9 was previously detected in *O. ficus indica* peels by **[11].**

Biological activities

Median lethal dose (LD_{50})

The results of median lethal dose (LD_{50}) of the ethanolic extracts of both plant peels *P. granatum* and *O. ficus-indica* are illustrated in Table 6.

The tested extracts were safe showing LD_{50} 5.5 g/kg b.wt. for *P. granatum* extract, while (7.1 g/kg) for *O. ficus-indica* ethanol extract.

Table (6): LD_{50} of total extracts of the two plants:

Plant Peels Extract	LD_{50} (g/kg b.wt.)
P. granatum	5.5
O. ficus-indica	7.1

In Vitro antioxidant activity

DPPH free radical scavenging assay was carried out on the ethanolic extracts of both plant peels in order to investigate their antioxidant activities using Trolox as a standard. Results were expressed as (mg Trolox equivalent TE/g) in Table 7.

Measuring the antioxidant capacity of the fruit peel total extracts was performed through evaluating their ability to convert the violet color of DPPH to the yellow and using Trolox as a standard **[34]**. The extent of discoloration reflects the activity of the tested extracts as free radical scavenging agents. Results revealed that *P. granatum* peel extract has relatively more antioxidant effect than that of *O. ficus-indica* peels. This

result could be attributed to their richness and diversity of many phytochemical classes such as flavonoids, triterpenes, pigments and tannins. These classes possess their antioxidant ability via neutralizing reactive oxygen species such as hydrogen peroxide. Thus, the ability of phytochemicals to inhibit free radical generation, by restoring the redox state of the internal tissue organs can possibly provide reasonable explanation for their prophylactic role as well **[35].**

Table 7: Antioxidant activities of the ethanolic extracts of the two tested plant peels:

Peel extract	DPPH (mg /g)
P. granatum	142.373
O. ficus-indica	110.800

Cytotoxic activity

Cytotoxic activities of the ethanolic extracts of *P. granatum* and *O. ficus-indica* peels against different cell lines are illustrated in Table 8.

Table 8 revealed that the ethanolic extract of *P. granatum* peels exhibited cytotoxic effect on all tested cancer cell lines. The most significant effect was recorded against HEPG2 (IC 50 =17µg/mL). As for the ethanolic extract of *O. ficus-indica peels* showed potent cytotoxic activity against colon cancer cell lines (HCT-9) at IC_{50} =14 µg/ml followed by liver cancer cell lines (HEPG2) at IC_{50} =18.5 µg/mL, while it has no activity on other cancer cell lines at the tested different concentrations. The American National Cancer Institute assigns a significant cytotoxic effect of any promising anticancer product for future bio guided studies if it exerts an IC_{50} value < 30 µg/mL **[36].**

Table 8: Cytotoxic activity against different cancer cell lines

cell lines	IC 50 (µg/mL)	
	P. granatum	*O. ficus-indica*
liver (HEPG2)	17.0	18.5
prostate (PC-3)	17.6	---
breast (MCF7)	22.5	---
colon (HCT)	19.0	14.0
lung (A549)	21.1	---

Antimutagenicity study (chromosome evaluation in somatic and germ cells)

1. Chromosome evaluation and percentage of inhibition of aberrations in bone marrow cells (somatic cells)

Different number and percentage of abnormalities in all treated groups are shown in Table 9. CP treated group (II) induced a high percentage of aberrations ($p<0.01$). The percentage of aberrations in the animal group treated with 150 mg/kg b. wt of each plant peel extract for 7 days (group III) was nearly close to the negative control group where they were statistically non-significant in comparing to the control group.

Punica granatum and *O. ficus-indica* peel extracts exhibited safe effect regarding the total abnormal metaphases comparing to the control group. This proved the safety of the tested extracts on chromosomes of somatic cells.

Pre-administration of CP-treated groups with the tested extracts at the doses 50, 100 and 150 mg/kg b. wt for 7 days (groups IV, V and VI) reduced the number of abnormalities in a statistically significant manner ($p<0.01$). This reduction of abnormalities is a dose dependent increased with increasing the dose of treatment. The percentage of the inhibitory index of the different plant extracts is listed in Table 9.

The percentage of inhibitory index in chromosome aberrations in bone marrow cells of *P. granatum* and *O. ficus-indica* peels extracts were greater than the negative control even at the lowest dose of the plant extract (50 mg/ kg b.wt) of *P. granatum* and *O. ficus-indica*, they recorded (12 and 4), respectively. The obtained results emphasized the antimutagenic effect of the tested extracts as they have the ability to repair the genotoxic effect of the anticancer drug cyclophosphamide.

Table 9: Percentage of inhibition of abnormalities in bone marrow cells after treatment with different doses of *P. granatum* and *O. ficus-indica* peel extracts.

Groups/Treatments (mg/kg b.wt)	Total abnormal metaphases		No. of different types of abnormal metaphases						Inhibitory index
	No.	Mean(%) ± SE	G.	Frag. and/or Br.	Del.	C.F.	M.A.	Polyp.	
I. Negative control	20	4.00±0.70	9	8	3	0	0	0	-
II. CP (20)	151	30.20±0.66 [a]	17	63	15	5	48	3	-
III. *P. granatum* (150)	21	4.20±0.80	10	8	3	0	0	0	-
O. ficus-indica (150)	27	5.40±0.45	9	15	2	0	0	1	-
IV. *P. granatum* (50) + CP	136	27.20±0.58[ab]	18	49	8	0	58	3	12
O. ficus-indica (50) + CP	154	30.80±0.50[a]	11	71	8	4	56	4	4
V. *P. granatum* (100)+ CP	125	25.00±0.89[ab]	19	47	3	0	56	0	20
O. ficus-indica (100) + CP	140	28.00±0.58 [ab]	17	36	4	0	51	5	19
VI.) *P. granatum* (150)+ CP	118	23.60±0.87[ab]	14	49	5	4	46	0	25
O. ficus-indica (150) + CP	122	24.40±0.38[ab]	14	52	3	3	48	2	14

Number of examined metaphases=500 (100 metaphase/animal, 5 animals/ group); G.: Gap; Frag. : Fragments;

Br.: Breaks; Del.: Deletions; C.F.: Centric Fusions; M.A.: Multiple Aberrations; Polyp: Polyploidy.

a: Significant compared to negative control ($p<0.01$); b : Significant compared to CP treatment ($p<0.01$); t-test.

2. Chromosome evaluation and percentage of inhibition of abnormalities in germ cells (spermatocyte cells):

Different number and percentage of abnormalities in spermatocyte cells of all treated groups after treatment with different doses of both plant ethanolic extracts with CP treated group are shown in Table (10).

CP induced a high percentage of aberrations ($p<0.01$). The percentage of aberrations in the animal groups treated with 150 mg/kg b. wt of both plant peel extracts for 7 days was nearly close to the negative control. This show the safety of all extracts on chromosomes of germ cells.

Pre-administration of CP-treated groups with plant extracts at the doses 50, 100 and 150 mg/kg b. wt for 7 days reduced in a statistically significant manner ($p<0.01$) the number of abnormalities. This reduction of abnormalities is a dose dependent increased with increasing the dose of treatment.

The percentage of inhibitory index in chromosome aberrations in bone marrow cells of *P. granatum* and *O. ficus-indica* peels extracts were greater than the negative control even at the lowest dose of the plant extract (50 mg/ kg b.wt) of *P. granatum* and *O. ficus-indica*, they recorded (26 and 5), respectively, which proves the antimutagenic effect of the tested extracts as their potentiality to repair the genotoxic effect caused by the anticancer drug.

The selected anticancer drug cyclophosphamide CP induced significant percentage of chromosomal aberrations. Its cytotoxic effects result from chemically reactive metabolites that alkylate DNA and protein, producing cross-links. The injury of normal tissues is the major limitation of using CP, which gives rise to numerous side effects **[37].**

Results from Tables 9 and 10 showed the antimutagenic effect of both tested extracts where they have the ability to repair the genotoxic effect of the anticancer drug cyclophosphamide. They possessed safe effect on genetic material (non-genotoxic) in both somatic and germ cells in the examined groups compared to the negative control group. In addition, they achieved antimutagenic activities in comparing to the positive control group and with CP treated groups administered with each extract, a statistically significant decrease in chromosomes abnormalities was found in the bone marrow cells and germ cells. The rate of protection was proportionally associated to the dose of the extracts.

Many studies proved that high flavonoid intake may be correlated with a decreased risk of cancer and showed strong antioxidant effect. They provide evidence for the protective roles of flavonoids against cancer **[38].** *In vitro* studies indicate that the anticancer activities of flavonoids may be related to inhibiting cell proliferation, adhesion, and invasion, inducing cell differentiation, cell cycle arrest, and apoptosis, etc.

[39], while *in vivo* studies demonstrated their ability to inhibit carcinogenesis by affecting the molecular events in the initiation, promotion, and progression stages **[40].** Based on these results, flavonoids could be developed as promising antioxidant agents as well as their chemopreventive effect.

Both P. granatum and *O. ficus-indica* polyphenols have possessed *in vitro* antioxidant effect, together with curable effects against cancer, and inflammatory diseases **[5 & 41].**

Table 10: Percentage of inhibition of abnormalities in spermatocyte cells after treatment with different doses of *P. granatum* and *O. ficus-indica* peel extracts.

Groups/Treatments (mg/kg b.wt)	Total abnormal metaphases		No. of different types of abnormal metaphases					Inhibitory index
	No.	Mean(%)±SE	XY-uni.	Auto.uni	XY-uni.+ Auto.uni.	Frag.	Chain (IV)	
I. Negative control	14	2.80±0.73	11	3	0	0	0	0
II. CP (20)	83	16.60±0.50[a]	44	30	4	2	3	0
III. *P. granatum* (150)	16	3.20±0.58	11	5	0	0	0	0
O. ficus-indica (150)	19	3.80±0.52	12	7	0	0	0	0
IV. *P. granatum* (50)+ CP	65	13.00±0.70[ab]	53	12	0	0	0	26
O. ficus-indicia (50) + CP	73	14.60±0.58[a]	48	21	1	1	2	5
V. *P. granatum* (100) + CP	60	12.00±0.70[ab]	46	14	0	1	0	33
O. ficus-indica (100) + CP	62	12.40±0.52[ab]	49	12	0	0	1	23
VI. *P. granatum* (150)+ CP	56	11.20±0.80[ab]	34	20	0	1	1	39
O. ficus-indica (150)+ CP	56	11.20±0.42[ab]	35	17	1	21	1	33

Number of examined metaphases=500 (100 metaphase/animal, 5 animals/ group); XY-uni: XY- univalent; Auto. uni.: Autosomal univalent; XY-uni.+ Auto. uni.: XY-univalent + Autosomal univalent; Frag.: Fragment. a: Significant compared to negative control ($p<0.01$); b: Significant compared to CP treatment ($p<0.01$); t-test.

Discussion

Mutagenesis is the of mutations in DNA molecules. Spontaneous mutations are essential to produce genetic variation necessary for natural selection. Contrarily, other mutations that cause changes in the DNA sequence or rearrangement of the chromosomes either as a result of a default in ***transcription*** that occur during DNA replication or mitosis or due to environmental exposure to genotoxins **[42].**

The mechanism of mutagenesis has been reported to be through the generation of reactive oxygen species which mainly act as endogenous promoters for degenerative processes, including DNA damage that probably lead to cancer, heart diseases, aging and others **[2].**

A plethora of studies proved the antioxidant, anticarcinogenic and other important bioactivities and correlated them to the richness of *P. granatum* and *O. ficus-indica* peels of a diversity of phytochemicals (such as hydrolysable tannins, polyphenolics, triterpenes and natural pigments) which hindered both mitochondrial signaling pathway modulations and vital carcinogenesis pathways at different stages **[11; 43 ; 4].** Nevertheless, methanol extract of *O. ficus-indica* peels possessed the cytotoxic mechanism of action by decreasing cell proliferation and apoptosis induction in the cancer cells. This was confirmed by enhancing the gene Bax expression of pre-apoptosis as well as reducing the gene Bcl-2 expression of anti-apoptosis **[44; 6 ; 45].**

Phytochemical studies performed on *P. granatum* peel extract revealed that the high percentage of ellagitannins could be responsible for the significant antioxidant and antimutagenic effects. Hence, the reported mechanism of antimutagenic behavior could be attributed to the presence of variety of polyphenolics present in methanol extract such as flavonoids including anthocyanins, catechins and other complex flavonoids besides hydrolyzable tannins (punicalin, pedunculagin, punicalagin, gallagic acid and ellagic acid esters of glucose) all together play an important role by interacting with the reactive intermediates or interfering with the metabolic activation of the pro-mutagen and consequently, result in different pathways of metabolism of mutagens and carcinogens, and guard the cells against chemically induced mutagenesis **[2; 7].**

On the other hand, many reports in the literature demonstrated the advantageous effects of betalains (the natural pigment found in *O. ficus-indica* peels) on the redox-regulated pathways involved in the cell growth and inflammation with no noticeable toxic effects in humans **[32; 46].** Additionally, another study proved that a cactus pear extract in a 0.1 mg/mL dose reduced the H_2O_2-induced DNA damage in human peripheral lymphocytes in the comet assay **[47]. Zorgui *et al.*, [48]** stated the ability of cactus cladodes to protect against the genotoxicity with an efficient prevention of micronuclei and chromosomal aberrations frequency in bone marrow cells and DNA fragmentation *in vivo*.

Conclusion

In the present study, employing natural plant wastes, as an important source of natural antioxidants is increasing due to consumers' preference being economic and possess a high level of safety. *Punica granatum* and *O. ficus-indica* peels have been phytochemically investigated in details and the structure activity relationship was correlated to the anti-mutagenesis and antioxidant activities revealed the high

significance and promising results of the 80% ethanolic extracts of both plant peels. The fore mentioned activities were attributed to the prevalence of phenolics, flavonoids, tannins, triterpenes and pigments.

Declarations

Conflict of interest

The authors declare that there is no conflict of interest in this research work.

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Figures

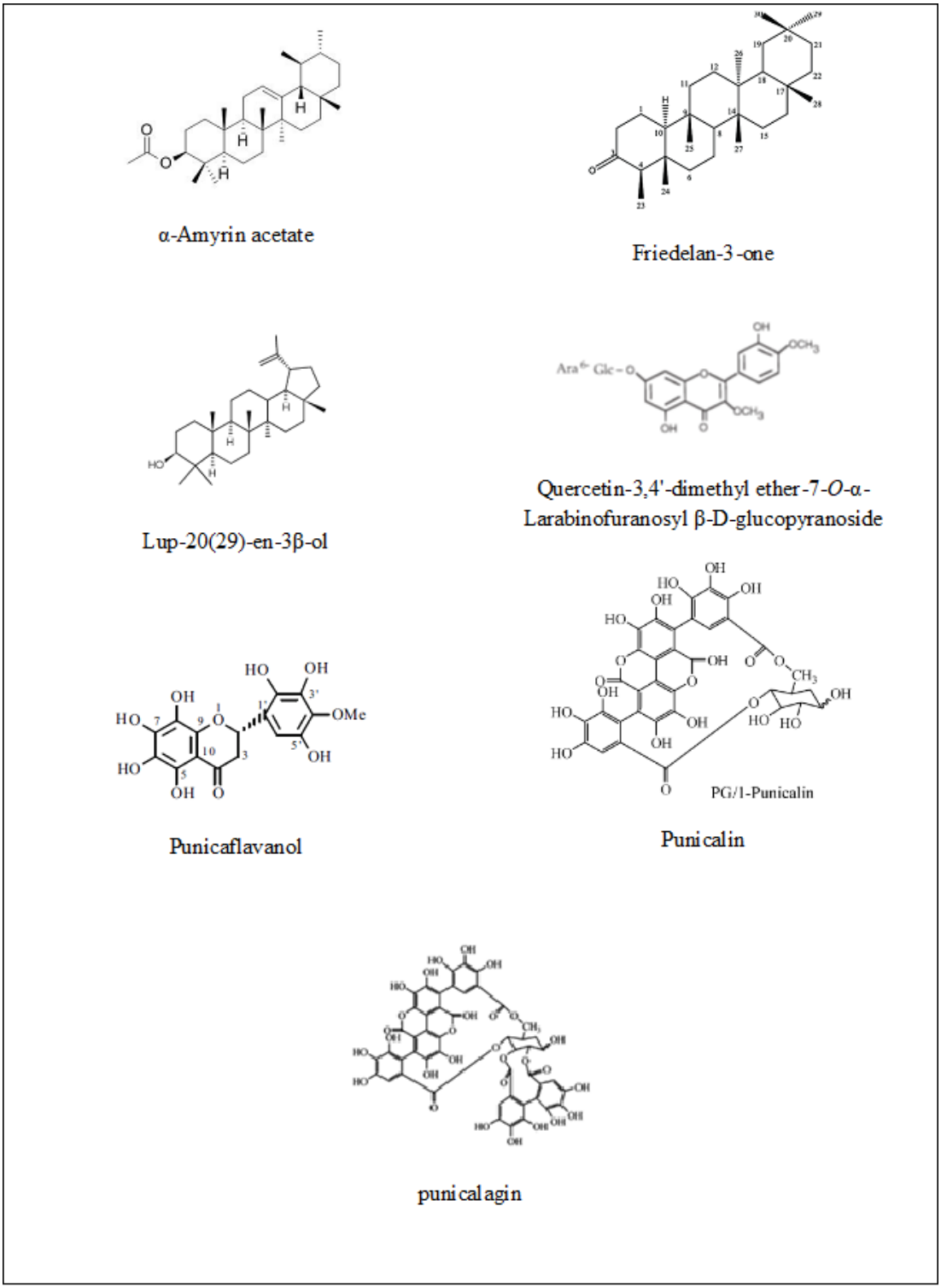
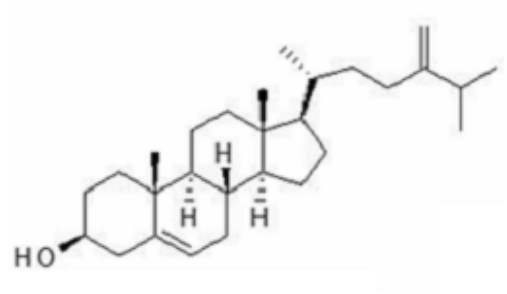
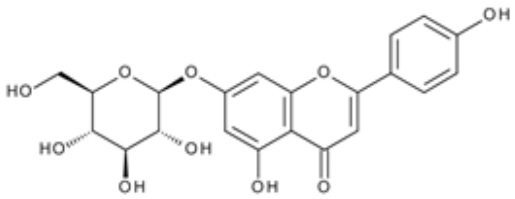


Figure 1

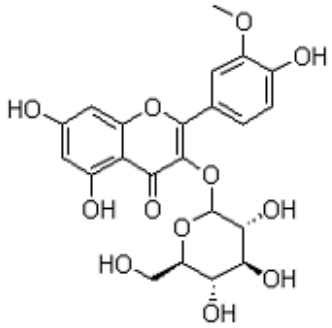
Structure of the isolated compounds from *P. granatum*



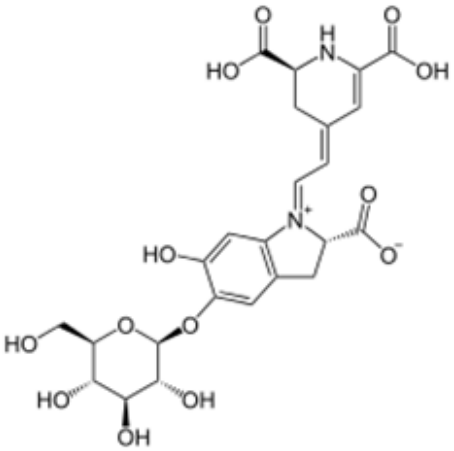
24-Methylene-ergosta-5-en-3β-ol



Apigenin-7-*O*-glucoside



Isorhamnetin 3-*O*-β-D-glucopyranoside



Betanin

Figure 2

Structure of the isolated compounds from *O.ficus-indica*.