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Research Article

GAS CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS AND ANTIOXIDANT ACTIVITY OF *PUNICA GRANATUM* L. PEELS AND ITS ROLE AS IMMUNOSTIMULANT AGAINST *SCHISTOSOMA MANSONI* INFECTION IN *BIOMPHALARIA ALEXANDRINA*

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ABSTRACT

Objective: To evaluate the antioxidant activity and chemical composition of *Punica granatum* L. and test it as immunostimulants against *Schistosoma mansoni* infection to *Biomphalaria alexandrina* snails.

Methods: Antioxidant activity was evaluated by measuring the free radical scavenging activity of the 90% defatted methanol extract (90% DM) of *P. granatum* peels and its sub-derived fractions was evaluated via 2,2'-diphenyl-1-picrylhydrazyl and its chemical constituents were identified via gas chromatography-mass spectrometry (GC-MS) analysis. *B. alexandrina* snails were exposed to pomegranate extracts (PEs) for 1 month before their challenging with *S. mansoni* miracidia. Infection rates, immunological and histological parameters were, then, evaluated in PE-exposed snails and compared to controls.

Results: The antioxidants activities of PE, expressed as scavenging concentration at 50%, were in the following order; 90% DM (12.45) >*n*-butanol (15.59) >ethyl acetate (21.36) >water (49.16) µg/ml, compared to 7.50 µg/ml for ascorbic acid. The infection rates of PE-exposed snails were 20%, 50%, 60%, 70%, and 80%, respectively, for 90% DM, *n*-butanol, ethyl acetate, water, and dichloromethane extracts compared to 95% in control snails. The number of amoebocytes showed a significant increase, clear differentiation, and size increment in exposed snails compared to controls. Moreover, hermaphrodite glands histology shows a full maturity in the formation of reproductive cells in PE-exposed snails. The GC-MS analysis of the 90% DM extract revealed the presence of 36 compounds representing 93.1% of the total composition. Piperidin-4-ol, 1,3-dimethyl-2,4,6-triphenyl (19.87%), and 6,11-dihydroxy-5,12 naphthacenequinone-1-carboxylic acid (7.80%) were the major components.

Conclusion: The identified compounds in 90% DM extract of *P. granatum* may be responsible for the high antioxidant activity of the fruit and it may account for its immunostimulatory effect against *S. mansoni* infection in *B. alexandrina*.

Keywords: *Punica granatum* L., Antioxidant activity, *Biomphalaria alexandrina, Schistosoma mansoni*, Infection rate, Gas chromatography-mass spectrometry.

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INTRODUCTION

Natural products from plants and herbs have been used extensively as alternative drugs for the treatment of a wide range of ailments and diseases [1,2]. Their use stem due to the undesired side effects and cost-prohibitive or limited effectiveness associated with the antibiotics, chemicals or drugs currently being used. Most of the well-known diseases are caused by oxidative stress occurring within the body as a result of chemical exposure or pathogen infection, whereby the affected organism cannot produce sufficient antioxidants to overcome the produced free radical, also known as reactive oxygen species [3,4]. Therefore, the body needs an exogenous source of antioxidants [5]. Plants or their byproducts are thus preferred because of their richness with phenolic, polyphenolic, alkaloid, quinone, terpenoid, lectin, and polypeptide compounds that proved to be effective alternatives to many synthetic compounds [6-8]. Natural plants have been used as immunostimulants to modulate the innate immune response and increase the body's resistance to infection and in prevention and treatment of various diseases [9,10]. Plant products such as phenolics and flavonoids play a major role in preventing or controlling infectious microbes [11]. *Punica granatum* L. (family Punicaceae) is usually recognized as pomegranate and is native to the Mediterranean area [12,13]. *P. granatum* has numerous applications in the traditional medicine worldwide for the treatment of many ailments and infectious diseases, i.e. helminthiasis, acidosis, dysentery, diarrhea, hemorrhage, acne, allergic dermatitis, and pile [14-16]. All parts of the pomegranate including fruits, bark, roots, and leaves reported to have antioxidant and therapeutic properties [17-19]. The most medically effective constituents of pomegranate are ellagitannins, punicic acid, anthocyanins, estrogenic flavonols, and flavones [20,21]. Many parasitic diseases depend on intermediate host organisms for the completeness of their life cycles. Controlling such diseases remains a great challenge, especially in developing countries. Most of the control initiatives are usually directed to the final host by administering drugs (chemotherapy) or application of chemical compounds to the intermediate host environments for eradication purposes. Either chemotherapy or chemical application toward intermediate hosts eradication is limited in approach and suffers many financial and logistical constraints [22]. An example of these diseases is schistosomiasis, which is considered a major public health problem in many tropical and sub-tropical countries with an estimated global burden of 240 million people infected [23] and close to 800 million at the risk of infection [24]. Schistosomiasis is caused by digenetic trematodes of the genus *Schistosoma* that depend on freshwater snails to complete their life cycle and infect human. Two main control strategies have been used to control schistosomiasis; (1) eliminating host snails using molluscicides, promoting good sanitation and health education [25-27] and (2) morbidity control by killing the worms in humans using chemotherapy approach [27,28]. However, schistosomiasis still prevalent due to the disadvantages

related to the application of these control strategies such as the high financial cost of molluscicides application and drug administration, in addition to the emerging drug resistance in the laboratory [29,30]. In Egypt, schistosomiasis *mansoni* is still endemic in some parts of the country supported by the abundance of *Biomphalaria alexandrina* snails in the Egyptian freshwaters [31-34]. With regard to molluscicides, a number of perceived disadvantages of using niclosamide have been reported such as the necessity for repeated reapplication, difficulty to achieve uniform dispersal and area coverage, high costs due to repeated treatments, and collateral effects on amphibians and fish [26]. The limitations of chemotherapy and molluscicide prompted the need to new innovative control strategies. One possible mechanism is the effective interruption of the *Schistosoma* life cycle in snails to block the parasite's transmission, and thus truly preventing human schistosomiasis over the long-term [26,35,36]. This study aims to assess the effect of methanol solvent extracts of *P. granatum* on innate immunity and resistance of *B. alexandrina* to *S. mansoni* infection, as a control mechanism that can be used to block schistosomiasis transmission.

METHODS

Plant materials

The fresh fruits of *P. granatum* L. (family Punicaceae) were collected from markets in Giza, Egypt during 2015. The identification and authentication of the collected fruits was carried out by Prof. Dr. Wafaa Amer, Professor of Plant Taxonomy, Faculty of Science, Cairo University, Giza, Egypt.

Extraction and fractionation

The air-dried powdered peels of *P. granatum* L. (1.5 kg) were soaked in 4 L of aqueous methanol (90%) for 3 days at room temperature (25±2°C). The crude methanolic extract was concentrated via rotatory evaporator to afford 220 g, after that 210 g from evaporated methanol extract were defatted using 1.5 L petroleum ether (60-80°C) to give petroleum ether extract (25 g) and 90% defatted methanol extract (diabetes mellitus [DM]; 175 g). The latter was suspended in distilled water, and extracted successively with dichloromethane (DCM) (1.5 L), ethyl acetate (2 L), and *n*-butanol (2 L), to obtain methylene chloride (40 g), ethyl acetate (15 g), *n*-butanol (50 g), and water (55 g) extracts. The obtained extracts were weighed and stored for further analyses.

Free radical scavenging antioxidant activity

The free radical scavenging antioxidant activity was determined according to Molyneux [37] and Ghareeb *et al*. [38]. Furthermore, the rapid screening of antioxidant by dot-blot and 2,2'-diphenyl-1-picrylhydrazyl (DPPH) staining was carried out according to Shoeb *et al*. [39]. All solvents and reagents used were of analytical grade. DPPH and ascorbic acid were obtained from Sigma-Aldrich, UK. All other solvents used in the current research work were obtained from El-Nasr Pharmaceutical Chemicals Co./Egypt.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was performed using a GC (Agilent Technologies 7890A) interfaced with a mass-selective detector (MSD, Agilent 7000) equipped with a polar Agilent HP-5ms (5%-phenyl methyl poly siloxane) capillary column (30 m × 0.25 mm i. d. and 0.25 µm film thickness). The carrier gas was helium with the linear velocity of 1 ml/minute. The identification of components was based on a comparison of their mass spectra and retention time with those of the authentic compounds and by computer matching with the database of the National Institute of Standard and Technique (NIST08s), WILEY8, and Adams [40].

Effect of the pomegranate extracts (PEs) on *B. alexandrina* susceptibility to *S. mansoni*

Biomphalaria collection and maintenance

B. alexandrina snails were collected from the irrigation canals of Giza Governorate, Egypt, transferred and maintained at Medical Malacology Laboratory, Theodor Bilharz Research Institute, Egypt. The snail's colonies were kept in polyvinyl plastic trays (8 cm × 21 cm × 31 cm) containing dechlorinated tap water of about 6 cm in depth at room temperature (25±1°C) for acclimation. The snails were supplemented with lettuce leaves daily and the blue green alga, *Nostoc muscorum*, was also added weekly as a dietary supplement. During the acclimation period, the snails were examined twice weekly for 6 successive weeks for any trematode infection. Only uninfected snails were used in the experiments (Shell diameter: 10-12 mm).

Exposure conditions and snails infection

Five groups of uninfected *Biomphalaria*, each of 30 snails in triplicates (10 snails/replicate), were exposed to 200 ppm (equivalent to the concentration at which the highest antioxidant activity was obtained) of each of PEs. Negative controls were also set up in triplicates using only dechlorinated water. The exposure of snails to the extracts was for 1 month and the solutions were changed every 3 days. PE-exposed and control snails were infected with miracidia hatched from *S. mansoni* ova obtained from Schistosome Biological Supply Center, Theodor Bilharz Research Institute. Exposure of snails to miracidia were done individually in 15 mm × 17 mm glass vials with 1.0 ml dechlorinated aerated tap water, under fluorescent light from 20 watt tubes, 30 cm far and temperature 24±1°C. On the next day, the exposed snails were transferred to and maintained in standard aquaria previously described. Starting from the day 21-post miracidial exposure, the snails were examined individually and repeatedly for cercarial shedding in multi dishes under artificial light for 2 hrs (stimulant period) in 2 ml of dechlorinated tape water/snail. All snails that died during the prepatent period were crushed between two slides and inspected under a microscope for immature parasite stages [41]. The snail's infection rate was calculated at the end of experiment by dividing number of shedding and positive crushed snails on the number of exposed snails [42].

Hemocytes investigation

Hemolymph samples were collected from each group of exposed snails by removing a small portion of the shell and inserting a capillary tube into the heart. The hemolymph pooled from 10 snails and collected in a 1.5 ml Eppendorf tube according to Michelson [43] and kept in an icebox for microscopic examinations. Blood films were stained with leishman, giemsa stains and examined according to Mossalem *et al*. [35]. The number of each blood cell type was calculated and represented as a percentage per 100 of cells. Histological sections were also prepared from the hermaphrodite glands and examined under light microscope. Three snails from each group were crushed gently and the hermaphrodite gland was removed, fixed in Bouin's fluid for 5 h and then transferred to 70% alcohol. Further procedures were followed including dehydration in an ascending series of alcohol, clearing in xylol, and embedding paraffin. 5 µm paraffin-embedded sections were stained with hematoxylin and eosin for general histological examination [44].

Statistical analysis

The antioxidant data were presented as mean±standard deviation of triplicates (n=3) according to Annegowda *et al*. [45] using SPSS 13.0 program (SPSS Inc. USA). Comparisons among infection rates of snails and hemocytes percentages were performed using Chi-square test (pairwise vs. control). Limit for statistical significance was set at $p \leq 0.05$ (significance level of 95%).

RESULTS

DPPH free radical scavenging activity

Analysis of the antioxidant activity of *P. granatum* extracts using free radical scavenging antioxidant activity assay with DPPH, revealed that the 90% DM and *n*-butanol are the most potent antioxidants followed by ethyl acetate and water while no activity was recorded with petroleum ether and DCM extracts. The order of activities (expressed as scavenging concentration at 50% [SC_{50}] values in µg/ml) was as follow; 90% DM (12.45) >*n*-butanol (15.59) >ethyl acetate (21.36) >water (49.16) compared to ascorbic acid as standard with SC_{50}=7.50 µg/ml (Table 1). Moreover, except petroleum ether and DCM, all the tested fractions qualitatively showed white wider zones upon the dark purple

background indicating their potential free radical scavenging activities compared with quercitin and ascorbic acid as standards (Fig. 1).

GC-MS analysis of 90% defatted methanol extract

Qualitative GC-MS analyses of the 90% DM of *P. granatum* peels (the highest antioxidant extract among all PE tested) identified thirty-six components (Table 2) representing 93.1% of the total extract composition. These compounds were identified qualitatively based on their retention times and mass spectral fragmentation patterns as;piperidin-4-ol, 1,3-dimethyl-2,4,6-triphenyl (19.87%), 6,11-dihydroxy-5,12 naphthacenequinone-1-carboxylic acid (7.80%), anthraquinone, 1,1'-iminodi- (7.70%), thiourea, 1,1-diethyl-3-(2-mercapto-5-benzoxazolyl) (6.22%), phloroglucinol (6.08%), 1H-benzimidazole (3.92%), coumarin-6-ol, 3,4-dihydro-4,4-dimethyl-5,7-dinitro (3.25%), pseudobaptigenin (3.18%), and 24,25-dihydroxyvitamin D3 (3.10%), as the major components and other constituents were detected as minors (Fig. 2).

Table 1: Free radical scavenging antioxidant activities (DPPH) of the 90% DM extract of *P. granatum* peel and its derived sub-fractions

Sample	DPPH (SC_{50}) (µg/ml)
90% DM	12.45±0.18
Petroleum ether	Not detected
DCM	Not detected
Ethyl acetate	21.36±0.06
n-butanol	15.59±0.17
Distilled water	49.16±0.08
Ascorbic acid	7.50±0.32

Results are expressed as mean values±standard deviation (n=3).
SC_{50}: Scavenging concentration at 50%, DPPH: 2,2'-diphenyl-1-picrylhydrazyl, DM: Diabetes mellitus, DCM: Dichloromethane

Effect of PE on the innate immunity of *B. alexandrina* and their susceptibility to *S. mansoni* infection

The effect of PE on the susceptibility of *B. alexandrina* snails to infection with *S. mansoni* parasite was evaluated after exposure of snails to these extracts for 1 month before infection. Snails exposed to an equivalent concentration for maximum antioxidant activity (200 ppm) of PE showed an infection rates of 20%, 50%, 60%, 70% and 80%, respectively, for 90% DM, *n*-butanol, ethyl acetate, water and DCM compared to 95% in control snails. Although all the extracts caused a significant decrease in the infection rate of snails ($p \leq 0.05$), the lowest infection rate was recorded with 90% DM (Table 3). Histological and microscopic examinations of the hemolymph and hermaphrodite glands before and after exposure to PE showed a significant increase in the hemocytes numbers and marked changes in their morphology after exposure. The number of hemocytes in the hemolymph of *B. alexandrina* snails was counted following 1 month in 90% DM-exposed snails, while amoebocytes were significantly increased in exposed snails (70%) compared to control (20%), the numbers of granulocytes, and

Table 2: Chemical constituents identified in the 90% DM of *P. granatum* peel using GC-MS

Peak No.	R_t	Area %	Identified compounds
1	3.11	4.35	Unknown
2	4.9	1.52	Unknown
3	10.09	6.08	Phloroglucinol
4	10.64	1.09	Anthraquinone, 1-(methylamino)-4-p-toluidino
5	11.48	0.41	Zapotin
6	11.93	0.54	Syringic acid
7	12.75	2.45	2-Iodohiistidine 2-([Bis (2 amino ethyl) amino] methyl) phenol
8	13.26	0.89	Thiocolchicine
9	13.7	0.67	Cinnamic acid, 3,5-dimethoxy Indole, 5-methyl-2-(4-pyridyl)
10	13.96	7.8	6,11-Dihydroxy-5,12 naphthacenequinone-1-carboxylic acid
11	14.19	1.43	Caffeic acid dimethyl ether
12	14.44	6.22	Thiourea, 1,1-diethyl-3-(2-mercapto-5-benzoxazolyl)
13	14.61	1.56	Flavone, 3-hydroxy-4',5,7-trimethoxy
14	14.69	1.82	Papaveroline
15	14.77	1.2	Bergenin
16	14.87	0.65	3-Hydroxyanthranilic acid
17	14.95	3.25	Coumarin-6-ol, 3,4-dihydro-4,4-dimethyl-5,7-dinitro
18	15.18	0.56	p-Menth-1-en-3-one, semicarbazone
19	15.33	1.0	Piperazine, 1-(4-methoxyphenyl)
20	15.66	0.75	Pyridine-3-carboxamide, 1,2-dihydro-4,6-dimethyl-2-thioxo
21	15.89	0.71	Isoelemicin
22	16.18	1	5-Acetyl-2-methylpyridine thiosemicarbazone
23	16.44	0.82	β-Asarone
24	16.71	0.68	4-[Ethylethanolamino]-1,2-naphthoquinone
25	16.92	19.87	Piperidin-4-ol, 1,3-dimethyl-2,4,6-triphenyl
26	17.14	1.01	β carotene
27	17.24	3.92	1H-Benzimidazole
28	17.4	0.92	Quinazolin-4 (3H)-one, 2-mercapto-3-(2-phenylethyl)
29	17.56	2.21	p-Aminobenzoic acid
30	17.63	1.78	Ethanone, 1-(6-methyl-3-pyridinyl)
31	18.03	3.1	24,25-Dihydroxyvitamin D3
32	18.37	2.2	Phenol, 4-amino-3,5-diethyl
33	18.61	7.70	Anthraquinone, 1,1'-iminodi
34	18.68	1.47	Coumarin, 4-hydroxy-3-nitro
35	19.15	2.46	Thiazolidine, 2-methyl-3-nitroso
36	19.28	0.51	Piperazine, 1-(2-methoxyphenyl)
37	20.13	3.18	Pseudobaptigenin
38	21.52	1.19	Phenol, 4-nitro-2-(6,7-dimethylbenzo[e] dithiepan-2-yl)
Total %		93.1	

DM: Defatted methanol, GC-MS: Gas chromatography-mass spectrometry

Table 3: Infection rates of *B. alexandrina* snails exposed to PE for 1 month before infection with *S. mansoni*

Sample/control	90% DM	*n*-butanol (%)	Ethyl acetate (%)	Water (%)	DCM (%)	Control (%)
Infection rate	20*	50*	60*	70*	80*	95

*Highly significant at p≤0.05 compared to control. DM: Diabetes mellitus, DCM: Dichloromethane

hyalinocytes were significantly decreased. Their percentages were 50% and 30% compared to 20% and 5% in control, respectively, as shown in Table 4. There was also an increase in the hemocytes size in 90% DM-exposed snails due to an increase in their internal vacuoles (Fig. 3). With regard to the effect of 90% DM extract on the hermaphrodite glands of exposed snails, the glands showed clear maturation and expelling of mature gametes. Moreover, intensive condensations of spermatids and intense of the female acini with ova and oocytes were also observed including clear developmental stages 1st and 2nd oogonia (Fig. 4).

Table 4: Percentage of hemocytes in the hemolymph of *B. alexandrina* snails exposed to 90% DM extract for 1-month infection with *S. mansoni*

Sample type	Granulocyte (%)	Amoebocyte (%)	Hyalinocyte (%)
Control	50	20	30
90% DM	20*	70*	5*

*Highly significant at p≤0.05 compared to control. *B. alexandrina: Biomphalaria alexandrina, S. mansoni: Schistosoma mansoni*, DM: Diabetes mellitus

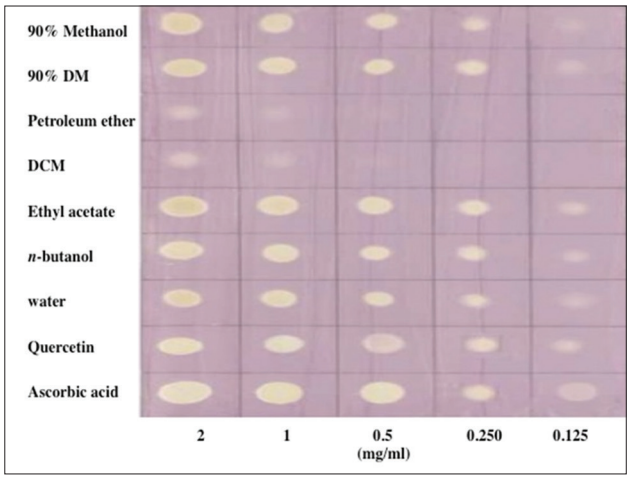

Fig. 1: Dot-blot qualitative antioxidant assay of different fractions of *Punica granatum* peels on silica sheet stained with 2,2'-diphenyl-1-picrylhydrazyl solution in methanol compared to quercetin and ascorbic acid as standards

DISCUSSION

In this study, the different extracts of *P. granatum* L. peels were evaluated for their free radical scavenging antioxidant activity using DPPH assay in order to test it as immunostimulant against *S. mansoni* infection in intermediate host snails, *B. alexandrina*. It is apparent from Table 1 and Fig. 1 that 90% DM and *n*-butanol are the most potent antioxidants. In the same context, the results of the DPPH antioxidant activity of *P. granatum* growing in India showed that the methanol extract exhibited highest activity followed by ethanol, acetone, and ethyl acetate extracts, moreover petroleum ether and benzene extracts showed a very weak activity [13]. Numerous studies have demonstrated that the high antioxidant activity of *P. granatum* may be due to the presence of a complex pattern of the bioactive polyphenolic compounds, i.e., phenolic acids, flavonoids, tannins, anthocyanins, quinones, and coumarins [13,46-48].

GC-MS analyses of the 90% DM of *P. granatum* peels identified 36 components representing 93.1% of the total extract composition. Based on their retention times and mass spectral fragmentation patterns, as the most prominent components were piperidin-4-ol, 1,3-dimethyl-2,4,6-triphenyl, 6,11-dihydroxy-5,12 naphthacenequinone-1-carboxylic acid, anthraquinone, 1,1'-iminodi, thiourea, 1,1-diethyl-3-(2-mercapto-5-benzoxazolyl), and phloroglucinol. Kumar and Vijayalakshmi [49] identified 26 constituents from ethanolic extract of *P. granatum* peels, of which glycerin (27.03%), hydroxymethylfurfurole (21.18%), guanosine (13.34%), and pyrogallol (6.45%) were the major constituents. Moreover, GC-MS analysis of ethanolic extract of *P. granatum* revealed that it contains nitroisobutylglycerol (19.02%), ethyl-α-D-glucopyranoside (12.65%), 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (11.83%), maltol (9.46%), and 3H-indole-3-carbaldehyde (4-amino-5-methyl-4H-1,2,4 triazol-3-yl) hydrazone (5.22%) as major constituents [50].

Following the evaluation the antioxidant activities of PE extracts and identification of its major constituents, their effects on *B. alexandrina* susceptibility to *S. mansoni* and changes in their hemocytes were investigated. The immune system of *Biomphalaria* is composed of cellular and humoral components acting independently or together to fight invading microbes or parasites [51,52]. The first line of defense is mediated by circulating phagocytic cells known as hemocytes (also known as amoebocytes) found in the hemolymph of the snail. These cells have an important role in phagocytosis and encapsulation reactions. During their course of development within snails, schistosomes larvae are in direct and intimate contact with the snail's tissues starts from miracidial penetration and ends with cercarial release within weeks after initial infection [53,54]. Many cellular and humoral factors have been studied with the hope that they might be used as indicators for measures of the effectiveness of potential immunostimulants [55]. Immunostimulants from medicinal plants can increase the resistance to disease by enhancing nonspecific and specific defense mechanisms [56-58]. The active compounds of plants activate several components of the immune system, such as phagocytes [59].

This study showed that all the extracts of *P. granatum* caused a significant decrease in the infection rate of snails (p≤0.05). However, the lowest infection rate was recorded with 90% DM extract which reduced the infection rate to 20%. This improvement in the resistance to infection in 90% DM-exposed snails could be attributed to its modulation to the hemocytes number and morphology of infected snails. A significant increase in the hemocytes numbers (p≤0.05) and marked changes in their morphology were reported following exposure to 90% DM extract. This increase in the hemocytes (amoebocytes) is of specific importance to snails since it represents the first line of defense and it is in direct contact with schistosomes larvae. The immune response of these blood cells is mediated by infiltration around the parasite, encapsulation, and phagocytosis. In a similar study, the phagocytosis activity of the fish *Paralichthys olivaceus* infected with lymphocystis disease virus significantly increased after the administration of aqueous, ethanol and methanol solvent extract of *P. granatum* at 50 and 100 mg/kg dose after 8 weeks. All the solvent extracts at 50 and 100 mg/kg doses increased the survival rate of infected fish [60]. The 90% DM extract has had an improving effect on the hermaphrodite glands of *B. alexandrina* as shown by intensive condensations of spermatids and female acini with ova and oocytes. In particular, this organ in the snails is sensitive for any change in optimum conditions. The beneficial effects of *Punica* 90% DM extract can be attributed to the antioxidant properties of the extract.

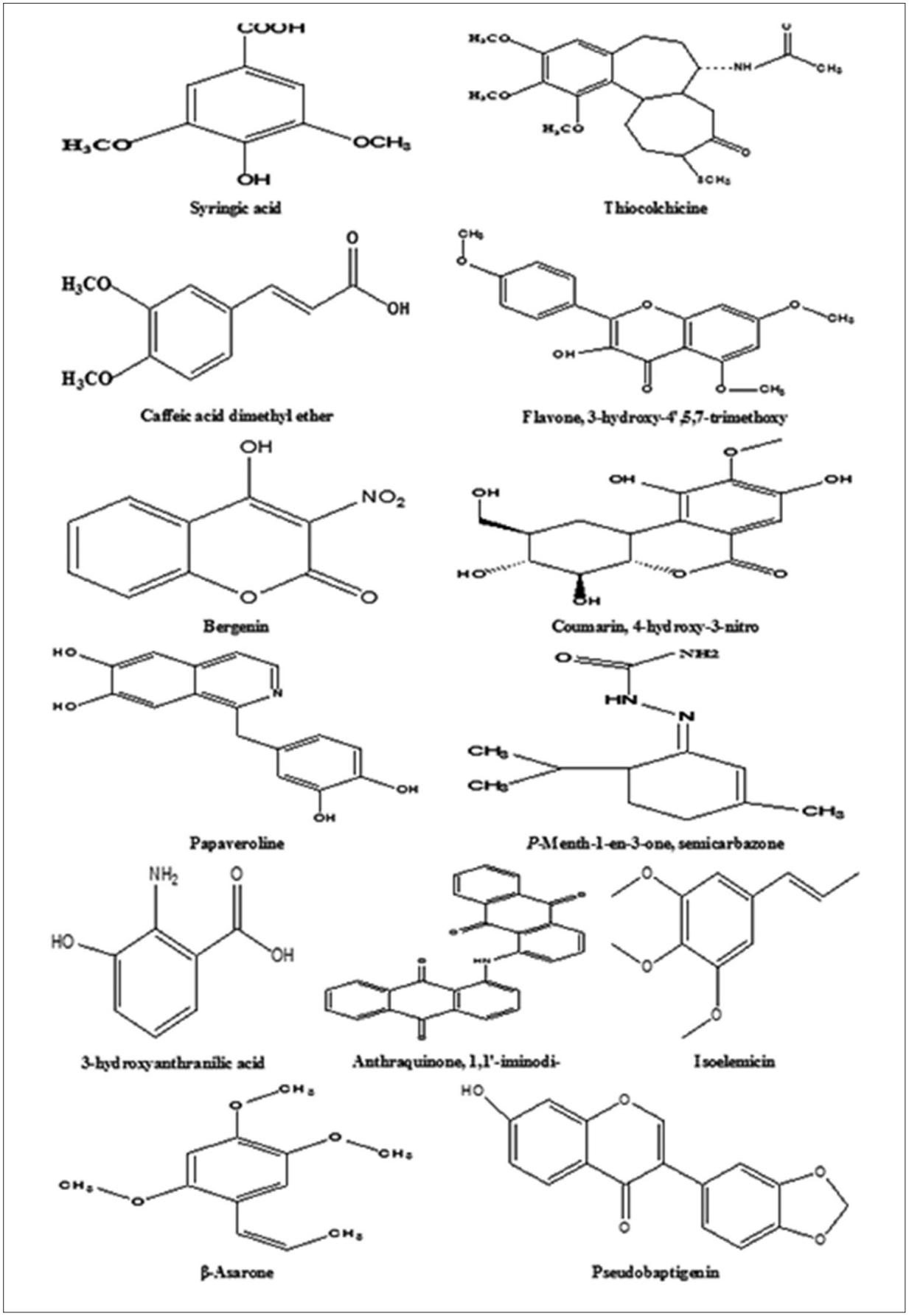

Fig. 2: Chemical structures of some identified compounds in the 90% diabetes mellitus of *Punica granatum* peels by gas chromatography-mass spectrometry analysis

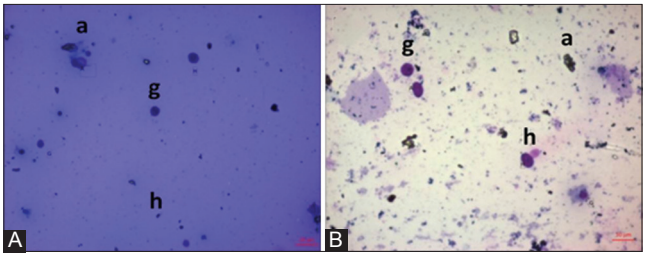

Fig. 3: Morphology of hemocytes in the hemolymph of *Biomphalaria alexandrina* in control and 90% diabetes mellitus-exposed snails. (A) Control snails showing normal shape of the three hemocytes types (a) amoebocyte, (h) hyalinocyte and (g) granulocyte. (B) Exposed snails showing size increase in the three types of hemocytes, magnification at ×400

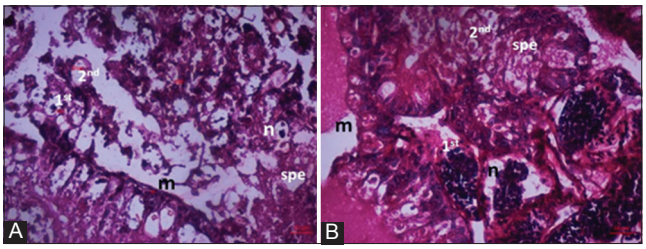

Fig. 4: Morphology of reproductive cells in the hermaphrodite glands of *Biomphalaria alexandrina* from control 90% diabetes mellitus-exposed snails. (A) Control snails (B) exposed snails showing healthy and clear developmental stages; (n) nucleus, (m) membrane, (spe) spermatogonia and 1st and 2nd oogonia, magnification at ×400

Other medicinal plants were used as adaptogens and showed a similar immunostimulatory and protective effect. Boon-Niermeijer *et al.* studied the protective role of the phytoadaptogens herbs *Acanthopanax senticosus* and *Rhodiola rosea* against high and toxic dose of different environmental stressors using the embryos of the pond snail *Lymnaea stagnalis* as a model. The application of aqueous or ethanolic extracts of *Acanthopanax* (0.68 mg/ml) or *Rhodiola* (40.5 µg/ml) for 20 hrs to 3-day old embryos significantly protected against the negative effect of superoxide radicals induced by heavy metals and heat shock indicating the ability of these plant extract to enhance the resistance of *Lymnaea* offspring to the various stress conditions tested. The protective potential of phytoadaptogens may be attributed to their antioxidative properties [61].

CONCLUSION

In conclusion, this study showed that most of *P. granatum* extracts significantly improved the innate immunity of *B. alexandrina* and increased their resistance to *S. mansoni* infection at 200 ppm concentration. This improvement in snails' resistance was positively correlated to the antioxidant activity of the tested extract. For example, 90% DM extract showed the highest antioxidant activity (SC_{50}=12.45) and reduced the snail's infection with *S. mansoni* to 20% compared to 95% in control. The extract can, therefore, be used as immunostimulant to hinder *S. mansoni* development within snails leading to inhibition of the parasite's life cycle and transmission. It also has an improving role on the reproductive organs of the snails as revealed by hermaphrodite glands histology. Finally, this activity may attribute to the chemical constituents that were identified via GC-MS analysis in 90% DM extract.

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