INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 13, No. 5, p. 230-238, 2018

RESEARCH PAPER OPEN ACCESS

Chemical analysis and assessment of the *in vivo* and *in vitro* bioactivities of seeds and peels extracts of pomegranate

Warda Ahmed, Maryam Shahid, Umara Afzal, Faryal Kabir, Muhammad Sheeraz Ahmad, Muhammad Gulfraz*

University Institute of Biochemistry and Biotechnology, PMAS Arid Agriculture University Rawalpindi, Pakistan

Key words: Pomegranate, Phytonutrients, Antioxidants, Antidepressant, *Silico* study.

http://dx.doi.org/10.12692/ijb/13.5.230-238 Article published on November 18, 2018

Abstract

Pomegranate (*Punica granatum* L) is commonly consumed as fresh fruit since ancient time, although its therapeutic potential is still not fully explored. We determined in *vivo* antidepressant, *in vitro* antioxidants and cytotoxicity activities of seeds and peels extracts of pomegranate collected from Northern areas of Pakistan. Chemical analysis of extracts has provided total phenols, flavonoids, tannins, vitamin C, thiamine, riboflavin and some essential metals. HPLC quantification revealed presence of higher amount of quercetin in pomegranate extracts. Antioxidants and cytotoxicity assays correlated well with flavonoids, vitamins and essential metals investigated from extracts. The antidepressant like study in animals indicates favorable effects and provided relaxation to animals from stressed condition in minimum time period after treatment with pomegranate extracts. Where as in *silico* study we have determined an important role of quercetin 3- glucosides ligand by docking with β 2 androgenic protein receptor that binds with epinephrine (hormone). A possible pathway is predicted to reduce hypertension linked with epinephrine in blood by minimizing condition of anxietyin human population.

* **Corresponding Author:** Muhammad Gulfraz ✉ gulfrazsatti@uaar.edu.pk

Introduction

Punica granatum L.is belong to family punica and considered as one of the oldest edible fruits (Singh, 2002). The pomegranate fruit has been used as medicine from ancient time (Gurib-Fakim, 2006) and for the production of fresh juices, beverages, jam and jelly as well as flavoring and coloring reagent in food industries**.** Pomegranate is relatively non-toxic even at high doses according to the preliminary toxicity assessments .Bark roots and leaves of pomegranate tree are medicinally beneficial.

The peel extracts contained higher level of phenolic constituents while ellagic tannins and ellagic acid are among the potent antioxidants (Miguel *et al.,* 2004; Murthy *et al.,* 2002). Ellagitannins have remarkable antimicrobial activity against *Staphylococcus aureus* and some other pathogenic bacteria (Bekir *et al.,* 2013) and useful for preparation of natural antibiotics. Ant proliferative effects of pomegranate on MCF-7, breast cancer cells and to treat the infection of male or female sexual organs, mastitis, acne, pile, allergic and dermatitis were reported earlier. Furthermore its effects on stress as indicated by increased hormone concentration and enzymatic activities were also reported by many research workers. Exposure to over-stress may induce depression and negatively alter behavioral, learning and biochemical processes. Mood disorders including major depression are potentially life-threatening illness. Side-effects or losses of desired are common with antidepressant drugs. Pomegranate seeds and peel has been shown to possess good antioxidant activity due to presence of polyphenols vitamin C and macro elements like potassium, calcium, magnesium, iron, znic and manganese (Aviram *et al.,* 2008). The purpose of current investigation was to evaluate the various bio activities of peels and seeds extracts of pomegranate the in *vivo* and *vitro* methods as well as quantification of phytonutrients with its possible correlation by minimizing hypertension levels through molecular docking.

Materials and methods

Sample collection and preparation

Fresh pomegranate fruit (*Punica granatum* L.) were collected from Hunza and Chitral region. (Pakistan). A specimen (voucher no.142) was deposited at Herbarium Department of Botany PMAS Arid Agriculture for future reference. The seed and peel samples of pomegranate were shad dried followed by sun and oven dried at 60 ^{0}C. The samples were ground to powder form sieve (80 msh). Total 100 grams of dried sample was extracted with distilled water, methanol, ethanol and chloroform using soxhlet and rotary evaporator procedure.

Quantitative analysis

Total phenols, flavonoid and tannins contents of peel and seed extracts of pomegranate were estimated by Folin's Ciocaletu and other colorimetric methods (Abbasi *et al.,* 2015; Harbone, 1984).

Oil from pomegranate samples were extracted with Soxhlet techniques by using methanol, ethanol and chloroform. Vitamin were determined by spectrophotometric methods. Macronutrients (Sodium, potassium calcium and magnesium) and micronutrients (Iron, zinc and manganese) were determined by using method (AOAC, 2000) with the help of Atomic Absorption Spectroscopy.

HPLC analysis of extracts was performed using Shimadzu HPLC system (Tokyo, Japan) attached with UV/visible detector and C18 column (25mm × 4.5mm, 5µm). The compounds were eluted using a gradient of acetonitrile and 0.1% phosphoric acid (36:64).The injection volume for all samples was 20 µl. Flavonoids were monitored at 280 nm and 285nm at a flow rate of 1 ml/min. Quercetin was used as a standard and all determinations were performed in triplicates.

Brine shrimps cytotoxic assay

Brine shrimps cytotoxicity assay was carried out to evaluate the cytotoxic effect of seeds and peels of extracts of pomegranate by using method already described (Ruch *et al.*,1989).

Determination of antioxidant capacity of extracts in vitro

The measurement of DPPH radical scavenging activity was performed according to methodology described (Moon and Shibamo to, 2009;Yu *et al.,*2005).2,2′-azino-bis-3-ethylbenzthiazoline-6 sulphonic acid (ABTS) assay (Ashafa *et al.,*2010). Hydroxyl radical, superoxide and Fe chelating assays were conducted by using reported method (Cefarelli *et al.,*2006). IC_{50}values were calculated from dose response curve.

Depression study (Tail suspension test TST)

Animals, 18 albino mice of either sex (body weight 52.5±1.6g) were purchased from National Institute of Health, Islamabad. Animals were housed per cage under a normal laboratory condition and had free access to water and food pellets.

The animals were acclimatized for at least three days before behavioral experiment and study was approved by ethical committee of University constituted for animal studies. Immobility induced by tail suspension was measured (Steru *et al.,* 1985). Briefly depression was produced by suspending tape on tip of the tail of animal .The test was carried out in 6 min duration and immobility period was recorded. Immobility period is defined as time spent an absent of limb or anybody movements (Except movement caused by breathing). Changes in the immobility duration were studied after administration of drugs to animals as mentioned below.

Experimental design

Animals were divided randomly into 6 groups of 3 each. Group I: Negative control (Animals of this group were given 1 mL of saline water 0.9 % with feed). Group 11: positive control (Animals received Fluoxetine 20 mg/kg). Group III was provided 100 mg/Kg b.w methanolic peels extracts. Group IV was given methanolic peels 200 mg/kg b.w. Animal of group V got 100 mg/kg methanolic seeds extracts .Group VI received methanolic seeds extracts 200 mg/kg b.w. Fluoxetine standard antidepressant drug was administrated intraperitoneally (i.p), whereas methanolic extracts were given to animals orally by gavage.

Molecular docking

The quercetin 3- glucosides ligand were docked with crystal structure of β- 2 and renergic protein receptor through Vina suit (PyRx, v 0.8) and Automatic server Patch dock (online server).

The interaction of ligands, 2-D structures (SDF format) Quercetin 3-glucoside (PubChem:44259229), The receptor structure was retrieved from Protein Data Bank (PDB) while ligands were retrieved from Pub Chem and converted into PDB using PyMol. The docking view and surface analysis of receptor was conducted using Avogadro tool for geometry optimization through energy minimization. Lig Plus for H bond and hydrophobic interaction and Chimera v 1.10.2 for docked receptor ligands were used (Morris *et al.,* 2009).

Statistical analysis

Data obtained after triplicate analysis of phytochemicals as well as bioactivities determination was further statistically analyzed for mean, standard deviation (SD) and average values using SPSS version 16.0.

Results

Chemical analysis of pomegranate extracts

Methanolic seeds extracts of pomegranate has provided higher level of total phenolics, total flavonoids and tannins, whereas peels extracts has imparted higher yields of total oils (Table 1). Water soluble vitamins, macro and micro nutrients contents analyzed from peel and seed extracts (Table 2).

Brine shrimps lethality assay

Four different dilutions of seeds and peels extracts of pomegranate (100, 200, 400 and 600 µg/mL) were tested during brine shrimps cytotoxicity assay (Table 3). The results revealed the better brine shrimps larvicidal potential and lethality was maximum at maximum concentration of extracts and was concentration dependent.

Table 1. Quantification of phytochemicals from Seeds and Peels extracts of pomegranate.

Extracts	Total flavonoids (mg Quercetin /100g)	Total phenols (mg Caffeic acid /100g	Tannins (mg/100g)	Oil (%)
Methanol seed extract	45.16 ± 1.68	228.35 ± 1.25	3.92 ± 0.58	3.9 ± 0.6
Ethanol seed extract	42.19± 1.02	219.73 ± 2.36	3.73± 0.64	3.2 ± 0.4
Chloroform seed extract	35.31 ± 0.64	215.28 ± 2.8	2.65 ± 0.51	2.6 ±0.1
Methanol peel extract	38.14 ± 1.53	122.34 ± 1.23	3.65 ± 0.53	4.5 ±0.5
Ethanol peel extract	35.15± 1.62	113.76 ± 1.34	3.21± 0.62	4.2±0.3
Chloroform peel extract	24.36 ± 0.54	114.26 ± 1.52	2.35 ± 0.57	2.8 ± 0.6

Mean ± SD (n=3).

It was assumed that extracts might be composed of antitumor components in the form of essential phytonutrients.

Antioxidant capacity in vitro

Five methods for the evaluation of antioxidant activities of pomegranate extracts were evaluated. However, free radical scavenging activity of methanol seed extract determined by DPPH assay has shown better results (IC50= 15.29±1.49µg/mL) as compared to other assays performed (Table 4).DPPH assay is a reliable method to determined antioxidant capacity of biological substrates. Higher antioxidant potential of seeds extracts might be due to combined action of all organic compounds present in seeds extracts including flavonoid (quercetin).

Table 2. Concentration level of Vitamins (mg/dL) and Metals ions (%) in peels and seed extracts of Pomegranate.

Analytes	Peel extract	Seed extract
Ascorbic acid	8.82 ± 0.56	9.73 ± 0.75
Thiamine	0.18± 0.61	0.43 ± 0.02
Riboflavin	0.16 ± 0.53	0.12 ± 0.01
Sodium	2.67± 0.56	3.07± 0.28
Potassium	205.6± 0.13	225.3± 0.18
Magnesium	11.84 ±1.46	12.34 ±1.24
Calcium	8.15 ± 0.62	9.76 ± 0.35
Iron	0.46 ± 0.04	0.67 ± 0.05
Znic	0.64 ± 0.01	0.89 ± 0.04
Manganese	0.15 ± 0.02	0.18 ± 0.05

Mean ± SD (n=3).

Analysis of methanolic extracts of *pomegranate* with HPLC revealed presence of higher quantity of quercetin followed by rutin(Fig. 1).

In Vivo antidepressant study

The results indicates that there was no mortality at higher doses of drugs after 72 hours. The dose of 100 mg/kg extracts had provided little change in immobility period, however, by increasing dose up to 200 mg/kg, especially methanolic seed extracts has provided significant antidepressant effects those were comparable to standard drug fluoxetine (Table 5). Tail suspension test (TST) is widely used to screen new antidepressant drugs.

Molecular docking

A ligand of quercetin glycosides was prepared, the active sides of ligand conformation in β- 2 and

erenergic receptor was based on its polar interaction (Fig.2 ABC). The docking results indicated binding of the ligand with active sides of the receptor at Glu 107, Cyst 106 , 184 and 191, Thr110, 174 and 185,Arg 175, Ala 181 and Asp 192. A protein cavity becomes active after interaction with a ligand that may create further activation or inhibition of protein activity.

Table 3. Cytotoxicity screening of various concentration (µg/ml) extracts of pomegranate and % mortality.

Extracts	100 mg/ml	200 mg/ml	400 mg/ml	600 mg/ml	LD_{50}
Methanol pees extract	40	50	60	80	270.45
Ethanolic peels extract	50	60	80	90	16.27
Chloroform peels extract	55	70	70	70	324.82
Methanolic seeds extract	10	30	50	60	14.77
Ethanolic seeds extract	20	25	55	65	319.77
Chloroform seeds extract	30	30	60	70	372.53

Values are Mean ±SD (n=3) and significantly different (P<0.05); positive control are saline sea salt.

The beta-2 adrenergic receptor also known as ADRB2binds with epinephrine (neurotransmitter) whose interaction to calcium channel possibly mediated through physiologic responses probably like smooth muscles relaxation. It was documented by some earlier studies that if quercetin, vitamin C and macro nutrients like potassium and calcium consumed through fruits and vegetables, may diminish level of hypertension and epinephrine in blood. That consequently reduces the level of anxiety (depression)in effected person and provides relief from prevailing stress condition.

Table 4. Antioxidant effects of peels and seeds extracts of Pomegranate (IC50 values µg/mL).

Extract 100 µg/mL	DPPH	H_2O_2	ABTS	Reducing power	Iron chelation
Ethanolic peel extract	28.25±2.21b	44.51±1.18b	42.92±1.51b	66.03± 2.14b	36.31± 1.38b
Methanolic peel extract	16.28±1.45[a]	38.25±2.17[a]	39.52±2.31[a]	63.19±2.54 a	25.52±2.34 a
Aqueous peel extract	36.35±0.48	46.62±2.81	47.21±1.73	71.45± 2.66	46.09± 1.17
Ethanolic seed extract	26.19±1.28b	43.56±2.18b	45.72±3.81b	68.53± 2.06b	36.32± 1.35b
Methanolic peel extract	15.29±1.49a	41.22±2.15[a]	38.52±2.33[a]	61.19±3.15 a	24.65±1.36 a
Aqueous seed extract	34.13±2.11	52.43±3.45	46.21±2.31	92.16± 3.64	41.35± 1.27
Ascorbic acid	8.25±0.72b	7.52 ±0.39b	6.34±0.35 Þ	11.35 ±1.17 Þ	35.06± 1.28b
Gallic acid	3.93±0.38 a	1.68±0.23 a	1.23±0.34 a	2.35±0.38 a	20.38±1.38 a

Means ± SD, (n = 3), where as[a] = p<0.01, p=p<0.05; a higher values, b lower values.

Discussion

Results obtained in present study indicates that seeds and peels extracts of pomegranate possess lower level of cytotoxicity which indicates suitability of these extracts for drugs preparation promising level of antioxidant activities of seed and peels extracts shows relationship of flavonoids, phenolics and tannins quantified from extracts. Whereas HPLC analysis

revealed that major compound was quercetin which together with tannins especially ellagic tannins are likely to be main antioxidants (Li *et al.,* 2006; Reddy *et al.,* 2007) whereas quantification of oil explored importance of pomegranate fruit that might be comprised of essential and non-essential fatty acids those have health benefits for consumer.

Antidepressant like activity of pomegranate extracts might be due to presence of water soluble vitamins (Vitamin C, thiamine and riboflavin), micro and macro nutrients those imports favorable effects on muscles relaxation and helpful to reduce level of anxiety in effective subject.

Table 5. Antidepressant like activity of seeds and peels extracts on immobility period (second) of rats using tail suspension test.

Group	Drugs	Pre treatment	Post treatment
1	Negative control (saline 0.9 %)	195.4 ± 2.5	193.5±2.7
2	Positive control Fluoxetine (20 mg/kg)	185.8 ±1.6	175.6 ±1.6 ^{S}
3	Methanolic peels extract 100 mg/kg	189.6 ±1.3	184.5 ±1.7
4	Methanolic peels extracts 200 mg /kg	187.5 ±2.6	181.6 ±2.1
5	Methanolic seeds extracts 100 mg/kg	185.7 ±1.5	179.5 ±1.8 ^{B}
6	Methanolic seeds extracts 200 mg/kg	182.6±2.6	176.8 ± 2.4 ^{A}

Values are Mean ± SD (n=3). Higher effects methanolic seed extracts (A and B) as compared to standard drugs (S) to overcome depression condition of animals.

Oxidative stress is considered as roadmap leading to different human disorders, however, toxic effects of reactive oxygen and nitrogen species is balance with antioxidants comes through foods especially through fruits and vegetables (Sing *et al.,* 2002; Yu *et al.,* 2005). Results obtained in current study about antioxidant potential of pomegranate extracts are comparable with results reported by other authors (Elfalleh *et al.* 2012; Negi and Jayaprakasha, 2003).

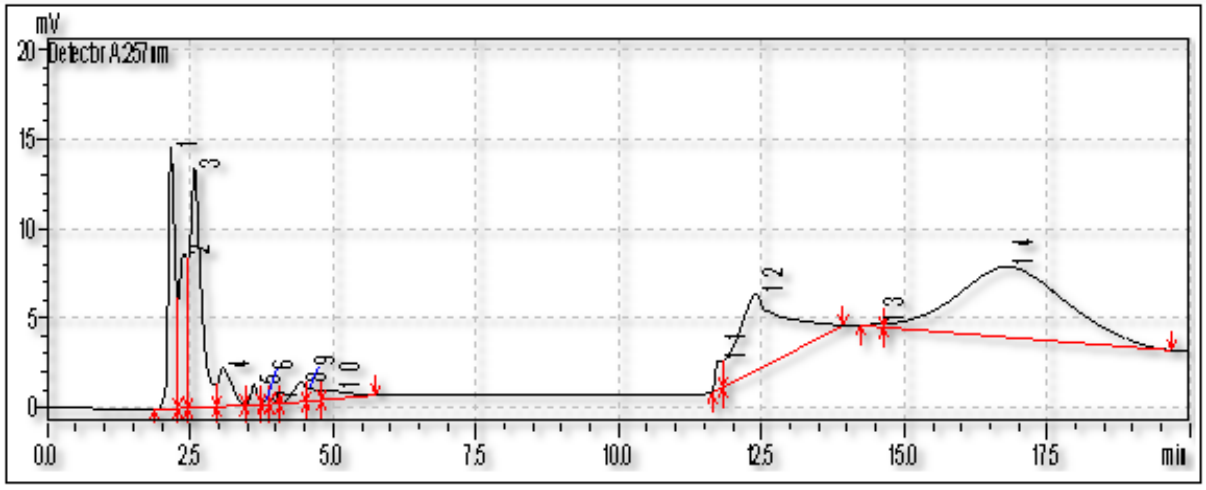

Fig. 1. HPLC analysis of Methanolic extracts of pomegranate showing peak 1 (querceitn), 2(rutin) etc.

Our results suggested correlation between antioxidant and antidepressant activities of pomegranate extracts. Flavonoids, vitamin C potassium and calcium possess many biochemical properties, can prevent damaging process caused by oxidative stress leading to development of cancer and similar other infection in human body (Morris *et al.,* 2009). There are different causes of stress in human body that could be due to

hypertension, environmental and hereditary factors those are producing symptoms of depression. Daily use of fruits those having higher levels of flavonoids, vitamin C and essential metals may provide antidepressant effects.

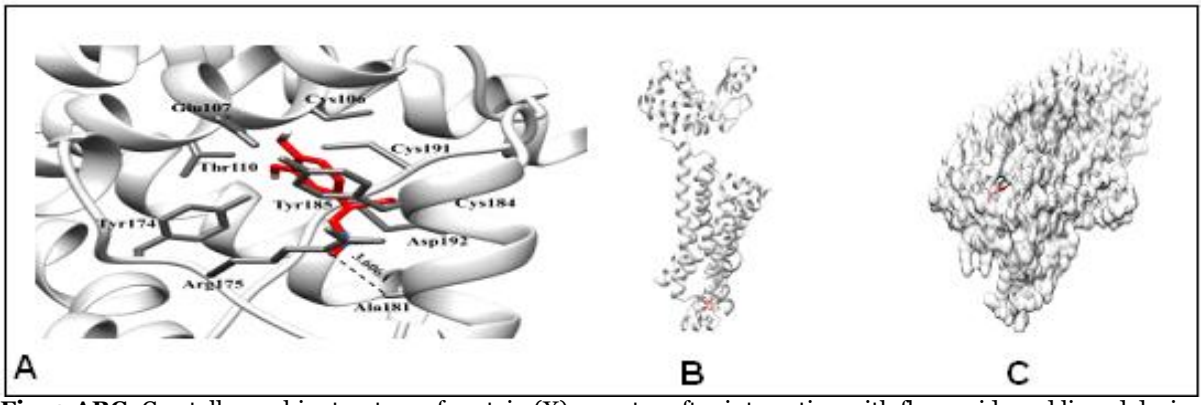

Fig. 2 ABC. Crystallographic structure of protein (X) receptor after interaction with flavonoids and ligand during Molecular docking study.

Molecular docking study indicates binding of quercetin with beta -2 adrenergic receptor that shows association with glutamine, cystine threonine, tyrosine, arginine and alanine indicating possible hydrophobic and hydrophilic sites for interaction between small molecules (ligands) and protein at the atomic level(Hina *et al.,* 2017). Therefore use of fruits that contained higher level of flavonoids (Quercetin) may help to minimize level of various parameters those create stress or hypertension (Acharya *et al.*, 2010).

Acknowledgement

We are thankful to department of Biochemistry PMAS Arid Agriculture University for providing funds for this study.

Conflict of interest

We declare no conflict of interest.

Conclusion

Pomegranate is an important fruit that contains flavonoids (quercetin), water soluble vitamins (vitamin C), essential fatty acids and metals that as a group act as antioxidants, inhibits growth of cancer cells, and may also provide antidepressant like activities. Our results suggests that consumption of pomegranate fresh fruits provides many health benefits and justified its use in ethno medicines.

References

Abbasi AM, Shah MH, Li T, Fu Guo X, Liu RH. 2015. Ethnomedicinal values, phenolic contents and antioxidant properties of wild culinary vegetable. Journal of Ethno pharmacology **162,** 333-345. https://doi.org/10.1016/j.jep.2014.12.051.

Acharya A, Das IS, Singh S, Saha T. 2010. Chemo preventive properties of indole-3-carbinol diindolylmethane and other constituents of cardamom against carcinogenesis. Patents on Food, Nutrition and Agriculture **2,** 166-177.

AOAC. 2000. Official Methods of Analysis of the Association of Official Analytical Chemists, (17th . ed) Washington Dc.

Ashafa AOT, Sunmonu TO, Afolayan AJ. 2010. Toxicological evaluation of aqueous leaf and berry extracts of Phytolaccadioical in male Wistar rats. Food and Chemical Toxicology **48,** 1886-1889. https://doi.org/10.1016/j.fct.2010.04.029.

Aviram M, Volkova N, Coleman R, Dreher M, Redd MK, Ferreira D, Rosenblat M. 2008. Pomegranate phenolics from the peels, arils, and flowers are antiatherogenic: studies in vivo in atherosclerotic apolipoprotein E- deficient (E0) mice and in vitro in cultured macrophages and

lipoproteins. Journal of Agriculture and Food Chemistry **56,** 1148-1157. https://doi.org/10.1021/jf071811q.

Bekir J, Mars M, Souchard JP, Bouajila J. 2013. Assessment of antioxidant, anti-inflammatory, anti-cholinesterase and cytotoxic activities of pomegranate (Punica granatum) leave. Food and Chemical Toxicology **55,** 470-475. https://doi.org/10.1016/j.fct.2013.01.036.

Cefarelli G, Abrosca BD, Fiorentino A, Izzo A, Mastellone C, Pacifico S, Piscopo V. 2006. Free-radical-scavenging and antioxidant activities of secondary metabolites from reddened cv. Annurca apple fruits. Journal of Agriculture and Food Chemistry **54,** 803-80. https://doi.org/10.1021/jf052632g.

Elfalleh W, Hannachi H, Tlili N, Yahia Y, Nasri N, Ferchichi A. 2012. Total phenolic contents and antioxidant activities of pomegranate peel, seed, leaf and flower. Journal of Medical Practice and Reviews **6,** 4724-4730. https://doi.org/10.5897/JMPR11.995.

Gurib-Fakim A. 2006. Medicinal plants: traditions of yesterday and drugs of tomorrow. Molecular Aspects of Medicine **27**, 1-93. http://dx.doi.org/10.1016/j.mam.2005.07.008.

Harborne JB. 1984. Phytochemical methods: A guide to modern techniques of plant analysis Chapman and Hall London.

Hina G, Mushtaq A, Zafar M, Muhammad SA, Ambreen A, Syeda H, Imam S, Gulfraz M. 2017. The invitro and invivo Biological activities of the leaf of Cape Myrtle Myrsine Africana L. Phytotherapy Research **31,** 1305-130.

Li Y, Guo C, Yang J, Wei J, Xu J, Cheng S. 2006. Evaluation of Antioxidant Properties of Pomegranate Peel Extract in Comparison with Pomegranate Pulp Extract. Food Chemistry **96,** 254-260. https://doi.org/10.1016/j.foodchem.2005.02.033.

Miguel G, Fontes C, Antunes D, Neves A, Martins D. 2004. Anthocyanin concentration of "Assaria" pomegranate fruits during different cold storage conditions. Bio Med Research International, 338-342. http://dx.doi.org/10.4067/S071858392010000100005.

Moon JK, Shibamoto T. 2009. Antioxidant assays for plant and food components. Journal of Agricultural and Food Chemistry **57,** 1655-1666. https://doi.org/10.1021/jf803537k.

Morris GM, Huey R, Lindstrom W, Sammer MF, Belew RK, Goodsell DS, Olson AJ. 2009. Auto Dock 4 and Auto Dock Tools 4: Automated docking with selective receptor flexibility. Journal of Computational Chemistry **30,** 2785-2791. https://doi.org/10.1002/jcc.21256.

Murthy KC, Jayaprakasha G, Singh R. 2002. Antioxidant Activity of Pomegranate Peel Extracts in Vivo Models. Journal of Agricultural and Food Chemistry **50,** 4791-4795. http://dx.doi.org/10.1021/jf010865b.

Negi P, Jayaprakasha J. 2003. Antioxidant and Antibacterial Activities of Punica granatum Peel Extracts. Journal of Food Sciences **68,** 1473-1477. https://doi.org/10.1111/j.1365-2621.2003.tb09669.x.

Reddy MK, Gupta SK, Jacob MR, Khan SI, Ferreira D. 2007. Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from Punica granatum L. Planta Medica **53,** 461-467. https://dx.doi.org/10.1007%2Fs13197-011-0351-y.

Ruch RJ, Cheng SJ, Klaunig JE. 1989. Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins

isolated from Chinese green tea. Carcinogenesis **10,** 1003-1008.

Singh RP, Chidambara Murthy KN, Jayaprakasha GK. 2002. Studies on the antioxidant activity of pomegranate (Punica granatum) peel and seed extracts using in vitro models. Journal of Agricultural and Food Chemistry **50,** 81-86. http://dx.doi.org/10.1021/jf010865b.

Steru L, Chermat R, Thierry B, Simon P. 1985. The tail suspension test: A new method for screening antidepressant in mice. Psychopharmacology **85,** 367-370.

Yu J, Wang L, Walzem RL, Miller EJ, Pike LM, Patil BS. 2005. Antioxidant activity of citrus limonoids, flavonoids and coumarins. Journal of Agricultural and Food Chemistry **53,** 2009–2014. https://doi.org/10.1021/jf0484632.