ORIGINAL ARTICLE

Antibiotic potential of phytochemicals in *Punica granatum* pericarp and their proposed mechanism of action by *in silico* studies

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Abstract

Aim: *Punica granatum* is a very important plant with commercial interest and is known for its antioxidant potential. The pericarp is a leftover unwanted part of the fruit that has been reported to have several medicinal uses in traditional medicine. This study focuses on analyzing the antibacterial potential of the pericarp extracts and predicts its mechanism of action by *in silico* studies. **Materials and Methods:** Antibacterial activity of *P. granatum* extracts was analyzed using agar-well-diffusion assay. The phytochemicals reported from pericarp of *P. granatum* were analyzed for ADMET properties using SwissADME tool. The molecules were subjected to protein-ligand docking study using AutoDock-4. **Results:** Polar extracts of the pericarp demonstrated significant antibacterial activity against Gram-positive bacteria *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA), i.e., acetone extract showed highest activity with 18 mm zone of inhibition against MRSA and ethanol extract showed 16 mm zone of inhibition against *S. aureus*. The non-polar extract had no significant antibacterial activity. All ten molecules were predicted to be suitable drug-like molecules, with biocompatible physiochemical parameters. Among the analyzed ten phytochemical molecules, flavogallol and ursolic acid demonstrated significant enzyme inhibition potential against dihydrofolate reductase and topoisomerase-IV with a free binding energy of −11.0 kcal/mol and −10.7 kcal/mol, respectively. **Conclusion:** This suggests that the phytochemicals in the polar extracts of *P. granatum* pericarp exhibit a synergistic antagonism against Gram-positive bacteria. Further purification of individual molecules and investigation of their antagonistic activity are currently IN progress.

Key words: ADMET, antibacterial activity, methicillin-resistant *Punica aureus*, protein-ligand docking, *Punica aureus*, *Punica granatum*

INTRODUCTION

Ever since decades, humans have used plants for their day to day needs such as food, fodder for animals, and shelter. Medicinal plants have been studied as a cure for innumerable ailments. In India, Ayurveda system of medicine has been in practice for decades. Ancient literature evidences various plants and their parts to be used in Ayurveda, Siddha, and Unani medicine for treatment and cure of many diseases.[1] In comparison with synthetic drugs, antimicrobials originated from plants are not linked with side effects and have a wide therapeutic potential to cure different infectious diseases.[2]

Punica granatum is commonly known as pomegranate. Primarily originated from Iran, but also found in Northern India, China, USA, and over the Mediterranean region.[3] The pomegranate as fruiting plant is anatomically divided into different compartments, including root, bark, flower, leaf, peel, juice, and seeds which are suggested to possess many pharmacological and toxicological activities. The edible fruit of pomegranate is believed to be used

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Received: 12-10-2019
Revised: 20-11-2019
Accepted: 03-12-2019

in many traditional remedies over decades, such as remedy for acidosis, microbial infections, dysentery, diarrhea, hemorrhage, helminths infection, and respiratory pathologies.[4] Moreover, the juice of the fruit and the dried pericarp are suggested to be beneficial for the treatment of dermatitis, headache, acne, colic, menorrhagia, colitis, oxyuriasis, diuretic, allergic dermatitis, piles, and for oral diseases.[5]

The pomegranate peel scientifically known as "pericarp" is generally considered as wastes but are truly antibiotic in nature, and has no side effects. These antimicrobials kill the antibiotic-resistant pathogens as well.[6] It is a rich source for flavonoids, tannins, polyphenols, and some anthocyanins as cyanidins, delphinidins, etc.[7] Pomegranate peel is also considered as a strong astringent and a remedy for oral aphthae and diarrhea.[8] Many scientists have reported that the water decocted pericarp extract is internally and externally beneficial for various problems draining astringents and/or germicides, mostly for diarrhea, aphthae, and ulcers. Moreover, the mixture of pomegranate seeds, peel, and juice products paradoxically being used to not only for counteracting abortion but also conception.[9-11]

The pericarp is studied widely and found to have flavonoids and tannins in a large quantity.[12] Phytochemical punicalagin originated from the pericarp is studied to have antioxidant capacity as compared to other parts of the pomegranate.[6]

The pomegranate pericarp is currently used in treating respiratory diseases and even in therapeutic formulae preparations. Pericarp peel is suggestive of possessing antibacterial activity due to the presence of broad-spectrum antimicrobial compounds or metabolic toxins that functions counter to Gram-positive and Gram-negative bacteria. In comparison with other extracts pomegranate pericarp, ethanolic extracts are observed to possess higher degree of antibacterial activity against bacteria that cause diarrhea, gut infection, and stomachache.[13]

The current study investigates the antibacterial activity of the pericarp extracts of *P. granatum* and predicts the possible mechanism of action of the phytochemicals that are reported from the pericarp extracts by *in silico* protein-ligand docking analysis.

MATERIALS AND METHODS

Plant Extraction

The fresh pericarp samples of *P. granatum* were procured from local markets, and the samples were shade-dried for 3 days until the samples were completely dry. The dried samples were then grounded and subjected to maceration. The plant powder (10 g) was mixed with 100 ml of respective solvents (ethanol, acetone, chloroform, and hexane) for 24 h at room temperature. The solvent is then filtered and concentrated to obtain the respective crude extract.[14,15]

Antibacterial Assay

The crude extracts were screened for their antibacterial activity using agar well diffusion assay against common pathogen strains such as *Staphylococcus aureus* (MTCC:7405), methicillin-resistant *S. aureus* (MRSA) (ATCC:43300), *Escherichia coli* (MTCC:1687), and *Proteus vulgaris* (MTCC:7299). The extracts were dissolved in 100% dimethyl sulfoxide at a concentration of 20 mg/ml from which 100 µl was added into each well in the agar plate, giving a final test concentration of 2 mg/well for all the solvent extracts. The plates were incubated at 37°C overnight and observed for zone of inhibition. The antibacterial activity was measured in terms of millimeter of zone of inhibition.[16-18]

ADMET Analysis

The list of retrieved phytochemicals from different literature sources was subjected to ADMET analysis using SwissADME online web tool (www.swissadme.ch). The structures of the molecules were sketched in using online structure sketch tool, and their biophysical parameters were calculated, which was then compiled into a final table for representation and visualization.[19,20]

Protein-ligand Docking

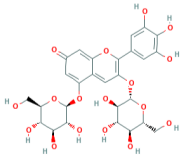
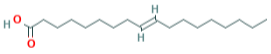
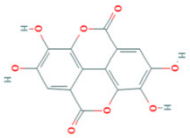
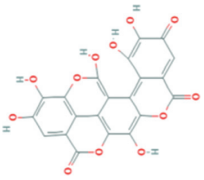
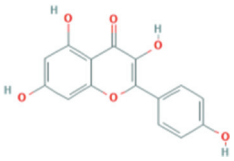
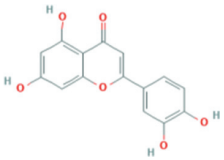
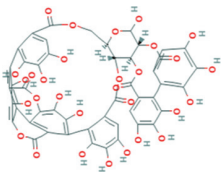
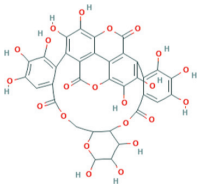
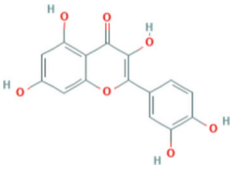
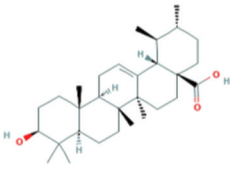
The *in silico* protein-ligand docking study was performed using AutoDock4 in the MGL tools downloaded from website (autodock.scripps.edu). The macromolecules

Table 1: Antibacterial activity of different extracts of *Punica granatum* pericarp against Gram-positive and Gram-negative pathogens

Zone of inhibition against test pathogens at 2 mg/well	*Staphylococcus aureus*	Methicillin resistant *Staphylococcus aureus*	*Escherichia coli*	*Proteus vulgaris*
Acetone extract	17 mm	18 mm	N.S	N.S
Ethanol extract	16 mm	15 mm	N.S	N.S
Chloroform extract	10 mm	10 mm	N.S	N.S
Hexane extract	N.S	N.S	N.S	N.S

N.S: No significant activity

Table 2: List of known phytochemical constituents in *Punica granatum* pericarp

Chemical	Molecular weight (g/mol)	Chemical structure	PubChem ID
Delphinidin-3,5-Diglucoside	626.5		25201902
Elaidic-acid	282.5		637517
Ellagic acid	302.19		5281855
Flavogallol	452.3		136794813
Kaempferol	286.2		5280863
Luteolin	286.2		5280445
Punicalagin	1084.7		44584733
Punicalin	782.5		5388496
Quercetin	302.2		5280343
Ursolic-Acid	456.7		64945

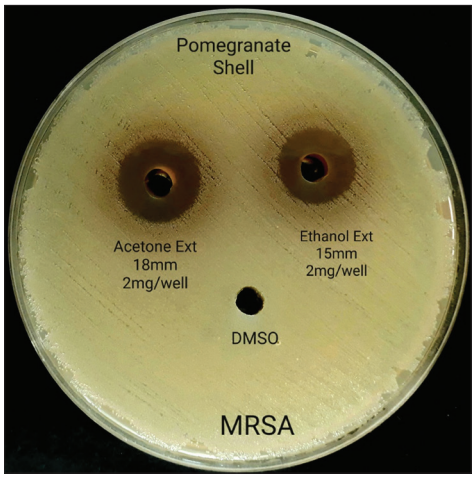


Figure 1: Polar extracts (acetone and ethanol) showing antibacterial activity against methicillin-resistant *Staphylococcus aureus*

were downloaded from the Protein Data Bank (PDB) file format from the www.rcsb.org website. The downloaded molecules were penicillin binding protein 2a (PBP2a) (PDB ID: 3ZG5), dihydrofolate reductase (DHFR) (PDB ID: 4FGG), dihydropteroate synthase (PDB ID: 1AD1), and topoisomerase-IV (PDB ID: 2INR).

The macromolecule (proteins) structures were cleaned of all the non-amino acid residues and were optimized for the docking study. The ligand (phytochemicals) molecules were retrieved from PubChem website with their respective IDs. The ligand molecules were downloaded in their sdf file format and were used in the MGL tools for docking study. Standard docking protocol was followed, with the grid box focusing on the active site of the protein molecules reported in their respected PDB entries.[21-23]

RESULTS

Antibacterial Activity

Among the four different extracts of pomegranate shell, i.e., acetone, ethanol, chloroform, and hexane extracts, only the two polar extracts (acetone and ethanol extracts) demonstrated significant antibacterial activity against the tested Gram-positive bacterial pathogens. Results of the agar well diffusion assay are tabulated in Table 1. The image of agar well diffusion assay against MRSA is shown in Figure 1.

Agar well diffusion assay strongly demonstrated that the polar extracts of the *P. granatum* pericarp, possess significant antibacterial activity against Gram-positive bacterial pathogens. This extract was further investigated to identify the possible mechanism of action of the observed activity.

ADMET of Phytochemicals

The phytochemical constituents of the *P. granatum* pericarp were retrieved from various literature sources, to construct a list of known compounds [Table 2].[24-26] These molecules are likely to be dissolved or extracted in acetone and ethanol as the molecules constitute significant carbon:oxygen ratio and also demonstrate significant total polarity surface area. Further, to investigate the drugability of these phytochemicals, ADMET analysis was performed for the listed phytochemicals. Results of the ADMET analysis are given in Table 3. All molecules demonstrated a significant druglikeness based on Lipinski's rule-of-five (RO5). Although three molecules (i.e., Delphinidin-3,5-Diglucoside, Punicalagin, and Punicalin) demonstrated three violations in the RO5. Overall, since they are all natural products, they can be accepted as drug-like molecules with three violations of RO5. All molecules were predicted to be BBB non-permeant (blood–brain barrier), suggesting no expected neurological side effects. All the molecules demonstrated significant bioavailability, suggesting that the molecules could be absorbed and delivered throughout the body in case of use as drug. Thus, all molecules were screened for their ADMET properties, and the molecules were confirmed to be suitable drug-like molecules.

Protein-ligand Docking Analysis

All the ten ligands were subjected for protein-ligand docking studies, against known drug targets of MRSA and *S. aureus* using AutoDock4. The results of the protein-ligand docking analysis are given in Table 4.

Among all the analyzed conformations, the flavogallol molecule demonstrated strong inhibition toward DHFR with a free binding energy of −11 kcal/mol and formation of two hydrogen bonds with Ser-49 amino acid. The molecule also demonstrated significant number of hydrophobic interactions with residues at the active site. Graphical representation of this protein-ligand interaction is shown in Figure 2 in both three-dimensional (3D) and two-dimensional (2D) visualization analysis. The molecule was located deep inside the active site, preventing the possibility of other molecules like the substrate entering the site, suggesting a stable protein-ligand complex resulting in inhibition of the target protein.

A second most significant interaction was demonstrated by ursolic acid against topoisomerase-IV protein with a free binding energy of −10.7 kcal/mol and formation of four hydrogen bonds with Ser-108, Pro-215, and Lys-266 residues at the active site of the protein. The ligand also demonstrated significant hydrophobic interactions suggesting a stable protein-ligand complex resulting in inhibition of the target protein. Graphical representation of the protein-ligand interaction is shown in Figure 3 in both 3D and 2D visualization.

Table 3: ADMET analysis of phytochemical constituents

ADMET parameters	Delphinidin-3 5-diglucoside	Elaidic acid	Ellagic acid	Flavogallol	Kaempferol	Luteolin	Punicalagin	Punicalin	Quercetin	Ursolic acid
Formula	$C_{27}H_{30}O_{17}$	$C_{18}H_{34}O_2$	$C_{14}H_6O_8$	$C_{21}H_8O_{12}$	$C_{15}H_{10}O_6$	$C_{15}H_{10}O_6$	$C_{48}H_{28}O_{30}$	$C_{34}H_{22}O_{22}$	$C_{15}H_{10}O_7$	$C_{30}H_{48}O_3$
Molecular weight (g/mol)	626.52	282.46	302.19	452.28	286.24	286.24	1084.72	782.53	302.24	456.7
TPSA	289.66	37.3	141.34	208.1	111.13	111.13	518.76	385.24	131.36	57.53
iLOGP	0.81	4.27	0.79	1.06	1.7	1.86	−0.08	−0.07	1.63	3.95
ESOL log S	−1.43	−5.41	−2.94	−2.21	−3.31	−3.71	−8.05	−4.88	−3.16	−7.23
ESOL class	Very soluble	Moderately soluble	Soluble	Soluble	Soluble	Soluble	Poorly soluble	Moderately soluble	Soluble	Poorly soluble
BBB permeant	No	No	No	No	No	No	No	No	No	No
Pgp substrate	No	No	No	No	No	No	Yes	Yes	No	No
Lipinski's violations	3	1	0	2	0	0	3	3	0	1
Bioavailability score	0.17	0.56	0.55	0.11	0.55	0.55	0.17	0.17	0.55	0.56

TPSA: Total polarity surface area

Table 4: Results of protein-ligand docking of 10 phytochemicals of *Punica granatum* pericarp against bacterial drug targets

Ligand name	PubChem ID	PBP2a	DHFR	DHPS	Topoisomerase IV
Delphinidin-3,5-diglucoside	44584733	−9.9	−8.5	−10	−10.3
Elaidic-acid	637517	−5.2	−6.1	−4.7	−4.5
Ellagic-acid	5280445	−7.8	−8.6	−7.5	−7.4
Flavogallol	136794813	−9.5	−11.0	−9.8	−8.9
Kaempferol	5280863	−7.7	−8.4	−7.3	−7.2
Luteolin	5281855	−8.1	−8.5	−7.9	−7.2
Punicalagin	25201902	−8.9	−8.8	−8.8	−8.2
Punicalin	5280343	−7.8	−8.7	−7.7	−7.7
Quercetin	5388496	−10	−7.7	−9.3	−9.6
Ursolic-acid	64945	−9.7	−9	−8.7	−10.7

DHFR: Dihydrofolate reductase, DHPS: Dihydropteroate synthase, PBP2a: Penicillin binding protein 2a

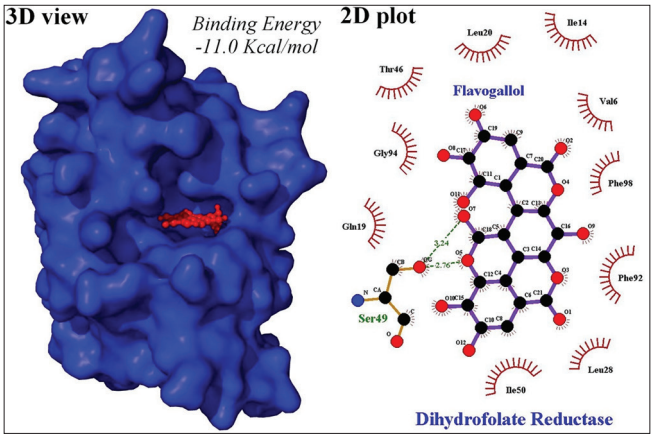


Figure 2: Protein-ligand interactions between DHFR and flavogallol

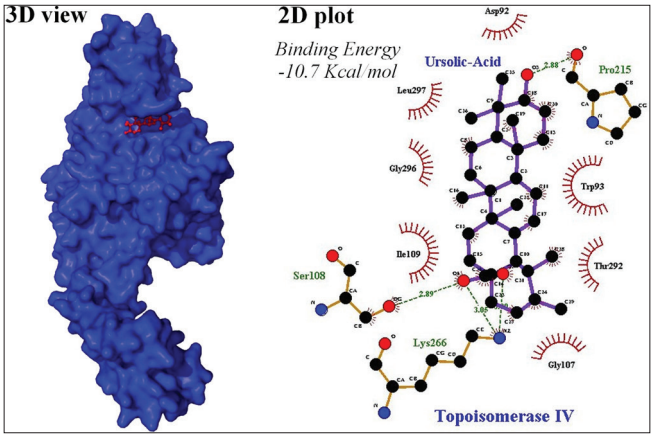


Figure 3: Protein-ligand interactions of topoisomerase-IV and ursolic acid

In addition, delphinidin-3-5-diglucoside also demonstrated significant inhibition potential against topoisomerase-IV protein with a free binding energy of −10.3 kcal/mol; delphinidin-3-5-diglucoside also demonstrated significant inhibition against dihydropteroate synthase (DHPS) enzyme with a binding energy of −10.0 kcal/mol; and quercetin demonstrated significant inhibition potential against PBP2a enzyme with a binding energy of −10.0 kcal/mol. Thus, all analyzed phytochemicals present in the pericarp of pomegranate demonstrated significant inhibition potential toward the known drug targets of *S. aureus* and MRSA pathogens. This suggests that the antibacterial activity observed from the acetone and ethanol extracts of *P. granatum* pericarp is due to synergistic effect of all phytochemicals present and simultaneously inhibiting the vital enzymes resulting in inhibition of bacterial growth/bacterial death. This protein-ligand docking analysis provides a preliminary understanding of how the observed antibacterial activity is justifiable in terms of its mechanism of action. Further studies are needed to justify and confirm the predicted mode of action.

DISCUSSION

The economically unwanted pericarp of pomegranate fruit has been reported to have several medicinal values in both *in vitro* researches and in traditional medicines.[6-9] In this study, the antibacterial activity of the pericarp was examined, and the potential mechanism of action of the phytochemical components was predicted by *in silico* studies. A total of 10 different phytochemicals were retrieved from different literature sources that are significantly polar in nature and are probable components of the acetone and ethanol extracts of *P. granatum* pericarp.

Common drug targets, i.e., DHFR, DHPS, PBP2a, and topoisomerase-IV were studied in this analysis. Among the 40 different combinations of protein-ligand complex, the most significant interaction was demonstrated by flavogallol toward DHFR enzyme with a free binding energy of −11.0 kcal/mol. This inhibition prevents the biosynthetic pathway of folic acid in the bacteria, leading to a bacteriostatic effect, preventing further multiplication of the bacteria, similar to that of the sulfa drugs.[27]

Ursolic acid demonstrated strong inhibition activity against topoisomerase-IV enzyme with a binding energy of −10.0 kcal/mol, suggested that the molecule prevents DNA replication in the bacteria, leading to cause a bacteriostatic effect.[28] Delphinidin-3, 5-diglucoside also demonstrated significant inhibition toward topoisomerase-IV enzyme with a binding energy of −10.3 kcal/mol. Similar to that of some reported naturally occurring tannins.[28]

Quercetin demonstrated the highest inhibition potential toward PBP2a with a binding energy of −10.0 kcal/mol. It is notable that quercetin derivatives have already been reported to have antibacterial activity against MRSA bacteria by Rani *et al*.[29] Since quercetin derivatives have already been proven for their *in vitro* anti-MRSA and anti-PBP2a activity, it supports the current prediction of quercetin being inhibitor of PBP2a. Inhibition of PBP2a would result in bactericidal activity, by disruption of the biosynthesis of bacterial cell wall.

Thus, a combination of the phytochemicals can produce a synergistic effect of both bactericidal and bacteriostatic activity against *S. aureus* and MRSA Gram-positive bacterial pathogens. Although further investigations are required for confirmation of the proposed activity, these results provide a basic understanding of how the phytochemicals exert the observed activity. Extraction and purification of individual phytochemicals and its mechanism of antibacterial activity can be studied further.

CONCLUSION

Antibacterial activity analysis suggests that the pericarp extract of *P. granatum* has significant antagonism toward Gram-positive bacterial pathogens such as *S. aureus* and MRSA. The *in silico* investigation of the possible mechanism of action for the observed activity suggests that, more than one active ingredient is involved in the activity, causing a synergistic effect by inhibition of multiple vital proteins resulting in an effective antibacterial activity. Among the observed interactions, interactions of flavogallol and ursolic acid were found to be most significant. Based on the observed results, it is evident that the polar compounds present in the pericarp of *P. granatum* have significant antibacterial activity; however, the activity of the individual phytochemicals has to be investigated for better understanding the mode of action. Further studies are currently in progress for purification and characterization of the individual phytochemicals.

ACKNOWLEDGMENT

The authors thank St. Joseph's College (Autonomous), Bengaluru, for supporting this research work.

CONFLICTS OF INTEREST

There are no known conflicts of interest for this research work. All authors have made scientific contributions toward the completion of this research work.

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Source of Support: Nil. **Conflicts of Interest:** None declared.