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RESEARCH ARTICLE

THE POLYPHENOLS IN POMEGRANATE PLAY IMPORTANT ROLE IN EXHIBITING ANTI-AGEING EFFECTS

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ABSTRACT

Pomegranate (*Punica granatum* L), in addition to its ancient historical uses, has been used in several systems of medicine for a variety of ailments. Pomegranate juice is a polyphenol-rich juice with high antioxidant capacity. It is evident from the previous studies that pomegranate juice has been shown to exert significant antiatherogenic, antioxidant, anti-carcinogenic, and anti-inflammatory effects. The aim of the present study was to validate the skin care properties of *P. granatum*. The extract exhibited high anti-elastase and collagenase activities at various concentrations indicating the significant anti-wrinkle effect (52.28 and 53.98 % respectively at 100 µg/ml concentration). Further, pomegranate extract was highly effective in protecting the DNA from hydroxyl radical mediated scission. The *in silico* calculations predicted that lowest energy docked poses of phenolic compounds can interact with catalysis-dependent residues, thus making them possible catalytic inhibitors and of course physiologically active. This study concludes that pomegranate can potentially be used as a readily accessible source of skincare anti-ageing agents and demand the need for extensive research in cosmeceutical applications.

INTRODUCTION

Skin aging is a multisystem degenerative process that involves the skin and the skin support system (Sjerobabski and Poduje, 2008). The process of skin aging may be divided as intrinsic and extrinsic ageing (Jenkins, 2002; Schlotmann *et al*., 2001). It may be caused by several factors, such as, UV irradiation, stress, ROS generation or smoking. Acute exposure of human skin to UV irradiation causes sunburn, inflammation, immune suppression, and dermal connective tissue damage (Fisher *et al*., 2002), and chronic exposure to UV over many years disrupts the normal skin architecture and ultimately causes photoaging and even skin cancer (Quan *et al*., 2009). Wrinkle formation is a striking feature of photoaged skin and is caused by the degradation of collagen fibrils and gelatin fibers. Pomegranate (*Punica granatum* L.) is a plant of family Lythraceae cultivated in India, Mediterranian countries, Malaysia, tropical Africa and to some extent in United States (Viuda-Martos *et al*., 2010). The plant has been valued since ancient times to treat many conditions such as diarrhea, dysentery, hemorrhage, parasitic infections, ulcers and microbial infections (Lihua Zhang *et al*., 2010). Pomegranate juice, which is rich in tannins, possesses anti-atherosclerotic, antihypertensive, and potent anti-oxidative characteristics (Stowe, 2011); hence, it provides cardioprotective benefits (Basu and Penugonda, 2009). Pomegranate juice may have cancer-chemopreventive as well as cancer-chemotherapeutic effects against prostate cancer, in humans (Malik *et al*., 2005). Pomegranate is a rich source of polyphenols and possesses an array of compounds that have been attributed to the pharmacological activities such as anti-tumor, astringent and antidiarrheal activities. The aim of this study was to investigate the skin care properties of pomegranate juice extract through *in vitro* and *in silico* approaches.

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MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma Aldrich Ltd. (India) unless otherwise stated.

Extraction of polyphenol rich fraction from *P. granatum*

100 ml of fresh *P. granatum* fruit juice was taken into a separating funnel and extracted into 50 ml of ethyl acetate (LR grade, Merck, India). The aqueous layer was washed twice with ethyl acetate and the total organic fraction collected. At the end of extraction, extracts were filtered under vacuum through a Whatman No. 1 filter paper and the process repeated

until all soluble compounds had been extracted. The filtrates obtained were concentrated *in vacuo* using a Rotavapor (Buchi Flawil, Switzerland). The ethyl acetate extract was stored at 4 °C in air tight bottle until further use.

Elastase inhibition assay

The assay employed was based on methods in the literature (Kim *et al*., 2004). This assay was performed in 0.2 mM Tris-HCL buffer (pH 8.0). Porcine pancreatic elastase (E.C. 3.4.21.36), was dissolved to make a 3.33 mg/mL stock solution in sterile water. The substrate N-Succinyl-Ala-Ala-Ala-p-nitroanilide (AAAPVN) was dissolved in buffer at 1.6 mM. Different concentrations of PE were incubated with the enzyme for 15 minutes before adding substrate to begin the reaction. The final reaction mixture (250 µL total volume) contained buffer, 0.8 mM AAAPVN, 1 µg/mL pancreatic elastase and various concentrations of PE. EGCG (1 mg/mL) was used as a positive control. Negative controls were performed using water. Absorbance values between 381 and 402 nm (following pre-screen scans) were measured immediately following addition of the substrate and then continuous for 20 minutes. The percent inhibition of elastase was calculated as follows:

Enzyme inhibition (%) = $[(OD_{control} - OD_{sample})/ OD_{control}] \times 100$,

Determination of anti-collagenase activity

The assay employed was based on spectrophotometric methods reported in the literature (Van Wart and Steinbrink, 1981). The assay was performed in 50 mM Tricine buffer (pH 7.5 with 400 mM NaCl and 10 mM $CaCl_2$). Collagenase from *Clostridium histolyticum* (ChC – EC.3.4.23.3) was dissolved in buffer for use at an initial concentration of 0.8 units/mL according to the supplier's activity data. The synthetic substrate N-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA) was dissolved in Tricine buffer to 2 mM. Different concentrations of PE were incubated with the enzyme in buffer for 15 minutes before adding substrate to start the reaction. The final reaction mixture (150 µL total volume) contained Tricine buffer, 0.8 mM FALGPA, 0.1 units ChC and 20-100 µg PE extract. Negative controls were performed with water. Absorbance at 335 nm was measured immediately after adding substrate and then continuous for 20 minutes. EGCG, 250 µM (0.114 mg/mL) was used as a positive control.

***In vitro* DNA protective activity**

The experiment was conducted using Calf thymus DNA following the method of Xican *et al*. (2013). Briefly, sample was dissolved in ethanol at 1 mg/mL. 50 µl of different concentrations of PE was then separately taken into mini tubes followed by addition of 400 µL phosphate buffer (0.2 mol/L, pH 7.4). Subsequently, 50 µL each of DNA sodium, H_2O_2 (50 mM/L), $FeCl_3$ (3.2 mM/L) and Na_2EDTA (1 mmol/L) were added. The reaction was initiated by adding 50 µL ascorbic acid (18 mmol/L) and the total volume of the reaction mixture was adjusted to 800 µL with buffer. After incubation in a water bath at 55 °C for 20 min, the reaction was terminated by adding 250 µL TCA. The color was then developed by addition of 150 µL of Thiobarbituric acid (TBA)(0.4M/L in 1.25 % NaOH) and heating in an oven at 105 °C for 15 min. The mixture was cooled and absorbance was measured at 532 nm against the buffer (as blank). The percent of protection against DNA damage is determined as follows:

% Protective effect = $(1 - A/A_0) \times 100$

Where A_0 is the absorbance of the mixture without sample; A is the absorbance of the mixture with sample.

***In silico* docking studies**

AutoDock tools was utilized to generate grids, calculate dock score and evaluate the conformers of inhibitors bound in the active site of enzymes elastase and collagenase as targets for anti-wrinkle activity. Automated docking is a graphical user interface. AutoDock 4.2 was employed to get docking and binding scores; which is implemented by Lamarckian genetic algorithm method. The ligand molecules i.e., the phenolic compounds from pomegranate juice (Figure 1), standard molecule epigallocatechin gallate were designed and the structure was analyzed using ACD/Chemsketch. The PRODRG server was used to minimise energy of drug compounds and 3D coordinates were prepared. The protein structure files (PDB ID: 1GVK and 1CGL) were taken from PDB and edited by removing the hetero atoms using Python molecule viewer. The grid map was centred at particular residues of the protein and was generated with AutoGrid. As per genetic algorithm all the torsions were allowed to rotate during docking. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization, using default parameters (Rodriguez and Infante, 2011).

RESULTS

The anti-wrinkle effect of Pomegranate juice extract was investigated *in vitro*. Results of anti-elastase activity of PE are presented in Figure 2A as percentage inhibition. The extract presented a strong inhibition of elastase at various concentrations. Interestingly, at all tested concentrations the extract showed better activity than the standard EGCG. The activity of PE was nearly one fold higher than EGCG with a maximum of 52.28 % at 100 µg/ml and IC50 value of 32.37 µg/ml. In the present study, appreciable anti-collagenase activity was found at tested concentrations of PE (Figure 2B) comparable to standard EGCG. This could be explained by the presence of pharmacologically active phytochemicals in pomegranate (Figure 1).

Further, in this study, the protective effect of PE on hydroxyl radical induced DNA scission was demonstrated. The DNA scission induced by hydroxyl radical was efficiently protected by various concentrations of PE (20-100 µg/ml). Interestingly, the activity was better than the standard BHA at all the concentrations (Figure 3). At low concentration of 10 µg/ml itself, PE exerted 60.2 % activity. The extract exhibited more than 75 % of protection at 100 µg/ml. The docking results showed compounds with more favorable interactions with the targets and also indicated that some of the compounds present certain structural motifs that could make them form extensive Van der Waals interactions and hydrogen bonding with targets.

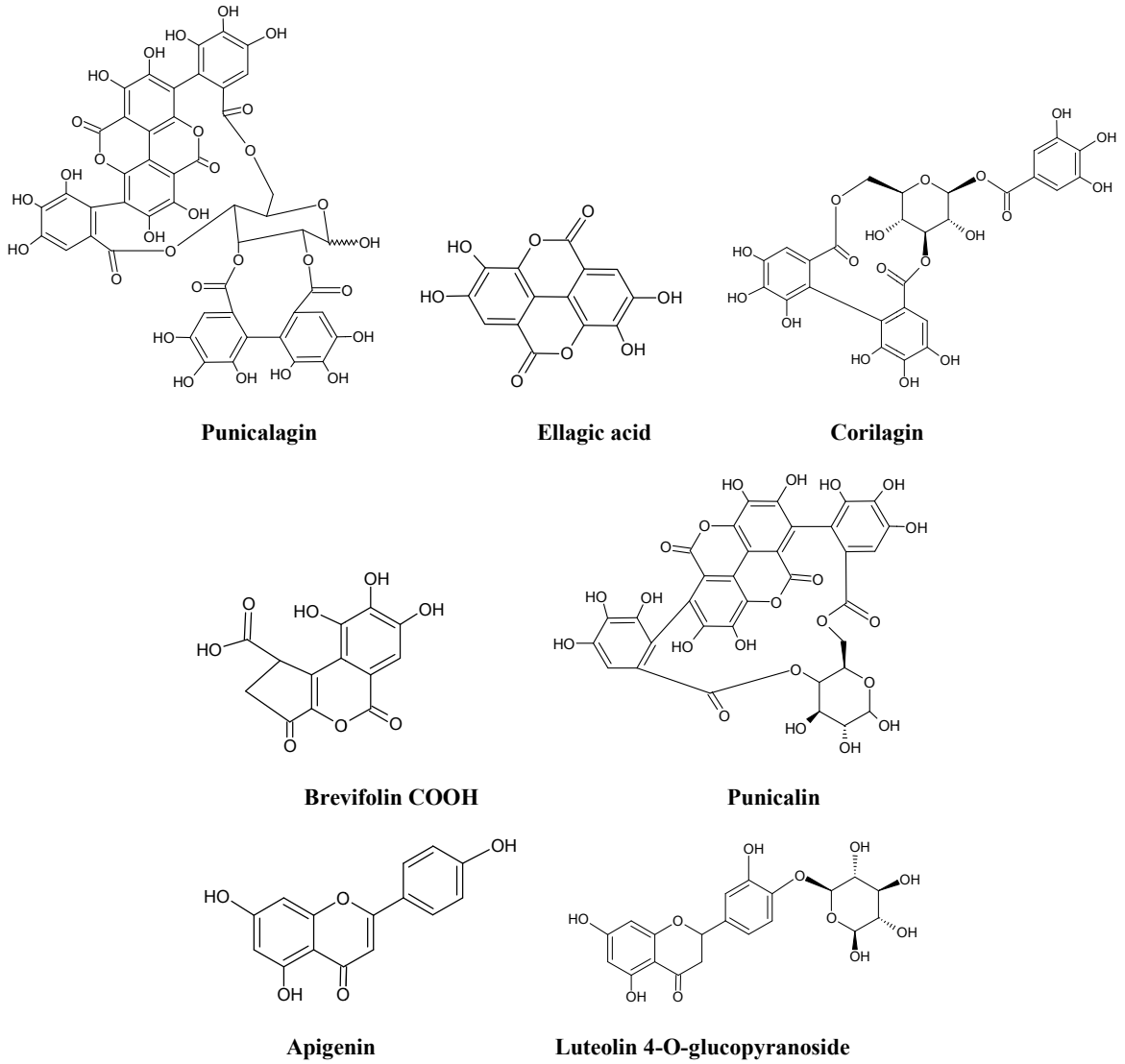


Fig 1. Structures of docked polyphenols discussed in this work

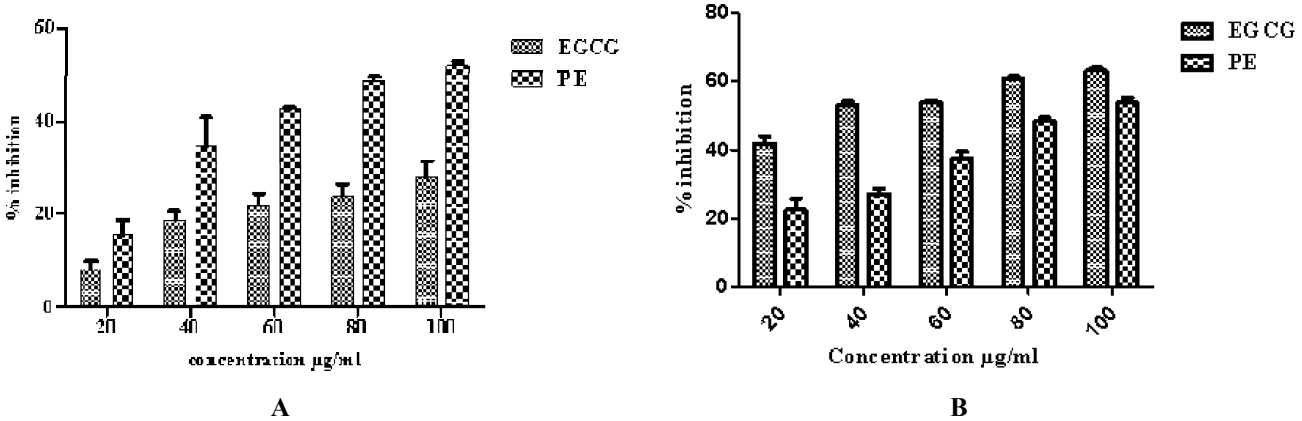


Fig 2. Anti-wrinkle effects of *P. granatum* juice extract. Values expressed as mean±SD (n=3). (A) Inhibition of elastase activity (B) Collagenase inhibitory effect of PE

The orientation of polyphenols in the catalytic sites of elastase and collagenase indicated profound interaction in the form of protein-ligand hydrogen bond formation (Table 1 & 2).

Brevifolin COOH showed better inhibition of enzyme elastase in comparison to other compounds, with binding energy of -8.28 $kJmol^{-1}$ and forming four hydrogen bonds with active

pocket residues; Leu130, Arg230, Val163, Cys182 (Figure 4A). After comparative docking analysis it was learnt that the active principles of PE extract more efficiently inhibited the enzyme collagenase than elastase. Among the tested molecules, Luteolin 4-O-glucopyranoside exhibited top pose with low binding energy (-8.85 kJmol^{-1}) and forming 6 hydrogen bonds with active pocket residues; Thr241, Ser243, Gln250, Asp245, Leu248 (Figure 4B).

DISCUSSION

PE exerted its anti-wrinkle effect by the inhibition of key enzymes involved in ageing. Elastase, a member of the chymotrypsin family of proteases, is responsible primarily for the breakdown of elastin, an insoluble elastic fibrous protein that, together with collagen, determines the mechanical properties of connective tissue (Antonicelli *et al*., 2007).

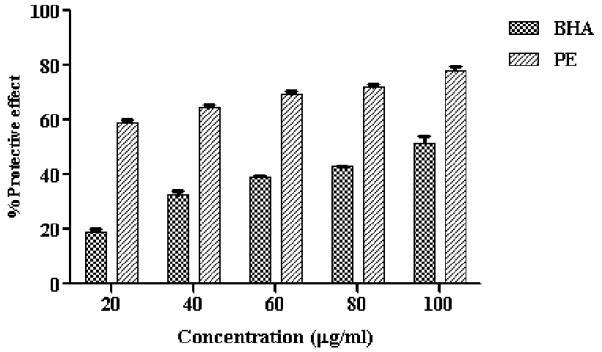
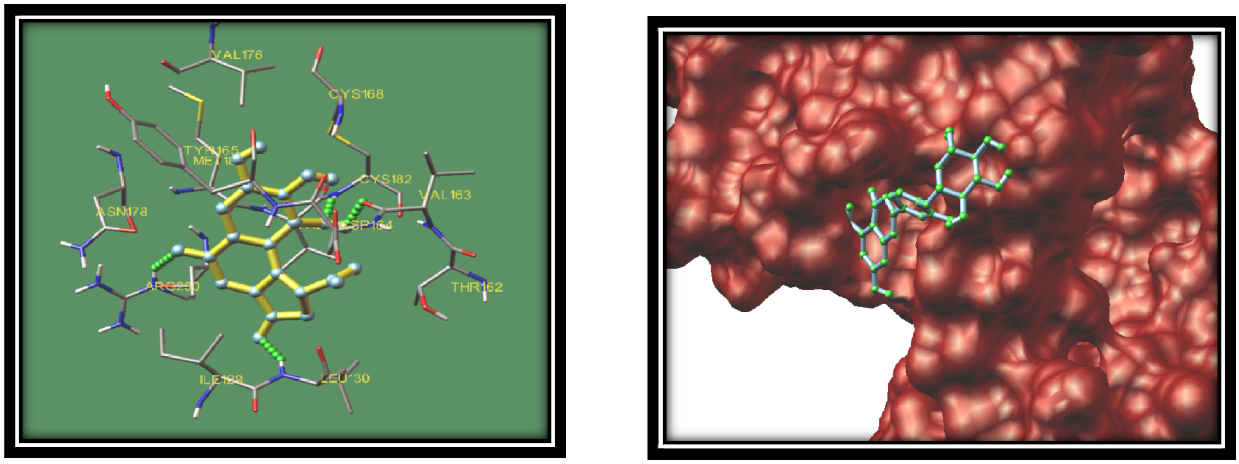


Fig 3. Protective effect of *P. granatum* juice extract on non-site specific hydroxyl radical mediated 2-deoxy-d-ribose degradation



A B

Fig 4. (A) Interaction of Brevifolin COOH with active pocket residues of Elastase; (B) Binding of Luteolin 4-O-glucopyranoside with active site of Collagenase

Table 1. *In silico* docking score of polyphenols from *P. granatum* and EGCG with Elastase

Molecule	Binding Energy (kJ/mol)	Ligand Efficiency (kJ/mol)	Inhibitory constant (µM)	H-Bond	Interaction
Punicalagin	-	-	-	-	-
Ellagic acid	-7.25	-0.33	4.82	3	Cys182, Asn178
Corilagin	3.16	0.51	18.98	6	Asn178, Asp164, Val163, Tyr165, Cys182
Brevifolin COOH	-8.28	-0.39	857.37	4	Leu130, Arg230, Val163, Cys182
Punicalin	-	-	-	-	-
Apigenin	-7.44	-0.37	3.52	2	Met180, Tyr165
Luteolin 4-O-glucopyranoside	-7.25	-0.23	4.88	4	Met180, Cys182, Leu130, Asn178
EGCG (Std)	-9.84	-0.3	61.17	6	Met180, Ile129, Asp164, Tyr165, Leu130, Asn178

Table 2. *In silico* docking score of polyphenols from *P. granatum* and EGCG with Collagenase

Molecule	Binding Energy (kJ/mol)	Ligand Efficiency (kJ/mol)	Inhibitory constant (µM)	H-Bond	Interaction
Punicalagin	-4.48	-0.06	522.07	4	Asp231, Ser243
Ellagic acid	-7.46	-0.34	3.4	6	Gln247, Ser243, Arg214, Ile232
Corilagin	-7.73	-0.17	2.17	6	Gln247, Ser243, Leu248, Asp245
Brevifolin COOH	-7.28	-0.35	4.61	5	Arg214, Gln247, Thr241, Ile232
Punicalin	-8.13	-0.15	1.11	4	Arg214, Gln247, Thr241,
Apigenin	-7.04	-0.35	6.95	3	Thr241, Ser243, Asp245, Gln247
Luteolin 4-O-glucopyranoside	-8.85	-0.29	328.4	6	Thr241, Ser243, Gln250, Asp245, Leu248
EGCG (Std)	-8.41	-0.25	689.45	6	Asp245, Ser243, Ile232, Gln247

Elastin, due to its unique elastic recoil properties, is vital for giving elasticity to arteries, lungs, ligaments and skin (Kim *et al*., 2004; Baylac and Racine, 2004). (Melzig *et al*., 2001; Siedle *et al*., 2002) Elastases can cleave elastin as well as having a broad substrate portfolio including ability to cleave collagen, fibronectin and other ECM proteins (Melzig *et al*. 2001; Siedle *et al*., 2002). During the recent past a number of studies have investigated the interactions between elastase and its inhibitors (Kim *et al*., 2007; Masuda *et al*., 2009; Thring *et al*., 2009). Because of the presence of phenolics, medicinal plant extracts exhibit promising inhibitory effect on elastase activity and hence may be good choices for cosmetic purposes because of their relatively low incidence of side effects. In the present study, due to its high phytochemical profile, the pomegranate extract exhibited intense activity.

Matrix Metalloproteinases (MMPs) are part of a group of transmembrane zinc containing endopeptidases which include collagenases and gelatinases. Collagenases are metalloproteinases capable of cleaving other molecules found within the cell for example collagenase-2 (MMP-8) can cleave aggrecan, elastin, fibronectin, gelatine and laminin as well as collagen (Raffetto and Khalil, 2008). Collagenase cleaves the X-gly bond of collagen and also synthetic peptides that contain the sequence -Pro-X-Gly-Pro where X is almost any amino acid provided that the amino terminus is blocked (Van Wart and Steinbrink, 1981). Plant secondary metabolites and whole extracts have been widely investigated and found to have anti-collagenase activity. Plants contain a vast variety of compounds such as polyphenols such as phenolic acids, flavanoids, tannins etc. which attribute to their anti-ageing and anti-oxidant effects. Polyphenols isolated from herbal extracts such as green tea, Aloe vera (*Aloe barbadensis*), Persimmon (*Diospyros kaki*) have profound collagenolytic activity (Barrantes and Guinea, 2003; Lee *et al*., 1999). In agreement with the previous reports, PE had shown better anti-collagenase activity indicating the possible mechanism of its action.

UV exposure causes physical changes to the skin due to alterations that occur in the connective tissue via the formation of lipid peroxides, cell contents and enzymes (Lee *et al*., 2006) and reactive oxygen species (ROS) (Benaiges *et al*., 1998). When ROS are overproduced, redox-active transition metal ions such as iron(II) or copper(II) can cause severe oxidative stress and thus damage tissues and the cellular biomolecules such as DNA, protein, lipid and carbohydrate constituents within (Kaur *et al*., 2006). In our study, a dose dependent increase in the protective effect of PE against hydroxyl radical mediated DNA scission reaching upto 89 % was observed. Interestingly, the activity was much higher than the standard BHA. This could be attributed to the synergistic effect of polyphenols in the extract. These results clearly indicate that pomegranate phytoconstituents are effective in scavenging the hydroxyl radicals and give protection against the hydroxyl radical mediated complications such as DNA damage.

This work also presents the molecular docking of polyphenols from pomegranate with the key metabolic enzymes responsible for skin wrinkle formation; with a view of obtaining structural motifs that preferentially interact with these molecules. The major polyphenols present in pomegranate were analyzed for their interaction with elastase and collagenase *in silico*. The docked conformations clearly reflected the inhibitory role of these polyphenols in PE. Several studies have been reported on docking studies of polyphenols from plants against major therapeutic targets as applications to rational drug design (Smith *et al*., 2004).

Conclusion

To meet the demand for cosmetic preparations with antiwrinkle properties, many cosmetics companies have been developing elastase inhibitors because of their potential as active agents. The present study was carried out at Vidya Herbs Pvt Ltd. in order to scientifically validate the skin care properties of pomegranate. The pomegranate extract was highly effective in inhibiting the major enzymes involved in ageing. The cosmeceutical application of pomegranate was further supported by the *in silico* interaction studies and the DNA protection by free radical scavenging. On the basis of the results, it is suggested that pomegranate can be used as a comprehensive source of cosmeceuticals for the maintenance of healthy skin.

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