The Pharma Innovation Journal 2018; 7(11): 173-177

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2018; 7(11): 173-177

www.thepharmajournal.com
Received: 25-09-2018
Accepted: 30-10-2018

K Durgadevi
Department of Biochemistry P.G & Research, Marudupandiyar College, Vallam Post, Thanjavur, Tamil Nadu, India

J Antonia Auxi Marueen
Department of Biochemistry P.G & Research, Marudupandiyar College, Vallam Post, Thanjavur, Tamil Nadu, India

R Gowri
Department of Biochemistry P.G & Research, Marudupandiyar College, Vallam Post, Thanjavur, Tamil Nadu, India

V Ramamurthy
Department of Biochemistry P.G & Research, Marudupandiyar College, Vallam Post, Thanjavur, Tamil Nadu, India

Correspondence
Sylvester O
V Ramamurthy
Department of Biochemistry P.G & Research, Marudupandiyar College, Vallam Post, Thanjavur, Tamil Nadu, India

A study of phytochemical screening and antibacterial activity of leaves extract of *Punica granatum*

K Durgadevi, J Antonia Auxi Marueen, R Gowri and V Ramamurthy

Abstract
Plant secondary metabolites provide a vital role for the medicinal activity of plant species. This study was conducted to assess the phytochemical constituents in *Punica granatum* L. Leaf extracts using standard methods. The qualitative analysis of bioactive compounds for the three extracts have been analysed in this study and there is wide range of phytochemical compounds present in the three extracts. Ethanolic extract and hydro alcoholic extract was found to have a wide range of bioactive compounds like alkaloids, carbohydrates, coumarins, flavonoids, proteins, phenols, reducing sugars, steroids and tannins. The ethanolic leaf extract was tested against Gram positive and Gram negative bacterial pathogens. Antimicrobial activity of six bacterial species and three fungal species were employed as test organism. These include *Streptococcus pyogenes, Salmonella typhi, Klebsiella pneumonia, Staphylococcus aureus, Heliocabacter pylori* and *Psudomonas auroginosa.* The fungal species such as *Aspergillus niger, Candida albicans* and *Trichoderma viride* were prevent by *Punica granatum.* The result of this proposed study enlightened the evidence that, *P. granatum* contains various bioactive compounds that may be useful in the synthesis of therapeutic drugs and also suggest the herbal medicine. The therapeutic effect of this plant may be accounted for its counteracting action on free radicals *in vivo*.

Keywords: *Punica granatum*, antimicrobial activity, Phytochemicals, scavenging activity

Introduction
Plants have been major source of medicine in all cultures from ancient times. In the traditional system, various indigenous plants are being used in the diagnosis, prevention and elimination of physical, mental or social imbalance. Phenolic compounds, ubiquitous in plants, are of considerable interest and have received more and more attention in recent years due to their bioactive functions. Polyphenols are amongst the most desirable phytochemicals because of their antioxidant activity. Natural therapy for various human ailments purified with plant products has gained much attention now days, due to various side effects associated with allopathic medicine these can be derived from any part of the plant like bark, leaves, stem, flowers, roots, seeds, etc., (Cragy and David, 2001) [5]. Medicinal plants are believed to be an important source of chemical substances with potential therapeutic effects (Farnsworth, 1989) [7].

Ethanol is the most commonly used organic solvent by herbal medicine manufacturers because the whole products can be safely used internally by consumers of herbal extracts (Low Dog, 2009) [11]. Furthermore, the bioactivity of plant extracts depends on the water and ethanol concentration used in the extraction process (Ganora, 2008) [8]. However a great amount of research has been performed to determine the antibacterial activity of medicinal plants, optimal extraction of bioactive compounds has not been well known for most plants. To maximize up take the recovery of plant antimicrobials for human consumption, establishing optimal and specific extraction condition using various solvent system.

Punica granatum belongs to the family Punicaceae, commonly known as pomegranate, is a shrub or small tree with several upright, thorny stems, the leaves are elliptic, roughly 2 inches, the flowers are white or red, double-flowered races, native of Asia and Mediterranean Europe (Egharevba and Kunle, 2010) [6]. It is also found in India and more arid regions of Southeast Asia (Naqvi *et al*., 1991) [12], the East Indies, and tropical Africa. For centuries, the barks, leaves, flowers, fruits and seeds of this plant have been used to ameliorate diseases (Jayaprakasha *et al*., 2006) [9]. The potential therapeutic properties of pomegranates are wide-ranging and include treatment and prevention of cancers, cardiovascular disease, diabetes, dental conditions, erectile dysfunction and prevention from ultra violet (UV) radiation. The pericarp of *P. granatum* is used to treat infections found in human sexual organs as well as

mastitis, acne, folliculitis, piles, allergic dermatitis, tympanitis, scalds, diarrhoea and dysentery (Singh *et al*., 2002) [14]. Hence, the objective of this study was to determine qualitative investigation was carried out to evaluate the presence of phytochemicals. In this report a comparative study on three different solvents used to screening of high yield of bioactive clusters. Furthermore, the ethanolic leaf extract as a good source for the determination of the antimicrobial activity against various human pathogens.

Materials and Methods

Collection and Identification

Leaves of pomegranate plant (*Punica granatum* L.) were obtained from around the Tiruchirappalli, Tamil Nadu. The *Punica granatum* leaf was authenticated by Director of National Institute of Herbal science, Plant anatomy research centre and the voucher specimen is deposited in our laboratory.

The leaves of the plant were carefully removed and thoroughly washed with distilled water to remove dust particles. They were dried in shade and finely powdered using an electric blender. Fifty grams of powdered material was subjected to Soxhlet extraction with 500 ml of n-Hexane, ethyl acetate, ethanol, hydroalcohol and water separately for 8 h. The extracts were evaporated to dryness under controlled temperature (35-40°C). The extracts were stored in air tight containers under refrigeration. These dried extracts were dissolved in respective solvents and used for further analysis.

Qualitative Phytochemical screening: Phytochemical screening of *Punica granatum* Leaf extracts was assessed by standard methods (Sofowara, 1993; Trease and Evans, 2002) [15, 16].

Test for alkaloids: A fraction of extract was treated with 3-5drops of Wagner's reagent [1.27g of iodine and 2g of potassium iodide in 100 ml of water] and formation of reddish brown precipitate (or colouration) indicates the presence of alkaloids.

Test for anthraquinone: To 1 ml of plant extract, few drops of 1% HCl were added. Appearance of red colour precipitate indicates the presence of anthraquinone.

Test for carbohydrates: To 1 ml of plant extract was mixed with alpha napthol solution and then to the sides of the test tube conc.H_2SO_4 is added. Appearance of violet ring indicates the presence of carbohydrates.

Test for reducing sugar: To 1 ml of plant extract was mixed with few drops of Benedict's reagent and kept in boiling water bath, observed for the formation of reddish brown precipitate. A positive result shows the presence of reducing sugar.

Test for flavanoids: To 2 ml of extracts was treated with few drops of 20% sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute hydrochloric acid, indicates the presence of flavonoids.

Test for phenols: A portion of the extract was treated with aqueous 5% ferric chloride and formation of deep blue or black colour indicates the presence of phenols.

Test for proteins: To the extract, 1 ml of distilled water was added which was then heated with Biuret reagent and observed for the formation of violet/pink colour.

Test for free amino acids: The extract was heated with 0.2 % solution of Ninhydrin which result in the formation of purple colour, suggesting the presence of free amino acid.

Test for coumarins: To 2 ml of the test solution, a few drops of 10% NaOH were added. Appearance of yellow colour indicates the presence of coumarins.

Test for saponins: To 2 ml of extract was added to 6 ml of distilled water in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of Saponins.

Test for steroids: To 1 ml of extract was treated with few drops of chloroform, acetic anhydride and conc. H_2SO_4 and formation of dark pinkor red colour indicated the presence of steroids.

Test for tannins: To 2 ml of extract was treated with 10% alcoholic ferric chloride solution and formation of blue or greenish colour solution indicated the presence of tannins.

Test for terpenoids: To 1 ml of chloroform was added to 2 ml of each extract followed by a few drops of concentrated sulphuric acid. A reddish brown precipitate produced immediately indicated the presence of terpenoids.

Quantitative phytochemical analysis

HPLC – UV analysis (Total phenols)

Ethanolic extract of *Punica granatum* was subjected to solid phase extraction using column 5mm (4.6.mm), & peptides, small molecules were removed; fractionation of neutral and acidic phenolic acids was also carried out simultaneously. The resulting fraction was then subjected to reverse phase high performance liquid chromatography (RP-HPLC). The total phenolics in ethanolic extract of *P. granatum* was detected using, Stationary phase octadecylsil. Silica and mobile phase (A phosphoric acid: water (0.5: 99.5v/v) B acetonitrile). The UV detector was set at 220 nm with the flow rate adjusted to 1.0ms / min. The major peaks were identified and the retention times were compared with these of standards.

Fractionation of total alkaloids

Ethanolic extract of *P. granatum* was detected using monobasic Phosphate as mobile phase (270ml. of Acetonitril). The liquid Chromatography is equipped with 235 nm detector & 4.6nm x 150 mm column. The flow rate was adjusted to 1.8ml / minute. The major peaks were identified and the total alkaloids concentration was determined.

Fractionation of total flavonoids

HPLC Chromatography (System Name: LACKROM L-7000 MERCK, Proc Method – HITECHI) total flavonoids. The total flavonoids in the extract was determined by using octadecysil silica gel as stationary phase and acetonitril, sodium dihydrogen phosphate with dilute orthophosphoric acid as mobile phase. UV detector was set at 350nm with flow rate of 0.5ml/min. The major peaks in ethanolic extract of *P. granatum* were determined in comparison to the retention time of standards run at identical conditions.

Microorganisms

Six bacterial species were employed as test organism. These include *Streptococcus pyogenes, Staphylococcus aureus, Salmonella typhi, Klebsiella pneumonia, Helicobacter pylori* and *Psudomonas auroginosa.* Three fungal species were employed as test organism. These include *Aspergillus niger, Candida albicans* and *Trichoderma viride* which were isolated from infected person.

Antibacterial Assay

The antibacterial assay was performed by agar well diffusion method (Perez et al., 1990) for solvent extract. The Muller Hinton Agar media was inoculated with the 100 µl of the inoculum ($1x10^8$ Cfu) and poured in to petriplates. In this method a well was prepared in the plate using a cork-borer (0.85) 50,100µg of test sample was introduced in to the well. The plates were incubated overnight at 37^0C and microbial growth was determined by measuring the diameter of zone of inhibition. The controls were maintained where pure solvent was used instead of the extract for each strain. The result was obtained by measuring zone of diameter.

Results and Discussion

Phytochemicals are naturally occurring biochemical compounds that plants developed, in order to protect themselves from oxidation, insect disease and other hazards in their environment. These phytochemicals give their characteristic colour, flavour, smell and texture. Epidemiological studies indicate that populations consuming high levels of plant derived foods have low incidence rates of various cancers.

The preliminary phytochemical tests are helpful in finding chemical constituents in the plant material that may lead to their qualitative analysis and also in locating the source of pharmacologically active chemical compound. The qualitative analysis of bioactive compounds for the three extracts have been analysed in this study and there is wide range of phytochemical compounds present in the three extracts as shown in table 1. The hexane being highly nonpolar in nature was able to extract very less compound characterized like carbohydrates, phenols, steroids and tannins. Ethanolic extract and hydro alcoholic extract was found to have a wide range of bioactive compounds like alkaloids, carbohydrates, coumarins, flavonoids, proteins, phenols, reducing sugars, steroids and tannins.

Table 1: Qualitative phytochemical analysis

S. No	Phytochemicals	Hexane extract	Ethanol extract	Hydroalcoholic extract
1	Alkaloid	-	+	+
2	Anthraquinone	-	-	-
3	Carbohydrate	+	+	+
4	Reducing sugar	+	+	+
5	Flavanoid	-	+	+
6	Phenol	+	+	+
7	Protein	-	+	+
8	Amino acid	-	+	+
9	Coumarin	-	+	+
10	Tannin	+	+	+
11	Saponin	-	-	-
12	Steroids	-	+	+
13	Terpenoids	-	+	+

In this investigation, presence of quantitative phytochemicals in ethanolic extract of *P. granatum* expressed the value in mg/g phenols was (7.76 mg/g), tannins (1.82 mg/g), flavonoids (5.15 mg/g) and alkaloids (6.18). Finding the natural substance of medicinal plant that decrease the inflammation and reduce oxidative stress and there by counteracting the macromolecular damage. Flavonoids and phenols in general are highly effective in scavenging free radical and providing antioxidant defense in living cells. Quantitative analysis of ethanolic extract of *P. granatum* was given in Table 2.

Table 2: Quantitative Phytochemical Analysis

S. No.	Phytochemical	Values in (mg/g)
1	Phenols (mg/g)	7.76
2	Tannins (mg/g)	1.82
3	Flavonoids (mg/g)	5.15
4	Alkaloids (mg/g)	6.18

HPLC Analysis of ethanolic leaves extract of *P. granatum:*

HPLC analysis reveals that the extract was found to be rich in Alkaloids (6.18 mg/g) terpenoids (1.82 mg/g) and phenols (7.76 mg/g). Ethanolic leaves extract of *P. granatum* also contain flavonoids such as Rutin (1.12 mg/g) and quercetin (1.19 mg/g) Fig. 1 (A) to (D) many reports demonstrate that antioxidant principle present in medicinal plants are responsible for their therapeutic potential (Larson, 1988) flavonoic compound such as quercetin and Rutin are formed to be responsible for anti-inflammatory and anticancer properties proliferates by their terminating action of free radicals. Alkaloids have many pharmacological activities including anticancer and anti-arthythmic effect (Cordell, 1983). Alkaloids are known to reduce the inflammation level significantly. These results shows that ethanolic leaves extract of *P. granatum* containing which could be accounted for the antioxidant and anti-inflammatory effects. In the present investigation, HPLC chromatrographics pattern of the ethanolic extract of *P. granatum* showed 2 peaks of flavonoids and 3 peaks of phenolic compounds. In HPLC Analysis of ethanolic extract of *P. granatum* was found to be rich in flavonoids such as quercetin, Rutin and phenols such as gallic acid, cinnamic acid and coumaric acid. The Natural phytonutrients presents in fruits and vegetables scavenge the free radicals and protect the cells from oxidative damages. The phytonutrients present in ethanolic leaves extract of *P. granatum* migrates the responsible for the traditional claim by the test drug.

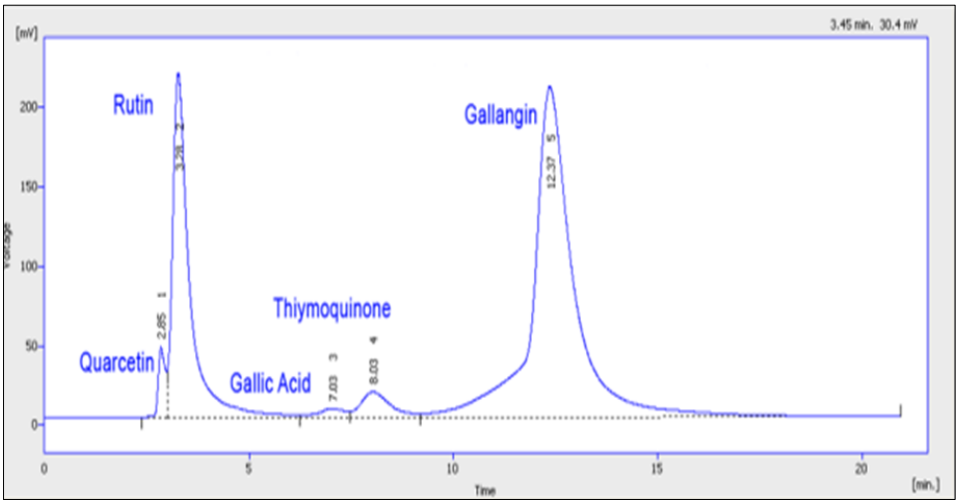

Fig 1A: HPLC Finger prints of standard flavonoids

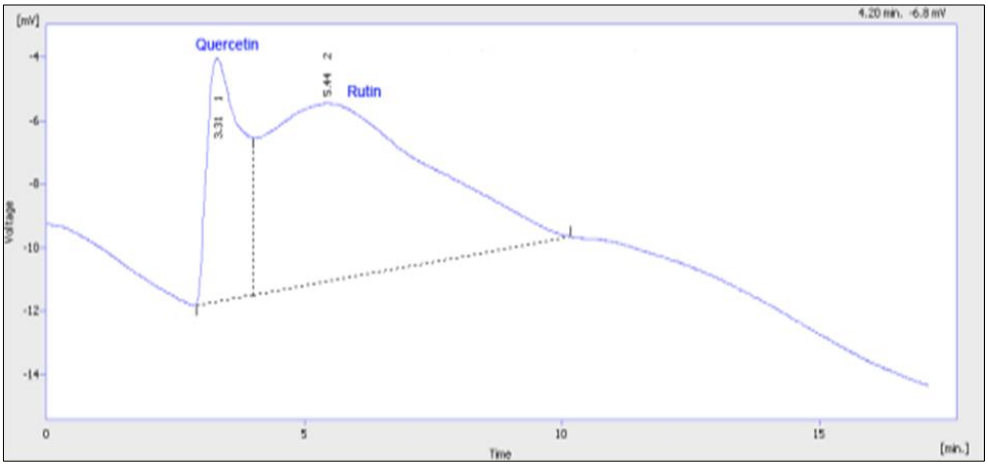

Fig 1B: HPLC Finger print of Flavonoids present in *P. granatum*

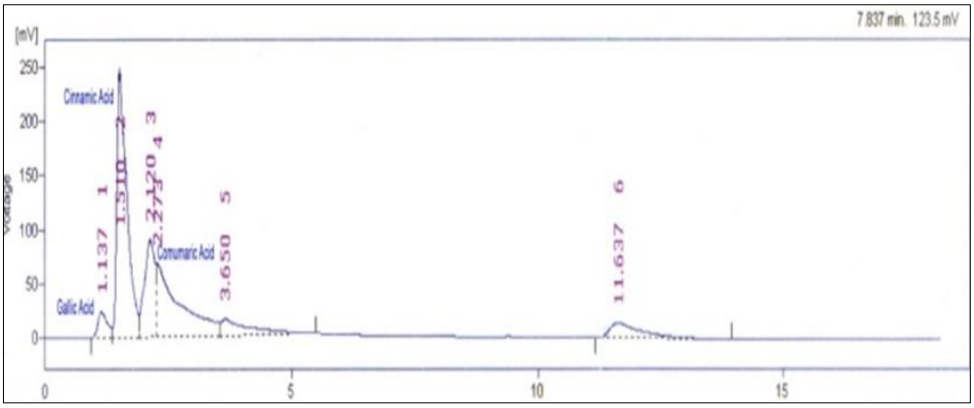

Fig 1C: HPLC Finger print of standard Phenols

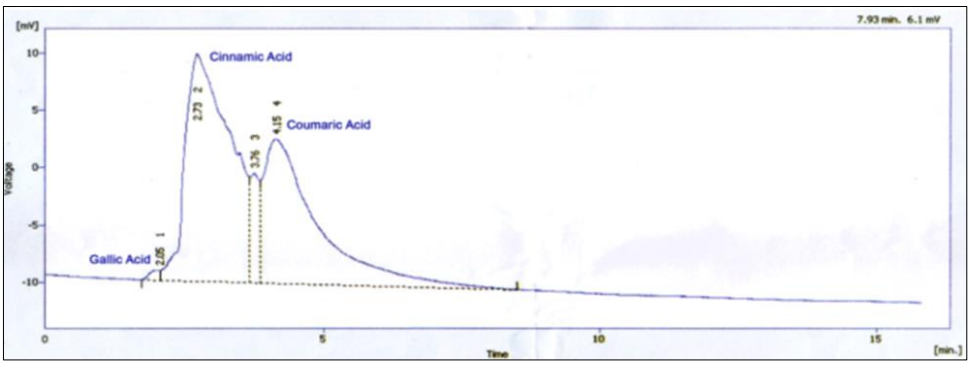

Fig 1D: HPLC Finger prints of Phenols Present in *P. granatum*

In this study, medicinal plants, i.e., *P. granatum* was extracted with water and 95% ethanol. The extracts were used to study antifungal and antibacterial effects by the agar diffusion technique. The bacterial strains such as *Streptococcus pyogenes, Staphylococcus aureus, Salmonella typhi, Klebsiella pneumonia, Helicobacter pylori, Pseudomonas auroginosa* and fungal strains such as *Aspergillus Niger, Candida albicans* and *Trichoderma viride* were used (Table 3). Until the late eighties, it was widely believed that superficial infection was no longer a threat to public health. Microbial infection seemss especially controllable due to good hygienic condition, but development of microbial resistance to antimicrobial drugs is almost an inevitable consequence of their application. Microorganism that acquired resistance to a particular antimicrobial agent becomes clinical important, particularly when the use of individual drug is wide spread. Mechanism of drug resistance by microorganisms to antimicrobial agents can be categorized into the enzymatic modification in activation of the antibodies or receptor modification as well as limiting access of the drug to its susceptible host of pathogen (Ramamurthy *et al*. 2013) [13].

Boominathan and Ramamurthy (2009) [2] reported that the ethanolic extracts were tested against bacteria and fungi. Among the extracts, the leaf extract of *Heliotrpium indicum* were effective against bacteria and fungi. The other three extracts have less inhibitory effect which has been noted in bacteria and fungi. In the present study it was interesting that the traditional method of treating a microbial infection was by administering a decoction of the plant, whereas according to our results an ethanolic extract was better; hence this may be more beneficial. Amongst the six bacterial and three fungal strains investigated *H. pylori* is the most resistant and *T. viride* is less resistant. These results are consistent with previous reports on related plants regarding Gram-positive bacteria (Cowan, 1999) [4]. The resistance of Gram-negative bacteria (*S. aureus*) to plant extracts was not unexpected as in general, this class of bacteria is more resistant than Gram-positive bacteria. Such resistance could be due to the permeability barrier provided by the cell wall or the membrane accumulation mechanism (Adwan and Abu-Hasan, 1998) [1].

Table 3: Antimicrobial bacterial effect of *P. granatum* leaf extracts

S. No	Test Organisms	Zone of inhibition (mm)	
		Ethanol extract	Aqueous extract
1	*Streptococcus pyogenes*	17	15
2	*Staphylococcus aureus*	12	10
3	*Pseudomonas auroginosa*	10	09
4	*Salmonella typhi*	17	14
5	*Klebsiella pneumoniae*	15	12
6	*Helicobacter pylori*	23	19
7	*Aspergillus niger*	9	6
8	*Candida albicans*	6	4
9	*Trichoderma viride*	4	3

Conclusions

Nowadays herbs are extensively used for the research purpose and it possesses more than one chemical entity so it has been widely used for the research investigations. The plant based compounds have the effective dosage response and minimal side effects when compared to the synthetic compounds Phytochemical screening of *Punica granatum* leaves reveals it as a valuable medicinal plant with numerous medicinal properties. Medicinal plants have the great therapeutic and economic values in all over the world. The present results offer a scientific basis for traditional use of *P. granatum* against various ailments. Further studies are required for this plant to validate their medicinal importance. In addition, isolation, characterization and elucidation of the structures of the bioactive compounds which may be responsible for their antimicrobial activity and other medicinal values of this widely available weed *P. granatum*.

References

1. Adwan K, Abu-Hasan N. Gentamicin resistance in clinical strains of Enterobacteriaceae associated with reduced gentamicin uptake. Folia Micro biol. 1998; 43:438-840.
2. Boominathan M, Ramamurthy V.Antimicrobial activity of *Heliotropium indicum* and *Coldenia procumbens*. J Eco biol. 2009; 24(1):11-15.
3. Cordell GA. Introduction to Alkaloids: A Biogenic Approach, Wiley, New York, 1983.
4. Cowan MM. Plant products as antimicrobial agents. Clin. Microbiol. Rev. 1999; 12:564-582
5. Cragy GM David. In Natural products drug discovery in the next millennium. J Pharm. Biol. 2001; 39:8-17.
6. Egharevba HO, Kunle OF. Preliminary phytochemical and proximate analysis of the leaves of *Piliostigma thionningii* (Schumach.): milne-redhead. Ethnobot Leaflets. 2010; 14:570-577.
7. Farnsworth NR. Screening plants for new medicines. In Biodiversity part II, Wilson Eo, Eds. National Academy Press, Washington, 1989, 83-97.
8. Ganora L. Herbal Constituents: Foundations of Phyto chemistry. Herbal Chem Press, Louisville, CO, 2008, 38-52
9. Jayaprakasha GK, Negi PS, Jena BS. Antimicrobial activities of pomegranates, in pomegranates; ancient roots to modern medicines, Eds., CRC Press: Boca Raton, FL, USA, 2006, 167-168.
10. Larson RA. The antioxidants of higher plants. Phyto chemistry. 1988; 27:96.
11. Low Dog T. Smart talk on supplements and botanicals. Alternative and complementary Therapies. 2009; 15:101-102
12. Naqvi SA, Khan MS, Vohora SB. Antibacterial, antifungal and anthelminthic investigations on Indian medicinal plants. Fitoterapia. 1991; 62: 221-228.
13. Ramamurthy V, Maria Rajeswari D, Vadivazhagi MK, Gowri R, Jayanthi G, Raveendran S. Study of the Phytochemical Analysis and Antimicrobial Activity of *Dodonaea viscose.* Int. J Pure Appl. Zool. 2013; 1(2):178-184.
14. Singh RP, Chidambara MKN, Jayaprakasha GK. Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extract using *in vitro* models. J Agric. Food Chem. 2002; 50:81-86.
15. Sofowara A. Screening plants for bioactive agents. In: Medicinal plants and traditional medicinal in Africa. 2nd edition. Spectrum Book Ltd, Sunshine House, Ibadan Nigeria, 1993, 134-56.
16. Trease GE, Evans WC. Pharmacognosy. 15th ed. Sounders Publishers. London, 2002, 42-44, 22-229, 246-249, 304-306, 331-332, 391-393.