A Study of Phytochemical Screening and Antioxidant Activity of Ethanolic Leaves Extract of *Punica granatum*

V. Ramamurthy & J. Antonia Auxi Marueen
P.G & Research Department of Biochemistry, Marudupandiyar College, Vallam Post, Thanjavur, 613 403, Tamil Nadu, India.

Received: July 01, 2018 Accepted: August 07, 2018

ABSTRACT *This study was conducted to assess the phytochemical constituents in Punica granatum L. Leaf extracts using standard methods. The ethanolic leaves extract of Punica granatum was analaysed by HPLC and GC to determine various Phytochemicals. The free radicals scavenging activity of extract by using DPPH, NO and Super oxide radicals generated in vitro. The ethanolic leaves extract of P. granatum was found to contain alkaloids (6.18 mg/gm), tannins (1.82mg/g), phenols (7.76 mg/gm) and flavonoids (5.15 mg/gm). The major flavonoids detected were kaempherol. The ethanolic extract of P. granatum was found to possess significant free radical scavenging activity against DPPH, NO and SOD anions the IC50 value of 39.0 µg/ml, 34.25 µg/ml and 30.05µg/ml respectively and comparable to that of their corresponding IC50 value. The medicinal property of P. granatum may be attributed to the presence of flavonoids and phenolic compounds with rich antioxidant potential. The therapeutic effect of this plant may be accounted for its counteracting action on free radicals in vivo.*

Keywords*: Punica granatum, free radical, 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). Medicinal plants are believed to be an important source of chemical substances with potential therapeutic effects (Farnsworth, 1989).

Free radicals play an important role in various pathological conditions such as tissue injury, inflammation, neurodegenerative diseases, cancer and aging. The Compound that can scavenge free radicals has great potential in ameliorating these diseases (Coban et al., 2003). Inflammation is a disorder characterized by invasion of leucocytes and production of proinflammatory cytokines (Mantri and Witiak, 1994).

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). It is also found in India and more arid regions of Southeast Asia (Naqvi *et al*., 1991), 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). The potential therapeutic propertiesof 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). Therefore the present study quantifies the secondary metabolites and the antioxidant potential of ethanolic extract of *P. granatum*. Considering all these facts, the present study was designed to investigate the presence of various phytochemicals in the different extracts of *Punica granatum* leaf, a plant which evokes various therapeutic effects.

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).

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.

Free radical scavenging activity

1. Diphenyl – 2- Picrylhydrazyl (DPPH) radical scavenging activity.

DPPH radical scavenging assay is a commonly recommended method for assessment of antioxidant potential of plant extracts. The assay is based on the ability of DPPH, a free radical which get decolorized in the presence of antioxidants. To 200ml. of ethanolic solution of DPPH (1 µg/ml) various concentration of (20mg – 100 µg/ml) in water were added and incubated at 37^0C for 30 min in dark and the absorbance was measured at 517nm. Ascorbic acid was used as the reference standard. The percentage scavenging of DPPH free radical was calculated and compared with that of the standard ascorbic acid. The IC_{50} value also determined.

2. Superoxide anion scavenging activity

The method of Nishkimi et al. (1972) was applied for the measurement of MIT superoxide anion scavenging activity, Briefly 312µm Nitroblue tetarzolium in 120 µm phosphate buffer pH 7.4 were added to an aliquots of MIT (20-100µg/ml) the reaction was started by adding 100ml of phenazine metho sulphate (120mm preaperd in phosphate buffer pH 7.4) and the colour change was monitored at 560nm against water blank querceutin was used as the positive control.

3. Nitric oxide scavenging activity

The nitric oxide scavenging activity of the aqueous extract was measured by taking various concentrations of MIT and standard. Ascorbic acid (20-100µg/ml) dissolved in phosphate buffer (0.025m, pH 7.4) and incubated with sodium nitroprusside (5mm) in standard phosphatebuffer at 25^0C for 5 hrs. After the incubation, 0.5ml of the reaction mixture was added with 0.5ml of Griess reagent (equal volume of 1% sulphanilamide in 2% phosphoric acid and 0.1% napthlthyl ethylene diamine dihydrochloride in water). The absorbance of the chromophore formed was read at 540nm. The activity was compared with that of similar concentration of Ascorbic acid (Sreejavan Rao, 1997).

Result 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	-	+	+

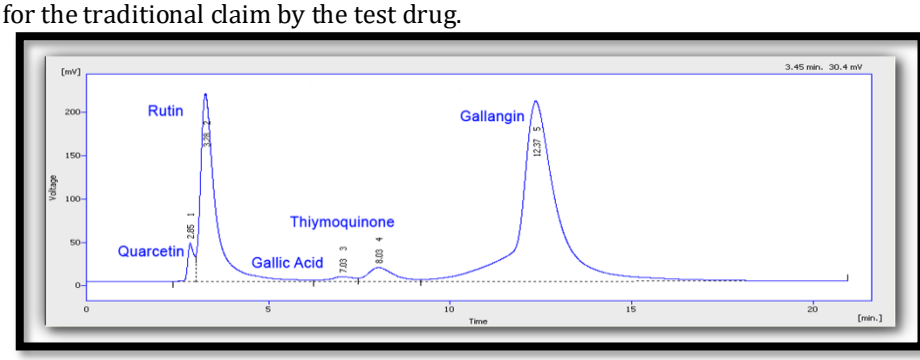
In this investigation, presence of quantitative phytochemicals in ethanolic extract of *P. granatum* expressed the value in mg/g phenols was (7.76 mg/g), tannins (1.82 mg/g), flavonoids (5.15 mg/g) and alkaloids (6.18). Finding the natural substance of medicinal plant that decrease the inflammation and 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

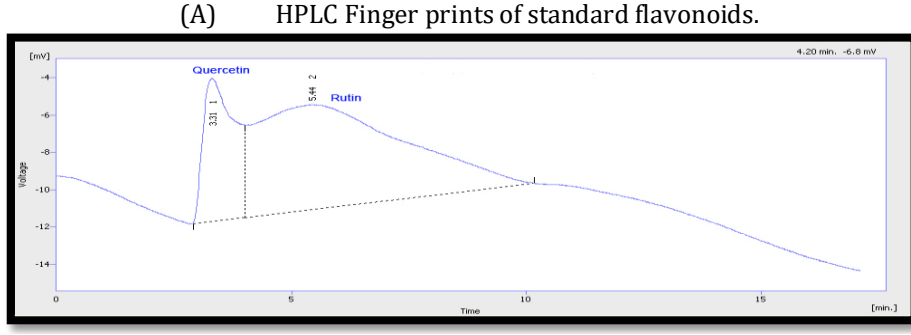
S. No.	Phytochemical	Values in (mg/g)
1	Phenols (mg/g)	7.76
2	Tannins (mg/g)	1.82
3	Flavonoids (mg/g)	5.15
4	Alkaloids (mg/g)	6.18

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

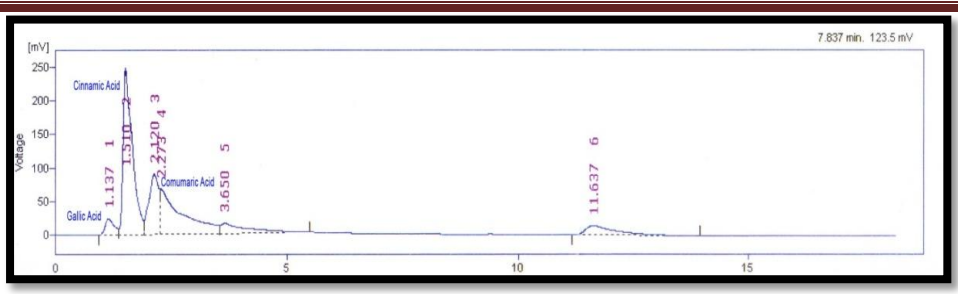
HPLC analysis reveals that the extract was found to be rich in Alkaloids (6.18 mg/g) terpenoids (1.82 mg/g) and phenols (7.76 mg/g). Ethanolic leaves extract of *P. granatum* also contain flavonoids such as Rutin (1.12 mg/g) and quercetin (1.19 mg/g) Fig. 1 (A) to (D) many reports demonstrate that antioxidant 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 anti cancer 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.



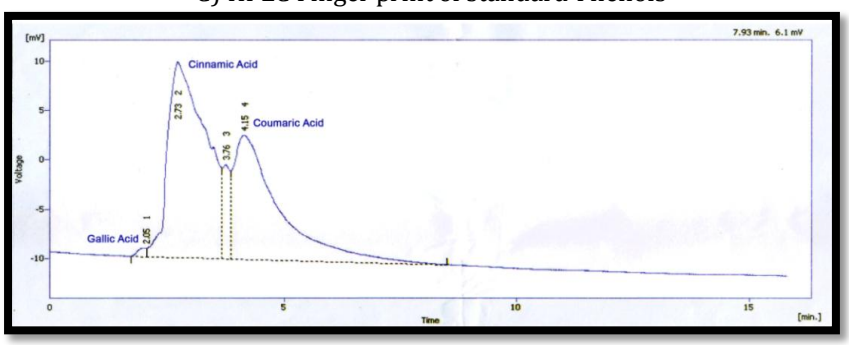
(A) HPLC Finger prints of standard flavonoids.



(B) HPLC Finger print of Flavonoids present in *P. granatum*.



C) HPLC Finger print of standard Phenols



(D) HPLC Finger prints of Phenols Present in *P. granatum*.

***Invitro* antioxidant assays**

Antioxidants are known to exhibit their biochemical effects through numerous mechanisms, including the prevention of chain initiation, reductive capacity and radical scavenging mechanisms. Several methods have been used to measure the antioxidant activity of biological materials. It is essential to use more than one method to evaluate antioxidant capacity of plant materials simply because of the complex nature of phytochemicals present in them. Therefore, in the present study, DPPH free radical scavenging activity and nitric oxide scavenging activity assessment were done.

The percent DPPH Scavenging activity ethanolic extract of *P. granatum* were depicted (Table 2). Ascorbic acid was used as a positive control for comparison of plant materials. The IC_{50} value of Ascorbic acid was found to be 52.25µg/ml. The IC_{50} value of DPPH scavenging activity was found to be ethanolic extract of *P. granatum* was 39.0 µg/ml. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The degree of discoloration indicated the scavenging potential of the antioxidants in the sample. The extract significantly inhibited the activities of DPPH radicals in a dose-dependent manner. The comparison of scavenging effects of ethanolic extract on DPPH radical showed consistently higher radical scavenging activity observed in seabuckthorm *Hippophae rhamnoidesl* seeds extracts of phenolic compounds (Heim et al., 2002), Flavonoids (Apati et al., 2003) and terpens (Saranya Panneerselvam et al., 2010) might be taken in to account.

Activity evaluation of *P. granatum* extract showed the capacity of the plant towards nitric oxide scavenging in a dose dependent manner. Ascorbic acid, a natural antioxidant was used as a positive control for comparison. Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with super oxides. These compounds are responsible for altering the structural and functional behaviour of many cellular components. Incubation of solutions of sodium nitroprusside in PBS at 25°C for 2 hours resulted in linear time dependent nitrite production, which is reduced by the ECH. The IC_{50} of ascorbic acid was found to be 39.35µg/ml. The order of the Scavenging activity was found to be ethanolic extract of *P. granatum* was IC_{50} 34.25 µg/ml. Nitric oxide scavenging activity of ethanolic leaves extract of *P. granatum* is on important chemical mediator generated by endothelial cells, macrophages, neuron and it is involved in the regulation of various physiological process like control of arthritis, cytotoxice effects alzhemer's disease (Sainani et al., 1997). Thirunavukkarasu et al. (2011) previously studied higher free radicals compared to the results of 73.89±4.22 at 2mg/ml concentration of ethanol extracts of mangrove *A. officinalis* in Cuddalore region. Higher nitric oxide scavenging activity was reported by Athiperumalsami et al. (2010) in the methanol and water extracts of *H. ovalis* but the IC_{50} values were much lower than that of standard tocopherol.

Superoxide anion is a free radical created from the normal process of energy generation in the human body. Superoxide anion is toxic to cells and tissues and plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical or singlet oxygen. The

concentration of standard gallic acid to inhibit 50% of superoxide formation was found to be 40.3µg/ml. The scavenging activity of *P. granatum* extract was found to be IC_{50} 30.05µg/ml. The superoxide onions are toxic intermediates formed during inflammatory process and found to enhance the risk of inflammation related disorders such as arthritis and atherosclerosis. Super oxide anion is a free radio that plays an important role in the formation of reactive oxygen species such as hydrogen periode, hydroxyl / radicals, or singlet oxygen in living organism. Korycka et al. (1978) reported that the therapeutic activity of medicinal plants can be determined by superoxide activity. Superoxide anion is a free radical created from the normal process of energy generation in the human body. Superoxide anion is toxic to cells and tissues and plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical or singlet oxygen (Nohl et al., 2003).

Table 2. Free Radicals scavenging activity in *P. granatum*

Free radicals		Concentration of Standard and extract in (µg/ml)				
		20	**40**	**60**	**80**	**100**
DPPH	**Ascorbic Acid**	35.6	41.7	62.8	72.4	81.8
	P. granatum	25.5	32.4	45.6	56.5	65.2
Nitric Acid	**Ascorbic Acid**	29.5	35.8	42.9	55.7	67.1
	P. granatum	26.8	31.7	36.8	47.9	57.5
Superoxide	**Gallic acid**	29.8	32.7	47.9	53.9	78.6
	P. granatum	18.8	24.8	35.3	42.2	58.4

Conclusion

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. Since the ethanolic extract of *P. granatum* leaves contains more constituents it can be considered beneficial for further investigation. A typical research and developmental work needs to be carried out for its better therapeutic and commercial utilization. The free radical scavenging activity of *P. granatum* revealed that they can be used for the Prevention or treatment of human diseases such as cancer, arthritis, diabetes mellitus which are associated with oxidative stress.

References

1. Apati, P., Szentmihalyi, K., Kristo, S.z.T., Papp, I., Vinkler, P., Szoke, E and A. Kery. 2003. Herbal remedies of Solidago, correlation of phytochemical characteristics and antioxidative properties. J. Pharmacol. Biomedical Anal.,**32:** 1045–1053.
2. Athiperumalsami, T., Devi, R.V., Hastha, P.S., Kumar, V and J.L. Louis. 2010. Antioxidant activity of seagrasses and seaweeds. Botanica Marina.**53:** 251-257.
3. Coban, T, G.S. Citoglu, B. Sever and M. Iscan, 2003, Antioxidant activities of plants used traditional medicine in Turkey, pharm. Bio 1., 41; 608 – 613.
4. CordellG. A. 1983, Introduction to Alkaloids: A Biogenic Approach, Wiley, New York.
5. Cragy GM, and David. 2001. In Natural products drug discovery in the next millennium. J. Pharm.Biol., **39:** 8-17.
6. Egharevba HO, Kunle OF. 2010. Preliminary phytochemical and proximate analysis of the leaves of Piliostigma thionningii (Schumach.): milne-redhead. Ethnobot Leaflets, **14:** 570-577.
7. Farnsworth NR. 1989. Screening plants for new medicines. In Biodiversity part II, Wilson Eo, Eds. National Academy Press, Washington. P. 83-97.
8. Heim, K.E., Taigliaferro, A.R and D.J. Bobilya. 2002. Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. The J. Nutritional Biochem., **13:** 572–584.
9. Jayaprakasha GK, Negi PS, Jena BS. 2006. Antimicrobial activities of pomegranates, in pomegranates; ancient roots to modern medicines, Eds., CRC Press: Boca Raton, FL, USA; Pg No. 167-168.

10. Korycka DM, Richardson T. Phytogeneration of super oxide anion in serum of Bovine milk and in model systems containing riboflavin and aminoacids. J Diary sci 1978; 61: 400-407.
11. Larson RA. The antioxidants of higher plants. Phytochmistry 1988; 27:96.
12. Mantri P, Witiak DT. Inhibition of Cycloxygenase & 5 - lipoxygenase, curr. Med. Chem. 1994; 328 – 355.
13. Naqvi SA, Khan MS, Vohora SB. 1991. Antibacterial, antifungal and antihelminthic investigations on Indian medicinal plants. Fitoterapia, **62:** 221-228.
14. Nishkimi M, Appaji N, Yagi K. 1972. The occurrence of Superoxide anion in the reaction of reduced Phenazine methosulphateand molecular oxygen. Biochem Biophy Res Beans Commun., **46:** 849-854.
15. Nohl, H., Kozlov, A.V., Gille, L and K. Staniek. 2003. Cell respiration and formation of reactive oxygen species: facts and artifacts. Biochem. Soc. Trans., **31:** 1308-1311.
16. Sainani G.S. Manika JS, Sainani RG. 1997. Oxidative Stress – a key factor in Pathoegenesis of Chronoic disease, med update 1:1.
17. Saranya Panneerselvam., Arumugam Geetha and Selvaperumal Munis Karthikeyan Narmadha Selvamathy. 2010. The antioxidant and H^+K^+ atpase inhibitory effect of andrographis paniculata and andrographolide –in vitro and in vivo studies. Pharmacologyonline, **1:** 356 - 376.
18. Singh RP, Chidambara MKN, Jayaprakasha GK. 2002. Studies on the antioxidant activity of pomegranate (Punica granatum) peel and seed extract using in vitro models. J. Agric. Food Chem., **50:** 81-86.
19. Sofowara, A. 1993. Screening plants for bioactive agents. In: Medicinal plants and traditional medicinal in Africa. 2nd edition. Spectrum Book Ltd, Sunshine House, Ibadan Nigeria. p. 134-56.
20. Sreejayan Rao MN. Nitric oxide scavenging by curcuminoids. J Pharm Pharmacol 1997; 49:105-107.
21. Thirunavukkarasu, P., Ramanathan, T., Ramkumar, L., Shanmugapriya, R and G. Renugadevi. 2011. The antioxidant and free radical scavenging effects of Avicennia officinalis. J. Medi. Plants Res., **5**(19): 4754-4758.
22. Trease GE and Evans WC. 2002. Pharmacognosy. 15th ed. Sounders Publishers. London. p. 42-44, 22-229, 246-249, 304-306, 331-332, 391-393.