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Research Article

Phytochemical Analysis and Evaluation of *In Vitro* Anti Oxidant Activity of *Punica Granatum* Leaves

Narender Boggula[1*], Himabindu Peddapalli[2]

[1]*Department of Pharmaceutical Chemistry, Anurag Group of Institutions, School of Pharmacy, Ghatkesar, Medchal, Telangana, India.*
[2]*Department of Pharmaceutics, Nalla Narasimha Reddy Education Society's Group of Institutions, School of Pharmacy, Medchal, Telangana, India*

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ABSTRACT
Pomegranate is a large compound of the eastern diets, yet no research has been performed on this product. The pomegranate, *Punica granatum* belongs to family Lythraceae is an ancient, mystical, unique fruit borne on a small long-living tree cultivated throughout the mediterranean region, as far north as the Himalayas, in Southeast Asia, and in California and Arizona in the United States. *Punica granatum* is a fruit bearing deciduous herb or small tree growing between 5-8m tall. *Punica granatum* leaves are used as bitter tonic in fever and used in pneumonia, flu, mouth and lip infections. In recent studies plant has shown anti fungal, immunosuppressant and anti diabetic activity. It is also used in treatment of heart problems, stomach disorders, dental care, cancer, anaemia, osteoarthritis. *Punica* including *Punica protopunica* are known to exhibit strong anti oxidant activity. The extraction was subjected to assay by Reducing power, Hydrogen peroxide scavenging assay, DPPH methods for evaluation of anti oxidant activity. Results revealed that the pomegranate can be categorized as a leave with extremely high anti oxidant potential.

Keywords: Reducing power, anti oxidant, free radicals, DPPH method, Hydrogen peroxide scavenging assay and *Punica granatum.*

INTRODUCTION

The plants have been utilized for basic and curative health care since time immemorial. The use of plants as food and medicines started ever since man started life on the planet. Plants play as important role in our life. Plants not only provide us nutrition but also they have medicinal values[1]. Herbs are being used by about 80% of the world population especially in the developing countries for primary health care. They have stood the test of time for their safety, efficacy, cultural acceptability and minimal side effects. Medicinal plants exhibit phytotherapeutic effects caused by biologically active compounds specific secondary metabolites[2]. The plants have been utilized for basic and curative health care since time immemorial. The use of plants as food and medicines started ever since man started life on the planet. The plant kingdom is a virtual goldmine of potential drug targets and other active drug molecules waiting to be discovered. During the last decade, use of traditional medicine has expanded globally and gained popularity. Plant based drugs are having a revived interest now-a-days because of awareness of deleterious effects of modern synthetic drugs. Natural products can play a very crucial role in pharmaceutical industry as drug them or as drug carrier or bio-enhancers or excipients[3]. The importance of herbal/plant medicines is well documented in Vedas, which proved to be the ancient literature. The properties of the plants and their remedies are given in detail and in fact Ayurveda is the very principle root for the emergence of Ancient medical science in India that gave origin to branches like Sushruta and Charka Samhita. In order to set up quality in production and products, research documentation is mandatory to supply to international requirements. By referring global standards and international pharmacopoeia like Herbal B.P, China, Japanese Herbal, Ayurvedic Formulary of India, WHO Guidelines on Herbal Medicines, this could be met with. If the Indian herbal industry, is to survive in the domestic and international markets steps have to be taken to establish a good quality control mechanism, for which the government should consider assisting the standardization of drugs to meet International requirements in the coming years. It is also necessary to integrate modern knowledge with traditional knowledge. The drugs and products of the industry are working on the scientifically defined techniques and explained with modern biological and chemical definitions and tools, and that alone will give a therapeutically active herbal original drug available for health care worldwide[4,5].

Punica granatum L. has been widely used by traditional medicine in America, Asia, Africa and Europe for the treatment of different types of diseases. Many Indian medicinal plants recommended for the treatment of diabetes mellitus lack rigorous scientific justification. *Punica granatum*, commonly known as

**Author for Correspondence: narender.b987@gmail.com*

pomegranate is one of the plants that have long been used in traditional herbal medicine against differ diseases. *Punica granatum* is native to a region from Iran to Northern India. The *Punica granatum* leaves have been used in natural and holistic medicine to treat sore throat, cough, urinary infections, digestive disorders, arthritis etc. clinical research shows that pomegranates when part of healthy diet might help prevent heart diseases, heart attacks and strokes[6]. Over the past decade, significant progress has been made in establishing the pharmacological mechanisms of *Punica granatum* leaves and the individual constituents responsible for them. The current research seems to indicate the most therapeutically beneficial *Punica granatum* leaves constituents are ellagic acid, ellagitammins, punicic acid, flavonoids, anthocyanidins, anthocyanins and estrogenic flavonols and flavones[7].

Epidemiological studies indicate that frequent consumption of fruits and vegetables is associated with low risk of chronic diseases such as diabetes, cardiovascular diseases and cancers. The increased intake of natural antioxidants, particularly the anti oxidative compounds present in fruits and vegetables contributes to the antioxidant capacity of plasma and these constituents are reported to mitigate the damage caused by the oxidative stress. Recent studies reveal that the defensive effect of fruits and vegetables is at least partially attributable to the phytochemicals such as vitamins A, C and E, flavonoids, phenolic acids, lignans and carotenoids. These constituents serve as free radical scavengers, hydrogen donating compounds, singlet oxygen quenchers and metal chelators. Therefore, it is imperative to evaluate the commonly consumed fruits and vegetables for their anti oxidative efficacy[8,9].

Antioxidants are the compounds that when added to food products, especially to lipids and lipid-containing foods, can increase the shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food products during processing and storage. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytolune (BHT), have restricted use in foods as these synthetic antioxidants are suspected to be carcinogenic. Therefore, the importance of the search for and exploitation of natural antioxidants, especially of plant origin, has greatly increased in recent years. Several disorders such as cardiovascular diseases, diabetes, metabolic syndrome, cancer have become more prevalent. Free radicals such as reactive oxygen species (ROS) have been implicated in their etiology. However, the defense mechanism of the body against ROS could be overwhelmed in many circumstances by an over-production of these species. Therefore, synthetic antioxidants and foods rich in antioxidants have been shown to boost our defense against ROS. The Middle Eastern diets contain many foods, among which the pomegranate molasses, are believed to have antioxidant effects but without much scientific evidence. This substance is an extract from pomegranate (*Punica granatum L.*). This species grows generally in Asia, the Mediterranean border, and the American continent. Pomegranate is considered one of the oldest fruits and one of the earliest to appear in human diet[10,11].

Plant profile

Botanical name- *Punica granatum*
Kingdom: Plantae (Angiosperms)
Order: Myrtales
Family: Lythraceae
Genus: *Punica*
Species: *P. granatum*

The pomegranate does best in well drained ordinary soil, but also thrives on calcareous or acidic loam as well as rock strewn gravel. Pomegranates prefer a semi-arid mild-temperate to subtropical climate and are naturally adapted to regions with cool winters and hot summers. A humid climate adversely affects the formation of fruit. The tree can be severely injured by temperatures below 12 ^{0}F. Once established, pomegranates can take considerable drought, but for good fruit production they must be irrigated. To establish new plants they should be watered every 2 to 4 weeks during the dry season. The plants are tolerant of moderately saline water and soil conditions.

Leaves

Colour: dark green
Size: 3-7 cm long and 2 cm broad
Shape: The pomegranate has glossy, leathery leaves that are narrow and lance-shaped.

The objectives of this study were to prepare antioxidant-rich fractions from pomegranate leaves extract and to evaluate their anti oxidant activity using various *in vitro* models.

MATERIALS AND METHODS

Plant Material

The leaves of *Punica granatum* were collected from surrounding places of Rangareddy Dist. The leaves collected were washed under running tap water, blotted dry and kept for drying in oven at temperature 40 ± 2 ^{0}C for five days. The dried leaves were powdered and stored in air tight container. The plant specimens for the proposed study were collected from ken to select healthy plants and for normal organs. The required samples of different organs were cut and removed from the plant and fixed in FAA (Farmalin-5ml + Acetic acid-5ml + 70% Ethyl alcohol-90ml). After 24 hrs the specimens were dehydrated with graded series of tertiary butyl alcohol as per the schedule given by Sass, 1940. Infiltration of the specimens was carried by gradual additional of paraffin wax (melting point 58-60 ^{0}C) until tertiary butyl alcohol solution attained super saturation. The specimens were cast into paraffin blocks.

Preparation of plant extract

Leaves of *Punica granatum* were washed and shade dried, then coarsely grounded. The shade-dried leaves of *Punica granatum* (214g) were subjected to cold extraction with ethanol in a round bottom flask. The solvent was evaporated under reduced pressure using a rotary evaporator. This process yields 10g of dried ethanol extract which is stored at ±20 ^{0}C until use.

Preliminary phytochemical screening

The different chemical tests were performed for establishing profile of the leaves extract for its chemical composition; the following chemical tests for various phytoconstituents in the ethanol extract was carried out as described below.

Test for alkaloids

Dragendroff's Test

In a test tube containing 1ml of extract, few drops of Dragendroff's reagent was added and the colour developed was noticed. Appearance of orange colour indicates the presence of alkaloids.

Wagner's Test

To the extract, 2 ml of Wagner's reagent was added; the formation of a reddish brown precipitate indicates the presence of alkaloids.

Mayer's Test

To the extract, 2 ml of Mayer's reagent was added, a dull white precipitate revealed the presence of alkaloids.

Hager's Test

To the extract, 2 ml of Hager's reagent was added; the formation of yellow precipitate confirmed the presence of alkaloids.

Test for terpenoids

Salkowski test

To 1 ml of extract, tin (one bit) and thionyl chloride were added. Appearance of pink colour indicates the presence of terpenoids.

Hirshonn reaction

When the substance was heated with trichloroacetic acid, red to purple colour was observed.

Test for steroids

Liebermann Burchard Test

To 1ml of extract, 1ml of glacial acetic acid and 1ml of acetic anhydride and two drops of concentrated sulphuric acid were added. The solution become red, then blue and finally bluish green indicates the presence of steroids.

Test for coumarins

To 1 ml of extract, 1 ml of 10% sodium hydroxide was added. The presence of coumarins is indicated by the formation of yellow colour.

Test for tannins

To few mg of extract, ferric chloride was added, formation of a dark blue or greenish black colour showed the presence of tannins.

The extract was mixed with basic lead acetate solution; formation of white precipitate indicated the presence of tannins.

Test for saponins

To 1 ml of the extract, 5 ml of water was added and the tube was shaken vigorously. Copious lather formation indicates the presence of saponins.

Test for flavones

Shinoda Test

To the extract, a few magnesium turnings and 2 drops of concentrated hydrochloric acid were added, formation of red colour showed the presence of flavones.

To the extract, 10% sodium hydroxide or ammonia was added; dark yellow colour shows the presence of flavones.

Test for quinones

To 1 ml of the extract 1 ml of concentrated sulphuric acid was added. Formation of red colour shows the presence of quinones.

Test for flavanones

To the extract, 10% sodium hydroxide was added and the colour changes from yellow to orange, which indicates the presence of flavanones.

To the extract, conc. sulphuric acid was added, and the colour changes from orange to crimson red, which indicates the presence of flavanones.

Test for anthocyanins

To the extract, 10% sodium hydroxide was added, and the blue color shows the presence of anthocyanins.

To the extract, conc. sulphuric acid was added, and the yellowish orange colour confirms the presence of anthocyanins.

Test for anthraquinones

Borntrager's test

The extract was macerated with ether and after filtration; aqueous ammonia or caustic soda was added. Pink red or violet colour in the aqueous layer after shaking indicates the presence of anthraquinones.

Test for phenols

Ferric chloride test

To the extract, few drops of 10 % aqueous ferric chloride were added. Appearance of blue or green colour indicates the presence of phenols.

Test for proteins

Biuret Test

To the extract, 1 ml of 40% sodium hydroxide solution and two drops of one percent copper sulphate solution were added. Formation of violet colour indicates the presence of proteins.

Xanthoprotein Test

To the extract, 1 ml of concentrated nitric acid was added. A white precipitate was formed; it is then boiled and cooled. Then 20% sodium hydroxide or ammonia was added. Orange colour indicates the presence of aromatic amino acids.

Tannic Acid Test

To the extract, 10% tannic acid was added. Formation of white precipitate indicates the presence of proteins.

Test for carbohydrates

Molisch's Test

To the extract, 1 ml of alpha-naphthol solution, and concentrated sulphuric acid through the sides of test tube were added. Purple or reddish violet colour at the junction of the two liquids revealed the presence of carbohydrates.

Fehling's Test

To the extract, equal quantities of fehling's solution A and B were added and on heating, formation of a brick red precipitate indicates the presence of carbohydrates.

Benedict's Test

To 5 ml of Benedict's reagent, extract was added and boiled for two minutes and cooled. Formation of red precipitate showed the presence of carbohydrates.

Test for amino acids

Ninhydrin test

Figure 1: *Punica granatum* plant.

Figure 2: *Punica granatum* leaves.

Table 1: Preliminary phytochemical screening of *Punica granatum* leaves.

Constituents	Ethanol extract
Terpenoids	+
Saponins	+
Steroids	+
Carbohydrates	-
Flavonoids	+
Alkaloids	+
Quinones	-
Tannins	-
Fixed oils and fats	-
Phenols	-
Glycosides	-
Amino acids	-
Anthraquinones	-

(+) Present, (-) Absent

Two drops of ninhydrin solution were added to the extract, a characteristic purple colour indicates the presence of amino acids.

Test for Fixed Oils and Fats

Spot Test

A small quantity of extract was pressed between two filter papers. Oil stains on the paper indicates the presence of fixed oils and fats.

Anti oxidant activity

The total anti oxidant activity of the extract was evaluated by the DPPH radical scavenging assay and Reducing power assay and Hydrogen peroxide scavenging assay.

DPPH radical scavenging assay

Procedure

The DPPH free radical scavenging was assessed according to the method with slight modification. 0.1mM DPPH radical solution in methanol was prepared and 1mL of this solution was added to 1 mL of different concentration of ethanol extract (50, 100, 150, 175 and 200 µg/mL) or standard solution of ascorbic acid (1, 2, 5, 8 and 9 µg/mL). A control was prepared with ethanol. After 30 mins, the absorbance was measured at 517nm on UV-Visible spectrophotometer (Multiskan spectrum, Thermo Scientific). Decrease in absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity. The radical scavenging activity, expressed as percentage of inhibition was calculated and this activity was expressed as an inhibition concentration 50 (IC50). The percentage of inhibition was calculated by using the equation:

% Inhibition = [(A-B)/A] x 100

Where A is the absorbance of control (DPPH solution without the sample),

B is the absorbance of DPPH solution in the presence of the sample.

Reducing power assay

Procedure

The reducing power was determined according to the method. Different concentration of ethanol extract (5, 10, 20, 30 and 40 µg/mL) and standard solution of ascorbic acid (1, 2, 4, 5, 6, 8 and 10 µg/mL) are prepared in different test tubes and made up to 1mL by adding distilled water. A control was prepared with distilled water. To this test tubes, 2.5mL of phosphate buffer (0.2M, pH 6.6) and 2.5mL potassium ferricyanide (1%) were added and incubated at 50 °C for 20min. After incubation, 2.5mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10min. The upper layer of the solutions (2.5mL) was mixed with 2.5mL distilled water and 0.5mL ferric chloride (0.1%). Absorbance was measured at 700nm on UV-Visible spectrophotometer (Multiskan spectrum, Thermo Scientific). Increased in absorbance of the reaction mixture indicates increase in reducing power. Percentage of reducing power inhibition was calculated and this activity was expressed as an inhibition concentration 50 (IC50). The percentage reducing power was calculated by using the formula.

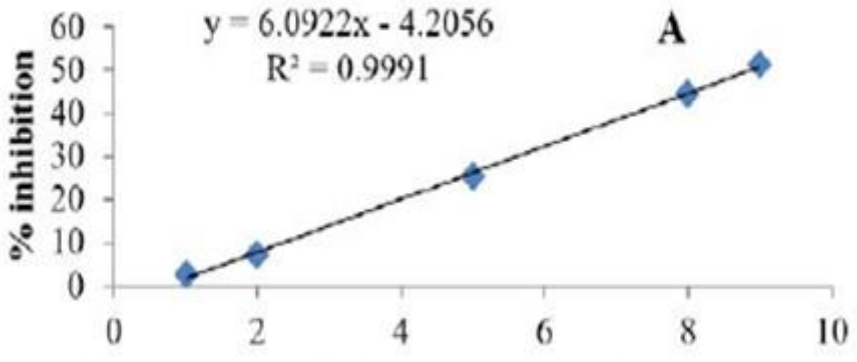
% Reducing power = [(B-A)/A] x 100

Where B is the absorbance of sample

A is the absorbance of control.

Hydrogen peroxide scavenging assay (H_2O_2 assay)

The scavenging activity of extract towards hydrogen peroxide radicals was determined by the modified method of Dehpour. Solution of hydrogen peroxide (40Mm) was prepared in phosphate buffer pH 7.4 and its concentration was determined by measuring the absorbance at 560nm using UV spectrophotometer. 0.1mg/ml of the extract was added to hydrogen peroxide solution and absorbance measured at 560nm using UV spectrophotometer against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extract and stand compound was calculated using the given formula:



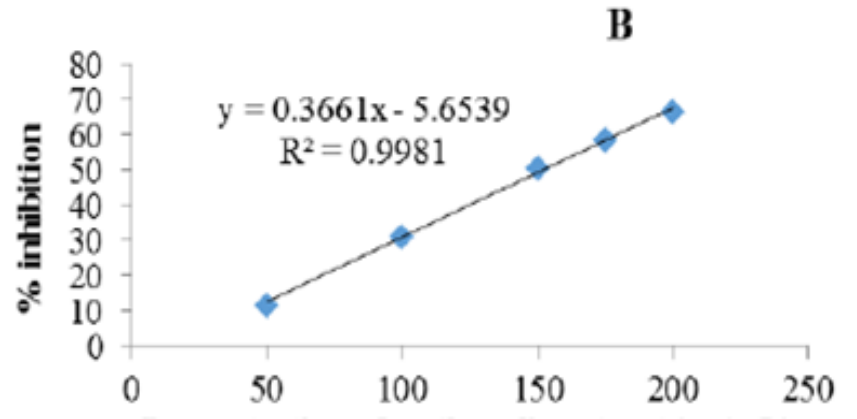


Figure 3: Percentage inhibition at different concentrations in DPPH assay (A) Ascorbic acid, (B) ethanol extract of *Punica granatum* leaves.

Table 2: IC50 value of *in vitro* anti oxidant activity of ethanol extract of *Punica granatum* leaves.

Sl. No.	sample	IC50 value (µg/mL) of *in vitro* Anti oxidant activities	IC50 value (µg/mL) of *in vitro* Anti oxidant activities
1.	Ascorbic acid	3.880±0.366	8.459±0.393
2.	Ethanol extract	10.052±2.365	153.509±3.476

Percentage scavenged (H_2O_2) = 1 – Abs standard / Abs (Control) x 100

Where, Abs (control) was the absorbance of the control (without extract) at 560nm;

Abs sample was the absorbance in the presence of the extract at 560nm.

RESULTS AND DISCUSSION

The extract of *Punica granatum* was assayed for anti oxidant activity for ethanol extract with ascorbic acid and results are presented in figure-3 and figure-4 respectively. The anti oxidants act either by scavenging various types of free radicals derived from oxidative processes, by preventing free radical formation through reduction precursors or by chelating agents. In this study the extracts significantly posses anti oxidant activity.

Preliminary Phytochemical Screening

Our observation revealed that in the preliminary phytochemical screening was found that the ethanolic dried leaves extract contain alkaloids, phytosterols, diterpenes, saponins. The preliminary phytochemical screening results are shown in Table 1.

Anti oxidant activity

DPPH radical scavenging assay

The study revealed that ethanolic extract of *Punica granatum* leaves shows a decrease in absorbance of DPPH solution. The decrease in absorbance of DPPH solution is because of reaction between antioxidant molecules present in methanol extract with DPPH, by hydrogen donation.

The scavenging activity was determined as percent inhibition of DPPH radical and expressed as IC50 value of the extract. The IC50 value for methanol extract was found to be 153.509±3.476 µg/mL (Fig. 3B and Table 2). Ascorbic acid was taken as reference which shows IC50 value of 8.459±0.393 µg/mL (Figure 3A and Table 2).

Reducing power assay

Similarly, the ethanol extract shows an increased in absorbance with increasing concentration of the extract indicating reductive ability of ethanol extract of *Punica granatum* leaves. The IC50 value of ethanol extract was

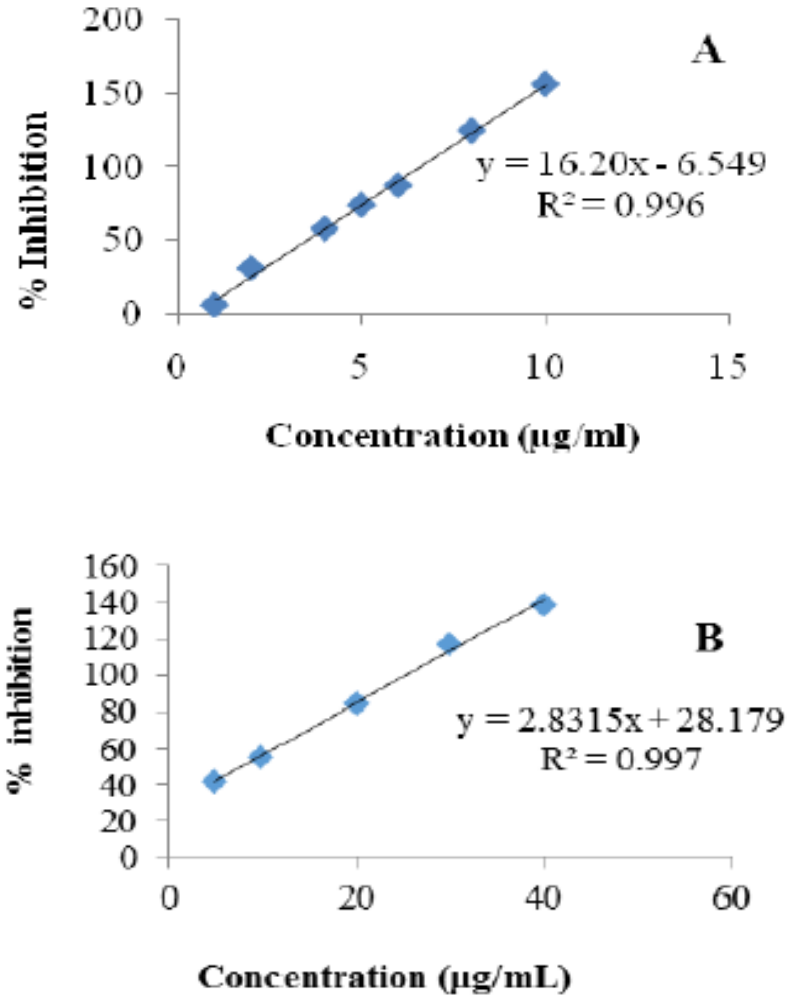


Figure 4: Percentage inhibition at different concentrations in reducing power assay (A) Ascorbic acid, (B) ethanol extract of *Punica granatum* leaves.

found to be 10.052±2.365 µg/mL (Fig. 4B and Table 2) which is comparatively close to the IC50 values of standard ascorbic acid 3.880±0.366 µg/mL (Figure 4A and Table 2).

Statistical Analysis

All the experiments were carried out in triplicate and the results are expressed as mean ± standard deviation.

Hydrogen peroxide scavenging assay (H_2O_2 assay)

The principle of this method is that there is a decrease in absorbance of Hydrogen peroxide upon oxidation. Hydrogen peroxide can arise normally or sometimes the immune cells create them purposefully to neutralize the foreign bodies. Ascorbic acid is a positive control which is commercially available as standard. From Table 3, the result of Hydrogen peroxide indicates that the percentage of scavenging activity by the standard Gallic acid was 58.31% and the test sample (*Punica granatum* leaves ethanol extract) yields 43.12% of scavenging activity.

Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. Most of the phytochemicals have anti oxidant activity and protect our cells against oxidative damage and reduce the risk of developing certain types of cancer. Antioxidants are secondary metabolites found naturally in plants. An antioxidant can be defined as anything that inhibits or prevents oxidation of a substrate. Free radicals are chemical species which contains one or more unpaired electrons due to which they are highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. Free radicals are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signalling and immune function. Free radicals can initiate the oxidation of bio molecules, such as protein, lipid, amino acids and DNA which will lead to cell injury and can induce numerous diseases. Antioxidants reduce the oxidative stress in cells and are therefore useful in the treatment of many human diseases, including cancer, cardiovascular diseases and inflammatory diseases. This activity is due to the ability of antioxidants to reduce oxidative stress by neutralizing or scavenging of reactive species by hydrogen donation. Medicinal plants contain some organic compounds which produce definite physiological action on the human body and these

Table 3: Hydrogen peroxide assay for ethanolic leaves of *Punica granatum.*

Sample	Percentage of scavenging activity
Standard (Ascorbic acid)	58.31%
Test sample	43.12%

bioactive substances include tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids.

The usage of herbal medicine has amplified dramatically for various diseases amongst general people over last few years not only because of their easy accessibility without prescription, low cost and appointment to the health care specialists and more with the belief that natural remedies have less lethal effects as compared to synthetic medicines. Secondary metabolites have been reported to exert wide range of various biological activities which include anti viral, anti- inflammatory, anti bacterial, anti diabetic, anti allergic, anti tumor, treatment of neurodegenerative diseases and vasodilatory actions. From the present study it was revealed that the isolated fraction was rich in secondary metabolites which aimed for anti oxidant activity. Anti oxidants act as a defense mechanism that protects against oxidative stress or repair damaged molecules, hence the said was proved by the ethanolic extract has anti oxidant power. A marked anti oxidant activity of *Punica granatum* was observed in this study.

CONCLUSION

The present study was conducted to determine the antioxidant activity of *Punica granatum* leaves. As synthetic antioxidants have side effects, search for natural source of antioxidants has gained attention recently. The study has proved that *Punica granatum* leaves are rich in antioxidants. Therefore it has the potential to be used as an alternate for synthetic antioxidants. The present study demonstrated that the evaluation of *in vitro* antioxidant of ethanolic leaf extract of *Punica granatum* exhibited anti oxidant activity and the results are compared with the standard ascorbic acid. The active compounds such as steroids, terpenoids, tannins, flavonoids, saponins have properties of promoting Antioxidant activity. Further work can be carried out to isolate the compounds and screen for their biological activities. Further studies (including the analysis and identification of the specific active compounds, toxicological and haematological studies) with this plant extract should be carried out using higher animal models, in order to authenticate it as a potent antiemetic agent. These local ethno medical preparations of plant sources should be scientifically evaluated and then disseminated properly. This knowledge about the medicinal plants usage can also be extended to other fields like field of pharmacology. A large scale isolation and further spectral techniques are required to isolate and identify a particular compound responsible for scavenging free radicals.

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CONFLICT OF INTEREST

There is no conflict of interest.

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