ANALYSIS OF POMEGRANATE PEEL EXTRACT (PPE) FOR ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES

by

Miss Nutan Vishnupant Manapure
(Reg. No. 12/272)

A Thesis submitted to the
MAHATMA PHULE KRISHI VIDYAPEETH,
RAHURI - 413 722, DIST.AHMEDNAGAR,
MAHARASHTRA, INDIA

In partial fulfilment of the requirements for the degree

of

MASTER OF SCIENCE (AGRICULTURE)

in

BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY

POST GRADUATE INSTITUTE
MAHATMA PHULE KRISHI VIDYAPEETH,
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CANDIDATE'S DECLARATION

I hereby declare that this thesis or part there of has not been submitted by me or other person to any other University or Institute for a Degree or Diploma

Place : M.P.K.V., Rahuri

Date : / /2014 **(Nutan V. Manapure)**

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C E R T I F I C A T E

This is to certify that, the thesis entitled, "**Analysis of pomegranate peel extract (PPE) for antioxidant and antimicrobial properties"** submitted to the MAHATMA PHULE KRISHI VIDYAPEETH, RAHURI, DIST. AHMEDNAGAR, for the award of degree of **MASTER OF SCIENCE (AGRICULTURE)** in **BIOCHEMISTRY**, embodies the results of a bonafide research work carried out by **Miss. MANAPURE NUTAN VISHNUPANT**, under my guidance and supervision and that no part of this thesis has been submitted for any other degree, diploma or publication in other form.

The assistance and help received during the cource of this investigation and sources of literature reffered have been duly acknowledged.

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C E R T I F I C A T E

This is to certify that, the thesis entitled, " **Analysis of pomegranate peel extract (PPE) for antioxidant and antimicrobial properties"** submitted to the MAHATMA PHULE KRISHI VIDYAPEETH, RAHURI, DIST. AHMEDNAGAR, for the award of degree of **MASTER OF SCIENCE (AGRICULTURE)** in **BIOCHEMISTRY,** is a record of bonafide research work carried out **by Miss. MANAPURE NUTAN VISHNUPANT** under guidance and supervision of **Dr. R.M. NAIK,** Professor and Head,Department of Biochemistry,M.P.K.V., Rahuri and that no part of the thesis has been submitted for any other degree or diploma.

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Place: MPKV, Rahuri

Date: / /2014 *(Miss. Nutan V. Manapure)*

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LIST OF ABBREVIATION

ABTS	:	3-ethyl-benzothiazoline-6-sulfonic-acid
BHA	:	Butylated hydroxyanisole
BHT	:	Butylated hydroxyltoluene
CE	:	Catechin Equivalent
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
FRAP	:	Ferric Reducing Antioxidant Power
C.D.	:	Critical difference
^{0}C	:	Degree Celsius
et al.	:	Et alli (and other)
Fig.	:	Figure
g	:	Gram
HCl	:	Hydrochloric acid
h	:	Hour (s)
i.e.	:	That is
LDL	:	Low Density Lipoprotein
M	:	Molar
mg	:	Milligram (10^{-3} g)
min.	:	Minute (s)
ml	:	Milliliter (10^{-3} liter)
N	:	Normality
OD/A	:	Optical density/Absorbance
%	:	Per cent
S.E.	:	Standard Error
µl	:	Microliter
TBARS	:	Thiobarbituric acid reactive substances
TBHQ	:	Tert-butylhydroquinone
TE	:	Trolox Equivalent
TFC	:	total flavonoid content
TPC	:	total phenolic content
TPTZ	:	2,4,6-tripyridyl-s-triazine
viz.,	:	Namely

ABSTRACT

ANALYSIS OF POMEGRANATE PEEL EXTRACT (PPE) FOR ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY

by

Miss. Manapure Nutan Vishnupant

A candidate for the degree
of
MASTER OF SCIENCE (AGRICULTURE)
in
BIOCHEMISTRY

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2014

Research Guide	**:**	**Dr. R.M. Naik**
Discipline	**:**	**Biochemistry**

Pomegranate peels are usually discarded as waste even though a significant portion of polyphenols are often present in high concentration in the peel of the fruits. The objective of the present investigation was to establish suitable solvent(s) for extraction of phytochemicals present in the fresh and dried pomegranate peel and to assess their potential radical scavenging activity as well as their inhibitory potential against some plant pathogenic bacteria and fungi. Three solvents methanol:water (80:20), acetone:water (80:20) and ethyl acetate were used for extracting the phytochemicals. The methanol:water (80:20) extract recorded maximum content of phenols and ethyl acetate was least effective. Out of the four

varieties, the fresh peel extract of Ganesh variety recorded maximum phenol content of 66.23 mg/g when extracted with methanol and the least phenol content of 9.04 mg/g was recorded in Mridula with a ethyl acetate. The superiority of the solvent was in the order methanol>acetone>ethyl acetate. The methanol:water (80:20) was also found to be effective for extracting proanthocyanidins and maximum proanthocyanidins 4.14mg/g and 12.88mg/g were extracted from fresh and dry peel respectively of pomegranate Mridula variety. Both methanol and acetone were found to be equally effective for extracting proanthocyanidins. The content of flavonoids were also maximum in the peel extract of Ganesh when extracted with methanol, which was maximum in Ganesh 5.32mg/g where as it was minimum 2.44mg/g in Jodhpuri Red. The antioxidant activity of the dry peel extract when analysed by DPPH and FRAP assay, it revealed that the antioxidant activity of the acetone:water (80:20) extract was higher than the synthetic antioxidant BHT and it was also observed that the antioxidant activity increased in a dose dependent manner. The maximum FRAP value of 46.15 μM/g was recorded in Ganesh and least in Jodhpuri Red. The methanolic extract recorded more FRAP value, when expressed as equivalent to that of 1mM $FeSO_4$+. The antibacterial activity of the extracts were tested by disc diffusion method and it was observed that methanolic extract of fresh peel showed highest zone of inhibition when tested against

Abstract contd.... **Miss. Nutan V. Manapure**

Xanthomonas spp. It was also observed that only methanolic extract showed antifungal activity against *Fusarium oxysporum*. When the antifungal activity against *Aspergillus flavous* was tested by methanolic extract of Ganesh at different concentration it was observed that inhibition of growth increased in a dose dependent manner.

The methanol:water (80:20) was found to be the best solvent mixture for extracting the phytochemicals from pomegranate peel and the peel extract of Ganesh variety of pomegranate recorded maximum content of phenols, proanthocyanidins, flavonoids and also had more antioxidant potential.

Pages 1 to 92

1.INTRODUCTION

Pomegranate (*Punica granatum* L.) is an important fruit crop of tropical and subtropical regions. It is extensively cultivated in Iran, Spain, Egypt, Russia, France, Argentina, China, Japan, USA and in India (Patil and Karade,1996). The total area under cultivation of pomegranate in India is 113.2 thousand ha with production is about 745 metric tonns and productivity is 6.6 metric tonns. In India it is mainly grown in the states of Maharashtra, Karnataka, Gujrat, Andhra Pradesh, Madhya Pradesh, Tamil Nadu and Rajastan. Maharashtra is one of the major pomegranate growing state occopying an area of 78 thousand ha with the production of 408 metric tonns and productivity is 5.2 metric tonns (Anonymous, 2013).

Pomegranate is proved to have high antioxidant activity and good potency for cancer prevention. The edible part of the fruit contains considerable amounts of acids, sugars, vitamins, saccharide, polysaccharides, polyphenols and important minerals (Kulkarni *et al.*, 2004). During the industrial processing of pomegranate, large volumes of industrial wastes are produced, which have a wide range of nutritional values. Therefore in the recent years, the attention has been focused on the industrial byproducts of pomegranate that have a high potential of antioxidant and antimicrobial properties (Orzuaa *et al.,* 2009).

Great interest has recently been focused on the addition of polyphenols to foods and biological systems, due to their well-known abilities to scavenge free radicals, i.e. antioxidant power.

The generation of free radicals plays an important role in the progression of numerous pathological disturbances, such as atherosclerosis and brain disfunction (Gordon,1996). Several studies have reported the reduced-risk efficacy of various extracts or pure compounds from the different parts of pomegranate plant against the growth of microbial pathogens (Jayaprakash *et al.*,2006). Pomegranate (*Punica granatum*) has been known to have considerable pharmacological properties with antimicrobial, antiviral, anticancer, potent antioxidant and antimutagenic effects (Seeram *et al.*, 2005) and been used in the markets in the preparation of tinctures, juice, cosmetics and therapeutic formulae (Kim *et al.*,2002).

The by-product of pomegranate marc contains about 78% peel and 22% seeds based on wet weight. At present, most of the studies focus on the extraction and product properties of antioxidants, oil and juice from pomegranate fruits (Guo *et al.*, 2003). Pomegranate marc is normally used as cattle feeds with low value or directly disposed in the field which could cause environmental problem. Previous research has shown that pomegranate marc is a good raw material for producing natural antioxidants because of its high content of antioxidants (Qu *et al.,* 2009). In general, extraction solvent, temperature, solid-liquid ratio, and particle size are influential parameters for extraction process (Bucic-Kojic *et al.*, 2007). It has been reported that the solvents may not affect the characteristics of antioxidants, but the composition of the pomegranate extract (Kulkarni *et al.*, 2004).

Pomegranate peel contains high quantities polyphenols including ellagitannins, gallotannins, ellagic acid, catechins, anthocyanins and quercetins. Prashanth *et al.* (2001) tested a number of extracts of pomegranates against a range of bacteria *S. aureus, E.coli, Klebsiella pneumoniae, Proteus vulgaris, Bacillus subtilis and Salmonella typhi.* Pomegranate extracts were able to inhibit not only the growth of *S. aures* but also the production of enterotoxin (Braga *et al.*, 2005).

Some studies reveal that pomegranate peel extract inhibits the growth of certain microorganism. For example, *P. granatum* peel ethanol extract acts as an antibacterial agent against a number of *Salmonella* serotype strains *in vitro* and also inhibits the growth of *Salmonella typhimurium* in *in vivo* tests, reducing mouse mortality (Choi *et al.*, 2011). Other studies reveal that how pomegranate peel extract possesses antibacterial qualities (Ibrahium, 2010).

The use of synthetic antioxidants in food has been decreased due to their suspected action as promoters of carcinogenesis, as well for the general consumer rejection of synthetic food additives (Namiki,1990). Several studies indicated that the use of synthetic antioxidants has been restricted because of their health risks and toxicity and therefore the importance of replacing synthetic antioxidants with natural ingredients from spices and other plant materials has greatly increased. Pomegranate is one of the important dietary source of antioxidant phenolics (Ozgen *et al.*, 2008). Pomegranate peel is recognized for its many health-promoting qualities and apparent

wound-healing properties (Chidambara *et al.*, 2004), antimicrobial activity (Braga *et al.*, 2005), anticancer property (Jeune *et al.*, 2005), antiatherosclerotic and antioxidative capacities (Tzulker *et al.*, 2007). This antioxidant capacity has been mainly attributed to the water-soluble polyphenols, anthocyanins and hydrolysable tannins.

Antioxidants have an important role in body's health. Recently, a series of studies had demonstrated that there is a positive correlation between prevention of many cancers and the amounts of absorption and consumption of vegetables and fruits that contain natural antioxidants (Casanova and Garcia-Mina, 2008).

Hydrolyzable tannins are found in the peels, membranes and piths of the fruit. Hydrolysable tannins are predominant polyphenols found in pomegranate juice and accounts for 97% of its antioxidant activity (Dahham *et al.*, 2010).

Extraction of antioxidants from pomegranate peel, using a mixture of ethanol, methanol and acetone and the antioxidant properties of the extract were investigated. It was found that pomegranate peels contain tannins, anthocynins, flavonoides, pectins (Nozire and Serpil, 1993).

Extraction is a key step for obtaining antioxidants with an acceptable yield. Solvent extraction is more frequently used for the isolation of antioxidants and the extraction yield and economic viability is dependent on the type of solvent and method of extraction, mostly due to the differing polarity of these

compounds. Several extraction techniques have been reported for the extraction of phenolic compounds from different matrices using solvents with different polarities, such as methanol, water, ethyl acetate and petrolium ether (Cheung *et al.*, 2003).

Nowadays, uncontrolled and frequent use of antibiotics may cause emergence of microbial resistance among pathogenic agents. Therefore, the use of new synthetic and natural antimicrobial compounds is inevitable. Methanolic extracts of sour and sweet pomegranate peels showed bactericidal effect and sour pomegranate peel extract was more than that for sweet pomegranate peel extract. The antibacterial effect was greater against gram-positive bacteria compared to that for the gram-negative bacteria. The methanolic extracts of pomegranate peels exhibited relatively good bacteriostatic and bactericidal effects. The antibacterial activity of ethanol extracts of *Punica granatum* fruit peels on *P. syringae* pv. tomato was studied both *in vitro* and *in vivo*. The minimum in vitro inhibition value for peel extract of *P. granatum* on the *P. syringae* pv. tomato Pt 1301 strain (from Turkey) was obtained at a concentration of 0.5%. The *in vivo* antibacterial action of this natural substance lasted at least 15 days, permitting the replacement or reduction of the commonly used copper compounds. The active components of the pomegranate peel extracts were ellagic and gallic acids (Quattrucci *et al.*, 2013).

In novelty of applying pomegranate peel extract (PPE) as an alternative, reduced-risk antifungal agent for controlling citrus green mould invasion has been reported. Preliminary

results indicated that the most potent antifungal PPEs against *Penicillium digitatum* isolates were those extracted with methanol, ethanol and water (Tayel *et al.*, 2009).

The present investigation entitled "Analysis of pomegranate peel extract (PPE) for antioxidant and antimicrobial properties" was therefore proposed with the following specific objectives.

1. To estimate phenols, tannins, flavonoids and antioxidant activity of the pomegranate peel extract.
2. To determine antimicrobial activity against plant pathogenic microorganisms.
3. To study the interaction effects of extraction method and concentration on antioxidant and antimicrobial activity.

2. REVIEW OF LITERATURE

Punica granatum L., commonly called as pomegranate, recently described as nature's power fruit. It has been reported that consumption of pomegranate fruits has nutritional and medical benefits, including reduced oxidative stress, atherogenic modifications to LDL and platelet aggregation as well as anticancer, antibacterial and antiviral activities. Pomegranate is an important source of anthocyanins, hydrolysable tannins punicalagin and punicalin, ellagic and gallic acids and also contains vitamin C

Prashanth *et al.*, (2001) tested a number of extracts of pomegranates against a range of bacteria *S. aureus, E.coli, Klebsiella pneumoniae, Proteus vulgaris, Bacillus subtilis and Salmonella typhi.* Pomegranate extracts were able to inhibit not only the growth of *S. aures* but also the production of enterotoxin (Braga *et al.,* 2005).

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The literature relevant to the present investigation entitled "Analysis of pomegranate peel extract for antioxidant and antimicrobial properties" has been reviewed in this chapter is grouped into the following sections

2.1 Pomegranate composition

2.2 Phytochemicals in pomegranate peel extract

2.3 Antioxidants

2.4 Measurment of antioxidant activity

2.5 Antioxidative properties of pomegranate peel extract

2.6 Antimicrobial activity

2.1 Pomegranate composition

According to El-Nemr *et al.,* (1990) edible portion of pomegranate fruit represents on average 52% (w/w) of total fruit, comprising 78% juice and 22% seeds. The fresh juice contains 85.4% water (w/v), 10.6% (w/v) total sugars, 1.4% (w/v) pectin, 0.1% (w/v) acid (expressed as citric acid) and 0.7 mg ascorbic acid, 19.6 mg free amino nitrogen and 50 mg ash per 100 ml. The seeds are rich source of total lipids, protein, crude fiber and ash representing 27.2, 13.2, 35.3 and 2.0% (w/w), respectively. Pomegranate seeds also contain 6.0% (w/w) pectin and 4.7% (w/w) total carbohydrates. The iron, copper, sodium, magnesium and zinc contents of the juice are lower than those of seeds, except potassium, of which 49.2 ppm is found in the juice.

The pomegranate, *Punica granatum*, an ancient, mystical and highly distinctive fruit, is the predominant member of Punicaceae family. The pomegranate tree typically grows 12-16 feet, has many spiny branches. The ripe pomegranate fruit can be up to five inches wide with a deep red, leathery skin, is grenade-shaped, and crowned by the pointed calyx. The fruit contains many seeds (arils) separated by white, membranous

pericarp and each is surrounded by small amounts of tart and red juice (Naqvi *et al.*,1991).

Pomegranate fruit contains several very potent antioxidants. Pomegranate juice (PJ), prepared from squeezing whole fruit, contains several unique polyphenolics and sugars. Pomegranate soluble polyphenols contain hydrolysable tannins such as the ellagitannin punicalagin, gallic and ellagic acids as well as anthocyanins and catechins (NarrBen *et al.*, 1998).

Since the whole fruit extract contains the components of both peel and seeds, it was found to contain more constituents. It would thus mean that, the whole fruit extract had the highest number of bioactive compounds. Since the yield of bioactive metabolites in a plant extract also varies considerably with the method/solvent of extraction (Marston *et al.,* 1993 and Clark *et al.*, 1997).

The pomegranate has been an inexhaustible source of research going from chemistry in the search for new compounds, techniques of production and conservation, biotechnology in search of more profitable varities, to the biological effect as anti-microbial, antioxidant, anti-inflammatory, anticancer, anti-diabetic, among other applications (Gross and Gross, 2009).

Extraction process is sensitive to the extraction time and temperature in the early stage (Pekic *et al.*, 1998). Factors that have been atributed to bringing variation include the method of extraction (Negi and Jayaprakash, 2003), mixture of different solvents (Li *et al.,* 2006), and use of different materials (Jayprakash *et al.,* 2006) among others. A possible factor for

higher content of phenolics, proanthocyanidins and flavonoids (%) in extract (g phenols/100 g dried extract) is due to the higher purity of extract associated with using ethyl acetate. The use of methanol, ethanol, acetone and water generally yields a significant co-extraction of concomitant substances and decreases the yield of target antioxidants (Pekic *et al.*, 1998).

Results for the total extract yields reported as percentage of g of extract per 100g pomegranate peel on dry basis indicated that the pomegranate peel extracted with methanol gave the highest total extract yield, followed by water, ethanol, acetone and ethyl acetate when the extraction were done with the ratio of solvent/sample of 15:1 (w/w) at 40^0 C for 4 h. It should be noted that, because of polarity differences between solvents, the solubility of the solute into the solvent is expected to be different. Water, methanol and ethanol are polar protic solvents of dielectric constants of 80, 33 and 24 respectively, while acetone and ethyl acetate are polar aprotic and non-polar solvents of dielectric constants of 21 and 6 respectively. It has been reported that pomegranate peel extract yield (%,w/w) were 9.38, 7.53 and 1.04 for methanol, water and ethyl acetate respectively under the following experimental conditions; peel powder (25g) extraction by mixing using a magnetic stirrer with 100 ml of the corresponding solvents at 30^0 C for 1 h, filtration through Whatman no. 41, residue re-extraction with the same solvent, extract pooling and concentration under vaccum at 40^0 C (Singh *et al.,* 2002).

The Fe content of pomegranate peels was 60.5 ± 2 ppm, which was highest after Na and K value. Iron is an important trace element in the human body. It plays crucial roles in haemopoiesis, control of infection and cell mediated immunity (Beared *et al.* 2001 and Bhaskaram, 2001). The Mn and Zn content were calculated 4.5 ± 0.8 ppm and 4 ± 0.65 ppm respectively. Zinc is an essential micronutrient for human growth and immune function (Brown and Wuehler, 2000 and Black, 2003).

The main active constituents in pomegranate extract are tannins and alkaloids. The main phytochemical constituents in the peel of *P. granatum* are gallotannins, ellagic acid derivatives, catechins and procyanidins and flavonols (Seeram *et al.*, 2006).

Values for minimum inhibition concentration (MIC) have been reported in a number of studies, ranging from 0.62 to 10 mg/ml against *S. aureus, E. coli* and *P. aeruginosa (*Navarro *et al.*, 1996) and up to 250 mg/L against *S.aureus* (Machado *et al.,* 2003). This differences could be due to the extraction method, freshness of the fruit, season and region of growth (McCarrelln *et al.*, 2008).

Siri *et al.,* (2008) found that both water and ethanol extracts of pomegranate fruit peels showed anti-*Aeromonas caviae* activity with more effectiveness in water extracted samples. In contrast, Al-Zoreky (2009) reported that only water-methanol extract of peels have marked inhibition (12-20 mm inhibition zones) and the water extract was inactive against

eleven microorganisms tested, such as *S. aureus* (2 strains), *B. subtilis, E. coli, Listeria monocytogenes, Pseudomonas aeruginosa, Klesiella pneumoniae, Yersinia enterocolitica, candida utilis, Saccharomyces cervisiae* and *Aspergillus niger.*

The interaction between material type and solvent type on antioxidant activity and phenolic content was significant. Aqueous extract of pomegranate leaves was the lowest amount of this trait, while methanolic extract of pomegranate peel had the highest antioxidant activity (85.4%) and phenolic content (423.5 mg/g) in comparision with other extracts (Tehranifar *et al.*, 2011).

2.2 Phytochemicals in pomegranate peel extract

The different types of phytochemicals that have been identified from various parts of the pomegranate tree and from pomegranate fruits and seeds. The major class of pomegranate phytochemical is the polyphenols that predominate in the fruit. Pomegranate polyphenols include flavonoids, condensed tannins and hydrolysable tannins (Dahham *et al.*, 2010).

2.2.1 Phenols

Pomegranate peel contains substantial amounts of polyphenols such as ellagic tannins, ellagic acid (Negi and Jayaprakash, 2003).

Generally, PPE exhibit good antioxidant capacity and is effective scavenger of several reactive oxygen species, due to its high levels of polyphenolic compounds (Kulkarni and Aradhdhya, 2005).

The pomegranate primarily peel extracts were the most powerful antioxidants, whereas pomegranate arils were much less potent, indicating that the peel polyphenols are the major contributors to pomegranate juice antioxidative capacity (Ricci *et al.* and 2006 Zhang *et al.*, 2007). The polyphenol content and the antibacterial effects of these polyphenolic compounds have been reported by several studies in recent years (Haslam, 1996; Machado *et al.*, 2002 and Naz *et al.*, 2007).

The antioxidant constituents of pomegranate mainly comprise the compounds with phenolic hydroxyl groups and double bonds including tannins, flavonoids and unsaturated fatty acids. Significant and positive linear correlations were found between total antioxidant capacities and phenolic contents, indicating that phenolics are the dominant antioxidant constituents of pomegranate (Surweswaran *et al.*, 2007).

2.2.2 Flavonoids

Flavonoids also make a great contribution to the antioxidant activity of pomegranate due to their effect in free radicals elimination (Wang *et al.*, 2006b and Suo *et al.*, 2009).

Flavonoids isolated from promegranate include flavones, flavonols, anthocyanidins and flavon-3-ols. The brilient colour of pericarp and juice are attributed to anthocyanidins and flavon-3-ols, of which the content decrease or increase with the time of ripening. Anthocyanidins reported in pomegranate usually present in the form of glycocide with aglycon of delphinidin, cyanidin and pelargonidin, while flavon-3-ols found

in this plant only present in the unglycocylated form including catechin, epicatechin, epigallocatechin and their derivatives. Flavons and flavonols constitute the major flavonoid of pericarp and leaves, which frequently exist as glycosides with aglycons of luteolin, kaempferol, quercetin, apigenin and naringin. (Wang *et al.*, 2010).

2.2.3 Tannins

Hydrolysable tannins of diverse structures including ellagic tannins and gallotannins constitute the most prevalent compounds presenting in various parts of pomegranate. Condensed tannins, however, are rarely found in this plant. Ellagitannins are mainly found in the pericarp, bark, seeds and flowers (Tanaka *et al.*1986 and Wang *et al.* 2004, 2006a). For example, punicalin and punicalagin are the major constituents of pericarp and also exist in the bark; however, they are practically undetected in the leaves. This kind of compounds is the darivatives of ellgic acid which is abundant in every part of pomegranate and its content can be as high as 0.1% in the flowers and up to 0.2% in the pericarp and leaves (Wang *et al.*, 2006b).

Gallotannins which are mostly found in the leaves and seldom reported in other parts of pomegranate, usually consist of a couple of galloyl groups and, therefore, can be considered as the darivatives of gallic acids (Li *et al.*, 2002).

2.2.4 Organic acids

The seeds are rich in unsaturated fatty acids including punicic acid, linoleic acid, oleic acid, palmitic acid, stearic acid, and linolenic acid, of the total content account for 15.26% of the weight of seeds (Li *et al.*, 1994; Schubert, 1999 and Wang *et al* 1999). The juice mainly contains straight chain fatty acids, of which citric acid and mallic acid are the major compounds with the content of up to 4.85 and 1.75 g/L, respectively. In addition, tartaric acid, oxalic acid and succinic acid were also found in the juice (Poyrazoglu *et al.,* 2002). Phenolic acids consisting of caffeic acid, fumalic acid, chlorogenic acid and *p*-coumaric acid usually present in the juice and pericarp (Artik 1998 and Amakura *et al.,* 2000).

2.3 Antioxidants

The antioxidant activity of the pomegranate fruit peel extract were investigated as compared with the pulp extract. The content of total phenolics, flavonoids, pro-anthocyanidins and ascorbic acid were also measured. The result showed that, pomegranate fruit peel extract had markedly higher antioxidant capacity than the pulp extract in scavenging or preventive capacity again superoxide anion, hydroxyl and peroxyl radicals as well as inhibiting $CuSO_4$ induced LDL oxidation (Li *et al.*, 2006).

Antioxidant is molecule capable of inhibiting the oxidation of others molecule. Oxidation is known as a chemical reaction that transfers the electron from a substance to an oxidizing agent. From the oxidation reaction, it can produce a free radical. This radical can initiate the chain of reaction that

tends to destroy the cell. The role of antioxidant is to terminate this chain of reaction through the removal of free radical intermidiats and antioxidant also inhibits other oxidation reaction. It is done by oxidized themselves. So that is why, it often act as reducing agents such as ascorbic acid, polyphenols or triols.

2.3.1 Natural antioxidants

During the last one hundred years the importance of antioxidants of oxidative degeneration of food products during processing, preservation and storage for a reasonable period of time was well noticed. According to Wojdylo *et al.* (2007), that classified the medicinal plant into five categories of FRAP Assay activity such as very low, low, good, high and very high. Sample that consists of FRAP value (<10 μM/100g) considered as very low, (10-50 M/100g) considered as low, (50-100 μM/100g) considered as good, (100-500 μM/100g) considered as high and (>500 μM/100g) considered as very high. It was found that Vitamins C and E were not produced in the human bodies and should be obtained from different sources. Other three antioxidants are produced by the human body at inadequate levels particularly during the process of aging. It was thus rightly concluded that all these antioxidants are to be supplemented (Mousavinejad *et al.*,2009).

The common natural antioxidants are vitamin E or known as tocopherol, vitamin C or known as ascorbic acid,

carotinoids and phenolic compounds. All of these natural antioxidants can be obtained in some fruits and vegetables.

2.3.2 Synthetic antioxidants

Nowadays, the synthetic antioxidant is widely used in order to extent the shelf life of food products. The synthetic antioxidant that are usually used in food industries such as Butylated hydroxytoluene (BHT), Butylated hydroxyanisole (BHA), Prophyl gallate (PG), Tert-butylhydroquinon (TBHQ) and many more. Synthetic antioxidant is lipophilic (fat-soluble) organic compound that is primarily used as an antioxidant food adititives as well as antioxidant in cosmetics, pharmaceutical, jet fuel, petroleum products, electrical transformer oil and embalming fluid (Peterson *et al.*, 2006). The synthetic antioxidant behaves as a synthetic analogue of vitamin E, which is primarily acting as a terminating agent that suppresses antioxidation, a process whereby unsaturated organic compounds are attacked by atmospheric oxygen. It stops this autocatalytic reaction by converting peroxy radicals to hydroperoxides (Bondet *et al.*, 1997).

2.3.3 Source of antioxidant

Sources of antioxidant include vitamin E, vitamin C and beta-carotene. These can all be found in many fruits such as elderberries and vegetables. In 2007, the United States Department of Agriculture looked at the oxygen radical absorbance capacity (ORAC) or overall antioxidant capability of

various berries. They studied the antioxidant capacity of various berries. They studied the antioxidant capacity of certain berries in term of how effective they were at attacking these damaging free radicals. The higher the figure obtained the more antioxidant capacity the berries had. Table below is the result for ORAC of selected fruits.

Table -The ORAC (mg TE/100g) and total phenolic content (mg GAE/100g) values of various berries

Fruit	ORAC mmol TE/100g			Total phenolic (mg GAE/100g)
	Hydrophilic ORAC	Lipophilic ORAC	Total ORAC	
Blackberry	5245	103	5347	660
Blueberry	6520	36	6552	531
Cranberry	9382	202	9584	718
Raspberry	4745	138	4882	502
strawberry	3541	36	3577	368

Source: United States Department of Agriculture Oxygen Radical Absorbance Capacity (ORAC) of selected foods,(2007).

2.3.4 Health benifits of antioxidant

Antioxidants are anti-aging. This means staying younger longer with better health. The benifits of antioxidant from food or a good quality of natural antioxidant supplement

will give the anti-aging which is protection against degenerative disease and help to slow down the aging process of skin, cell, tissues and organs. Antioxidants are the naturally occurring nutrients found in certain fruits and vegetables. They have been proven that antioxidant can protect human cells from oxidative damage and provide anti-aging of cells and overall body, greatly reduced incidence of all cancers and molecular degeneration prevention and resistance to flues, viruses and infections.

2.3.5 Agrowaste as antioxidant

Agro waste such as pomegranate peel, apple peel and citrus peel have been analysed for yield of extract, total phenolic content, total flavonoid content and DPPH remaining.

Table- Yield of extract (g/100g of dry matter), total phenolic contents (g GAE/100g dry matter), total flavonoid contents (g CE/100g dry matter) and present DPPH remaining.

Agro waste	Yield	TPC	TFC	DPPH
Pomegranate peel	29.9 ±0.8	36.4 ±0.7	4.89 ±0.15	11.2 ±0.8
Apple peel	16.4 ±0.9	3.9 ±0.6	2.21 ±0.13	15.6 ±0.5
Banana peel	24.6 ±0.4	1.1 ±0.3	0.62 ±0.03	23.6 ±1.8
Citrus peel	21.5 ±0.5	2.4 ±0.3	1.92 ±0.12	21.8 ±1.4

2.4 Measurment of antioxidant activity

Antioxidant activity cannot be measured directly but rather by the effects of the antioxidant in controlling the extent of oxidation. Methods show extreme diversity. Some methods Involve a distinct oxidation step followed by measurement of the outcome as, for example, oxidation of linoleic acid followed by determination of diene conjugation. In other instances, there is no clear distinction between the various steps in the procedure. The features of an oxidation are a substrate, 43 an oxidant and an initiator, intermediates and final products and measurement of any one of these can be used to assess antioxidant activity.

2.4.1 DPPH (Diphenylpicrylhydrazyl radical)

The DPPH radical absorbs at 517 nm and in a second substrate-free system, antioxidant activity can be determined by monitoring the decrease in this absorbance. Results are reported as the EC_{50}, that is, the amount of antioxidant necessary to decrease by 50% the initial DPPH concentration.

Phenolic compounds generally exhibited significant scavenging effects against the DPPH free radical. DPPH reduction has been compared with other methods including the ABTS assay, superoxide-anion scavenging and lipid oxidation. The antioxidant activity of pomegranate juices was evaluated by DPPH and ABTS and the results were compared with those of red wine and tea infusions. Hydrolysable tannins accounted for the high activity of juices. The antioxidant activity of plant

biophenols has been attributed to trapping of ROS and regeneration of endogenous membrane bound a-tocopherol. The phenols form *o*-quinone intermediates upon H-atom abstraction from DPPH and subsequent radical disproportionation (Silva *et al.*, 2000).

2.4.2 FRAP assay

The ferric reducing antioxidant power (FRAP) method is based on the reduction of a ferroin analog, the Fe^{3+} complex of tripyridyltriazine $Fe(TPTZ)^{3+}$, to the intensely blue coloured Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants in acidic medium. Results are obtained as absorbance increases at 593 nm and can be expressed as micromolar Fe^{2+} equivalents or relative to an antioxidant standard. The authors claim the method to be simple and rapid and both manual and automated procedures have been described. We are in agreement with Frankel and Meyer, however, that the measured reducing capacity does not necessarily reflect antioxidant activity. It provides instead a very useful 'total' antioxidant concentration, without measurement and summation of the concentration of all antioxidants involved. The method was originally applied to plasma but has been extended to other biological fluids, foods, plant extracts, juices, *etc.*(Benzie and Strain, 1999)

2.4.3 ABTS assay

The procedure based on inhibition of the production of the ABTS radical cation did not involve a substrate. ABTS with

an absorption maximum at 342 nm has high water solubility and chemical stability. It is a peroxidase substrate which, when oxidized in the presence of H_2O_2 generates a metastable radical cation with a characteristic absorption spectrum and high molar absorptivity at 414 nm. The accumulation of $ABTS^{4+}$ can be inhibited by the presence of an antioxidant in the reaction medium, to an extent and on a time scale dependent on the antioxidant activity. The relative ability of hydrogen-donating antioxidants to scavenge $ABTS^{4+}$ generated in the aqueous phase, can be measured spectrophotometrically, by measurement in the near-infrared region at 734 nm.

The results were expressed by comparison with standard amounts of the synthetic antioxidant trolox (a water-soluble vitamin E analogue) to give rise to the TEAC (Arnao *et al.*,1996).

2.4.4 Thiobarbituric acid reactive substances (TBARS) assay

The TBARS assay was proposed over 40 years ago and is now the most commonly used method to detect lipid oxidation. This procedure measures the MDA formed as the split product of an endoperoxide of unsaturated fatty acids resulting from oxidation of a lipid substrate. It is postulated that the formation of MDA from fatty acids with less than three double bonds (*e.g.*, linoleic acid) occurs *via* the secondary oxidation of primar carbonyl compounds (*e.g.*, non-2-enal).135 The MDA is reacted with thiobarbituric acid (TBA) to form a pink pigment (TBARS) (Fig. 1) that is measured spectrophotometrically at its absorption

maximum of 532–535 nm (Guillensans and Guzmanchozas, 1998).

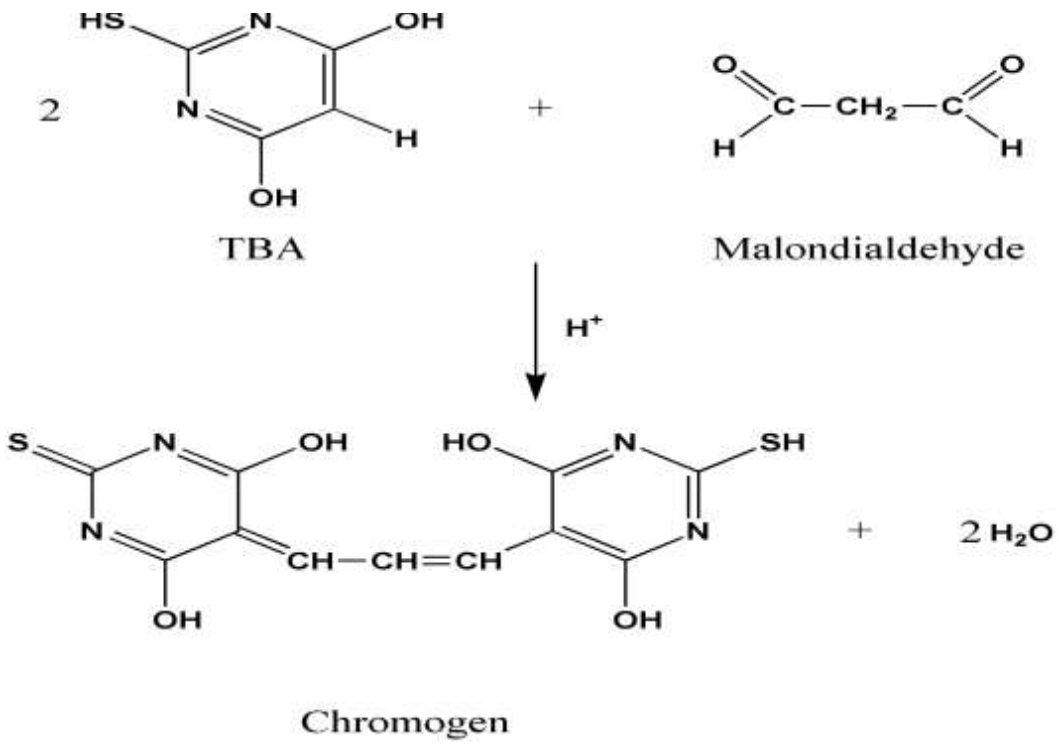

Fig.1 Chromophore formed by condensation of MDA with TBA.

2.5 Antioxidative properties of pomegranate peel extract

The extraction yield of antioxidant compounds from plant material is influenced mainly by the conditions under which the process of liquid-solid extraction is carried out to separate a soluble fraction from a permeable solid (Singh *et al.,* 2002).

It is known that antioxidants are mainly constituted by phenolic compounds and have strong potential in scavenging free radicals (Gil and Tomas, 2000 and Noda *et al.*, 2002). The potential of scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH)

radical has been widely used to evaluate the activity of antioxidants (Awika *et al.*, 2003).

It was shown that the antioxidant activities of punicalagin, ellagic acid and total pomegranate tannin extract are enhanced when present in combination with other polyphenols, as exists in pomegranate juice (Seeram *et al.*, 2005).

Recently, it was shown that the antioxidative action of punicalagin, a major hydrolyzable tannin polyphenolic antioxidant, is the result of its free radicals scavenging capacity, as well its action as a metal chelator (Kulkarni *et al.*, 2007).

Two tannins, namely, ellagic acid and punicalagin are considered to play an important role in the antioxidant activity of tannins in pomegranate (Sestili *et al.*, 2007).

The antioxidant activity was almost determined *in vitro* conditions and several methods could be used for its determination (Stangeland *et al.*, 2009). Generally pomegranate possessed the best antioxidant activity, independent on the antioxidant test assayed and generally with significant linear correlation between phenolics concentration and antioxidant capacity (Elfalleh *et al.,* 2009).

By-product from fruit processing may be an economic source of natural antioxidants replacing easily the synthetic antioxidants. Without cooking, pomegranate rind powder revealed also to be also a good antioxidant on the preservation of raw ground goat meat during refrigerated storage (Devatkal and Naveena, 2010).

2.4 Antimicrobial activity

2.4.1 Antibacterial activity

Prashanth *et al.* (2001) reported that methanol extracts of peel were more active than water extracts against *E. coli, S. aureus* and *B. Subtilies.*

The polyphenol content and the antibacterial effects of these polyphenolic compounds have been reported by several studies in recent years (Haslam, 1996; Machado et al., 2002 and Naz *et al.*, 2007). Effect of tannins on bacterial metabolism are identified by their effect on bacterial membrane, because tannins can pass through cell walls, which contain polysaccharides and proteins and bind to their surface, preventing their normal activity (Scalbert, 1991; Chung *et al.*, 1993 and Vasconcelos *et al.*, 2006).

Nascimento *et al.*, (2000) showed that pomegranate extract inhibited *Pseudomonas aeroginosa* growth and has a synergistic effect against bacteria resistant to the known antibiotics.

Besides the *Helicobacter pylori*, *Escherichia coli, Salmonella typhi* and microorganisms of *shigella,* the extract of pomegranate also exhibit significant inhibiting effect against the common pathogenic bacteria especilly Gram positive pathogens. It was reported that both methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) strains of *Staphyloccocus aureus* were susceptible to the extract of pericarp or fruit and the subsequent enterotoxin production was inhibited by these

extracts, indicating the therapeutic use of pomegranate for bacterial infection (Machado *et al.*, 2003 and Braga *et al.*, 2005).

Vasconcelos *et al.,* (2003) and Singh *et al.,* (2002) also reported that extracts of Punica granatum peel in different concentrations were effective against *S. epidermis, S. aureus, S. mutans, S. sanguinis* and *S. salivarius.* It is demonstreted that this antibacterial activity may be related to the presence of hydrolysable tannins and polyphenolics in the pomegranate extract specifically punicalagin and gallic acid (Vasconcelos *et al.*, 2006 and Reddy *et al.*, 2007). It means that the antimicrobial effect of tannins is related to its toxicity and molecular structure. Tannins may act on the cell wall and across the cell membrane because they can precipitate proteins (Vascaoncelos *et al.*,2003; Vascaoncelos *et al.*, 2006). They may also suppress many enzymes such as gycosyltransferases (Vascaoncelos *et al.*, 2003).

Synergistic effects of methanolic pomegranate extract and chloramphenicol, gentamicin, ampicilin, tetracycline and oxacillin against *Staphylococcus aureus* are also reported by Braga *et al.*, (2005b).

Melendez and Capriles (2006) found that methanol extract of pomegranate fruit was active against *E. coli*, *S. aureus* and *B. subtilis* with the diameter of inhibition zone 12, 22, and 12 mm, respectively.

Reddy *et al.* (2007) and Naz *et al.* (2007) demonstrated that gallic acid (a tannic acid) has the highest antibacterial effect against tested sensitive strains even at low concentrations. Hence, the antibacterial activity of *Punica granatum* may be

related to polyphenol structures because polyphenols may affect the bacterial cell wall, inhibit enzymes by oxidized agents, interact with proteins and disturb co-aggregation of micro-organisms (Naz *et al.*, 2007 and Vasconcelos *et al.*, 2003).

Naz *et al.* (2007) isolated various compounds from methanolic extract of pomegranate fruit and showed that pomegranate's phenolic compounds, especially gallic acid, exert certain antibactrial effects against *coryne-bacterium, Staphylococcus, Streptococcus, Bacillus subtilis, Shigella, Salmonella, Escherichia* and *Vibrio* species.

The *in vivo* antibacterial activity of this natural substance continued to be effective for at least 15 days, offering the possibility of replacing, reducing or even alternating treatments involving copper compounds. The identification and characterization of the *P. granatum* active compounds and a better understanding of their active mechanisms might contribute to a better utilization of them either alone or in combination with other natural extracts. Study investigated the presence and the content of total phenolic substances and phenolic acids such as ellagic and gallic acids, which are known to have antibacterial effects (Arulmozhi and Mirunalini, 2010).

The plant extract tested proved useful for effective biocontrol of *P. syringae pv. tomato* on tomato plants. The *P. granatum* peel extract successfully reduced the disease incidence and disease severity caused by a highly virulent strain of *P. syringae pv. tomato* and no negative (phytotoxic) effects were recorded on the tomato plants tested (Quattrucci *et al.*,2013).

2.4.2 Antifungal activity

According to the type of tested microorganism, the reports about antifungal properties of the pomegranate peel extract are various. For example, this extract can prevent the growth of *Penicillium citrinum* and *Aspergillus ochraceus* for 8 and 3 days respectively. In some cases in China the peel extract of pomegranate is used as a fungicide (Seeram *et al.*, 2006).

Vasconcelos *et al.* showed that *punica granatum* may be used as a topical antifungal drug against *C. albicans* in two reports. The real mechanism of the antifungal effect of tannins (the major components of punica granatum extract) against *candida albicans* is not clear; however, it may be related to their toxicity, astringent, molecular structure or other ways (Vasconcelos *et al.,* 2006).

Pomegranate peel extract showed various degrees of inhibition against the growth of investigated microorganisms (Ibrahium, 2010). The antibacterial and antifungal activities of pomegranate peel extract (rind), seed extract, juice and whole fruit on the selected bacteria and fungi. The peel extract has shown highest antimicrobial activity compared to other extracts (Dahham *et al.*, 2010).

The methanolic peel extract has shown highest antimicrobial activity compared to aquas extracts. Staphylococcus aureus and among fungi high activity against *Aspergillus niger* was recorded (Saad *et al.*, 2010).

The methanolic extract showed the highest inhibitory effects on the mycelia growth and spore germination with 49.82 and 41.25%, respectively. On the other hand, peel and seed extracts had more inhibitory effect than leaf extract. The phenolic content of peel extract were also measured 2.8 fold higher than pomegranate leaf extract and antioxidant capacity of peel, seed and leaf extracts of pomegranate were 55.3, 35.7 and 16.4%, respectively. Therefore, it seems that the high percentage of phenolic content in the peel and seed of pomegranate could cause the high antifungal and antioxidant activity of there extracts (Tehranifar *et al.*, 2011).

2.4.3 Antiviral activity

Polyphenols espesially tannins of pomegranate play a key role in the antiviral effect because of their special property of protein precipitation which adversely affect the enzymes involved in the life cycle of virus. (Zhang *et al.*,1995 and Zhang *et al.,* 1997) reported that the aqueous extract of pericarp containing high content of tannins significantly inactivated HSV-2 and HBV via inhibiting the DNA polymerase in a does dependent manner in *in vitro.*

Four major polyphenols in pomegranate, namely ellagic acid, caffeic acid, luteolin and punicalagin were evaluated for anti-influenza effect and the result demonstreted that punicalagin is the most effective one. Punicalagin blocked replication of the virus RNA, inhibited agglutination of chicken RBC induced by virus and had a virucidal effect (Haidari *et al.*, 2009).

3. MATERIAL AND METHODS

The material required and methods adopted during the present investigations entitled "Analysis of pomegranate peel extract for antioxidant and antimicrobial properties" are described under here.

3.1 Material

3.1.1 Fruits

The pomegranate fruits of three cultivars *viz.*, Ganesh, Bhagawa and Mridula and one wild germplasm *viz.*, Jodhpuri Red used in this study were obtained from the Horticultural farm, MPKV, Rahuri.

3.1.2 Pathogens

The following bacterial strains and fungal cultures available in the Department of Plant Pathology and Microbiology, MPKV, Rahuri were used.

Bacteria : 1) *Xanthomonas axonopodis pv. Punicae* – causal organism of oily spot of pomegranate

2) *Xanthomonas axonopodis pv. Malvacearum*- causal organism of bacterial blight of cotton

3) *Xanthomonas axonopodis pv. Citri* - causal organism of citrus cancker

Fungi : 1) *Aspergillus flavous* – produces aflatoxins in groundnut seed

2) *Fusarium oxysporum* f.sp. *ciceri* – causal organism of wilt of gram

3.1.3 Chemicals

Most of chemicals used in this study were of analytical grade and procured from Himedia, SRL and S.D. Fine Chemicals, Mumbai.

3.2 Methods

The peel of fruits were manually removed, shade dried and powdered in grinder to 40-mesh. Three different solvents *viz.* Methanol:Water (80:20), Acetone:Water (80:20) and Ethyl acetate were used for extraction. Dried powder of peels (15 g) were extracted with100 mL of each solvent at room temperature for 1 hour. The extract was filtered through Whatman No.42 filter paper to remove fine particles. The residue was re-extracted with the same solvent and the extracts were added to each other. After extraction, the solvent was evaporated on a rotary evaporator under vacuum and at 30^0 C upto 20 ml and the concentrated extracts were stored at -20^0 C until use. The extract were used for estimation of phytochemicals, total phenols, proanthocyanidins, flavonoids and antioxidant activity both by DPPH and FRAP assay. The antimicrobial activity of the extract were tested against plant pathogenic fungi (*Fusarium oxysporum* and *Aspergillus flavous*) and bacteria (*Xanthmonas* sp.). The interaction effect of extraction method and concentration were evaluated. The same procedure was followed for fresh peel of pomegranate fruits of same cultivars and wild germplasm.

3.2.1 Phytochemical analysis

The extracts were tested by standard qualitative tests for the presence of different phytochemicals as described by Trease and Evans, (1989).

a) Test for tannins

To 0.5 ml of extract solution, 1 ml of distilled water and 1-2 drops of ferric chloride solution were added and observed for brownish green or a blue black coloration.

b) Test for terpenoids

Five ml of extract was mixed with 2 ml of $CHCl_3$ in a test tube. 3 ml of concentrated H_2SO_4 was carefully added to the mixture to form a layer. An interface with a reddish brown coloration was formed for the presence of terpenoids.

c) Test for saponins

Five ml of extract was shaken vigorously to obtain a stable persistent froth. The frothing was then mixed with 3 drops of olive oil and observed for the formation of emulsion, which indicated the presence of saponins.

d) Test for flavonoids

A few drops of 1% NH_3 solution was added to the extract in a test tube. A yellow coloration was observed for the presence of flavonoids.

e) Test for alkaloids

One ml of 1% HCl was added to 3ml of extract in a test tube and was treated with few drop of Meyer's reagent. A creamy white precipitate indicted the presence of alkaloids.

f) Test for glycosides

Ten ml of 50% H_2SO_4 was added to 1ml of extract in a boiling tube. The mixture was heated in boiling water for 5min. Ten ml of Fehling's solution (5ml of each solution A and B) was added and boiled. A brick red precipitate indicated presence of glycosides.

3.2.2 Estimation of total phenols

Estimation of total phenols was carried out with Folin Ciocalteu reagent (Jayaprakash *et al.*, 2001).

Reagents

1. Folin-Ciocalteu reagent : 10-fold diluted – 1 ml Folin-Ciocalteu reagent + 9 ml distilled water
2. Sodium carbonate: 7.5%
3. Standard tannic acid solution - 100 mg of tannic acid was dissolved in distilled water and volume was made to 100 ml.
4. Working solution – Ten ml of stock solution was diluted to 100 ml with distilled water. This solution contained 100µg tannic acid per ml.

Method

1. The concentrated peel extract from different solvents was diluted 50 fold in distilled water and 0.1ml of the diluted extract was taken for estimation of phenols.
2. The 0.1ml of the diluted aliquots of samples were mixed with 2.5 ml of 10-fold diluted Folin-Ciocalteu reagent and 2 ml of 7.5% sodium carbonate.
3. The mixture was allowed to stand for 30 min at room temperature and the absorbance was measured at 760 nm
4. The final results were expressed as tannic acid equivalents

from standard curve.

Calculation - From the standard curve the concentration of phenols in the test sample was determined and expressed as mg/g material.

Calibration of standard curve - The calibration curve was prepared by taking 0, 0.1, 0.2, 0.3.....,1ml of working standard solution of tannic acid (0.1mg/ml=100µg/ml) corresponding to 0, 10. 20, 30, 40.....100µg. (Fig. 1).

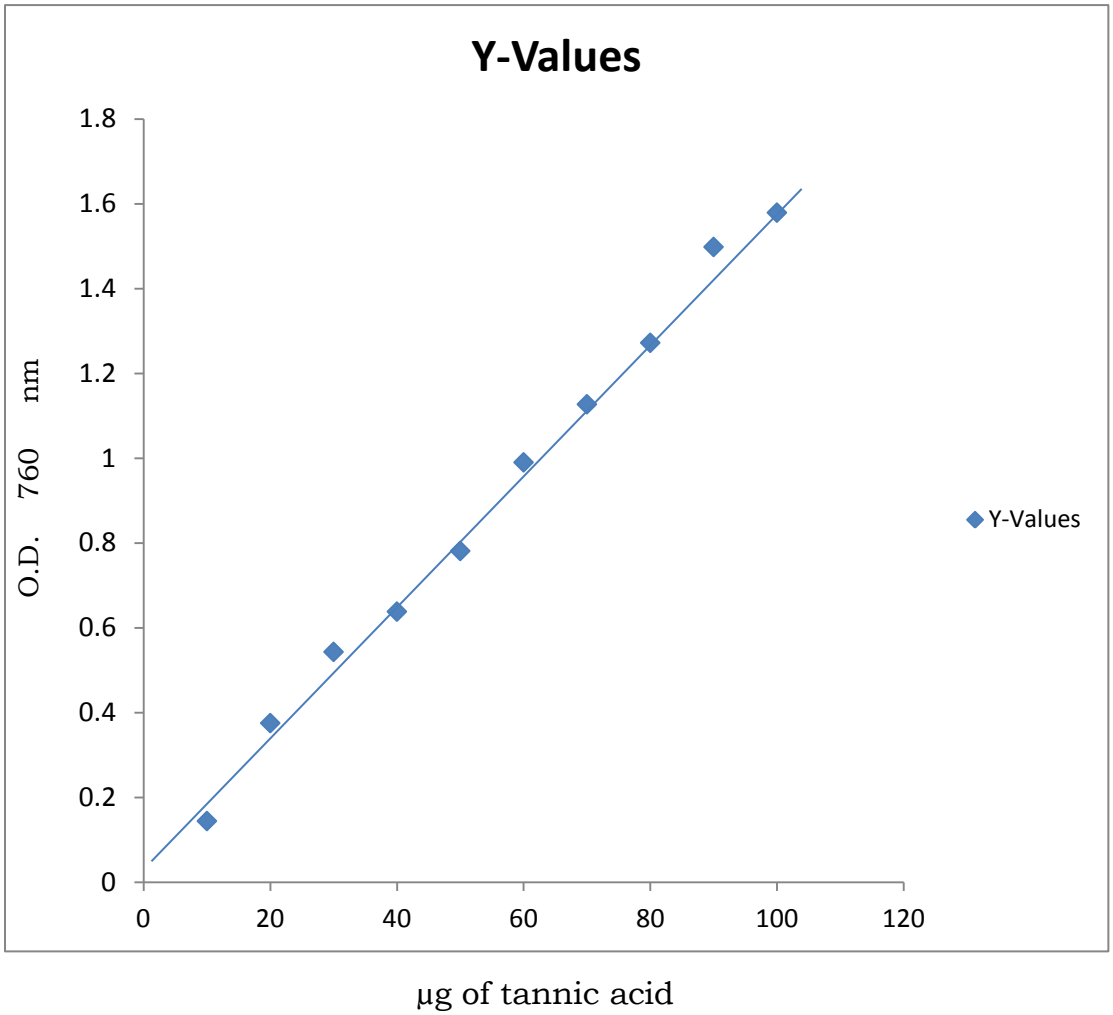

Fig .1- Calibration of standard curve for the estimation of phenols

3.2.2 Estimation of proathocyanidins content

Determination of proanthocyanidins was based on the procedure reported by Sun *et al.* (1998).

Reagents

1. Vanillin-methanol solution: 4%
2. Hydrochloric acid:1N

Method

1. A volume of 250µl of the concentrated extract was mixed with 750 µl water to make a total volume of 1ml.
2. The above solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml 3.6N hydrochloric acid.
3. The mixture was allowed to stand for 15 min. and the absorbance was measured at 500 nm.
4. The final results were expressed as catechin equivalents, based on the standard curve, prepared by using 0,30,60,90 and 120 µg of catechin (Fig.2).
5. The standard curve with 3.6 N HCl was taken into consideration for calculating proanthocyanidins in the sample to express as catechin equivalent.

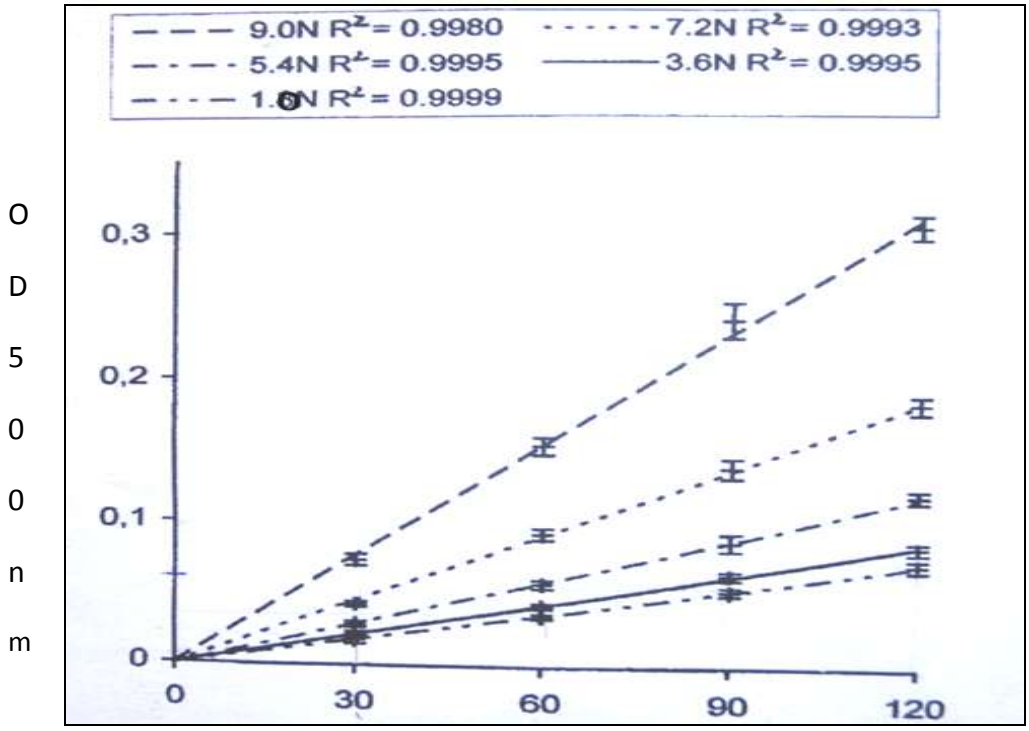

Fig.2 - Calibration of a standard curve for the estimation of proanthocyanidins

3.2.3 Estimation of flavonoids content

The flavonoids content was measured using a modified colorimetric method (Li *et al.*, 2006).

Reagents

1. Methanol
2. Ethanol 30%
3. Sodium nitrite 5%
4. Aluminium nitrate 10%
5. Sodium hydroxide 1 M
6. Rutin was weighed (10 mg) and dissolved in 100 ml of methanol to get 100 µg/ml stock solution. Lower concentrations (2, 4, 6, 8 or 10 µg/ml) for rutin were prepared by diluting serially with methanol. Equal volume of different concentrations of standard were added to methanolic solution of DPPH and kept in dark for 30 min at room temperature. After 30 min, the absorbance was recorded at 510 nm using spectrophotometer against methanol as blank.

Method

1. The sample extract was diluted (1:10) with 9 ml respective solvent *viz*; methanol, acetone and ethyl acetate.
2. From this 1:10 diluted extract 0.4 ml of the solution was transferred to a 25 ml flask containing 5 ml of 30% ethanol and mixed with 0.75 ml of 5% sodium nitrite for 5 min.

3. Then, 0.75 ml of 10% aluminum nitrate was added.
4. After 6 min, the reaction was stopped by adding 5 ml of 1M sodium hydroxide.
5. The mixture was further diluted with 30% ethanol up to 25ml.
6. The absorbance of the mixture was immediately measured at 510 nm.
7. The flavonoids content was calculated and expressed as rutin equivalents from the standard curve (fig.3).

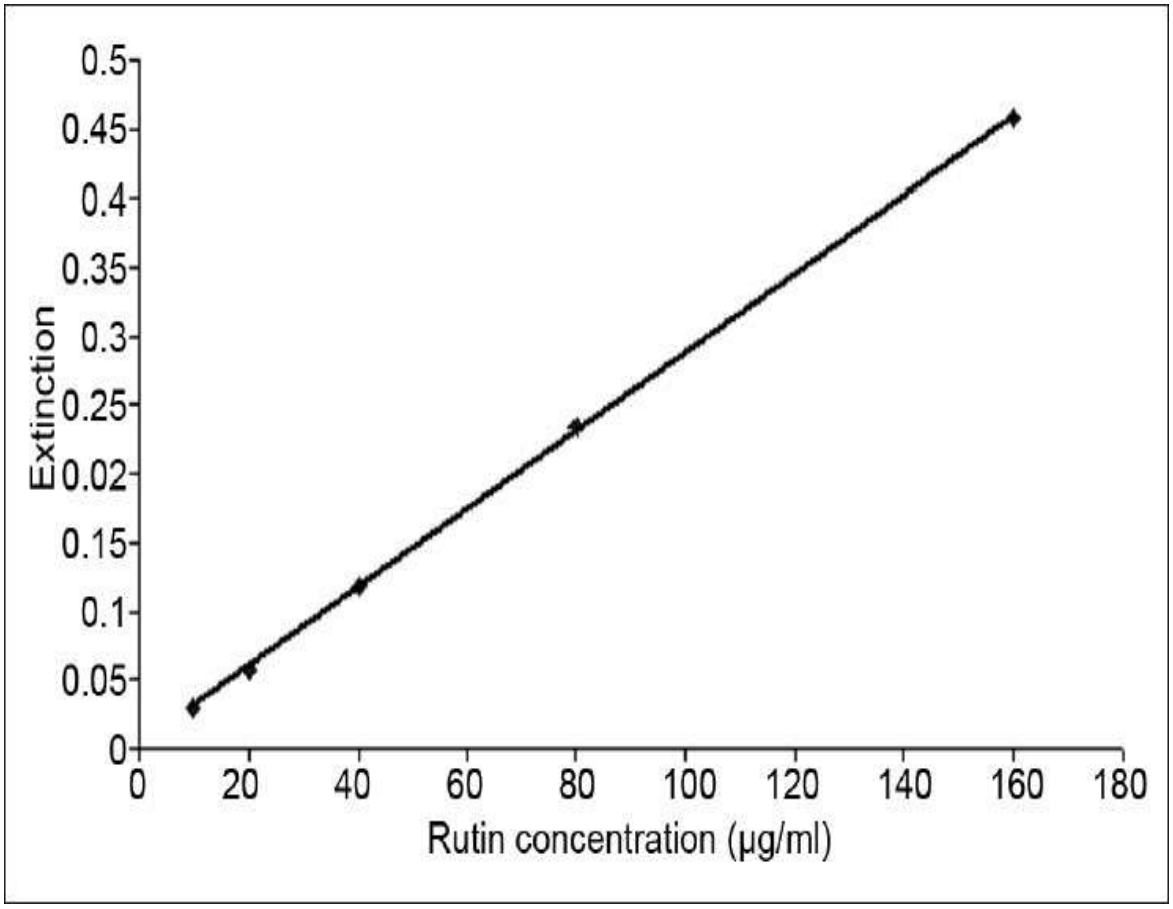

Fig. 3- Calibration of standard curve for the estimation of flavonoids

3.3.4 FRAP (Ferric Reducing Antioxidant Power) assay-

The procedure described by Benzie and Strain (1999) was followed.

Reagents :-

1. **FRAP reagent**- 2.5 ml of 10 mM TPTZ solution in 40 mM HCl plus 2.5 ml of 20 mM $FeCl_3$ and 25 ml of 0.3M acetate buffer, pH 3.6 and was prepared fresh and warmed at 37˚ C prior to use.
2. 1 mM $FeSo_4$

Method :-

1. Aliquots of 40 µl samples were diluted (1:25) with respective solvents and the volume was made 1ml. 40µl of the diluted extract was mixed with 0.2 ml distilled water and 1.8 ml FRAP reagent.
2. The absorbance of reaction mixture was measured at 593 nm after incubation at 37^0C for 10min.
3. The standard 1 mM $FeSO_4$ solution was used to take the absorbance readings at 593 nm in a similar way to express the results of FRAP activity.
4. The final result were expressed as the concentration of the antioxidants having a ferric reducing ability (µM/g) equivalent to that of 1 mM $FeSO_4$.

3.3.5 Estimation of antioxodant activity (DPPH Method)

The free radical scavenging activity of the peel extract and the standard reference compound (BHT) was analysed by the DPPH assay.

Reagents :-

1. 0.1% methanolic 2,2-diphenylpicrylhydrazyl (DPPH)
2. 0.16% of Butylated hydroxytoluene (BHT)

Method:-

1. In quantitative assay 40 µl of concentrated extract was dilutedto 1ml with respective solvent. From this, a volume of 39 (1.17 mg), 52 (1.56 mg) and 100 µl (3.0 mg) was combined with 2.7 ml of methanol and 1ml of 0.1% of methanolic DPPH.
2. Incubated for 30 min. in dark and the absorption maxima were measured at 517 nm.
3. The control contained all reagents except the extract fraction while methanol was used as blank.
4. Positive samples with observed discoloration from purple to yellow were used for quantitative analysis.
5. 0.16% BHT was used as the standard.

$$\text{Antioxidant Activity (\%)} = \frac{\text{O.D. of control} - \text{O.D. of sample}}{\text{O.D. of control}} \times 100$$

3.3.6 Determination of antibacterial and antifungal activity

The antibacterial activity of the pomegranate peel extract was tested on different strains of *Xanthomonas* spp. using the disc diffusion method. Active cultures for the experiments were prepared by taking suspension of bacterial strains and mixed with sterilized Nutrient Agar (NA). This NA containing bacterial strains was poured in petri plates. The sterile discs (4-5 mm diameter) dipped in different sample extracts having 4-5µl volume of extract and placed on the surface of NA medium. Plates were incubated at 25 ± 2^0 C. After 48 hr the inhibition zones around the disc were measured. One disc with streptocycline placed in middle and one disc of control also placed on surface of NA in petri plates. The same procedure was followed for *Fusarium oxysporum* using Potato Dextrose Agar (PDA) to determine antifungal activity.

The antifungal activity of extract also tested on *Aspergillus flavous* by mixing different concentrations of extract (15µl/100ml PDA, 50µl/100ml PDA) in PDA. Poured this mixture in petri plates and a bit of culture placed in the centre of petri plate. Allow it to grow in incubator at 25 ± 2^0 C. After 4 days growth of the zone measured in mm and same procedure was followed for control.

4. RESULTS AND DISCUSSION

The pomegranate peel contain flavonoids and phenols which are significantly higher than the pulp. The pomegranate peel extract also has antioxidant and antimicrobial properties. The pomegranate peel extract has shown very good antimicrobial activity against *Salmonella typhi, Bacillus subtillis, E.coli, P. aeroginosa* and *Aspergillus flavous* and other human pathogenic microorganisms also. The high content of antioxidant is being utilized as a natural antioxidant rather than to use synthetic antioxidant due to suspected action of the synthetic antioxidant as promoter of carcinogenesis. Different solvents have been tried to extract phytochemicals from the pomegranate peel. In the present investigation three solvents differing in their polarity,

Solvent	Dielectric constant	Dipole moment	polarity	
Methanol	33	1.70 D	12.3	Polar protic
Acetone	20.7	2.88 D	10.4	Polar aprotic
Ethyl acetate	6.02	1.78 D	5.3	Polar aprotic

were tried to extract and to evaluate the antioxidant potential by DPPH assay and FRAP assay and to analyse total phenols, pronthocyanidins and flavonoids. The extracts were also tested for their antibacterial and antifungal activity of plant pathogenic microorganisms. The results obtained during the cource of investigation are presented and discussed in the light of available literature.

4.1 Phytochemical analysis

The extracts were subjected to phytochemical analysis to detect the presence of different metabolites using the standard qualitative procedures as described in material and methods and presented in Table 1.

Table 1: Phytochemicals constituents in peel of pomegranate extracted with different solvent

Phytochemicals	**Methanol (80:20)**	**Acetone (80:20)**	**Ethyl acetate**
Tannins	+	+	+
Terpenoids	_	_	_
Saponins	+	+	+
Flavonoids	+	+	+
Alkaloids	_	_	_
Glycosides	+	+	_

4.2 Estimation of total phenols content in the fresh and dried peel extracted with different solvents (as tannic acid equivalent)

Phenolic compound are secondary metabolites which are synthesized in plant. They possess biological properties such as antioxidant, anti-aging, anti-carcinogen, anti-inflammation, cardiovascular protection, improvement of the endothelial function as well as inhibition of angiogenesis and cell proliferation activity. Most of these biological actions have been attributed to their intrinsic reducing capabilies (Han et al. 2007).

Some typical phenolic compound which are highly correlated with antioxidant activity are phenolic acids; for example gallic acid and polyphenol; for example flavonoids. There were several studies showing that antioxidant activity was strongly correlated with the content of the total phenolic compound.

Three different solvents were used for the extraction of phenols which recorded differential response. Methanol:Water (80:20) extract recorded significantly maximum content of phenols with a mean value of (53.76 mg/g) whereas ethyl acetate recorded the minimum content of (14.93 mg/g). As regard to the content of phenols in different varieties is concerned, significantly maximum content of phenols were recorded in Ganesh (39.64 mg/g) and the least content of phenols (21.96 mg/g) were recorded in Mridula . Both variety and solvent recorded significant differences in the total phenols and solvent into variety interaction also were significant. The maximum phenol content of (66.23mg/g) was recorded in Ganesh with methanol whereas the least phenol content of 9.04 mg/g was recorded in Mridula with ethyl acetate (Table 2, fig.1.a).

When the dried peel of the pomegranate was extracted with the same three solvents, it was observed that methanol:water (80:20) and acetone:water (80:20) were equally effective in extracting the phenol whereas the ethyl acetate was least effective. As regard to the different varieties, variety Ganesh recorded a mean phenols content of 64.04 mg/g whereas variety Mridula recorded the mean phenol content of 45.92 mg/g. The

Table 2 : The content of phenols (mg/g) in **fresh** peel of pomegranate extracted with different solvents

Solvents → / Varieties↓	**Ethyl Acetate**	**Acetone**	**Methanol**	**Mean**
Ganesh	22.42	30.27	66.23	**39.64**
Bhagawa	10.50	28.02	56.10	**31.54**
Mridula	9.04	15.78	41.05	**21.96**
Jodhpuri Red	17.77	23.16	51.65	**30.86**
Mean	**14.93**	**24.31**	**53.76**	**31.00**
	Solvent	**Variety**	**S x V**	**CV %**
SE(±)	0.33	0.12	0.65	3.68
CD at 5%	0.96	0.35	1.92	

Table 3 : The content of phenols (mg/g) in **dry** peel of pomegranate extracted with different solvents

Solvents → / Varieties↓	**Ethyl Acetate**	**Acetone**	**Methanol**	**Mean**
Ganesh	22.23	87.54	82.39	**64.06**
Bhagawa	19.57	84.95	81.25	**61.92**
Mridula	13.30	66.69	57.76	**45.92**
Jodhpuri Red	15.07	69.54	60.45	**48.35**
Mean	**17.54**	**72.68**	**74.97**	**55.06**
	Solvent	**Variety**	**S x V**	**CV %**
SE(±)	0.69	0.25	1.39	4.37
CD at 5%	2.02	0.73	4.05	

solvent, variety and their interaction effects were also significant (Table 3, Fig.1.b).

The total phenol content of the dried peel extract with methanol was 1.39 times higher than that of the fresh peel extract whereas with acetone the total phenol content of the dried peel extract was 2.99 times higher than that of the fresh peel. However, with ethyl acetate a marginal increase of 1.17 fold was recorded.

The phenolics content of 249.4 ± 17.2 mg/g as tannic acid equivalent have been recorded in the dried pomegranate peel extract when extracted with combination of methanol, ethanol and acetone. (Yunfeng *et al.*, 2006). The maximum phenolic content of 8.26% has been reported in the pomegranate peel extract with methanol whereas ethyl acetate was found to be least effective with a yield of total phenolics of 1.18 % (Wang *et al.,* 2011). In general methanol had a higher capacity for extracting phenolic compounds from dried peel than water and ethanol (Rawashi *et al.*, 2013). The results of the present investigation are in accordance with the reported values and methanol is a better solvent is in comformity with the earlier reports. The phenols were extracted from pomegranate peel in acetone : water (70:30), ethanol : water (50:50) and ethyl acetate. It was observed that highest polyphenol were extracted with acetone : water (70:30), which were 28.47mg/10ml and the least with ethyl acetate i.e. 4.55 mg/10ml (Zam et al., 2012). It has also been reported that water gave the highest extract yield of

polyphenols (17.78%) and ethyl acetate was least effective giving extract yield of 0.75% (Anokwurtu *et al.*, 2011).

4.3 Estimation of proanthocyanidins in the fresh and dried peel extracted with different solvents (as catechin equivalent)

Proanthocyanidins are potent free radical scavengers and are belived to be contributors to the health benifits of fruits and vegetables (Prior and Gu, 2005). The studies showed that proanthocyanins antioxidant capabilities are 20 times more potent than vit.E (Lambert, 2005).

Three different solvents were used for the extraction of proanthocyanidins which recorded differential response. The methanol:water (80:20) extract recorded maximum content of proanthocyanidins with a mean value of 3.19 mg/g whereas ethyl acetate recorded the minimum content of 2.30 mg/g. As regard to the content of proanthocyanidins in different varieties is concerned, maximum proanthocyanidin content were recorded in Mridula (3.62 mg/g) and the least content of proanthocyanidin (1.59 mg/g) were recorded in Jodhpuri Red. Both variety and solvent recorded significant differences in the proanthocyanidin and solvent into variety interaction were also significant. The maximum content of proanthocyanidin (4.14 mg/g) was recorded in Mridula with methanol whereas the least proanthocyanidin content of 1.18 mg/g was recorded in Jodhpuri Red with ethyl acetate (Table 4, Fig.2.a).

When the dried peel of the pomegranate was extracted with the same three solvents, it was observed that methanol and acetone were equally effective in extracting the proanthocyanidins whereas the ethyl acetate was significantly least effective. As regard to the different varieties, variety Mridula recorded a mean proanthocyanidin content of 14.26 mg/g whereas variety Jodhpuri Red recorded the mean proanthocyanidins content of 4.26 mg/g. The solvent, variety and their interaction effects were significant.(Table 5, fig.2.b.)

The proanthocyanidin content of the dried peel extract with methanol was 2.26 times higher than that of the fresh peel extract whereas with acetone the proanthocyanidin content of the dried peel extract was 4.40 times higher than that of the fresh peel. However, with ethyl acetate a marginal increase of 1.45 fold was recorded.

The proanthocyanidin content of 10.9 ± 0.5 mg/g as catechin equivalent have been recorded in the dried pomegranate peel extract when extracted with combination of methanol, ethanol and acetone (Yunfeng *et al.* 2006). The proanthocyanidin content of acetone extract were higher than the contents of the water extract and methanol extract (Wang *et al.*, 2011). The proanthocyanidins were extracted from pomegranate peel in acetone:water (70:30), ethanol:water (50:50) and ethyl acetate. It was observed that acetone:water (70:30) contain maximum proanthocyanidins of 5.82 ± 0.13 mg/10 ml (Zam *et al.* 2012).

Table 4 : The content of proanthocyanidins (mg/g) in **fresh** peel of pomegranate extracted with different solvents

Solvents → / Varieties↓	**Ethyl Acetate**	**Acetone**	**Methanol**	**Mean**
Ganesh	2.72	3.37	3.94	**3.34**
Bhagawa	2.28	2.74	2.92	**2.65**
Mridula	3.03	3.70	4.14	**3.62**
Jodhpuri Red	1.18	1.86	1.74	**1.59**
Mean	**2.30**	**2.92**	**3.19**	**2.80**
	Solvent	**Variety**	**S x V**	**CV %**
SE(±)	0.06	0.02	0.12	7.50
CD at 5%	0.18	0.06	NS	

Table 5 : The content of proanthocyanidins (mg/g) in **dry** peel of pomegranate extracted with different solvents

Solvents → / Varieties↓	**Ethyl Acetate**	**Acetone**	**Methanol**	**Mean**
Ganesh	3.99	10.12	8.85	**7.65**
Bhagawa	3.20	7.86	4.05	**5.04**
Mridula	4.82	25.07	12.88	**14.26**
Jodhpuri Red	1.33	8.40	3.05	**4.26**
Mean	**3.34**	**12.87**	**7.21**	**7.80**
	Solvent	**Variety**	**S x V**	**CV %**
SE(±)	0.22	0.08	0.44	9.72
CD at 5%	0.64	0.23	1.28	

4.4 Flavonoids content in the fresh and dried peel extracted with different solvents (as rutin equivalent)

Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized according to chemical structure into flavonols, flavones, flavonones, isoflavones, catechins, anthocyanidins and chalcones. Over 4,000 flavonoids have been identified, many of which occur in fruits, vegetables and beverages (tea, coffee, beer, wine and fruit drinks). The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health-they have been reported to have antiviral, anti-allergic, antiplate, anti-inflammatory, antitumor and antioxidant activities.

The capacity of flavonoids to act as antioxidant depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities. Quercetin, the most abundant diatary flavonol, is a potent antioxidant because it has all the right structural features for free radical scavenging activity.

Three solvents were used for the extraction of flavonoids. The methanolic extract recorded maximum content of flavonoids with a mean value of 4.94 mg/g whereas ethyl acetate recorded the minimum content of 0.51 mg/g. As regard to the content of flavonoids in different varieties is concerned significantly maximum content of flavonoids were recorded in Ganesh (3.67 mg/g) and the least content of flavonoids (2.08 mg/g) were recorded in Jodhpuri Red. Both variety and solvent

recorded significant differences in the flavonoids and solvent into variety interactions also were significant. Maximum content of flavonoids (6.59 mg/g) was recorded in Ganesh with methanol whereas the least flavonoids content of 0.32 mg/g was recorded in Jodhpuri Red with ethyl acetate (Table 6, fig.3.a).

When the dried peel of the pomegranate was extracted with the same three solvents, it was observed that methanol and acetone were more effective in extracting the flavonoids whereas the ethyl acetate was least effective. As regard to the different varieties, variety Ganesh recorded a mean flavonoids content of 5.32 mg/g whereas variety Jodhpuri Red recorded the mean flavonoids content of 2.44 mg/g. The solvent, variety and their interaction effects were significant (Table 7, fig.3.b).

The flavonoids content of 59.1 ± 4.8 mg/g as rutin equivalent have been recorded in the dried pomegranate peel extract when extracted with combination of methanol, ethanol and acetone (Yunfeng *et al.*, 2006). The maximum flavonoid content have been reported in non pungent peppers when extracted with methanol (Bac *et al.*, 2012).

Table 6 : The content of flavonoids (mg/g) in **fresh** peel of pomegranate extracted with different solvents

Solvents → / Varieties↓	**Ethyl Acetate**	**Acetone**	**Methanol**	**Mean**
Ganesh	0.69	3.71	6.59	**3.67**
Bhagawa	0.57	3.61	5.04	**3.07**
Mridula	0.41	2.97	4.56	**2.65**
Jodhpuri Red	0.35	2.33	3.57	**2.08**
Mean	**0.51**	**3.16**	**4.94**	**2.87**
	Solvent	**Variety**	**S x V**	**CV %**
SE(±)	0.05	0.01	0.11	6.33
CD at 5%	0.15	0.05	0.31	

Table 7 : The content of flavonoids (mg/g) in **dry** peel of pomegranate extracted with different solvents

Solvents / Varieties	**Ethyl Acetate**	**Acetone**	**Methanol**	**Mean**
Ganesh	0.98	10.58	4.41	**5.32**
Bhagawa	0.87	10.13	4.10	**5.04**
Mridula	0.48	5.78	3.78	**3.35**
Jodhpuri Red	0.23	4.71	3.27	**2.74**
Mean	**0.64**	**7.80**	**3.89**	**4.11**
	Solvent	**Variety**	**S x V**	**CV %**
SE(±)	0.04	0.01	0.08	3.38
CD at 5%	0.12	0.04	0.23	

4.5 Ferric reducing antioxidant power (FRAP) of fresh and dried peel extracted with different solvents

FRAP assay uses antioxidants as reductants in a redox linked colorimetric assay. At low pH, the reduction of (Fe III TPTZ) complex to (Fe II TPTZ) which has an intense blue colour can be monitored by measuring the change in absorption at 593 nm.

Three different solvents were used for the estimation of ferric reducing antioxidant power recorded differential response. Methanol:water (80:20) extract recorded maximum ferric reducing antioxidant power with a mean value of 54.97 µM/g whereas ethyl acetate recorded the minimum of 39.39 µM/g equivalent to that of 1mM FeSO4. As regard to the ferric reducing antioxidant power in different varieties is concerned significantly maximum ferric reducing antioxidant power were observed in Ganesh (46.15 µM/g) and the least ferric reducing antioxidant power (44.89 µM/g) were recorded in Jodhpuri Red. Both variety and solvent recorded significant differences and solvent into variety interaction were also significant. Maximum ferric reducing antioxidant power (55.67 µM/g) was observed in Ganesh with methanol whereas the least ferric reducing antioxidant power 38.67 µM/g was recorded in Jodhpuri Red with ethyl acetate (Table 8).

When the dried peel of the pomegranate was extracted with the same three solvents, it was observed that methanol and acetone were equally effective in ferric reducing antioxidant power whereas the ethyl acetate was least effective. As regard to

the different varieties, variety Ganesh recorded a mean ferric reducing antioxidant power of 54.74 μM/g whereas variety Jodhpuri Red recorded the ferric reducing antioxidant power 53.85 μM/g. The solvent,variety and their interaction effects were significant.(Table 9).

The ferric reducing antioxidant power of the dried peel extract with methanol was 2.0 times higher than that of the fresh peel extract whereas with acetone the ferric reducing antioxidant power of the dried peel extract was 16.21 times more than that of the fresh peel.

The peel extract obtained by use of mixture composed of methanol, ethanol, acetone and water was significantly higher in ferric reducing antioxidant power than those obtained using individual solvents, namely using methanol, ethanol or acetone (Yunfeng *et al.,* 2006).

Table 8 : FRAP value (µM/g) equivalent to that of 1mM $FeSO_4$ of **fresh** peel of pomegranate extracted with different solvents

Solvent → / Varieties↓	**Ethyl Acetate**	**Acetone**	**Methanol**	**Mean**
Ganesh	40.56	42.22	55.67	**46.15**
Bhagawa	39.33	41.49	55.11	**45.31**
Mridula	39.00	41.00	54.67	**44.89**
Jodhpuri Red	38.67	40.67	54.44	**44.59**
Mean	**39.39**	**41.35**	**54.97**	**45.24**
	Solvent	**Variety**	**S x V**	**CV %**
SE(±)	0.04	0.01	0.07	0.27
CD at 5%	0.10	0.04	0.25	

Table 9 : FRAP value (µM/g) equivalent to that of 1mM $FeSO_4$ of **dry** peel of pomegranate extracted with different solvents

Solvents → / Varieties ↓	**Ethyl Acetate**	**Acetone**	**Methanol**	**Mean**
Ganesh	48.33	58.56	57.33	**54.74**
Bhagawa	47.89	57.67	56.89	**54.15**
Mridula	44.78	57.00	56.78	**52.85**
Jodhpuri Red	44.67	57.00	56.89	**52.85**
Mean	**46.42**	**57.56**	**56.97**	**53.65**
	Solvent	**Variety**	**S x V**	**CV %**
SE(±)	0.04	0.01	0.08	0.25
CD at 5%	0.11	0.04	0.23	

4.6 Estimation of antioxidant activity by DPPH assay of dry peel extracted with different solvents

DPPH (2,2-diphenyl-1-picrylhydrazyl) has two major applications. One is monitoring of chemical reactions involving radicals most notably in common antioxidant assay and as a standard in electron paramagnetic resonance signal.

DPPH is a well known radical and a trap (scavenger) for other radicals. The rate reduction of a chemical reaction upon addition of a DPPH is used as an indicator of the radical nature of that reaction.

Three different solvents were used for the estimation of antioxidant activity of dry peel of pomegranate by DPPH assay. Acetone:water (80:20) extract of dried peel of pomegranate recorded maximum % antioxidant activity whereas the ethyl acetate recorded the minimum % antioxidant activity. As regards to % antioxidant activity in different varieties is concerned

maximum % antioxidant activity was observed in Ganesh for all concentrations (Table 10,11,12). Both variety and solvent recorded a significant difference in % antioxidant activity in methanol:water (80:20) extract and solvent into variety interaction also were significant in methanol:water (80:20) extract. Maximum antioxidant activity (77.47%) was observed in Ganesh with acetone for 52 µl (1.56 mg) of diluted extract whereas the least % antioxidant activity of 0.32% was recorded in Jodhpuri Red with ethyl acetate for 39µl (1.17mg) of diluted extract.

The % antioxidant activity of Butylated Hydroxyltoluene (BHT)(0.16%) was recorded 74.16%. From the results it was observed that % antioxidant activity of acetone:water (80:20) extract is higher than Butylated Hydroxytoluene. % antioxidant activity of methanol:water (80:20) extract with a mean value is 74.29% for 100 µl of diluted extract, containing 3.0mg of peel powder.

The antioxidant activity of 85.4± 3.1 % have been recorded in the dried pomegranate peel extract with a methanol and 25.2 ± 2.0 % with a aqueous extract of dried peel of pomegranate (Tehranifar *et al.*, 2011). Study of antioxidant activities of the extracts was carried to investigate the correlations between the antioxidant activity and the content of phenolics, proanthocyanidins and flavonoids. The results indicate a strong correlation between DPPH and total phenolics, but no correlation existed with proanthocyanidins and flavonoids (Wang *et al.*,2011).

Sing *et al.*,(2002) reported that the antioxidant activity of pomegranate peel correlated to the total phenolics. Comparing methanol with water as the solvent in pomegranate antioxidant extraction, the total extract yield (dried extract/100g PPE) were 43.18% and 46.51%, the yield of total phenolics (g total calculated weight of phenolic/100g PPE) were 5.90% and 8.26%, the content of phenolics (g phenol/100g dried extract) were 13.63% and 17.78% and the DPPH antioxidant activities were 53.74% and 65.30%, respectively (Wang et al., 2011). By investigating the phenolic content and antioxidant capacity of pomegranate peel extracts, it was observed that the presence of phenolic compounds in this extract is an important factor in creating the antioxidant activity. In other words, more phenolic content causes more antioxidant activity (Tehranifar *et al.*, 2011).

The DPPH radical scavenging activity and phenolic compound content in different leaf extracts of blackberry was evaluated. Water has been reported as the best solvent with strongest antioxidative activity. The study also confirmed a strong correlation between antioxidant activity and the content of phenolics in the extracts. The leaf extract are good source of antioxidants compared to synthetic antioxidant BHA and about 7 times less active than vit. C (Gzella *et al.*, 2012).

Antioxidative components from pomegranate peel were extracted by using different solvents like methanol, ethanol, acetone, chloroform and ethyl acetate. All the extracts were tested for total phenolic content and the methanol which proved

Table 10 : Antioxidant activity (%) of dry peel of pomegranate extracted with **ethyl acetate**

Concentration (mg) ↓ / varieties →	Ganesh	Bhagawa	Mridula	Jodhpuri Red	**Mean**
1.17mg	1.16	1.11	0.42	0.32	**0.75**
1.56mg	6.24	6.13	4.58	5.06	**5.51**
3.0mg	10.95	10.61	6.06	6.55	**8.54**
Mean	**6.12**	**5.95**	**3.69**	**3.98**	**4.93**
BHT (0.16%)	**74.16**				
	V	**E**	**V x E**		CV%
SE	7.40	6.40	12.82		450.02
CD5%	NS	NS	NS		

Table 11 : Antioxidant activity (%) of dry peel of pomegranate extracted with **acetone**

Concentration (mg) ↓ / varieties →	Ganesh	Bhagawa	Mridula	Jodhpuri Red	**Mean**
1.17mg	76.47	75.15	75.67	75.46	**75.69**
1.56mg	77.47	77.00	76.66	76.47	**76.90**
Mean	**76.97**	**76.07**	**76.17**	**75.97**	**76.29**
BHT (0.16%)	**74.16**				
	V	**E**	**V x E**		CV%
SE	0.32	0.22	0.45		1.01
CD5%	NS	0.66	NS		

Table 12 : Antioxidant activity (%) of dry peel of pomegranate extracted with **methanol**

Concentration (mg) ↓ / Varieties →	Ganesh	Bhagawa	Mridula	Jodhpuri Red	**Mean**
1.17mg	20.98	5.61	0.95	1.06	**7.15**
1.56mg	64.09	53.51	1.90	4.55	**31.01**
3.0mg	81.54	80.36	63.56	71.69	**74.29**
Mean	**55.54**	**46.49**	**22.14**	**25.77**	**37.48**
BHT (0.16%)	**74.16**				
	V	**E**	**V x E**		CV%
SE	3.95	3.42	6.84		31.62
CD5%	11.46	9.93	19.86		

to be best in extracting the phenolic compounds was used to assess the antioxidant property (Padmaja and Prasad, 2011).

4.7 Determination of antibacterial and antifungal activity of fresh peel extracted with different solvent

Different solvents used for the determination of antibacterial and antifungal activity of fresh peel of pomegranate extract by disc diffusion method. Methanolic extract of fresh peel showed the highest inhibition zone than acetonic and ethyl acetate extract.

As ragards to the antibacterial activity of different varieties is concerned maximum zone of inhibition in *Xanthomonas* spp. were observed by Ganesh with methanol *(X. citri-* 31.67 mm, *X. punicae-* 31.67mm, *X. malvacearum*-32.33 mm). Both variety and solvent recorded significance differences in zone of inhibition with all three solvent (Plate 1& 2).

When the pomegranate peel extract of same three solvent tested for antifungal activity on *Fusarium oxysporum* f.sp.*ciceri* it observed that only methanol:water (80:20) extract showed zone of inhibition and there is no zone of inhibition with acetone and ethyl acetate extract of fresh peel (Plate 3).

Antifungal activity on *Aspergillus flavous* was determined by methanol:water (80:20) extract of Ganesh with different concentrations of extract. It was observed that mean value of zone of growth of *Aspergillus flavous* is 38.17 mm at a concentration of 15 µl/100 ml PDA and there is a least zone of growth of *Aspergillus flavous* (18.00 mm) at a concentration of 50

µl/100 ml PDA. When the control was tested it was observed that zone of growth of *Aspergillus flavous* was 64.0 mm.

The methanolic peel extract has shown highest antimicrobial activity compared to aquas extract (Saad *et al.,* 2010). The pomegranate peel extract alone did not exhibit antimicrobial activity against any of the isolates. Variation in results between studies on pomegranate extracts are not only seen in disc diffusion assay, but have also been recorded with minimum inhibition concentration determination (Yehia *et a*l., 2011). Methanolic extract of pomegranate peels exhibit good bacteriostatic and bactericidal effects and could potentially be a good substitute for the synthetic antibiotic against pathogenic resistant bacteria (Naziri *et al.*, 2012). Pomegranate ethanol extract group was tested with concentrations of 12.5, 25, 50 and 100 µl/ml. The results showed 60% motility of *Trichomonas tenax* trophozoite after treatment with 12.5 µg/100 ml of pomegranate ethanol extract group and 25 µg/ml showed higher anti-*T. Tenax*. The ethanol extract of pomegranate peel showed remarkable effect on *T. Tenax* (El-Sherbini *et al.*, 2012). The antifungal effect of petroleum ether, ethyl acetate and n-butanol fractions isolated from pomegranate pericarp and flower against Candida albicans. The maximum inhibition zone of Candida albicans was obtained by peel's n-butanol fraction, 35 mm. Petroleum ether fraction had no any antifungal activities (Shafigi *et al.*, 2012).

Table 13 : Effect of pomegranate peel extract (PPE) extracted with different solvents on zone of inhibition **(mm)** in *Xanthomonas axonopodis pv.citri* by disc diffusion method

Solvents / Varieties	**Ethyl Acetate**	**Acetone**	**Methanol**	**Mean**
Ganesh	25.33	27.00	31.67	**28.00**
Bhagawa	20.67	25.00	29.33	**25.00**
Mridula	19.33	21.00	28.00	**22.78**
Jodhpuri Red	21.00	24.67	27.00	**24.22**
Control	Nil	nil	nil	Nil
Mean	**21.58**	**24.42**	**29.00**	**25.00**
	Solvent	**Variety**	**S x V**	**CV %**
SE(±)	0.514	0.186	1.027	7.12
CD at 5%	1.499	0.542	NS	

Table 14 : Effect of pomegranate peel extract (PPE) extracted with different solvents on zone of inhibition (**mm**) in *Xanthomonas axonopodis pv.punicae* by disc diffusion method

Solvents / Varieties	**Ethyl Acetate**	**Acetone**	**Methanol**	**Mean**
Ganesh	17.00	19.33	31.67	**22.67**
Bhagawa	15.67	18.00	30.33	**21.33**
Mridula	13.33	16.67	28.00	**19.33**
Jodhpuri Red	13.67	17.67	30.08	**20.56**
Control	Nil	nil	Nil	Nil
Mean	**14.92**	**17.92**	**30.08**	**20.97**
	Solvent	**Variety**	**S x V**	**CV %**
SE(±)	0.363	0.131	0.736	6.00
CD at 5%	1.060	0.383	NS	

Table 15 : Effect of pomegranate peel extract (PPE) extracted with different solvents on zone of inhibition (**mm**)in *Xanthomonas axonopodis pv. malvacearum* by disc diffusion method

Solvent / Varieties	**Ethyl Acetate**	**Acetone**	**Methanol**	**Mean**
Ganesh	23.00	26.00	32.33	**27.11**
Bhagawa	21.00	26.00	28.67	**25.22**
Mridula	18.67	20.33	28.33	**22.44**
Jodhpuri Red	20.67	24.00	26.67	**23.78**
Control	nil	nil	Nil	nil
Mean	**20.83**	**24.08**	**29.00**	**24.64**
	Solvent	**Variety**	**S x V**	**CV %**
SE(±)	0.516	0.186	1.032	7.25
CD at 5%	1.506	0.544	NS	

Table 16 : Effect of methanolic extract of Ganesh on *Aspergillus flavous* (growth in **mm**)

Treat/ Replication	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**Mean**
Control	65.00	63.00	64.00	65.00	64.00	63.00	**64.00**
15µl	39.00	38.00	39.00	37.00	39.00	37.00	**38.17**
50 µl	19.00	18.00	19.00	17.00	18.00	17.00	**18.00**
						GM	40.27
						SE(+)	0.39
						CD @5%	1.22
						CV (%)	1.68

5. SUMMARY AND CONCLUSIONS

During the industrial processing of pomegranate, large volume of industrial wastes are produced, which have a wide range of nutritional values. Therefore in the recent years, the attention has been focused on the industrial byproduct of pomegranate that have a high potential of antioxidant and antimicrobial properties. The use of synthetic antioxidants in food has been decreased due to their suspected action as promoters of carcinogenesis. Pomegranate peel is recognized for its many health-promoting qualities and apparent antimicrobial activity, anticancer activity, antioxidative properties, etc. Recent studies indicate that the peel yields 1000 folds more phenolic compounds than pulp. The present investigation was therefore undertaken to analyze the antioxidant and antimicrobial properties of pomegranate peel extract. The results obtained during the analysis have been briefly summarized and concluded as under.

1. Phytochemical analysis of pomegranate peel extract when analysed by qualitative test revealed presence of tannins, flavonoids, saponins and glycosides.

2. Three solvents were used for the extraction of phenols. The methanol:water (80:20) extract recorded maximum content of phenols with a mean value of 53.76 mg/g whereas ethyl acetate recorded the minimum content of 14.93 mg/g. Maximum mean phenol content were observed in Ganesh (39.64 mg/g) and the least mean phenol content (21.96 mg/g) were recorded in

Mridula.. The maximum phenol content of 66.23mg/g was recorded in Ganesh with methanol whereas the least phenol content of 9.04 mg/g was recorded in Mridula with ethyl acetate. When the dried peel of the pomegranate was extracted with the same three solvents, it was observed that methanol:water (80:20) and acetone:water (80:20) were equally effective in extracting the phenols whereas the ethyl acetate was least effective. Variety Ganesh recorded a mean total phenol content of 64.04 mg/g whereas variety Mridula recorded the mean phenolic content of 45.92 mg/g.

3. The methanol:water (80:20) extract recorded maximum content of proanthocyanidins with a mean value of 3.19 mg/g whereas ethyl acetate recorded the minimum content of 2.30 mg/g. Maximum proanthocyanidins content were observed in Mridula (3.62 mg/g) and the least content of proanthocyanidins (1.59 mg/g) were recorded in Jodhpuri Red. The highest content of proanthocyanidins (4.14 mg/g) was in Mridula with methanol whereas minimum proanthocyanidins of 1.18 mg/g was noticed in Jodhpuri Red with ethyl acetate. When the dried peel of the pomegranate was extracted with the same three solvents, it was observed that methanol and acetone were equally effective in extracting the proanthocyanidins whereas the ethyl acetate was least effective. Mridula variety recorded maximum mean proanthocyanidins of 14.26 mg/g whereas variety Jodhpuri Red had the mean proanthocyanidins of 4.26 mg/g.

4. Three different solvents were used for the extraction of flavonoids, which recorded differential response. The methanol:water (80:20) extract recorded maximum content of flavonoids with a mean value of 4.94 mg/g whereas ethyl acetate recorded the minimum content of 0.51 mg/g. Maximum flavonoids were recorded in Ganesh (3.67 mg/g) and minimum flavonoids were recorded in Jodhpuri Red (2.08 mg/g). Maximum flavonoids (6.59 mg/g) was observed in Ganesh with methanol whereas the least flavonoids content of 0.32 mg/g was recorded in Jodhpuri Red with ethyl acetate. When the dried peel of the pomegranate was extracted with the same three solvents, it was observed that methanol and acetone were more effective in extracting the flavonoids whereas the ethyl acetate was least effective. Variety Ganesh recorded a mean flavonoids content of 5.32 mg/g, whereas variety Jodhpuri Red recorded the mean flavonoids content of 2.44 mg/g.

5. Three different solvents were used for the estimation of ferric reducing antioxidant power recorded differential response. Methanolic extract recorded maximum ferric reducing antioxidant power with a mean value of 54.97 μM/g whereas ethyl acetate recorded the minimum of 39.39 μM/g equivalent to that of 1mM FeSO4. Maximum ferric reducing antioxidant power (FRAP) were observed in Ganesh (46.15 μM/g) and the minimum FRAP of (44.89 μM/g) were recorded in Jodhpuri Red. Maximum FRAP of (55.67 μM/g) was recorded in Ganesh with methanol:water (80:20), whereas the minimum FRAP of 38.67 μM/g was recorded in Jodhpuri Red with ethyl acetate. When the

dried peels of the pomegranate were extracted with the same three solvents, it was observed that methanol:water (80:20) and acetone:water (80:20) were equally effective in FRAP whereas the ethyl acetate was least effective. Variety Ganesh recorded a mean FRAP of 54.74 µM/g whereas variety Jodhpuri Red recorded the FRAP of 53.85 µM/g.

6. Antioxidant activity of dry peel of pomegranate by DPPH assay recorded differential response. Acetone:water (80:20) extract of dried peel of pomegranate recorded maximum antioxidant activity whereas the ethyl acetate recorded the minimum antioxidant activity. Maximum antioxidant activity (%) were observed in Ganesh for all concentrations. Maximum % antioxidant activity (77.47%) was observed in Ganesh with acetone for 52 µl (1.56mg) of diluted extract whereas the least antioxidant activity of 0.32% was recorded in Jodhpuri Red with ethyl acetate for 39µl (1.17mg) of diluted extract. The synthetic antioxidant Butylated Hydroxyltoluene at 0.16% concentration recorded antioxidant activity of 74.16%. It is evident that % antioxidant activity of acetonic extract of dry peel is higher than Butylated Hydroxyltoluene. The antioxidant activity of methanolic extract recorded a mean value is 74.29% for 100 µl (3.0mg) of diluted extract.

7. Extract of different solvents were used for the determination of antibacterial and antifungal activity of fresh peel of pomegranate extract by disc diffusion method. Methanol:water (80:20) extract of fresh peel showed the highest zone of inhibition than

acetone:water (80:20) and ethyl acetate extract. Maximum zone of inhibition in *Xanthomonas* spp. were observed *(X. citri*-31.67 mm, *X. punicae*- 31.67mm, *X. malvacearum*-32.33 mm) when the peel of Ganesh variety was extracted with methanol:water (80:20). When the pomegranate peel extract of same three solvents were tested for antifungal activity on *Fusarium oxysporum* f.sp.*ciceri* it was observed that only methanol:water (80:20) extract showed zone of inhibition. When the antifungal activity on *Aspergillus flavous* was tested by methanolic extract of Ganesh at different concentrations, it was observed that mean zone of growth of *Aspergillus flavous* was 38.17 mm at a concentration of 15 µl/100ml PDA and there is a least zone of growth of *Aspergillus flavous* (18.00 mm) at a concentration of 50 µl/100ml PDA. The zone of growth of *Aspergillus flavous* was 64.0 mm in control.

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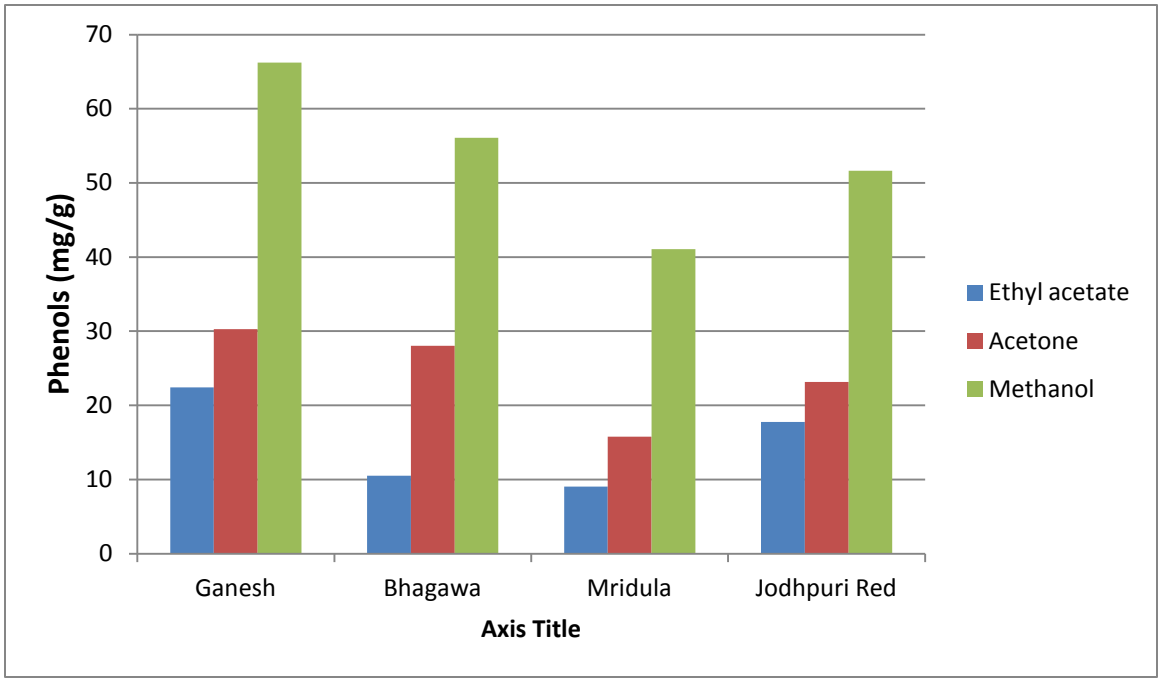

Fig.1(a)- Phenols (mg/g) in fresh peel of pomegranate extracted with different solvents

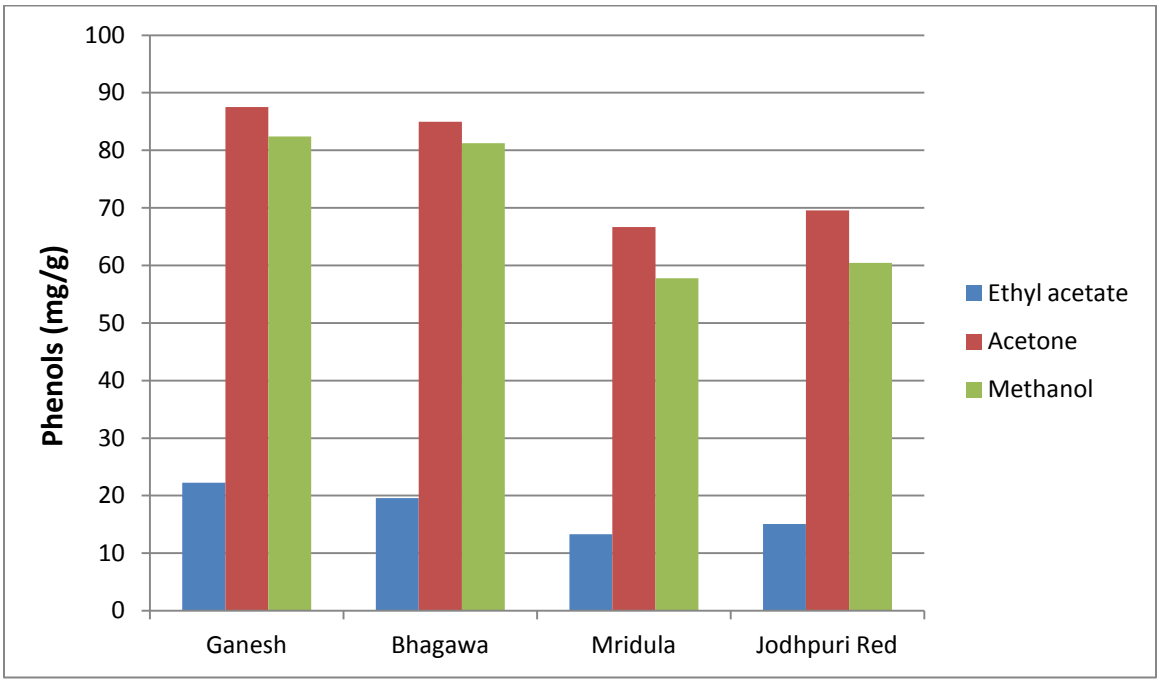

Fig.1(b)- Phenols (mg/g) in dry peel of pomegranate extracted with different solvents

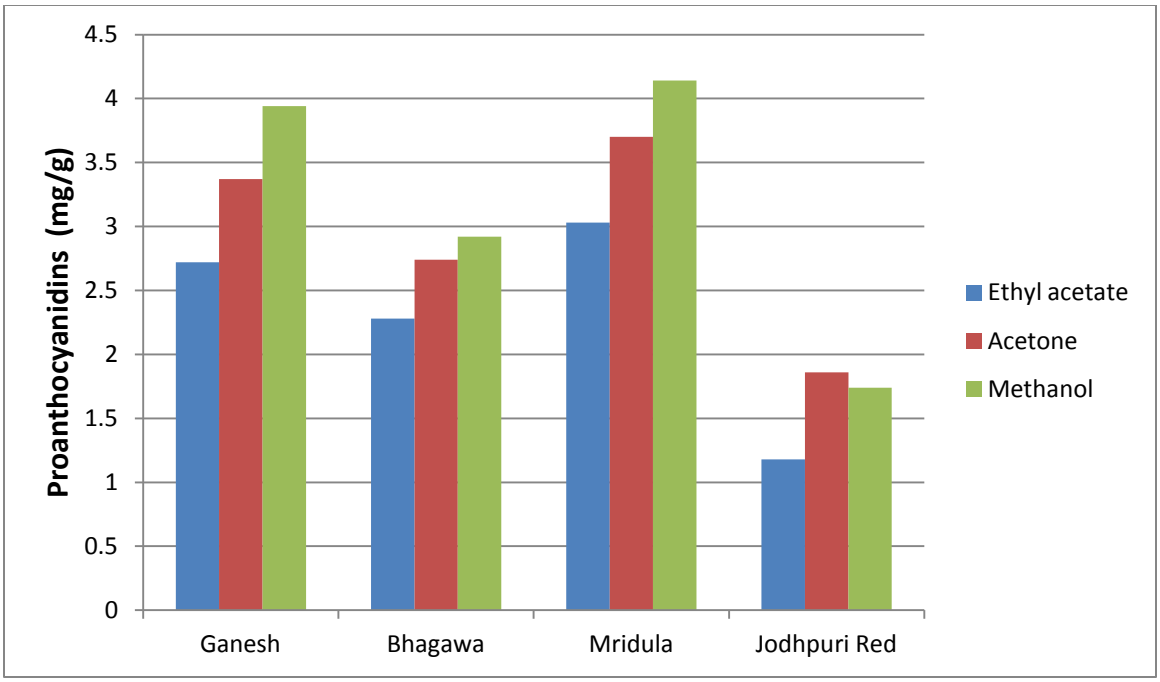

Fig.2(a)-Proanthocyanidins (mg/g) in fresh peel of pomegranate extracted with different solvents

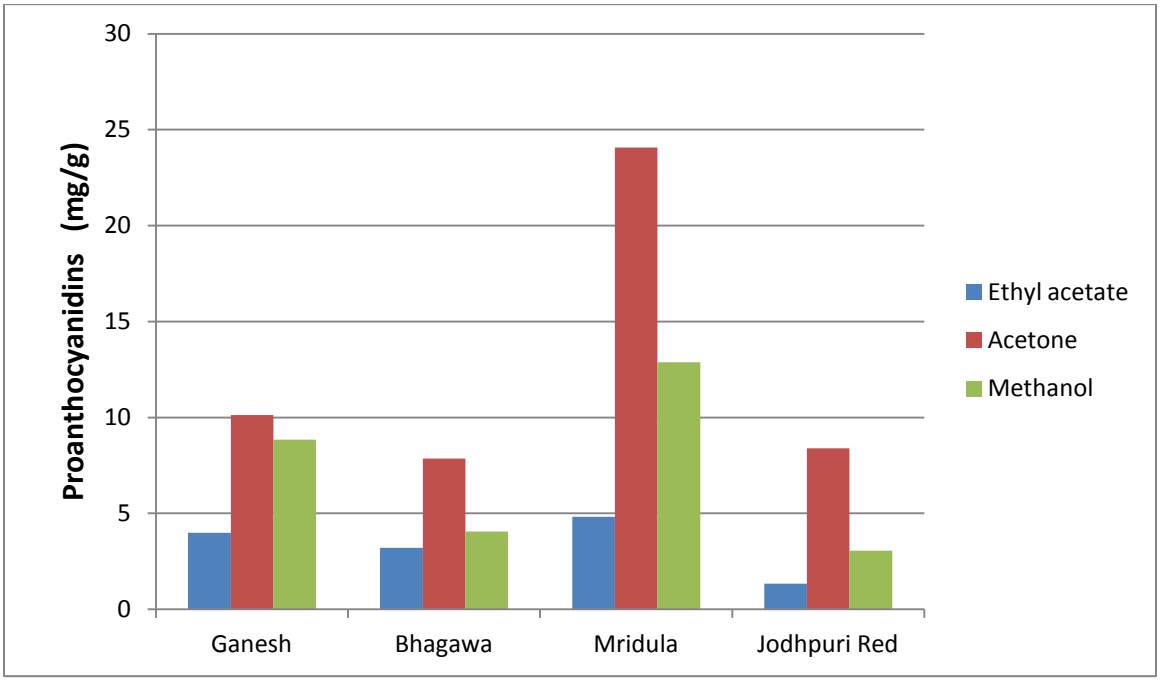

Fig.2(b)-Proanthocyanidins (mg/g) in dry peel of pomegranate extracted with different solvents

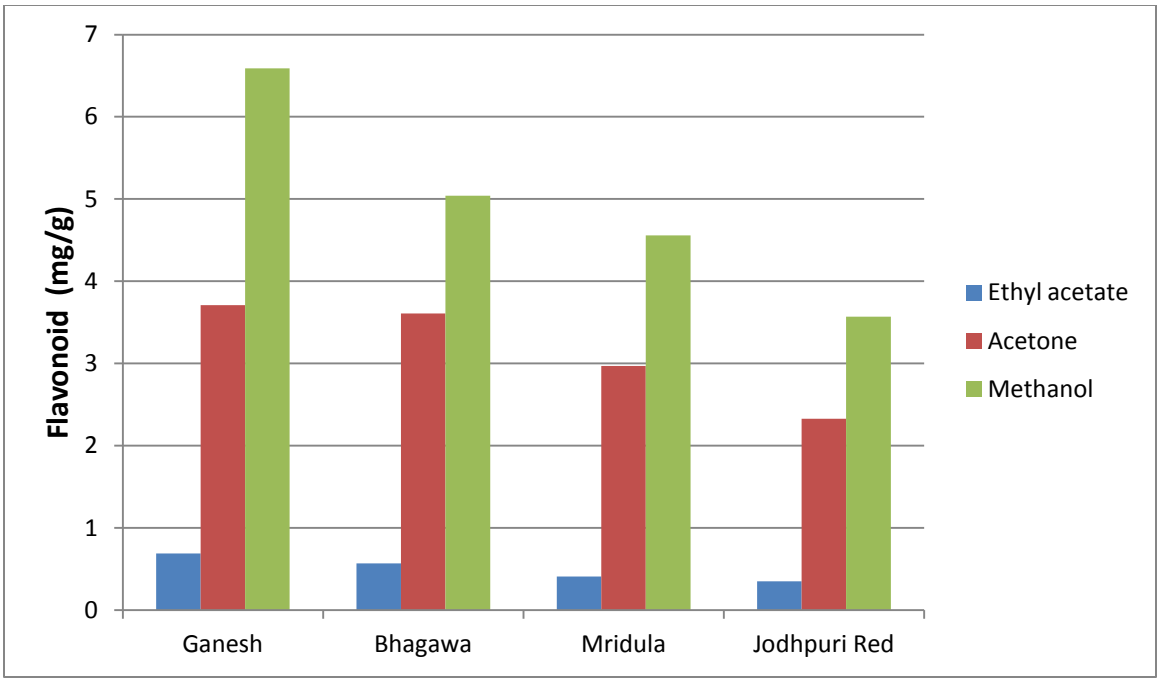

Fig.3(a)- Flavonoid (mg/g) in fresh peel of pomegranate extracted with different solvents

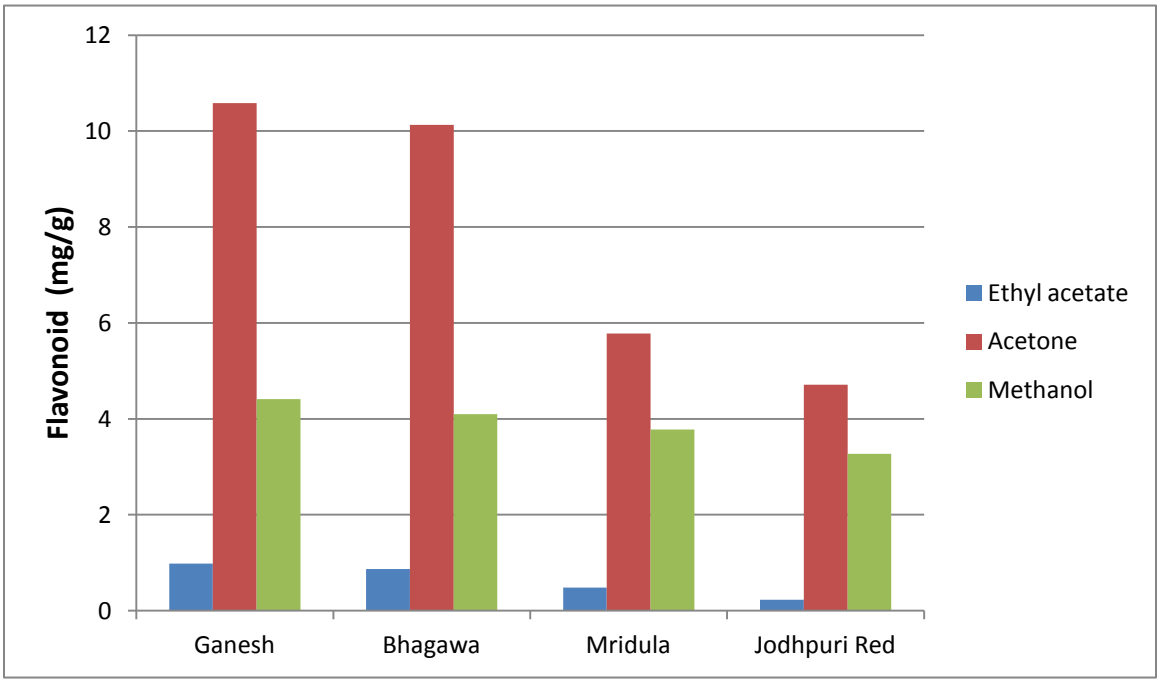

Fig.3(b)-Flavonoids (mg/g) in dry peel of pomegranate extracted with different solvents