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Comparative studies on antioxidant and antibacterial activity of pomegranate leaves (*Punica granatum* L.) grown in different soil and environmental conditions

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Abstract
Leaves of Pomegranate have been used traditionally in Ayurveda medicine for their antioxidant and antimicrobial properties. However, the effectiveness of these properties may be influenced by several environmental factors, such as soil quality, climate condition, and level of pollution. This study aimed to compare the antioxidant and antimicrobial activity of leaves of pomegranate grown in towns and villages in around North 24 Parganas district of West Bengal, India. The antioxidant activity was evaluated using two solvent extract of leaves; water and ethanol, and DPPH free radical scavenging activity, total Polyphenol, total Flavonoid, Tannin and Ascorbic acid content were used to compare antioxidant activity, while the antimicrobial activity was evaluated and compared against three bacterial strain; *Staphylococcus aureus, Bacillus subtilis,* and *Escherichia coli*. The pomegranate leaves grown in villages had significantly higher amount of antioxidant and antimicrobial activity compared to those grown in towns. The village-grown leaves have relatively higher levels of total Polyphenol, Tannin and Ascorbic acid which contributed to their enhanced antioxidant activity. However total Flavonoids and DPPH free radical scavenging activity have been found to be relatively higher in Town-grown leaves. The village-grown leaves had also shown higher antimicrobial activity compare to town-grown leaves. Overall, this study suggested that environmental factors such as soil quality, temperature and pollution may influence on the presence of bioactive compounds and overall efficacy of pomegranate leaves. Therefore, pomegranate leaves grown in villages may be a better source of natural antioxidants and antimicrobials components.

Keywords: Antibacterial activity, antioxidant activity, pomegranate leaves, DPPH free radical scavenging activity, environmental factors, total polyphenol, flavonoids, tannin

Introduction
The practices of Ayurveda medicine have gained popular since ancient time to cure and prevent various types of illness and attributed overall positive effect on health. The Indian subcontinent has been gain attention due the home of richest diversity of various plant species, most of which have medicinal values and have be used in traditional treatments. The *Punica granatum L.*, commonly called Pomegranate plant, is a fruit-bearing deciduous shrub or small tree, belongs to the family *Lythraceae* [1]. Originally, Pomegranate is a native of Iran, Afghanistan and Baluchistan but now cultivated in India, China, USA, and Turkey [2, 3]. The pomegranate is one of the oldest edible fruits [4] and very popular in Asian subcontinent. The production as well as consumption of pomegranate fruits had been increased many fold because of it's versatile uses in many field of food preparation due to its taste and aroma. This can be used for the preparation of refreshing drinks, starter and desert, jam, fruit cakes, wines, etc [5, 6]. Not only that, various part of this plant such as bark, leaves, immature fruits and fruit rind have been gained importance due to their medicinal properties and extensively used for the treatment of diarrhea, dysentery, acidosis, helminthiasis, hemorrhage and respiratory problem [2, 7, 8]. The medicinal properties of various parts of the pomegranate plants could be due to the present of several antioxidant, antimicrobial, anti-inflammatory and antipyretic photochemical [2, 8, 9, 10, 11, 12, 13, 14, 15, 16].
Leaves of Pomegranate have been used for centuries as a traditional medicine, and current studies have been confirmed their potential health benefits [15, 17, 18, 19]. Pomegranate leaves contain several antioxidants, such as ellagic acid, punicalagins, and anthocyanins, that can help to protect against oxidative stress and inflammation [20]. The various polyphenols present in pomegranate leaves have been related to anti-inflammatory properties, and may help to reduce inflammation and alleviate conditions such as arthritis [21]. Pomegranate leaves also

exhibited antimicrobial activity against wide range of microorganisms, including bacteria, viruses, and fungi [22]. The presence of numerous antioxidants including polyphenols in the pomegranate leaves may attribute to reduce the risk of cardiovascular disease (CVD) by improving lipid profiles, reducing blood pressure, and preventing platelet aggregation [23] and protect against neurodegenerative disorder, such as Alzheimer's and Parkinson's diseases, by reducing oxidative stress and inflammation [26]. Pomegranate leaves also contains several phytochemicals that have been shown to inhibit cancer cell growth and also induce apoptosis (cell death) against various types of cancer, such as breast, prostate, and colon cancer [24, 25]. Leaves of Pomegranate have also been used traditionally to treat various type of digestive issues, including low appetite, diarrhea, dysentery and skin problems, which may attribute due to the presence of wide range of phytochemicals which have antimicrobial and anti-inflammatory properties [15, 17, 27]. The pomegranate leaves helped to accelerate wound healing by promoting collagen synthesis, improving tissue strength, and reducing inflammation [28] and have anti-diabetic effects by means of reducing glucose levels, improving insulin sensitivity, and also protecting against oxidative stress [20]. The presence of various phytochemicals in pomegranate leaves may also helped to protect against skin damage and improve skin elasticity [29].

Considering that leaves are not consumed and rarely approached, the high amount of bioactive compounds present in Pomegranate leaves considered to be the main reasons for various health benefits [18, 30, 31, 32]. However, the effectiveness of these properties may be influenced by several environmental factors such as soil quality, climate condition, and environmental pollution. There are very few studies have been reported regarding this issue. So, primary aim of the present studies is to analyses and compares the presence of various antioxidant compounds and evaluates antimicrobial activity of pomegranate leave extract, which are collected from towns and villages area.

Materials and Methods

Chemicals and experiments: All the chemicals and reagents which were used in these studies are either AR or GR grade. All experiments were done in triplicate manner.

Plant material: The fresh Pomegranate leaves (*Punica granatum* L.) were collected from Shyamnagar area, North 24 Parganas District of West Bengal, India which was marked as a sample from Town and the fresh leaves of Pomegranate which was collected from Aronghata area, Nadia District of West Bengal, India as marked as sample from village. The Fresh and whole leaves of Pomegranate were used in all these studies.

Ethanol and water extraction of Pomegranate Leaf

The ethanol and water extracts of fresh leaves of Pomegranate was prepared by taking 1gm of fresh leaves in mortar and ground with pestle using 100mL of respective solvents (water and ethanol) separately. The extracts were centrifuged at 5000g (Model: Microspin TC 4815, Eltek) and then filtered through Whatman no.1 filter paper and stored in at 4ºC for analysis.

Antioxidant measurement assay methods

a) DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging activity: DPPH free radical scavenging activities were measured according to the method described by Sasidharan *et al*. [33] with some modification. 50µl of leaf extract (different solvents) at a concentration ranging from 1 to 10mg/ml were mixed with 200µl of 100µM DPPH solution of DPPH (80% methanol) and incubated at room temperature for 30 mints (in dark). The OD was measured at 517 nm wavelength of light by using spectrophotometer (Systronics spectrophotometer Model: 2200). The DPPH solution and 80% methanol (without leaves) was used as control. The known antioxidant, butylated hydroxyl toluene (BHT) at the range of 1 to 20µl/ml was used as a positive control. The percentage of the DPPH free radical was calculated using the following equation [34]:

DPPH scavenging effect (%) = $((A_0 - A_1)/ A_0) \times 100$

A_0 means the absorbance of the control and A_1, the absorbance of experiment. The decrease of OD, induced by the test was compared with the positive controls. The IC_{50} values (concentration providing 50% inhibition) were calculated use the dose inhibition curve (extract concentration versus the corresponding scavenging effect).

b) Total Polyphenol content assay: Total Polyphenol content of leaf extract Pomegranate was measured using Folin-Ciocalteus reagent as described by Singleton *et al*. [35], with some modifications. Initially, 500ml of leaf extract (10mg/ml) was mixed with 1500ml of Folin-Ciocalteau's reagent (1:10 v/v diluted with ddH_2O) and incubated for 5 min at 22°C. 2000ml of sodium carbonate (Na_2CO_3, 7.5%, w/v) was added thereafter and mixed well and incubate for 90 min (in the dark) with intermittent staring. The OD of the blue color developed in the mixture was measured at 725nm using spectrophotometer (Systronics spectrophotometer Model: 220). Total Polyphenol content in the leaf extract was expressed as milligrams of Gallic acid equivalent per gram of leaf (mg GAE/g) by using the standard curve Gallic acid (25 to 150µg/ml).

c) Total Flavonoid content assay: Total Flavonoid content of leaf extract Pomegranate was determined according to colorimetric method described by Zhishen *et al*. [36], with some modification. Briefly 0.5 µl leaf extract (10mg/ml) was added in three bijoux bottles and mixed with 2000ml of distilled water. 150ml of sodium nitrite ($NaNO_2$, 5% w/v) was added into each bottles and allowed to stand for 6 min. 150ml of Aluminum tri-chloride ($AlCl_3$, 10%) was added and again allowed to stand for 6 min, followed by addition of 2000ml of sodium hydroxide (NaOH, 4% w/v). Then final volume of the mixture was maintained to 5000ml by adding distilled water and mixed thoroughly and incubated for 15 min. Then OD was measured at 510 nm using spectrophotometer (Systronics spectrophotometer Model: 220). The total Flavonoid content was expressed in mg of Quercetin (using standard curve of Quercetin) per gram of leaf.

c) Tannin content assay: Tannin content in the leaf extracts was determined by Follin Denis reagent [37]. Briefly 200ml of leaf extract or standard solution of Tannic acid (5µg/ml - 40µg/ml) was mixed with 250ml Follin Denis reagent, 500ml of saturated Na_2CO_3 solution were added after that and incubated for 30 min. Then the volume of the final mixture was made up to 5000ml with distilled water and OD was measured at 700 nm (Systronics spectrophotometer Model: 220. The Tannic content expressed as mg of tannic acid equivalent per gram of leaf [38].

d) Vitamin C assay: Vitamin C of the leaf extract was measured titrimatrically by 2, 6-dichloroindophenol (DCIP) solution [39, 40]. 5000ml of the leaf extract or standard of ascorbic acid, 2000 ml 3% Meta-phosphoric acid and 25 mL of distilled water mixture was titrated with the DCIP solution (52 mg of 2, 6-dichloroindophenol and 42 mg of Sodium bicarbonate in about 1000ml of water) until a permanent (lasting more than 30 sec) light red or pink colour appears. The volume of DCIP needed to oxidize the sample and standard ascorbic acid was correlated to find out ascorbic acid content in the sample. The result was expressed in mg of ascorbic acid per gram of leaf sample.

Antibacterial Properties Assay methods

a) Bacterial strains: The *Bacillus subtilis (B.S), Staphylococcus aurous (S.A) and Escherichia coli (E.C)* strains were collected from Department of Biotechnology, Bengal Institute of Technology (BIT), Kolkata, West Bengal and sub-cultured at 37ºC for 24 hrs in Agar slant of Nutrient agar media [Ingredients in gm/ L: Peptic digest of animal tissue 5.0, Sodium chloride 5.0, Beef extract 1.5, Yeast extract 1.5, Agar power 25 gm, Final pH- 7.4±0.2]. Fresh 24 hrs Nutrient broth culture was used as inoculum for all antimicrobial activity assays.

b) Spread plate disc diffusion method: For antimicrobial assay of Pomegranate leaf extract, spread plate disc diffusion method was used as described by Ghose *et al* [41]. Initially, 200ml of Bacterial suspension of each strain was spread on a lawn of nutrient agar containing Petri dish with glass spreader. Sterile 6-mm diameter of Whatman® no.1 filter paper disc was aseptically placed on the Petri dish (2 or 3 or 4 no. per Petri dish). 20µl of various solvent extract of leaf with dilution with water (different ratio with water; 1:1, 1:2 and 1:5) were aseptically poured on each disc along with sterile double distilled water (ddH_2O) as negative and various concentration of streptomycin (2.5, 5, 7.5 and 10µg/ml) as positive control. Petri dishes were then allowed to stand for 30 minutes at 4ºC and incubation at 37 ºC. The diameter of zone of inhibition (DIZ) was measured in nearest mm after 24 hrs of Incubation.

c) Activity Index determination (A.I): The activity index of the antimicrobial activity of the leaf extract of Pomegranate against *Bacillus subtilis (B.S), Staphylococcus aurous (S.A) and Escherichia coli (E.C)* were determined by using the following formula [41]:

The activity index of the combined solvent extract against a specific microbe = [DIZ in mm measured against a sample / DIZ in mm of the reference against same microbes]

Here, DIZ in mm of Streptomycin (concentration of 10µg/ml) is used as reference positive control against various solvent extract of leaf of Pomegranate.

Statistical analysis: All experiments were performed in triplicate and the results were expressed as mean±SD. One-way analysis of variance (ANOVA) were performed. Significant differences were analyzed by Duncan's multiple range tests. P values less than 0.05 were considered statistically significant.

Results and Discussion

DPPH free radical scavenging activity

The free radical scavenging activity of the water and ethanolic extracts of Pomegranate leaves (*Punica granatum* L.) was measured by DPPH and the results of free radical scavenging activity (given in Figure 1) was expressed as IC_{50} value in µg/ml (half-maximal inhibitory concentration). Lower IC_{50} value indicates higher antioxidant capability. From Figure 1, the highest free radical scavenging effect (IC_{50} value 213.12±3.05 µg/ml of BHT equivalent) was observed in ethanolic leaf extract of Pomegranate, collected from town in compare to free radical scavenging effect (IC_{50} value 417.2±6.64 µg/ml of BHT equivalent) of leaf extract collected from village. This observation indicates its highest antioxidant potential was found in leaf extract of Town. IC_{50} value of the water extract of leaf of Pomegranate collected from Town (IC_{50} value 299.4 ±1.42 µg/ml of BHT equivalent) was also lower than free radical scavenging activity (IC_{50} value 328.8±9.40 µg/ml of BHT equivalent) of leaf extract collected from village.

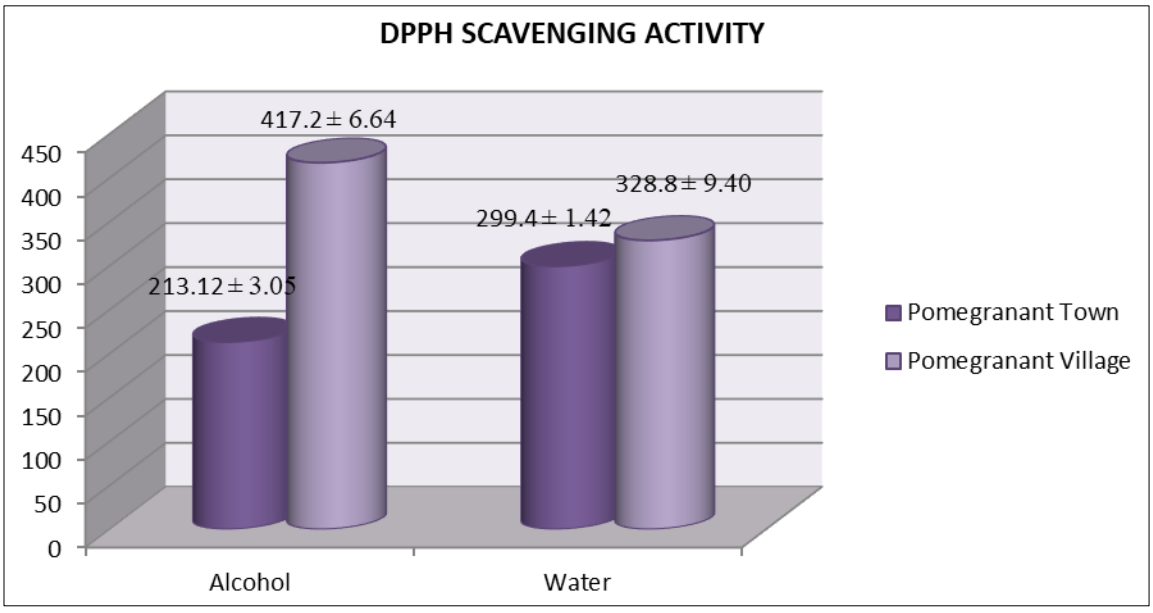

Fig 1: Estimation of total DPPH Scavenging activity (IC_{50} values in µg/ml of BHT equivalent) of Pomegranate leaf (Punica granatum L.) collected from Town and Village area.

Total Polyphenol content

The total Polyphenol content of the water and ethanol extracts of Pomegranate leaf (*Punica granatum* L.) was expressed as equivalent of mg of Galic acid per g of leaf sample (mg/g of GAE) and the results were presented in Figure 2. From Figure 2, the highest total Polyphenol activity (125.5±2.34mg/g of GAE) was observed in ethanolic leaf extract of Pomegranate collected from Village area in compare to total Polyphenol activity (83±2.08 mg/g of GAE) of leaf collected from Town area. The total Polyphenol content of the water extract of leaf of Pomegranate collected from Village area (121±2.08 mg/g of GAE) was also higher in compare to total Polyphenol content (102.5 ±4.04 mg/g of GAE) of leaf collected from Town area. This observation indicates that highest antioxidant potential regarding total polyphenol content in both solvent was found in leaf extract, collected from Village area in compare to leaf collected from Town area.

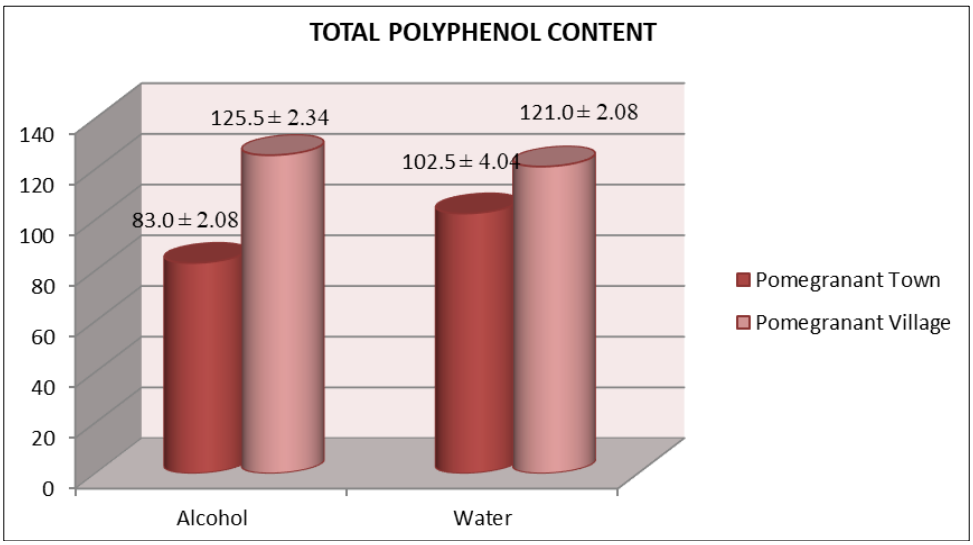

Fig 2: Estimation of total Polyphenol Content (mg/g of GAE) of Pomegranate leaf (*Punica granatum* L.) collected from Town and Village area.

Total Flavonoid content:

The total Flavonoid of the water and ethanol extracts of leaf of Pomegranate (*Punica granatum* L.) was measured by according to colorimetric method described by Zhishen *et al.* [36] expressed as equivalent of mg of Quercetin per g of leaf sample (mg/g of Quercetin). The results of total Flavonoid activity were presented in Figure 3. The highest total Flavonoid content (96.52±2.40 mg/g of Quercetin) was observed in water extract of leaf of Pomegranate collected from Town area in compare to total Flavonoid content (90.86±1.47 mg/g of Quercetin) of leaf collected from Village area. However the total Flavonoid content of the ethanolic extract of leaf of Pomegranate (67.94±1.13 mg/g of Quercetin) collected from Village area was higher in compare to total Flavonoid content (60.11±1.92 mg/g of Quercetin) of leaf extract collected from Town area. This observation indicates its highest antioxidant potential in compare to total Flavonoid content was found in leaf extract of Town.

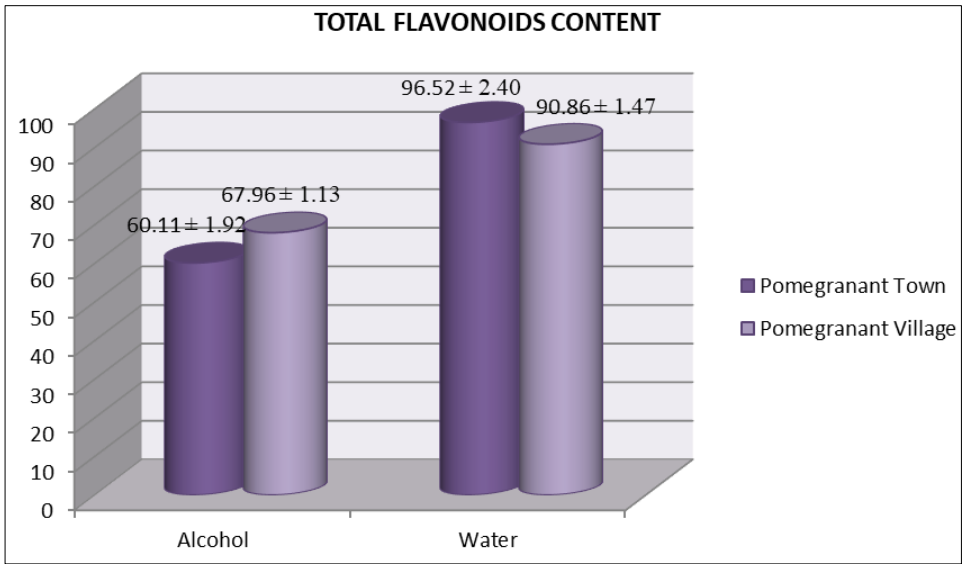

Fig 3: Estimation of total Flavonoid Content (mg of Quercetin/g of sample) of Pomegranate leaf (*Punica granatum* L.) collected from Town and Village area.

Total Tannin content

The total Tannin content of the water and ethanol extracts of leaf of Pomegranate (*Punica granatum* L.) was measured by Follin Denis reagent [37] and expressed as equivalent of mg of Tannic Acid per g of sample (mg of TA /g of leaf sample). The results of total Tannin content were presented in Figure 4. From Figure 4, the highest total Tannin content (108.16±1.08 mg of TA/g) was observed in water extract of leaf of

Pomegranate collected from Village area, in compare to total Tannin content (64.8±1.52 mg of TA/g) of leaf extract collected from Town area. Total Tannin content (69.27±1.82 mg of TA/g) of the ethanolic extract of leaf of Pomegranate collected from Village area was also higher in compare to total Tannin content (64.13±1.44 mg of TA/g) of leaf collected from Town area. This observation indicates that, highest antioxidant potential in context to Tannin content was found in leaf extract collected from Village area in compare to leaf collected from Town area.

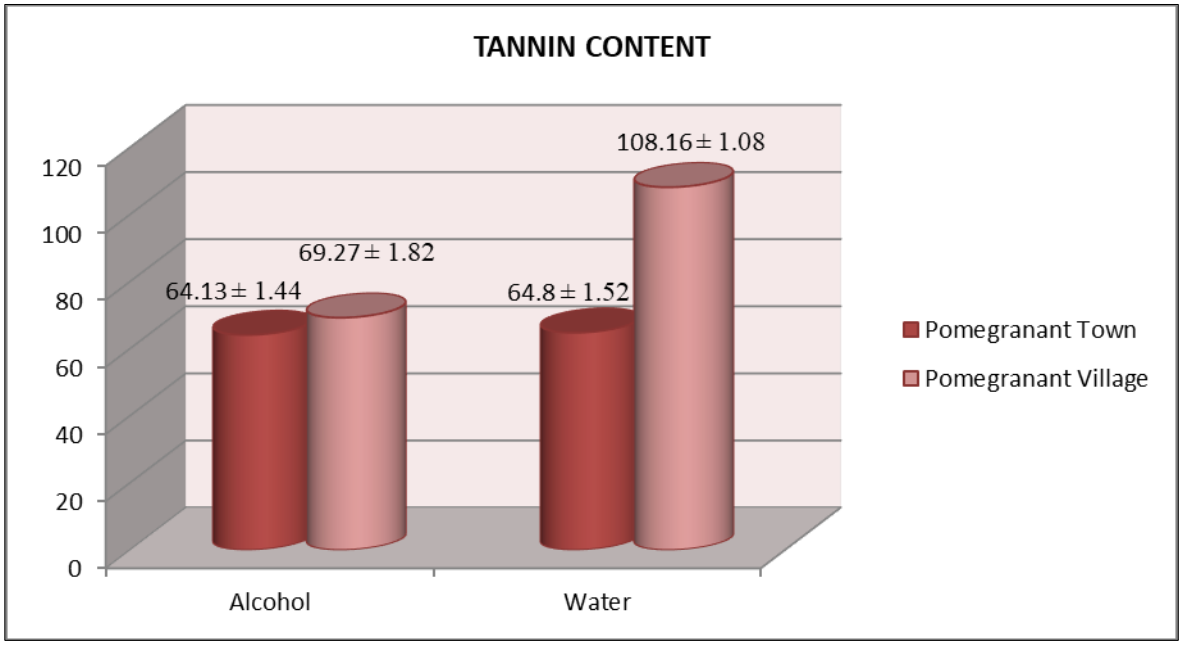

Fig 4: Estimation of total Tannin Content (mg of Tannic Acid /g of leaf sample) of Pomegranate leaf (*Punica granatum* L.) collected from Town and Village.

Vitamin C content

The Vitamin C content of the water and ethanol extracts of leaf of Pomegranate (*Punica granatum* L.) was measured titrematrically by 2, 6-dichloroindophenol (DCIP) solution [39, 40] and expressed as equivalent of mg of Vitamin C per g of leaf sample (mg/g of leaf sample). The results of Vitamin C content were presented in **Figure 5**. From **Figure 5**, the highest Vitamin C content (8.18±0.78 mg/g) was observed in ethanolic extract of leaf of Pomegranate collected from Village are, in compare to Vitamin C content (7.29±1.01 mg/g) of leaf collected from Town area. However, in case of water extract, Vitamin C content (7.04±1.15 mg/g) of leaf of Pomegranate collected from Town area has no significantly difference in compare to Vitamin C content (6.95±1.25 mg/g) of leaf collected from Village area. So overall data fiend in these experiments indicates that, antioxidant potential in context to Vitamin C content was found in leaf extract collected from Village area are relatively highest in compare to leaf, collected from Town area.

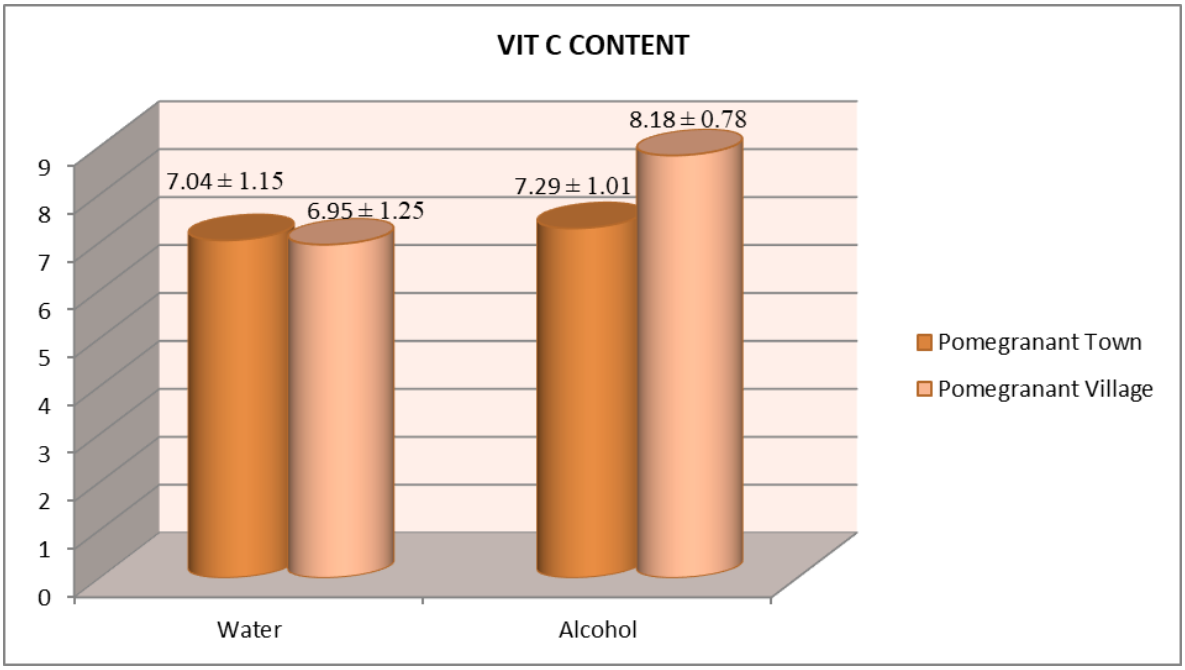

Fig 5: Estimation of Vitamin C Content (mg of Vitamin C /g of leaf sample) of Pomegranate leaf (*Punica granatum* L.) collected from Town and Village.

Antibacterial properties of leaf of Pomegranate

The fresh leaf extract of different solvents of pomegranate collected from town and village area are used for antibacterial properties analysis against two gram positive (+ve) strain; *Bacillus subtilis* and *Staphylococcus aureus* and one gram negative (-ve) bacterial strain; *Escherichia coli*. During antibacterial activity assay, various concentration of leaf extract (1:1; 1:2 and 1:5 diluted with sterile water) was used to determine the zone of inhibition (mm) and to calculate activity index (A.I). The antibiotic; streptomycin at various concentrations (2.5, 5, 7.5 and 10µg/ml) against *Staphylococcus aurous* are used as a standard. The results are represented in Table 1 and Figure 6. The Diameter of Inhibitory Zones (DIZ) in mm against *Staphylococcus aureus* was found to be 11.3mm, 13mm, 15.3mm and 16.9mm respectively for streptomycin concentration, 2.5, 5, 7.5 and 10µg/ml respectively. DIZ of 5µg/ml concentration of streptomycin is used as a reference to calculate the activity index (A.I.) of antibacterial activity of leaf of Pomegranate.

Table 1: Antibacterial Activity (DIZ in mm) of Streptomycin at various concentration against *Staphylococcus aureus.*

Microorganisms	Concentration of Streptomycin (µg/ml)	DIZ (mm)
Staphylococcus aurous	2.5	11.3
	5	13
	7.5	15.3
	10	16.9

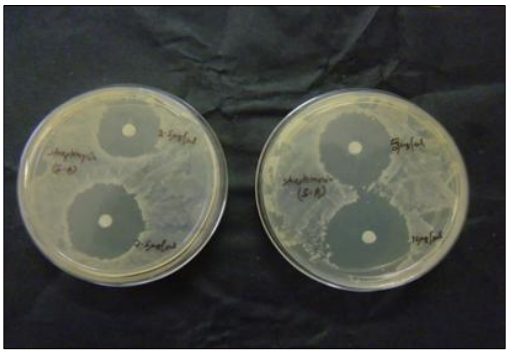

Fig 6: Antibacterial Activity (zone of inhibition) of Streptomycin at 2.5, 5, 7.5 and 10µg/ml concentration against *Staphylococcus aureus*.

The results of the diameter of zone of inhibition (DIZ in mm) and activity index (A.I) of the Antimicrobial activity of leaf extract of pomegranate (water and ethanol) against *Bacillus subtilis, Staphylococcus aurous and Escherichia coli* was presented in Table 2 and Figure 7.

Table 2: Antibacterial Activity of Pomegranate leaf (*Punica granatum* L.) collected from Town and Village area against *Bacillus subtilis, Staphylococcus aurous* and *Escherichia coli.*

Place of Leaves	Extracting Solvent	Ratio of Extract used	DIZ (mm)					
			B.S		S.A		E.C	
			I.Z	A.I	I.Z	A.I	I.Z	A.I
Town	Alcohol	1:1	2.2	0.17	2.1	0.16	2.1	0.16
		1:2	1.6	0.12	1.5	0.11	1.8	0.14
		1:5	1.2	0.09	1.1	0.08	1.3	0.10
	Water	1:1	N.I	-	N.I	-	N.I	-
		1:2	N.I	-	N.I	-	N.I	-
		1:5	N.I	-	N,I	-	N.I	-
Village	Alcohol	1:1	2.5	0.19	2.8	0.22	2.6	0.20
		1:2	1.9	0.15	1.7	0.13	2.0	0.15
		1:5	1.6	0.12	1.3	0.10	1.4	0.12
	Water	1:1	Nil	-	Nil	-	Nil	-
		1:2	Nil	-	Nil	-	Nil	-
		1:5	Nil	-	Nil	-	Nil	-

*DIZ-Diameter of zone of inhibition, *B.S-*Bacillus subtilis*, *S.A-*Staphylococcus aureus*,*E.C- *Escherichia coli* *I.Z- Inhibitory Zone (in mm) *N.I-No inhibition zone, *A.I- Activity index

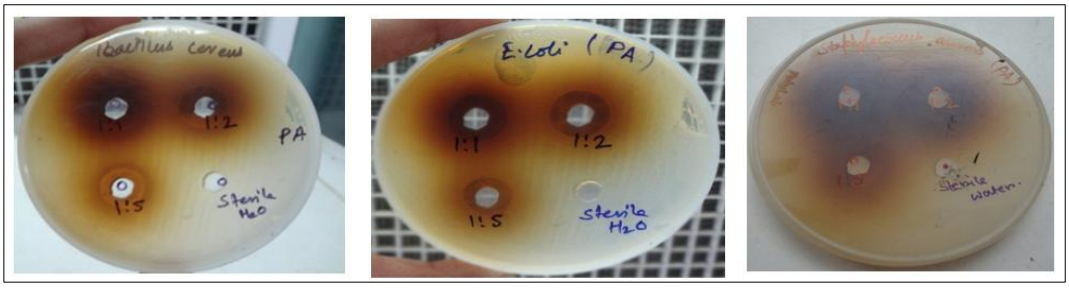

Fig 7: Antibacterial Activity (zone of inhibition) of Pomegranate leaf (*Punica granatum* L.) collected from Town and Village area against *Bacillus subtilis*, *Staphylococcus aurou*s and *Escherichia coli.*

From these results, it was observed that all samples of water leaf extract of pomegranate (collected from Town or Village area) did not show any measurable DIZ for all combination ratios (1:1, 1:2 and 1:5) against *Bacillus subtilis* (B.S), *Staphylococcus aurous* (S.A) and *Escherichia coli* (E.C). All ethanol leaf extract have shown measurable DIZ values at various dilution ratios and consequently activity index (AI). The highest activity index (AI) is measured to be 0.22 for 1:1dilution of ethanol extract of the leaf of Pomegranate collected from Village area against S.A, which is followed by 0.20 for E.C and 0.19 for B.S against same dilution ratio (1:1) and leaf sample. Whereas the highest activity index (AI) is measured to be 0.17 for 1:1 dilution ratio of ethanol extract of leaf of Pomegranate collected from Town area against B.S, followed by 0.16 each against S.A and E.C for both 1:1 dilution ratio. From this observation, it may be concluded that ethanol extracts of leaf of Pomegranate, collected from Village area have shown relatively higher antibacterial activity at different dilution in compare to ethanolic extracts of leaf of Pomegranate collected from Town area against all tested microorganism. Various studies on Pomegranate leaves have been shown to exhibit antimicrobial activity against a range of microorganisms, including bacteria and fungi [22]. The antimicrobial activity of pomegranate fruit peels has also explored against some food-borne pathogens [42].

Conclusion

So the present study revealed that, fresh leaf extract of Pomegranate, collected from Village area have relatively higher levels of total Polyphenol, Tannin, Ascorbic acid and antimicrobial components, whereas flavonoids and DPPH free radical scavenging activity have been found to be relatively higher in leaf collected from Town area. Kumar *et al*., [20] reported the presence of ellagic acid, punicalagins, and anthocyanins in the leaf of Pomegranate. Alcohol extracts of lead of Pomegranate collected either from Town or Village were shown the antibacterial activity in different dilution whereas water extracts of leaf of Pomegranate from both sources were not shown any measurable antibacterial activity against *S.A, B. S* and *E.C.* Hence most of the Antimicrobial components are present in ethanolic extract of leaf. These findings are also supported by the work of Singh *et al*., and Alam *et al*., [22, 27]. The environmental factors such as soil quality and pollution may impact on the presence of bioactive compounds and overall efficacy of pomegranate leaves. The presence of different antioxidant constituents in the fresh leaf extract of Pomegranate may influence nutritional as well as medicinal uses. Therefore, pomegranate leaves grown in village area may be a better source of natural antioxidants and antimicrobials components. However direct studies using more fractioned extract could provide better evidence for this claim.

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