Int. J. Adv. Res. Biol. Sci. 2(12): (2015): 178–187

International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
Coden: IJARQG(USA)

Research Article

SOI: http://s-o-i.org/ 1.15/ijarbs-2-12-20

In-vitro Antioxidant and Antimicrobial Activity of *Allium tuberosum* Rottler. ex Spreng

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Abstract

The present study is aimed to investigate the comprehensive *in-vitro* antioxidant and antimicrobial activity of *Allium tuberosum* Rottler. ex Spreng collected from western Himalayan region of India. Water extract and ethanolic extracts of *A. tuberosum* were compared in this study. Antioxidant activity of *A. tuberosum* in terms of IC_{50} (mg/ml) was determined against two type of free radicals i.e. DPPH (1, 1-diphenyl-2-picrylhydrazyl) and ABTS [2,2'-azinobis (3-ethylenebenzothiazoline-6-sulphonic acid)] radicals. The concentration of various polyphenolic compounds like total phenolic content, flavonoid content and proanthocyanidin content were also determined in *A. tuberosum* extracts due to their potent antioxidant behaviour in the plants. All extracts showed significant antioxidant activity, and maximum scavenging was observed in case of ethanolic extracts of *A. tuberosum* with minimum IC_{50} values 0.736 mg/ml and 0.651 mg/ml for DPPH and ABTS radicals. The antimicrobial activity of *A. tuberosum* was determined using zone of inhibition and Minimum Inhibitory Concentration (MIC) methods. Results clearly revealed the efficacy of *A. tuberosum* as a potent antimicrobial agent against the bacteria *E. coli a*nd P. *aeruginosa.* Ethanolic extract of *A. tuberosum* showed better antimicrobial activity, while compared with water extract. Ethanolic extract has potency to check the growth of *E. coli* at 64.0 µg/ml and *P. aeruginosa* at 32.0 µg/ml. The efficacy of *A. tuberosum* as a natural antioxidant and antimicrobial agent has been established in this study.

Keywords: *Allium tuberosum*, antioxidant activity, antimicrobial activity, polyphenolic contents.

1. Introduction

Medicinal and aromatic plants are the eventual resource of bio-molecules that are essentially required to cure various human ailments. As per the data available with World Health Organization (WHO), there are more than 20,000 such plant species that are currently being used in different therapeutic formulations all around the world [Pandey et al., 2007]. In Indian traditional medicinal system, the Himalayan region has been documented as a treasure of medicinal and aromatic plants. In India, Himalayan region alone supports about 18,440 species of plants, out of which 45% plants have been reported to have medicinal properties [Kumari et al., 2009]. In recent years most investigations have been oriented towards the standardization of the active principles in pharmaceutical preparations from these plants [Moyers, 1996]. *Allium* family, being a part of this enormous treasure has a unique position in our culture and literature as perennial edible plants and herbal medicine since the dawn of human civilization. *Allium* plants are generally consumed for their flavors along with their appreciable nutritive values. Garlic, onions, and leeks are well known for their multiple beneficial effects such as hypoglycemic, hypo-lipidemic, anti-arthritic, antimicrobial, antithrombotic and antitumor activities. The active components in garlic help to reduce serum cholesterol, blood sugar level, hypertension, respiratory infections and the aging process [Lawson and Bauer, 1998, Orekhv and Grunwald, 1997].

It is believed that consumption of one clove of garlic everyday leads to significant reduction in cardiovascular complications [Roman et al., 1989]. Beneficial effect of lowering elevated serum cholesterol level for prevention of coronary heart diseases and hypertension is well established [Simons, 2002]. The cardiovascular protective effects of garlic have been evaluated extensively in past and recent years [Banerjee and Maulik, 2002]. Oral feeding of garlic extracts has been shown to reduce the incidence and growth of transplantable and spontaneous tumors in experimental animals and the active components were found to influence a number of physiological and immunological functions which account for their anti-carcinogenic and antitumor effects [Kendler, 1997]. Antifungal activities have also been reported in *Alliums* [Cavallito and Bailey, 1944].

Allium tuberosum Rottler. ex Spreng (Chinese chive, Alliaceae) from Western Himalayan region of India is a high medicinal value herbal plant, which is well recognized as a potential medicinal herb due to its effectiveness in treating broad range of diseases and disorders including its hypo-lipidemic and hypoglycemic attributes. Chinese chive is widely cultivated in China, whose seeds have been reputedly used as a traditional Chinese medicine for treating both impotence and nocturnal emissions. Tender leaves of *A. tuberosum* with a light taste of garlic can be used, only chopped, to flavor vegetables, salads, omelets, etc. The flowers are also used for the same purposes [Shah, 2014]. It is also cultivated in many other parts of Asia including the Himalayan region of India and Nepal. Besides this, it is also found in Cambodia, Indonesia, Japan, Korea, Laos, Malaysia, Myanmar, Singapore, Thailand and Vietnam and outside of Asia in Cuba and the US [Alizadeh, 2013].

The major aspect of *A. tuberosum* is considered to be the inhibition of Reactive Oxygen Species (ROS). The scavenging of free radicals is the prime factor for controlling degenerative or pathological processes of various serious ailments in human body, such as aging [Burns, 2001], Cancer[Cai, 2004], Alzheimer's disease[Smith, 1996], and heart diseases[Luximon-Ramma, 2002], Plant derived antioxidants, such as ascorbic acid, α-tocopherol, polyphenols and flavonoids, are becoming increasingly popular as important dietary factors [Ferguson, 2001], due to the low risk associated with them. *A. tuberosum*, as a high medicinal value herb, is very potent source of several natural antioxidants.

The present investigation was aimed to assess the unexplored in-vitro antioxidant and antimicrobial activity of *Allium tuberosum* collected from cold and humid region of Western Himalayas. Antioxidant activity of *A. tuberosum* was determined against DPPH (1, 1-diphenyl-2-picrylhydrazyl) and ABTS [2,2'-azinobis (3-ethylenebenzothiazoline-6-sulphonic acid)] radicals. Total phenolic contents (TPC), total flavonoid contents (TFC) and proanthocyanidins were also determined, to evaluate their effects on free radical scavenging capabilities of *A. tuberosum*. The antimicrobial activity of *A. tuberosum* was determined using zone of inhibition and Minimum Inhibitory Concentration (MIC) methods against the bacteria *E. coli a*nd P. *aeruginosa.*

2. Materials and Methods

2.1 Collection of Plant Samples and Preparation of Extracts

Allium tuberosum Rottler. ex Spreng was collected from Munsayari, Pithoragarh, located in Western Himalayan region of India. The Plants were identified and authenticated from Botanical Survey of India (BSI) Northern Regional Centre, Dehradun, India.

Leaves of the plant were allowed for air-drying in the oven at ambient temperature (30-35℃), and then crushed. Fifty grams of the sample was extracted with 200.0 ml of Ethanol and water, separately, at room temperature, with agitation for 24–30 h. Alcoholic extracts were filtered off using Whatman no. 1 filter paper, and concentrated under reduced pressure up to dryness below 35°C. Water extracts were freeze-dried. Antioxidant activities and polyphenolic contents were evaluated for the *A. tuberosum* in terms of sample dry weight.

2.2 Chemicals used for Experimentation

2,2'-azino*bis*-3-ethylbenzothiazoline-6-sulfonate ammonium salt (ABTS), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), catechin, quercetin and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu phenol reagent, ascorbic acid, vanillin and sodium carbonate were from Merck Chemical Supplies (Darmstadt, Germany). All the other chemicals including the solvents were of analytical grade.

2.3 Determination of Total Phenolic Contents:

Total phenolic contents in the extracts were determined by the modified Folin-Ciocalteu Method [Wolfe, 2003]. Each sample extract (1.0 mg/ml) was mixed with 5.0 ml Folin-Ciocalteu reagent (earlier diluted with water 1:10, v/v) and 4.0 ml (75 g/l) of sodium carbonate. The tubes were vortexed for 30 s, and allowed to stand for 30 minutes, at room temperature. Absorbance was then measured at 765 nm using the Double beam PC based Labomed UV-Vis spectrophotometer (Culver City, CA 90232, USA). Total phenolic contents (TPC) were expressed as milligram Gallic acid equivalent/g (mgGAE/g) of sample dry weight.

2.4 Determination of Total Flavonoids:

Flavonoids contents were estimated using Aluminum chloride colorimetric method [Chang, 2002]. Sample extract (1.0 mg/ml) was mixed with 0.1 ml of 10% aluminum chloride, 0.1 ml of 1.0 M CH_3COOK and 2.8 ml of distilled water. The reaction mixture was vortexed for 30s, allowed to remain at room temperature for 30 minutes, and then the absorbance of the reaction mixture was measured at 415 nm. The calibration curve was prepared using quercetin as standard. Flavonoid contents were expressed as mg Quercetin equivalent/g (mgQE/g) of sample dry weight.

2.5 Determination of Proanthocyanidins:

Proanthocyanidin contents or condensed tannins were determined based on the procedure reported by Sun et al., 1998. A volume of 0.5 ml of 0.1 mg/ml of extract solution was mixed with 3.0 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid. The reaction mixture was vortexed for 30s, and then allowed to stand for 15 minutes. The absorbance was then measured at 500 nm. Total proanthocyanidin contents were expressed as mg Catechin equivalents/g (mgCE/g).

2.6 DPPH Radical-Scavenging Activity:

The stable free radical 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for evaluation of free radical-scavenging activities of *A. tuberosum* extracts, as per the method described by Hatano et al., 1989. Different concentration of each extract was added to an equal volume of methanolic solution of DPPH (0.1mM). Subsequently, after 30 minutes, at room temperature, the absorbance of the resulting samples was recorded at 517 nm. The experiment was repeated for three times. Ascorbic acid and quercetin were used as standard antioxidants. Results were expressed in terms of IC_{50} value. IC_{50} value represents the inhibitory concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Percentage inhibition by the sample was calculated as,

$$\text{Inhibition (\%)} = [(A_{control} - A_{sample})] / (A_{control})] \times 100$$

Where, $A_{control}$ is the absorbance of DPPH radical + methanol; A_{sample} is the absorbance of DPPH radical + sample extract/standard

2.7 ABTS Radical-Scavenging Activity:

Free radical scavenging capacity of *A. tuberosum* was also assessed with the help of 2, 2´-azino*bis* [3-ethylbenzthiazoline] -6-sulfonate (ABTS) assay [Re et al., 1999]. ABTS solution (7.0 mM) in methanol and potassium persulfate (2.45 mM) solution in water were prepared separately as stock solutions. The working solution was then prepared by mixing the two stock solutions in equal quantities, and allowed to react for 12 hrs, at room temperature, in the dark. This solution was then diluted by mixing 1.0 ml of $ABTS^{\cdot+}$ solution and appropriate volume of methanol, to achieve absorbance in the range of 0.702 ± 0.001 units, at 734 nm. *A. tuberosum* extracts (1.0 ml) were added with 1.0 ml of the ABTS solution, and the absorbance was taken at 734 nm, after 10-minute reaction time. The $ABTS^{\cdot+}$ scavenging capacity for the extracts was also compared with standard antioxidants like ascorbic acid and quercetin.

Percentage inhibition was calculated as,

$$\text{Inhibition (\%)} = [(A_{control} - A_{sample})] / (A_{control})] \times 100$$

Where, $A_{control}$ is the absorbance of ABTS radical + methanol; A_{sample} is the absorbance of ABTS radical + sample extract/standard

2.8 Antibacterial screening by zone of inhibition method

Extracts of *A. tuberosum* were screened for their antibacterial activities against *Escherichia coli* (MTCC-40) and *Pseudomonas aeruginosa* (MTCC-424) using the filter paper scrap disc method [Bauer et al., 1966]. Culture of *Escherichia coli* and *Pseudomonas aeruginosa* was carried out on growth medium (0.2% Yeast extract, 0.5% Peptone, 0.1% Beef extract, 0.5% NaCl and 1.5% Agar in 1.0 L double distilled autoclaved water). Culture media were prepared using aseptic and sterilization techniques.

Incubation period for bacterial strains was 48 hours at 37°C. Initially bacterial strains were grown in a broth media, there after the bacteria were swabbed uniformly across a culture plate. A filter-paper disk (5 mm), impregnated with the extract (50 ppm/100 ppm/150 ppm), is then placed on the surface of the agar and incubated for 48 hrs at 37°C, and the diameter of the inhibition zone in the bacterial lawn was measured.

2.9 Minimum Inhibitory Concentration (MIC)

MIC is expressed as the lowest dilution, which inhibited growth judged by lack of turbidity in the tube. The MIC of extract was measured using Broth dilution method [Tyagi and Malik., 2010]. The bacterial strains were incorporated into broth media having extract of double dilution in serial order i.e. 1024/512/256/128/64/32/16/8/4/2 ppm. The test mixture was having 10^6 organism/ml. Tubes were incubated for 18 hours at 37°C. Inoculation was also carried out of a tube containing 5ml broth with the organism and keep at +4°C in a refrigerator overnight for using it as standard for evaluating the inhibition process.

3. Results and Discussion

The present investigation has been focused on determination of antioxidant and antimicrobial activity of *A. tuberosum*. Efforts have also been made to quantify the Polyphenols, flavonoids and proanthocyanidin content as potent bio-molecules responsible for antioxidant activities. The antibacterial effect of *A. tuberosum* was evaluated against *E. coli* and *P. aeruginosa*, and the results were found very encouraging in order to establish the antibacterial activity of this medicinal plant. The findings of this study are summarized below;

3.1 Estimation of Total Phenolic Contents (TPC)

Phenolic compounds are well established bio-active components of the medicinal plants which are responsible for inhibiting oxidative damage caused by free radicals in the human body. Total phenolic contents (TPC) has been expressed as milligram Gallic acid equivalent/g (mgGAE/g) of sample dry weight using the straight line equation based on the calibration curve: $y = 0.0106x$, $R^2 = 0.999$; where, x was the absorbance and y was the mgGAE/g of sample dry weight (Fig. 1). The water extract was found to contain 157.606 mg/100g of phenolic contents in *Allium* dried weight. While the ethanolic extract has shown 229.079 mg phenolics/100g dried weight.

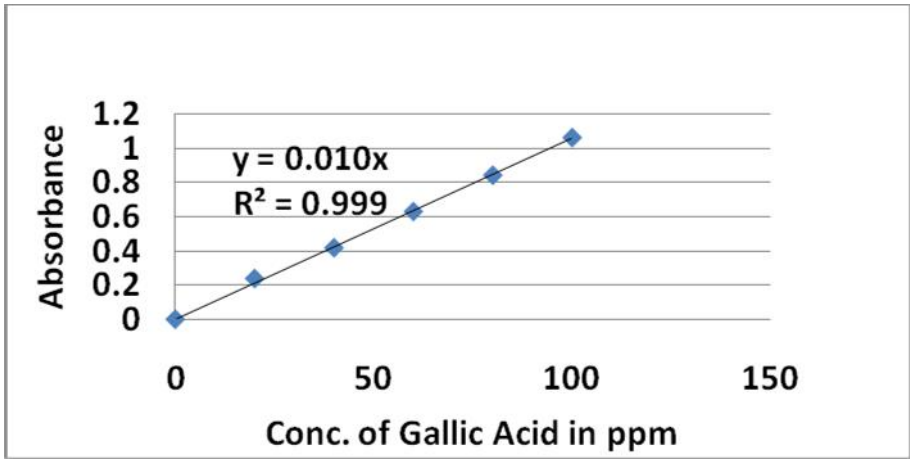


Fig.1 Standard curve for estimation of total phenolic Content

3.2 Estimation of Flavonoids

Flavonoids are well-known dietary constituents in the *Allium*, which show pH dependent radical scavenging behavior in human body. The calibration curve was prepared using quercetin as standard. Flavonoid contents (FC) were expressed as mg Quercetin equivalent/g (mgQE/g) of sample dry weight using the straight line equation based on the calibration curve: $y = 0.0066x$, $R^2 = 0.997$; where, x was the absorbance and y was the quercetin equivalent (mg/g) (Fig 2). The water extract was found to contain 106.310 mg of flavonoids/100g of *Allium* dried weight. While the ethanolic extract has shown 143.467 mg flavonoids/100g dried weight.

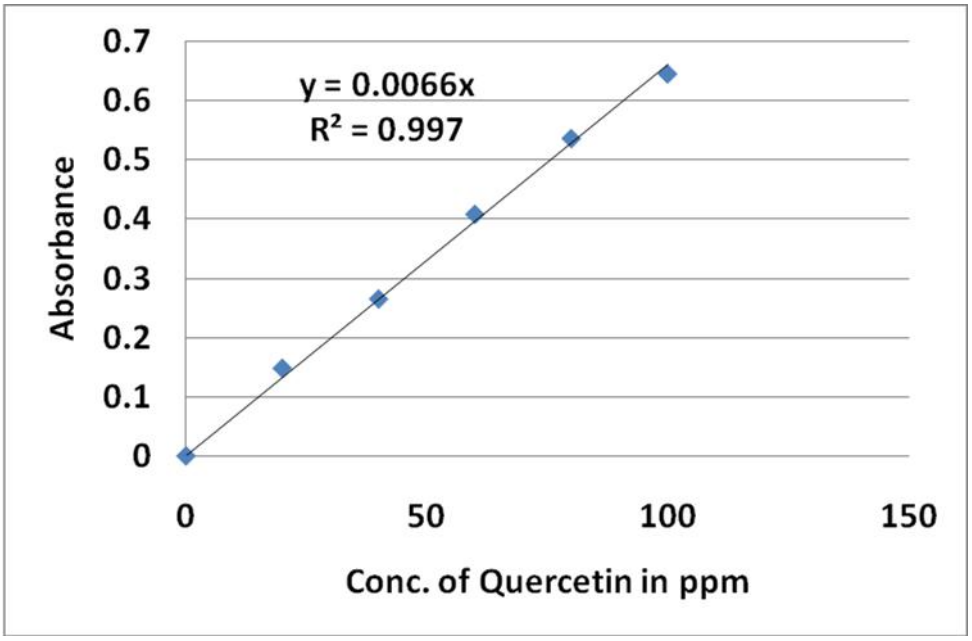


Fig.2 Standard curve for estimation of total flavonoids

3.3 Estimation of Tannins (Proanthocyanidin)

Proanthocyanidins (PA) are also known as condensed tannins or Oligomeric Proanthocyanidin (OPC). Studies have shown that proanthocyanidins have the ability to prevent cardiovascular diseases by suppressing the negative effects of high cholesterol on the heart and all related blood vessels. Proanthocyanidins have the antioxidant activities, and have the ability to scavenge the free radicals more than vitamin C & E. Proanthocyanidin contents were expressed as mg Catechin equivalents/g (mgCE/g) using the straight line equation based on the calibration curve: $y = 1.2415x$, $R^2 = 0.999$; where, y was the absorbance and x was the concentration of catechin (mg/g) (Fig 3). The water extract was found to contain 29.67 mg of proanthocyanidins/100g of *Allium* dried weight, while the ethanolic extract has shown 41.04 mg proanthocyanidins /100g *Allium* dried weight.

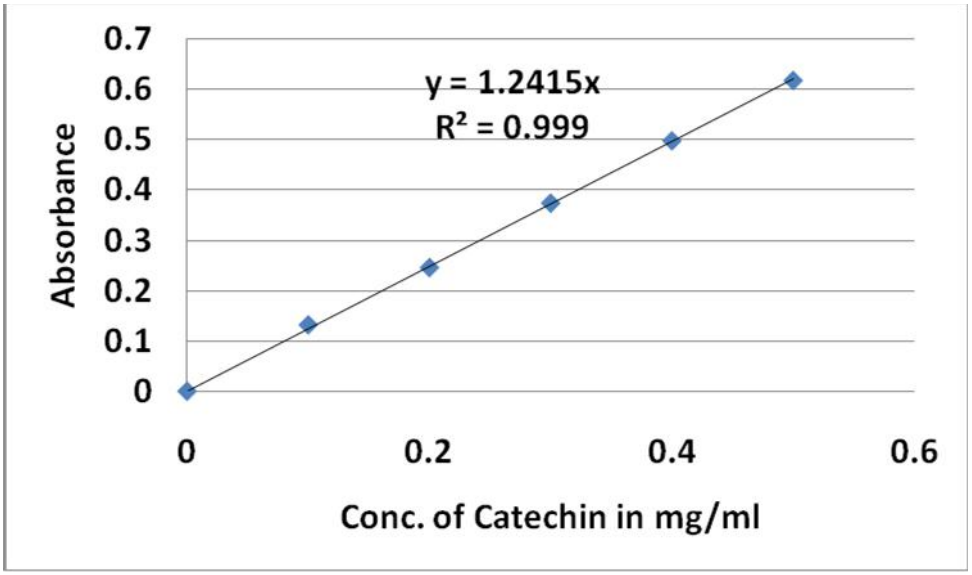


Fig.3 Standard curve for estimation of proanthocyanidins

The comparative analysis of the total phenolic contents, total flavonoids and tannin contents in *A. tuberosum* extracts is summarized below in Table-1.

Table 1- Quantitative estimation of phyto-constituents present in *A. tuberosum*

Sample	Extracts	Total Phenols in mg/100g Dry wt	Total Flavonoid in mg/100g Dry wt	Tannins in mg/100g Dry wt
A. tuberosum	Water	157.606	106.310	29.67
	Alcohol	229.079	143.467	41.04

3.4 DPPH Radical-Scavenging Activity

The scavenging activities of all the standard antioxidants and extracts against DPPH radical were found to be improved in dose dependent manner (Fig 4 & 5). The IC_{50} values of ascorbic acid and quercetin as standard antioxidants were evaluated as 7.85 µg/ml and 10.05 µg/ml respectively. While, the IC_{50} of the *A. tuberosum* were found 822.92 µg/ml and 735.96 µg/ml in water and ethanol extracts. Therefore, it is concluded that *A. tuberosum* is having excellent antioxidant activity against DPPH radical.

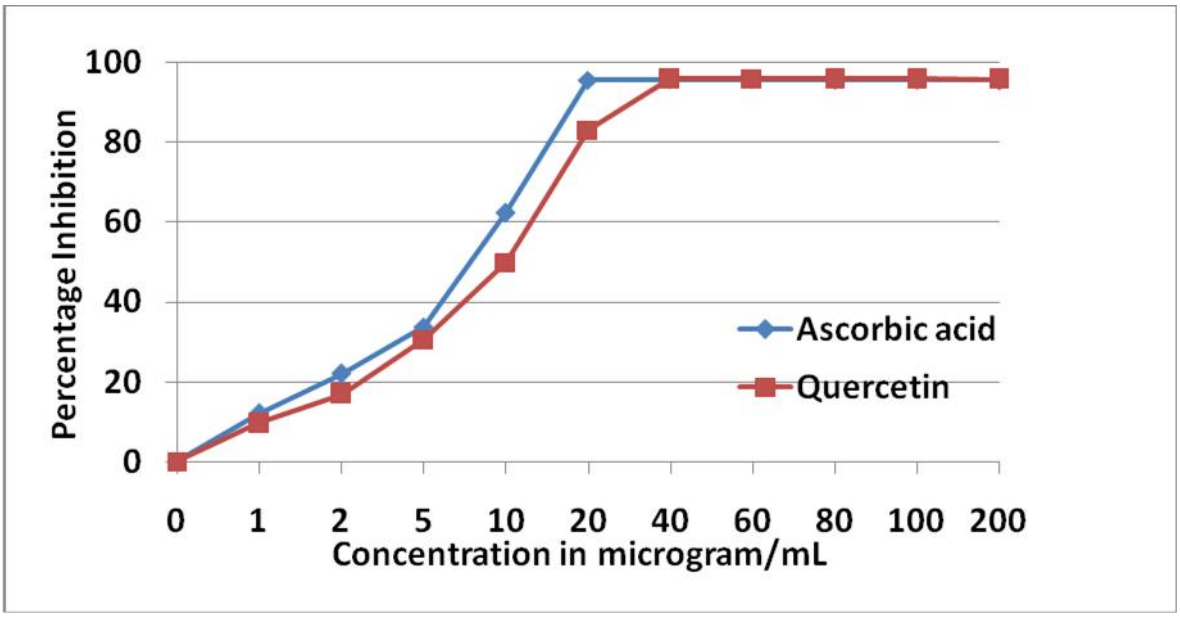


Fig.4 DPPH Radical Scavenging activity of Ascorbic acid and Quercetin

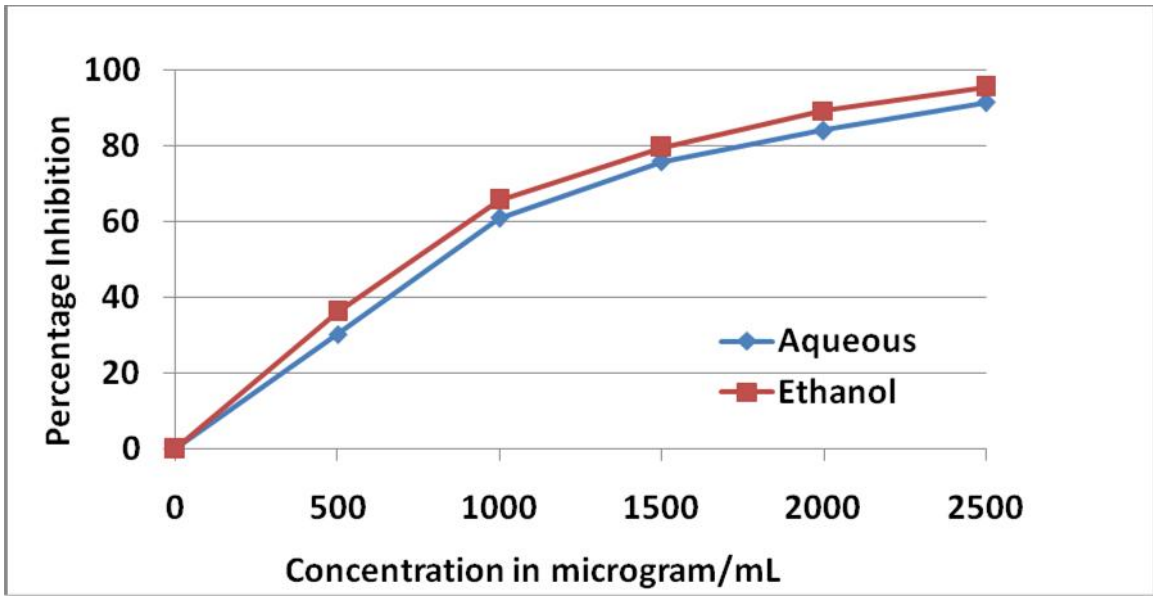


Fig.5 DPPH Radical Scavenging activity of *A. tuberosum* extracts.

3.5 ABTS Radical-Scavenging Activity

A. tuberosum was also analyzed for free radical scavenging activity against ABTS free radical system. Water and ethanol extracts of *A. tuberosum* were evaluated comparatively for their scavenging capacities. All the extracts showed significant ABTS scavenging capacity in dosage dependent manner (Fig 6 & 7). The IC_{50} values of the standard antioxidants i.e. ascorbic acid and quercetin were evaluated as 7.76 µg/ml and 7.28 µg/ml respectively. The IC_{50} of the *A. tuberosum* was calculated in various solvents *viz*. water (757.0 µg/ml) and ethanol (650.6 µg/ml). Therefore, it is worthwhile to mention that *A. tuberosum* is having excellent antioxidant activity against ABTS radical also.

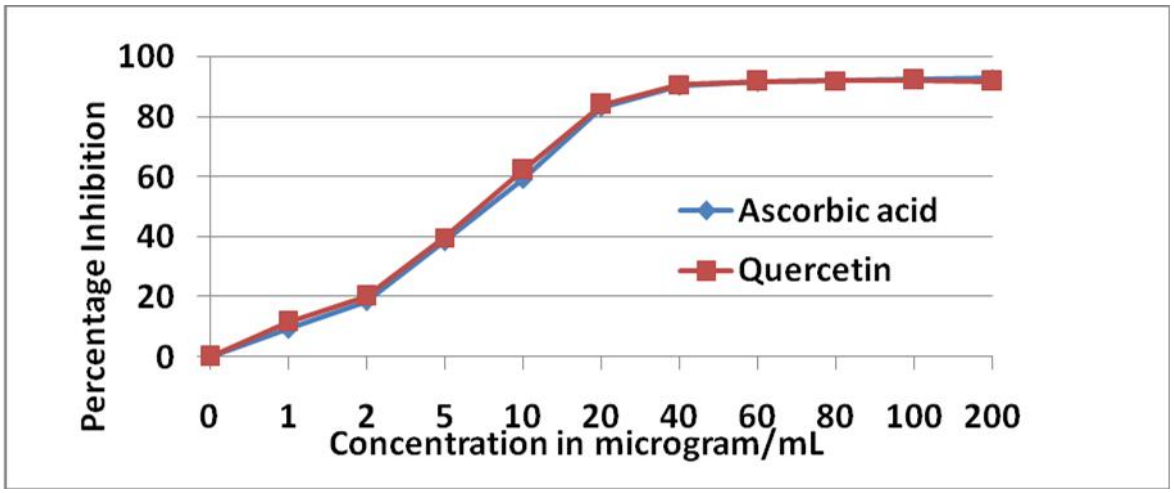


Fig.6 ABTS Radical Scavenging activity of Ascorbic acid and Quercetin

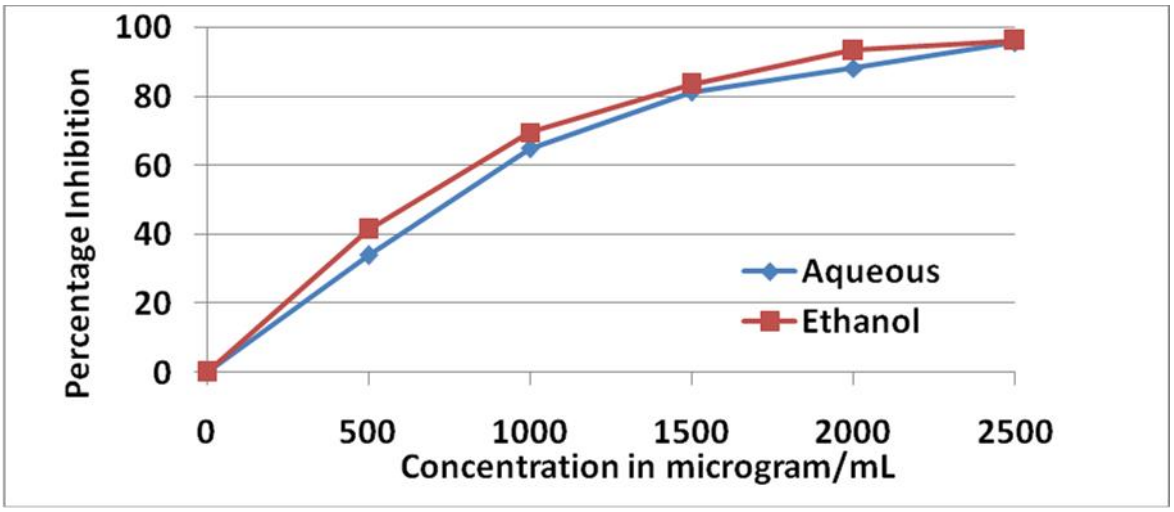


Fig.7 ABTS Radical Scavenging activity of *A. tuberosum* extracts.

3.6 Antimicrobial Activity

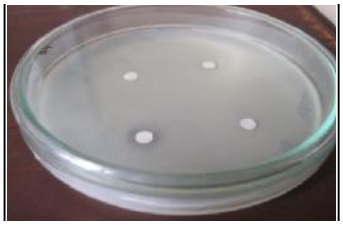
Health risks associated with bacterial diseases have resulted in mortality from treatment failures and it has become indispensible to evaluate the phytochemical preparations for their antimicrobial activities. To identify the precise public health risk and estimating the increase in costs is not a simple task and accordingly the easy availability of antimicrobial phytochemicals in the herbal formulation orients us to evaluate the antibacterial activity of *Allium tuberosum.*

A. tuberosum was found to have excellent antibacterial activities. Antibacterial screening by inhibition zone method clearly indicates that alcoholic extract of *A. tuberosum* is having more potency than the water extract against both bacteria *E. coli a*nd P. *aeruginosa.* The zone of inhibition is given below (Table-2 and Fig 8).

Table 2 - Antibacterial screening by inhibition zone method

S.N.	*A. tuberosum* Extract	*E. coli* (MTCC-40)			*P. aeruginosa* (MTCC-424)		
		50µg/ml	100µg/ml	150µg/ml	50µg/ml	100µg/ml	150µg/ml
1	Alcohol	6	9	11	8	10	13
2	Water	5	7	9	5	7	8

Antibacterial activity of ethanol extract against MTCC-40



Antibacterial activity of water extract against MTCC-

Fig.8 Zone of Inhibition for *Allium tuberosum* Extract Against *E. coli*

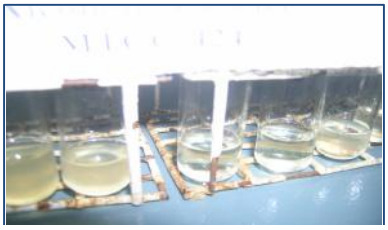
3.6.1 Minimum Inhibitory concentration (MIC):

MIC is the minimum concentration of a drug which is required to check the growth of micro-organism. The MIC of the *A. tuberosum* extract based on the serial double dilution method has indicated that both water and ethanolic extract has potency to check the growth of *E. coli* at 64.0µg/ml. While alcoholic extract was found to exhibit the MIC 32.0 µg/ml against *P. aeruginosa*. (Table-3 and Fig 9)

Table 3-Minimum Inhibitory Concentration (MIC)

S.N.	*A. tuberosum* Extract	MIC in µg/ml	
		E. coli (MTCC-40)	*P. aeruginosa* (MTCC-424)
1	Alcohol	64	32
2	Water	64	128

Alcoholic Extract MTCC-40 (MIC 64µg/ml)



Water Extract MTCC-40 (MIC 64µg/ml)

Fig. 9 MIC of *Allium tuberosum* Extract Against *E. coli*

Conclusion

The present investigation has shown the appreciable antioxidant and antimicrobial activity in *A. tuberosum* extracts. During the evaluation of antioxidant activity, maximum scavenging was observed in case of ethanolic extracts of *A. tuberosum* with minimum IC_{50} values 0.736 mg/ml and 0.651mg/ml against DPPH and ABTS radicals. Ethanolic extract of *A. tuberosum* was found to contain higher amount of polyphenolic contents in comparison to water extract and therefore the antioxidant activity of ethanolic extract can be attributed to these polyphenolic compounds. Findings of this study suggest that the extract of *A. tuberosum* can be potentially utilized as an antimicrobial agent against the bacteria *E. coli a*nd P. *aeruginosa.* Ethanolic extract of *A. tuberosum* was found to exhibit better antimicrobial activity in comparison to water extract. Water and ethanolic extract were found to inhibit the growth of *E. coli* at 64.0 µg/ml, while the alcoholic extract has shown better and lower MIC *i.e.* 32.0 µg/ml against *P. aeruginosa.* The excellent antioxidant and antibacterial properties of *A. tuberosum* has clearly revealed the importance of this medicinal plant for the nutraceutical and pharmaceutical purposes.

Acknowledgments

Authors pay their sincere gratitude to Dr. M. Nasim, Director, Defence Institute of Bio-Energy Research, Haldwani for providing research facilities. Encouragement from Principal, Kumaun University, Nainital is also well acknowledged for this study. Authors are also thankful to MTCC, Chandigarh (India) for providing bacterial cultures for this study. Extraordinary thanks goes to Dr. P. S. Negi, Scientist 'E', Dr. H. K. Pandey, Scientist 'D', Ms. Seema Singh, Senior Research Fellow and Mr. Subir Ranjan, Technical Officer 'A' Defence Institute of Bio-Energy Research, Field Station, Pithoragarh, Uttarakhand, India for their outstanding support to this study.

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How to cite this article:
Farha Sultana, M. Mohsin A.N. Sah (2015). *In-vitro* Antioxidant and Antimicrobial Activity of *Allium tuberosum* Rottler. ex Spreng. Int. J. Adv. Res. Biol. Sci. 2(12): 178–187.