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Qualitative and quantitative phytochemical analysis of Allium cepa L springs with a focus on its biological activity: A pilot study

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Abstract

Background: A preliminary work was carried out on phytochemical analysis and biological importance of *Allium cepa* springs.
Materials and Methods: Three different extractions were made from *Allium cepa* springs using hexane, ethyl acetate and ethanol. Antioxidant, anti-hemolytic, antibacterial and sun protection factor properties were performed and compared among the three extracts.
Results: The extracts contained alkaloids, phenolics, saponins, flavonoids, carbohydrates, tannins and glycosides, based on phytochemical screening. Among the three solvents used, and from all the tests, the highest biological activity was observed in ethyl acetate extract for antioxidant (1.2mM), sun protection factor (14.1) and anti-hemolytic (95%). Likewise, for antibacterial assay, *Pseudomonas aeruginosa* was selected and among the three prepared extracts tested, ethyl acetate extract exhibited the maximum zone of inhibition of 22 mm than the other two extracts. The extracts also have RBC protective activity when given as antileukemic to the patients and can be mixed with other formulations in preparing herbal sun protection lotions or creams. *In vivo* studies by using ethyl acetate *A. cepa* spring's extract will guide to develop new drug for the studied parameters. Further research is encouraged on the pharmacokinetics of the active components of ethyl acetate extract that contribute potential biological activity.
Conclusion: The quantitative determination of *A. cepa* springs has revealed abundance of flavonoids among all the other phytochemicals. The ethyl acetate extracts of *A. cepa* springs possessed potential antioxidant, SPF, anti-hemolytic and antibacterial activities and proved to have good pharmacological properties.
Key words: *Allium cepa*, antioxidant, anti-hemolytic, antibacterial, Sun Protection Factor

Introduction

Since pre-historic times, plants are known to exert remedial resources against human infections and defend organisms from the effects of free radicals, bacteria and viruses. The multifunctional properties of plants are majorly due to their phytochemicals produced by primary or secondary metabolism which are alkaloids, flavonoids, terpenoids, tannins, proteins, carbohydrates, lipids, gums and resins [1, 2]. Keeping in view of these inherent properties, there is a need to discover plants for their biological potential. *Allium cepa* L. (onion) belongs to Amaryllidaceae family with 250 genera and 3700 species. It is the oldest plant cultivated with abundant phytochemicals contributing to human health. Flavonoids are the most dominant phytochemicals present and quercetin (flavonol) accounts for more than 85% of all metabolites. Next to flavonoids are anthocyanins with cyanindin, peonidin, petunidin and delphidin as major groups. [3, 4]. To our knowledge, the chemical composition and biological activity of *A. cepa* springs with its therapeutic potential have not been studied so far. This has drawn attention to assess its biological activity.

Oxidative stress creates disturbance between production of reactive oxygen species and antioxidant defenses. Important biomolecules like DNA, proteins, carbohydrates and lipids get damaged due to over production of reactive oxygen species and hence suppression of oxidative stress can be done by dietary antioxidants. Diseases like β thalassemia, anemia are majorly occurred due to lysis of red blood cells with the aid of reactive oxygen species. With the advancement in literature it is believed that plant compounds have anti-hemolytic properties and hence *A. cepa* springs are screened for its anti-hemolytic property [5]. As bacterial infections are the second leading cause of mortality and current antibiotics are in serious threat of acquiring resistance, there is a need to screen novel antimicrobial agents. The role of onion bulb was proven to have anti-

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microbial effects and hence, the therapeutic activity of the leaves of *A. cepa* could be screened to find out the antimicrobial properties. [6, 7]

Natural products are recently considered as potential sunscreen resources due to their potential UV absorption and antioxidant capacity. [8, 9] As onions are rich sources of antioxidants, they may be beneficial for skin which triggers the collagen of the skin and protects the skin from harmful UV rays. [10, 11] Screening of Sun Protection factor activity of *A. cepa* springs will be in demand for inventing new sunscreen formulations.

Taking all these biological requirements into consideration, the present study is designed to screen phytochemical content, antioxidant activity, Sun Protection Factor, antibacterial and anti-hemolytic activity of various extracts of *A. cepa* springs.

Material and methods

Chemicals and reagents

All the solvents, chemicals and the media in the present study used were of analytical grade obtained from Sigma Aldrich.

The microorganism, *Pseudomonas aeruginosa* (ATCC 27853) is obtained from HiMedia Laboratory and the pure cultures were sub-cultured on nutrient agar slants to have fresh culture the day before the experiment.

Plant Collection and identification

The purchased (red onion) *A. cepa* seeds from Kolar market were sown in Dhanvanthari Herbal garden of Sri Devaraj Urs Academy of Higher Education and Research, Kolar. The plants were authenticated by College of Horticulture, Kolar. Herbarium has been made and submitted to Dr. Madhava Chetty, Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupati. Voucher specimen number of the submitted specimen is 1203. During vegetative stage, the pesticide free leaves were washed with clean water and made dry under shade for about 2-3 weeks for further experimental use.

Extraction of plant material

The air dried leaves were ground into fine powder. The extracts were prepared into a sequential procedure by soaking 25 grams of dried leaf powder in 500 ml of different solvents like hexane (non-polar), ethyl acetate (moderately polar) and ethanol (polar) for 48 hr. At the end of respective extraction, the plant material was filtered using Whatman No1 filter paper. Allowed the solvent to evaporate at 37^0C and obtained the dry weight of the extract was used to arrive at the concentration in mg/ml.

Preparation of inoculum

About 18 hour broth culture of the test organism was suspended into sterile nutrient broth. It was standardized according to National Committee for Clinical Laboratory standards and compared turbidity with 0.5 Mc Farland standards.

Phytochemical analysis

By following established qualitative methods [12], phytochemical screening of different solvent extractions of *A. cepa* springs for carbohydrates, flavonoids, gums, alkaloids, phenols, glycosides, proteins, tannins, saponins, steroids and fixed oils were carried out.

Quantitative determination of phytochemical constituents

Quantitative Estimation of Alkaloids

About 1mg of the extract was dissolved in dimethyl sulphoxide (DMSO), and added 1ml of 2N Hcl and filtered. The filtrate was aliqoted to a new tube and 5ml of bromocresol green and 5ml of phosphate buffer were added and were shaken by additing 4ml of chloroform. The extracts were collected in 10ml tube and adjusted the volume with chloroform. Standard solutions of atropine (AE), 20µg/ml, 40, 60, 80 and100µg/ml were prepared similar to the method mentioned above. For measuring the absorbance of test and standards against reagent black, the UV-Vis spectrophotometer instrument was set to 470 nm and noted the values. The total alkaloid content was expressed as mg of AE / g of extract. [13]

Quantitative estimation of flavonoids

To determine total flavonoid, Aluminium chloride method was obtained, using quercetin as a standard. Test sample (1mg/ml) was taken and added to 4 ml of water, incubated for 5min, further incubated for 6 min at room temperature followed by the addition of 0.3ml of 5% sodium nitrate, 0.3ml of 10% Aluminium chloride. To the reaction mixture, 2ml of 1M sodium hydroxide and measured the absorbance at 510 nm against reagent blank. The total flavonoid content was expressed as mg of Quercetin (QE) /g of extract. [14]

Quantitative estimation of Phenolic compounds

The total phenolic content was determined with Folin-Ciocalteus reagent (FCR). All the three extracts at a concentration of 1mg/ml were made and mixed with 0.4ml of FCR. After 5 min, 4ml of sodium carbonate was added. To make the final volume, 10 ml distilled water was added and allowed to stand for 90 min at room temperature. A set of standard solutions of gallic acid (GAE) 20 µg/ml, 40, 60, 80 and 100µg/ml was made in the similar manner. Absorbance was measured at 550nm with UV/Visible spectrophotometer. A calibration curve was constructed using standard and total phenolic content was expressed in terms of mg of GAE/g of extract. [15]

Quantitative estimation of Tannin content

The tannins were determined by Folin-Ciocalteu method. About 100 microliters of the extract was added to a volumetric flask of 10ml containing 7.5ml of distilled water and 0.5ml of Folin-Ciocalteu phenol reagent, 1ml of 35% Na_2CO_3 solution and diluted to 10ml with distilled water. Shaken well the mixture and left undisturbed for 30 min at room temperature. Similarly, standards of gallic acid (GAE)

were prepared. Absorbance for test and standard was measured at 725 nm with UV-Visible spectrophotometer. The tannin content was expressed in terms of mg of GAE/g of extract. [16]

Quantitative determination of Carbohydrates

About 100 mg of the extract was taken into a boiling tube and was hydrolyzed by keeping it in boiling water bath for 3 h with 5ml of 2.5 N Hcl and brought to room temperature, neutralized with solid sodium carbonate, and made up the volume to 100ml and centrifuged. Working standards of glucose were taken as 0.2 mg/ ml, 0.4, 0.6, 0.8 and 1 mg/ ml. The volumes were made to 1mL for both sample and standard with distilled water. Then 1ml of phenol solution was added to each tube followed by 96% sulphuric acid and shaken well. After 10 min, the contents were mixed and placed in water bath at 25^{0}C – 30^{0}C for 20 min and absorbance was read at 490 nm. The amount of total carbohydrate was calculated using the standard graph.

Antioxidant activity of A. cepa springs

Antioxidant activity of the extracts was carried out according to the protocol given by Prieto et al., [17]. Stock of 1mg/ml was dissolved in respective solvents (ethanol, hexane and ethyl acetate). Working concentration of 0.1mL of the extracts from stock was mixed with 1mL of solution containing 0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate. All the components were mixed properly and incubated at 90^{0}C for 90min. measured the absorbance at 695 nm after the samples were brought to room temperature. The amount of total antioxidant capacity was expressed in mM α-tocopherol acetate equivalent/ g dry mass.

SPF activity of *Allium* cepa springs

SPF was determined by More. [18] Working concentrations of 50 µg/ml, 100 and 150µg/ml were made from 1mg/ mL stock solution with the respective solvents. Spectrophotometric readings of these solutions were taken in wavelength ranging from 290-320 nm at 5 nm interval and the readings were noted down. All the readings were taken in quadruplicate at each point. Mansur equation was used to determine the SPF values of the formulations.

Mansur et al., [19] developed a very simple mathematical equation which is as follows.

In this equation, CF = 10 (Correction Factor), EE(λ) =Erythemogenic Effect of radiation at wavelength λ, I(λ) = Intensity of solar light at wavelength λ, and abs(λ) = absorbance of wavelength λ by a solution of the preparation. The obtained absorbance values were multiplied by the EE(λ) values; their summation was taken and multiplied by the correction factor 10.

Anti-hemolytic activity of *Allium* cepa springs

After obtaining Ethics clearance, (No. DMC/KLR/ IEC/88/2016-17), healthy volunteer's 1ml of blood was collected in heparin vacutainer. The supernatant was discarded after centrifugation for 5 minutes at 1500 rpm at room temperature. PBS (pH 7.2) was used to wash the pellet. Finally 0.5% erythrocyte suspension was made and used for the experiment. Erythrocyte suspension of 0.5ml was mixed with 0.5 ml of 5 concentrations of the extract (25µg/ml, 50, 75, 100, 125 µg/ml), incubated at 37^{0}C for 30 min. Later the mixture was centrifuged at 1500 rpm for 10 min at room temperature and the absorbance was measured at 540 nm. [20]

Screening for Antibacterial activity

Antibacterial activity was performed according to the method described by Vamshi et al., [21] Nutrient agar plates were made by sterilization and inoculated with 24 h culture of *Pseudomonas aeruginosa*. Wells were made with sterile cork borer. The extracts of *A. cepa* obtained from different solvents were made into various dilutions from 5µg, 10, 15, 20 and 25 µg separately and 20 µL of each extract was poured into respective wells. The plates were then kept for incubation at 37^{o}C for 24 h and recorded the zone of inhibition.

All the experiments were conducted in triplicates and standard errors were calculated wherever it is necessary by using latest SPSS software. The MIC for each solvent, SPF and anti-hemolysis activity was analyzed using one-way analysis of variance (ANOVA).

Results and Discussion

Phytochemical investigation of *Allium cepa* springs

The phytochemical investigation of three different solvent extractions of *A. cepa* springs revealed the differential expression of various secondary metabolites. Phenols, saponins, flavonoids, tannins and carbohydrates were uniformly present in all the extracts; ethyl acetate and ethanol extractions contained alkaloids; hexane and ethanol showed glycosides; gums, fixed oils and proteins are absent in all the three extracts; and steroids are present only in ethanol. The flavonoid content of various plant studies were compared with *A. cepa* springs and results were noted in table 1. [22] With respect to phytochemical investigation, ethanol shows the best polarity, contained maximum number of phytochemicals studied. Ethanol, ethyl acetate and hexane extracts were prepared to examine total phenolic, flavonoid, alkaloid, tannins and carbohydrate content and calibration graph were shown in figure 1 and table 2.

Total phenol, flavonoid, alkaloid and tannin content of *A. cepa* springs

The total phenolic contents of the examined plant extracts using FC reagent is expressed in terms of mg of GA/g of the extract and it was ranged from 10mg to 50mg.The highest concentration of phenols was measured in ethanol extract than ethyl acetate and hexane The flavonoid content of *A. cepa* springs was determined by spectrophotometric method using aluminium chloride. The total flavonoid con-

tent was expressed in terms of quercetin equivalent. The highest flavonoid content and carbohydrate were observed in ethyl acetate extract which shows that concentration of the extract depends on the polarity of the solvent used for extraction. The alkaloid content was examined in plant extracts and expressed in terms of capsaicin equivalent as mg of CP/g of extract. The tannin content was examined in plant extracts using FC reagent is expressed in terms of gallic acid equivalent. The highest amount of alkaloid and tannin contents was observed in hexane extract.

A. cepa springs as antioxidant agents

The determination of total secondary metabolites in the *A.cepa* springs has proven that, flavonoids occupy the first place in their availability and could be the major contributing factor towards the biological activity. Many reports emphasize that intake of fruits and vegetables prevent DNA alteration by ROS, which is an antioxidant activity of foods. Plants of *Allium* family are an important source of such dietary flavonols, quercetin and kaempferol. [23] The results obtained from this study revealed higher antioxidant activity and was compared with the standard alpha tocopherol. The standard α-tocopherol value was 0.25mM at 1mg/ml concentration and the antioxidant capacity of *Allium* springs of three extracts ranged from 0.2-1.2 mM with ethyl acetate being the highest (1.2mM) followed by hexane (0.28mM) and the least activity was observed in ethanol extract (0.14mM). The antioxidant activity of *A. cepa* springs of the three solvent extractions showed good antioxidant activity (Table 3).

Zhang and his co-workers proved that the flavonoid rich flowers of *Paenia ostii* exhibited high antioxidant activity. [24] The study conducted by Rathabhai and Bhaskaran has proven that flavonoid content of leaves of *Carica papaya, Murraya koenigii* is responsible for the antioxidant protection system. [25] Pater Chirag in his review demonstrated the antioxidant activities of various medicinal plants which are in coordination with the present study that major component held in antioxidant activity is flavonoid [26]. Another study by Nemanja Stankovic demonstrated antioxidant and antibacterial activity from traditional medicinal plants and found that aerial parts of *Achillea* species demonstrate antioxidant activity with the aid of its predominant flavonoid component [27]. The phenomenal antioxidant capacity is to delay or prevent oxidation of substances if they are present in less concentration when compared to substrates. Along with the synthesized antioxidants, dietary antioxidants also influence the oxidation process and one among those dietary antioxidants is onion.

Anti-hemolytic activity of *A. cepa* springs

In vitro anti-hemolytic activity of the extracts of *A. cepa* springs were studied by using human erythrocytes. Different onion extracts at different concentrations showed differential pattern of hemolysis. Increase in concentration showed increased hemolysis. In our study, the solvent extracts resulted moderate protective activity at a very low concentration i.e., from 25µg/ml to 125µg/ml. Among the three extracts used to screen for anti-hemolytic activity, maximum exhibited protection was by ethyl acetate extract when compared to the other two extracts (Table 4). Study conducted by Urbańska et al [28] has shown that saponins are known to have undesirable hemolytic effect when compared to other phytochemicals. In spite the exact mechanism for moderate stabilizing effect of the extracts is not yet known, a few studies have shown that the action of tannins and flavonoids towards anti-hemolysis lays a major role which is coping up with the current study. The major target for free radicals is erythrocytes and cause oxidative damage to the erythrocyte membrane. Our findings with ethyl acetate extract of onion springs exhibited effective anti-hemolytic action due to the evidenced role of phytochemicals that interact with lipids, part of the outer membrane of erythrocytes. [28,29] Hemolysis happens when the lipid bilayer of RBCs undergo destruction. When treated with plant extracts, this destruction depends upon the chemical composition and concentration of the extracts. [30]

Allium cepa springs as Sun protection factor agents

SPF is the purely quantitative measurement of effectiveness of any sun screen product. To be shown effective, each sun screen product should have a range of absorbance from 29 to 320 nm. A study conducted by Lesões cutâneas malignas et. al., [31] showed that there is a clear indication of increase in skin lesions related to sun exposure. Hence control of these lesions by natural formulations is mandatory. The SPF activity of the three solvent extracts of *A. cepa* springs revealed good SPF activity at very minimal concentrations i.e., 50 µg/ml, 100 and 150 µg/ml and the values were between 4.23 and 14.1 (Table 5). Among all, ethyl acetate extract has shown the highest SPF activity i.e., 14. Presence of phenolic content might be the possible reason behind the highest SPF activity of the extracts. It is also reported that antioxidant activity plays an important role in UV protection ability of the plant. Further, reports noted that the high SPF value of *D. moldavica* and *V. tricolor* is due to high phenolic content which correlates with the present study results which showed high concentration of flavonoids. Hence, phytochemicals play a major role in determining the Sun Protection Factor ability of medicinal plants. [32] Research revealed that topical application of gel with *A. cepa*, pentaglycan and allantoin showed skin lesion improvement and hence *A. cepa* spring extract might be another novel source for fighting against several skin problems. [33]

A. cepa springs as antibacterial agents

In the present study, we tried investigating antibacterial activity against *Pseudomonas aeruginosa.* Concentration dependent antibacterial property was noted when different concentrations (5 µg/ml -25 µg/ml) of the three solvent extracts of *A. cepa*. Among the three extracts, ethyl acetate extract showed maximum zone of inhibition compared

with hexane and ethanol (Table 6, Figure 2).The result was in consistent with the work carried out by Jonathan et. al., where in, the ethyl acetate extract showed antimicrobial activity on *Pseudomonas aeruginosa* and *E. coli*. [34] According to Hendrich, the onion juice contained flavonoids and polyphenols are reported to have broad spectrum antibacterial activity. [35] Another study conducted by Mohammed Eltaweel demonstrated that the antibacterial properties of methanolic extract of *A. cepa* bulbs showed potential zone of inhibition (29mm) at 1000µg/ml [36], in our study ethyl acetate extract showed 22mm zone of inhibition at minimal concentration.

Vamshi et al. [21] reported that hexane and ethanol extracts of scale leaves of *A. cepa* at a concentration of 1000µg/ml showed an inhibition zone of 8 mm of each extract against Gram positive bacterium, S*taphylococcus aureus*. In contrast, our study showed good zone of inhibition of 22 mm at a minimal concentration of 25µg/mL against *Pseudomona aeruginosa*. The abundant content of flavonoids in ethyl acetate extract of the onion springs are contributing for the growth inhibition of pathogenic bacteria.

Thus, in the obtained study results, ethyl acetate showed the best activity than the other two solvent extractions and the flavonoids might be the causing factor for all the tested parameters.

Table 4. Anti-hemolytic activity of the *A. cepa* extracts on human erythrocytes.

	Percentage anti-hemolysis		
Concentration (µg/ mL)	Hexane	Ethanol	Ethyl acetate
25	14	43	88
50	27	52	80
75	59	69	82
100	58	69	95
125	57	46	69

µg: microgram

Table 1: Phytochemical content of leaves of medicinal plants in comparison with *A. cepa* L. springs

S.No	Plant species	Flavonoids
01	Punica granatum	+(presence)
02	Psidium gujauva	+(presence)
03	Morusnigra	-(absence)
04	Morus alba	+(presence)
05	Ficus palmate	+(presence)
06	Momordica charantia	-(absence)
07	Allium cepa	+++(abundantly present)

Table 2. Total flavonoid, alkaloid, tannin, and carbohydrate contents of the extracts were expressed in terms of milligram equivalent per gram of extract of onion leaf.

Solvents	Phenol (mg/GAE/gm)	Flavonoids (mg/QE/gm)	Alkaloids (mg/AE/gm)	Tannin (mg/GAE/gm)	Carbohydrates (mg/G/gm)
Ethanol	50	40	49	55	12
Ethyl acetate	18	75	42	28	58
Hexane	10	50	50	62	24

GAE: gallic acid, QE: Quercetin, AE: atropine, C: Carbohydrate, mg: milligram, gm: gram

Table 3: Antioxidant activity of various extracts of *A. cepa* springs.

S.No	Compound (1mg/ mL)	Total antioxidant activity (695nm)
01	Alpha tocopherol	0.25mM
02	Hexane extract	0.28mM
03	Ethanol extract	0.14mM
04	Ethyl acetate extract	1.2mM

mg: milligram, ml: milliliter, nm: nanometer

Table 5. SPF activity of different concentrations of *A. cepa* extracts from 290 nm to 320 nm with 5 nm variation.

nm	EE*1 (Normalized)	Hexane (µg/mL)			Ethanol (µg/mL)			Ethyl acetate(µg/mL)		
		50	100	150	50	100	150	50	100	150
290	0.01	0.01	0.02	0.04	0.03	0.05	0.07	0.01	0.01	0.02
295	0.08	0.08	0.15	0.24	0.66	0.26	0.43	0.03	0.24	0.43
300	0.28	0.27	0.52	0.84	0.48	0.86	1.46	0.27	1.12	1.82
305	0.32	0.30	0.59	0.54	0.54	0.97	1.65	0.39	1.39	2.22
310	0.18	0.16	0.31	0.50	0.31	0.55	0.94	0.25	0.83	1.29
315	0.08	0.07	0.14	0.22	0.14	0.25	0.43	0.13	0.40	0.61
320	0.01	0.01	0.13	0.22	0.03	0.05	0.09	0.14	0.07	0.06
SPF	1	4.23	6.24	7.10	6.54	7.14	10.8	5.02	8.02	14.1

Table 6. Antibacterial activity of different concentrations of the three *A. cepa* extracts against Pseudomonas aeruginosa.

S.No.	Concentration (µg/ml)	ZI of Ethanol (mm)	ZI of Hexane (mm)	ZI of Ethyl acetate (mm)
1	5	7±1.23	6±1.11	10±3.6
2	10	8±1.23	8±1.11	11±3.6
3	15	10±1.23	10±1.11	16±3.6
4	20	13±1.23	12±1.11	20±3.6
5	25	15±1.23	14±1.11	22±3.6

mm: millimeter; ZI: zone of inhinition

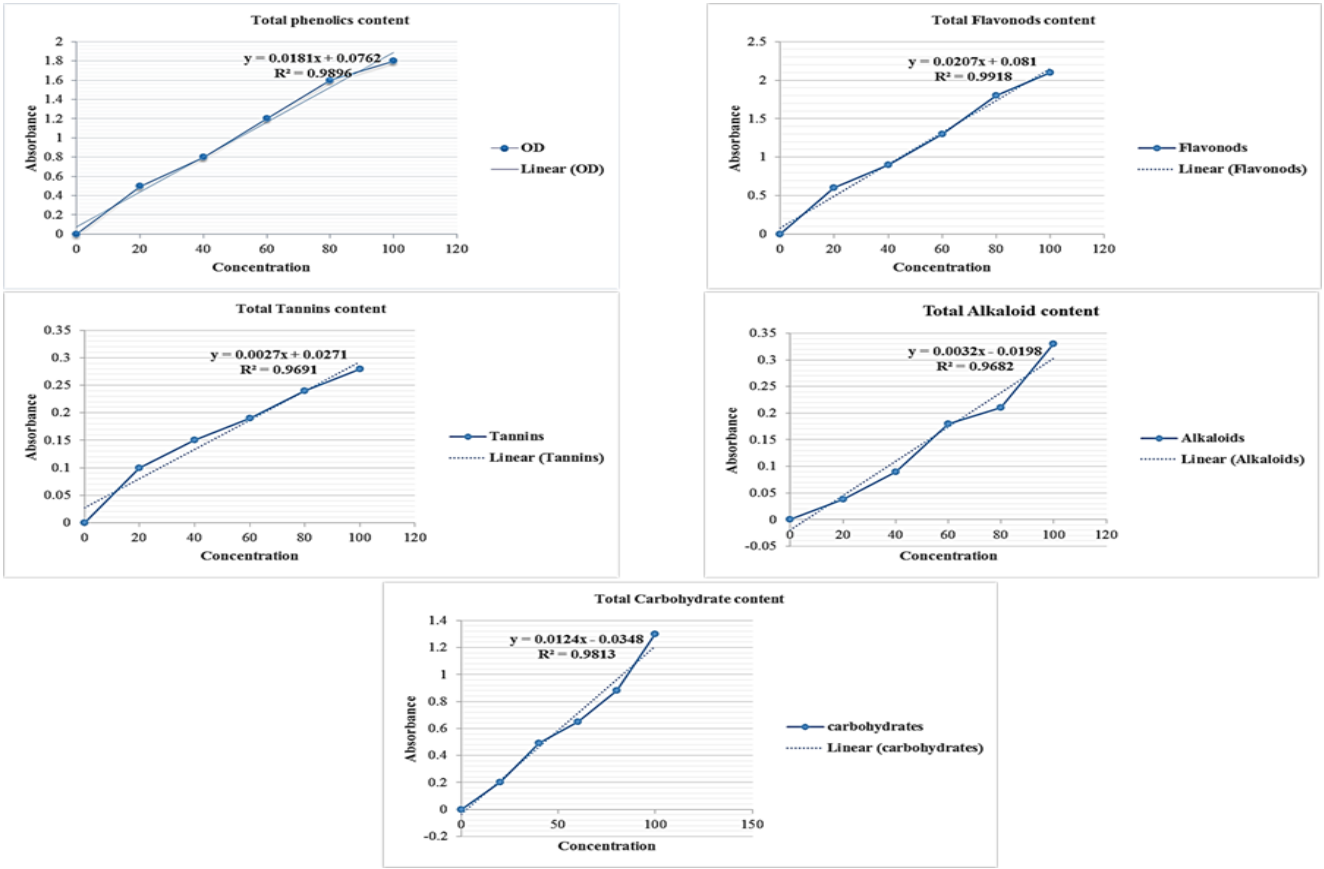

Figure 1. The total phenolic, flavonoid, tannin, alkaloid and carbohydrate content of *A. cepa* springs. X-axis shows the concentration and Y-axis shows the absorbance.

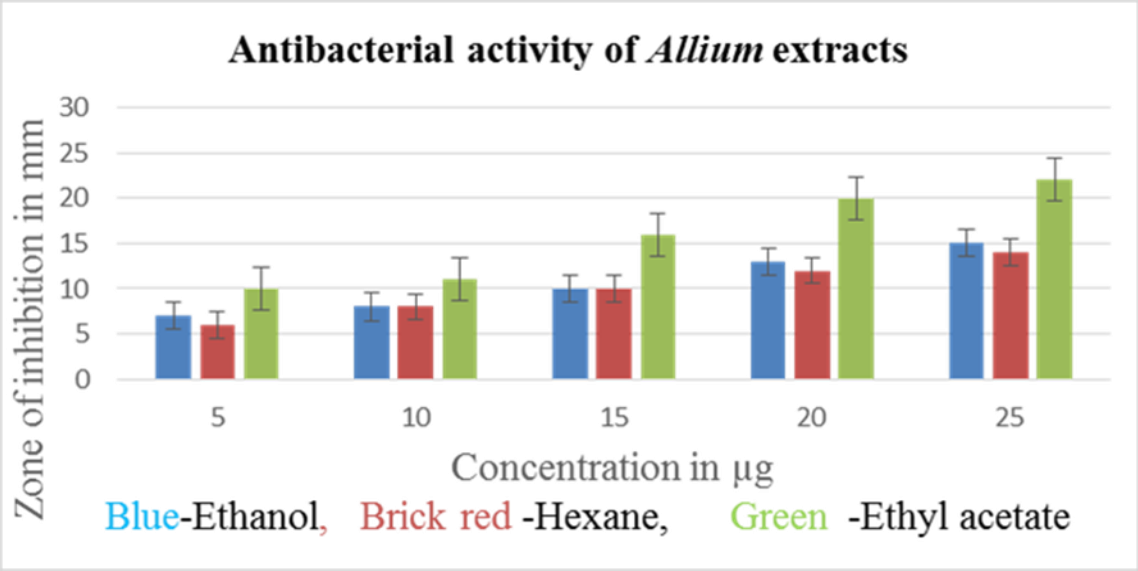

Figure 2: Comparison of antibacterial activity of solvents and different concentrations of *A. cepa* springs on *Pseudomonas aeruginosa*

Conclusion

This is one of the seldom studies which showed the phytochemical investigation of ethanol, ethyl acetate and hexane solvent extracts of *Allium* springs and their pharmacological activities. The quantitative determination of *A. cepa* springs has revealed abundance of flavonoids among all the other phytochemicals. The ethyl acetate extracts of *A. cepa* springs possessed potential antioxidant, SPF, anti-hemolytic and antibacterial activities and proved to have good pharmacological properties. Therefore, importance is given on ethyl acetate extracts of *A. cepa* springs in order to know its active components and exact mechanism of action.

Medicinal properties of *Allium* sp. is well documented for various ailments and our studies supported that the extracts also have RBC protective activity when given as anti-leukemic to the patients and can be mixed with other formulations in preparing herbal sun protection lotions or creams. *In vivo* studies by using ethyl acetate *A. cepa* spring's extract will guide to focus on new drugs for the studied parameters.

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Conflict of interest

There are no conflict of interest.

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