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Quantification of protein content and determined its antioxidant activity in *Azadirachta indica*

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Abstract: In this study, we measured the effect of these antioxidants i.e. vitamins (C and E) and total antioxidants in lysed human whole blood using aqueous leaves extract of *Azadirachta indica* (10 mg/ml; stock solution). All these antioxidants in this medicinal plant were measured using NanoDrop method and also isolated the protein content using Tris HCl and ice cold acetone and determined through SDS PAGE. For these studies, capsules of antioxidants {Vitamin C (Celin); vitamin E (Evion); and total antioxidants, A to Z multivitamin) were used as standard. The results showed that *Azadirachta indica* along with serially diluted vitamin E markedly increased in total cellular content in the form of Vitamin E production as compared to antioxidant (Vitamin E capsule, Evion) control dissolved in phosphate buffered saline (PBS, pH 7.2). In addition, there is slightly enhancement in vitamin C production at higher doses as compared to antioxidant (vitamin C capsule, Celin) control whereas total antioxidant capsule (A to Z, multivitamin) showed no effect at all. Capsules of antioxidants (i.e. vitamins C, E and total antioxidants) used separately dissolved in PBS as standard and showed enhancement at higher doses in case of vitamin C and E but in case of antioxidant capsule at lower doses in lysed human whole blood. In addition, two bands of protein (45 and 60 kDa) are observed in aqueous leaves extract of *Azadirachta indica.* Over all, the studies claimed that aqueous leaves extract of *Azadirachta indica* showed more enhancement of vitamin E production and it provides a scientific data of particular importance for the local people using these plant products especially related to vitamin E production for a variety of body immunological disorders.

Key words: Antioxidants; protein; vitamin; *Azadirachta indica*

INTRODUCTION

Antioxidants are believed to be the first line of defense against free radical damage and these are vital requirement for maintaining human health. Now a day, demand for the need of antioxidants becomes even more unfavourable with increased or enhanced exposure to free radicals [1, 2]. Due to healthy lifestyle and well-balanced wholesome diet, antioxidant supplementation is now being recognized and considered as an important means of improving free radical protection [3]. In other words, these antioxidant compounds that are present abundantly or enormously in food (simple or complex) material that played an important role as a health protecting factor [1-3]. Lot of scientific evidences which suggests that antioxidants reduce the risk or burden rate of various chronic diseases especially cancer and heart [4]. The major source of naturally occurring antioxidants i.e. whole grains, fruits and vegetables that are already reported and most of the medicinal plants have been recognized to enhance antioxidant activity and showed some immunological potential to reduce the risk rate or burden of disease [4]. In medicinal plants, antioxidant compounds e.g. phenolic acids, polyphenols and flavonoids scavenge free radicals i.e. peroxide, hydroperoxide or lipid peroxyl

and thus diminished the oxidative mechanisms that lead to degenerative diseases [2-5]. Number of clinical studies suggesting and proved that the presence of antioxidants in fruits, vegetables, tea and red wine are main factors for observed its efficacy of these foods in reducing the incidence of chronic diseases including cardiovascular disease and cancer [6].

Human body does not synthesize or produced Vitamin E but it has to be supplied externally. This vitamin especially E is extremely important and useful for anti-aging benefits. One of the most familiar example of vitamin E is that when we add into any skin product, it makes more effective pertaining to diminish wrinkles and possible signs of aging [7, 8]. One of the antioxidants i.e. Vitamin E is a group of related compounds called tocopherols, which normally reported in four major forms i.e. alpha, beta, delta and gamma tocopherols. Out of these, Alpha-tocopherol is the most common and potent form of vitamin E. As per the literature, vitamin E may play a crucial role in preventing cancer and protect against cardiovascular (heart) diseases including stroke [7, 8]. Recent research findings suggest and claimed that taking vitamin E and C may help to block some harmful effects of fatty meal [8, 9]. In contrast, vitamin C is generally used in medicines or it may be used as antioxidant that can preserve the product for long period. However, excess of vitamin C may create health problems as well such as diarrhea, gastric irritation etc. In the present study, we analyzed the protein content of medicinal plant i.e. *Azadirachta indica* and also measured the quantity of vitamin C, E and total antioxidants in lysed human whole blood using capsules C, E and antioxidants as standard for these studies.

Azadirachta indica (Neem), medicinal plant widely used as well as utilized in Ayurveda, Unani and Homoeopathic systems of medicine pertaining to treat health-related problems and ailments. In contrast, this medicinal plant also showed anti-cancer, wound healing and anti-microbial properties [10-12]. In this study, we focused on antioxidant properties of *Azadirachta indica.* Lot of research work is already done in case of *Azadirachta indica* related to antioxidant property. Recently, one study showed antioxidant properties of *Azadirachta indica* in inducing apoptosis of cervical cancer cells and proved to be a potent inducer or stimulator of apoptosis (cell death) in biopsies of cervical cancer patients [10-12]. In the present study, we discussed about the antioxidant properties of *Azadirachta indica* with respect to vitamin E, vitamin C and total antioxidants.

MATERIALS AND METHODS

Plant material

Medicinal plant products especially leaves of *Azadirachta indica* were collected during August 2016 from nakshatra udyan of Vidya Pratishthan. This medicinal plant material was taxonomically identified by Botanist, Dr. Bharat Shinde, Principal, Vidya Pratishthan Arts, Science and Commerce College.

Extraction of plant material

Shaded dried leaves (2 g) of *Azadirachta indica* were macerated in liquid nitrogen and prepared coarse powder. The powdered sample was dissolved and macerated in PBS (pH, 7.2) at room temperature. Finally, sample of *Azadirachta indica* was centrifuged at high speed (10000 rpm, 4 ^{o}C) pertaining to collect the supernatant in the form of stock solution (10 mg/ml) for immunological analysis.

Estimation of protein content and determined through SDS PAGE

For antioxidant studies, leaves powder (2 g) of *Azadirachta indica* were added in conical flask and then add extraction buffer (i.e. 20 mM Tris HCl) dissolved in PBS. Incubate leaves powder of *Azadirachta indica* along with extraction buffer for 7 minutes at room temperature and then centrifuged (6000 rpm; 10 minutes at 4°C). Supernatant was collected after centrifugation and then add similar volume of ice cold acetone. Incubate the solution for 10-15 minutes at room temperature and then centrifuged. Collect the pellet and then properly washed with acetone (ice cold) to remove the pigments including lipids as well. Finally, protein concentration of leaves powder of *Azadirachta indica* was determined by using Nano drop method [10].

In SDS PAGE, gels (resolving, 15 % and stacking, 4 %) were used for isolation of protein from aqueous leaves extract of *Azadirachta indica*. About 20 µl of protein sample of *Azadirachta indica* was loaded into the wells and voltage (80 Volts) was normally required to run the gel. After the separation of protein bands of *Azadirachta indica* through electrophoresis, staining solution was utilized to stain the gel in order to make bands visible. Afterwards the gel was placed in to a destaining solution for 36 hours on shaker and was changed frequently until clear gel was obtained [13].

Estimation of vitamin C and E including total antioxidants in *Azadirachta indica* using human whole blood

In order to determine its antioxidant studies of aqueous leaves extract of *Azadirachta indica*, EDTA normal human whole blood (n = 10; 2 ml) samples were collected from Mangal Pathology laboratory, Baramati. In these studies, lysed human whole blood (100 µl) were taken in Eppendorf tube and then add fixed concentration of aqueous leaves extract of *Azadirachta indica* (10 mg/ml; 100 µl) including vitamin C, E and total antioxidants (6.25 - 500 µg/ml, 1 ml; serially diluted) simultaneously in three different sets. Incubate these blood samples along with antioxidants for 2 h at 4ºC. After incubation, centrifuged these samples at high speed (10000 rpm, 4 ºC) and collect the supernatant for estimation of antioxidants and determined through Nanodrop method. Vitamin C, E and total antioxidants capsules are used as standard for these studies and diluted serially in PBS and used as negative control. On the basis of these studies, readings of aqueous leaves extract of *Azadirachta indica* treated with various antioxidants of different concentration and compared with various antioxidants (C, E and total antioxidants) diluted serially which is dissolved in PBS. So, calculations were done on the basis of slope intercept equation ($y = mx+c$) where m and c represents slope and intercept; y is optical density). All these readings or calculations were determined through Nanodrop method and expressed the readings in mg/ml.

Statistical analysis

All values were mentioned as Mean ± S.E. Data was represented by one-way ANOVA test (Bonferroni multiple comparison test).

RESULTS

Estimation of protein content and SDS PAGE

For measuring the quantity of protein present in aqueous leaves extract of *Azadirachta indica*, using Nanodrop. The quantity of protein in *Azadirachta indica* was found to be 8.4 mg/ml (data not shown). In SDS-PAGE, protein bands of *Azadirachta indica* (45 kDa and 60 kDa) were observed as shown in Fig.1.

Estimation of antioxidants

The effect of antioxidants (vitamin C and E including total antioxidant content) as shown in Fig.2, 3 and 4. The results of these studies claimed that *Azadirachta indica* along with vitamin E showed increased in total cellular content related to vitamin E production in lysed human whole blood and also slightly enhancement in vitamin C production but there is no effect in total antioxidant content as compared to antioxidant capsule used as standard dissolved in PBS. Overall, these studies claimed that *Azadirachta indica* showed potent enhancer of vitamin E production.

DISCUSSION

Due to the current scenario in the health issue we propose the use of these medicinal plant products as an option to provide nutritional benefits and tried to reduce the burden of chronic degenerative diseases. Lot of scientific data as well as evidences claimed that chronic degenerative diseases e.g. diabetes is influenced by numerous factors such as metabolic redox imbalance (14, 15). As per the literature, various compounds are reported in medicinal plants and showed various immunopharmacological properties, antioxidants are one of them and showed particular importance because they might serve as leads for the development of novel drugs. Several medicinal plants used as anti-inflammatory, neuroprotective and hepatoprotective properties and also showed antioxidant and/or antiradical scavenging mechanism as part of their activity (16, 17). The search for natural sources of medicinal products that have activity is on the rise. In this study, we focused on one of the medicinal plant i.e. *Azadirachta indica* and determined vitamins (C and E) and total antioxidant content in lysed human whole blood.

The quantitative estimation of protein and antioxidants (Vitamin C, E and total antioxidants) content in aqueous leaves extract of *Azadirachta indica* which generally provides the information about the percentage yields of these constituents that are present. In this study, our results claimed that *Azadirachta indica* showed the presence of primary metabolite i.e. protein content and also showed enhancement in antioxidants content especially vitamin E in lysed human whole blood as compared to capsules of these antioxidants dissolved in PBS which is determined through NanoDrop method. In addition, low molecular weight protein bands (approximately 45 and 60 kDa) were observed and this could be due to

protein's high molecular weight breakdown by enzyme. The finding of this immunological study of *Azadirachta indica* shows a significant increase in vitamin E production, which is consistent with the normal physiological features as a result of normal metabolic processes in human. In contrast, there was no significant enhancement in vitamin C and total antioxidant production but significant increase in group vitamin E content. Due to the enhancement of these antioxidants (especially vitamin E) containing aqueous leaves extract of *Azadirachta indica* may help to prevent damage that is associated with cancer, heart disease and other related human diseases.

Generally, diets of human do not contain the required proportion of vitamins (C and E) for normal growth and cannot be able to produce large quantity for their body metabolism. The most familiar example of Vitamin C content is reported in *B. vulgaris* and lowest in *E. hirta*. Normally, vitamin C is required for prevention of human disease i.e. scurvy (7-9). From these studies, our data showed that there is slightly enhancement in vitamin C production as compared to control. In addition, aqueous leaves extract of *Azadirachta indica* reported higher amount of vitamin E production and is generally able to prevent arteriosclerosis. In the literature, vitamin E showed various beneficial benefits to our immune system especially to prevent heart and blood vessels diseases. In other words, Vitamin E may act as a powerful antioxidant that has the power to reduce free radical damage generated in our immune system, fight inflammation, and therefore to help to naturally slow aging in your cells and fight off health issues like heart disease

CONCLUSION

In this study, aqueous leaves extract was and found to be more potent because of higher amount of vitamin E production but there is slightly enhancement in Vitamin C content and no effect in antioxidant content. Over all, these studies claimed that leaves can be used in various immunopharmacological applications as a valuable antioxidant natural source and medicine. Likewise, the presence of significant amount of azadirachtin and nimbin in all the parts of neem grown in this region showed its potential use as natural insecticide.

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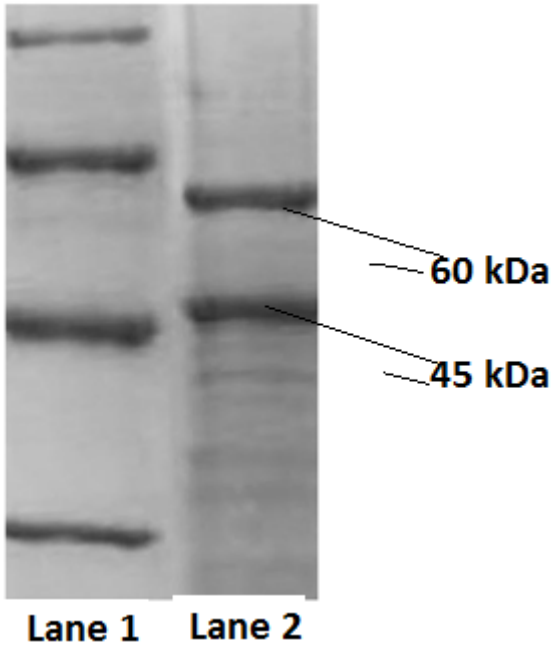
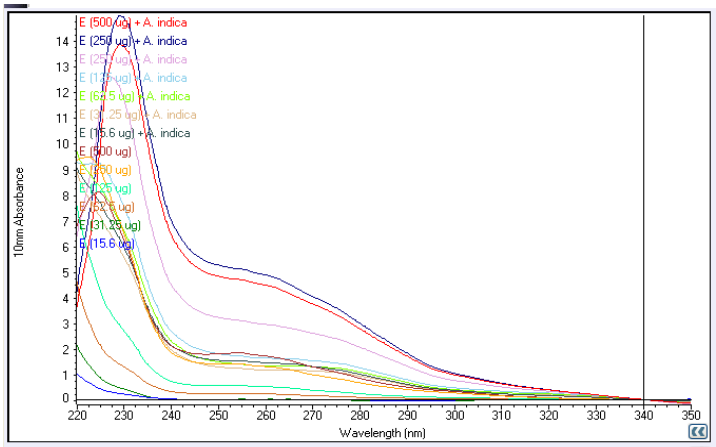


Fig.1. SDS-PAGE. Lane 1: Marker protein solution .Lane 2: Aqueous leaves extract of *Azadirachta indica*



#	Sample ID	User name	Date and Time	Protein Conc.	Unit	A280
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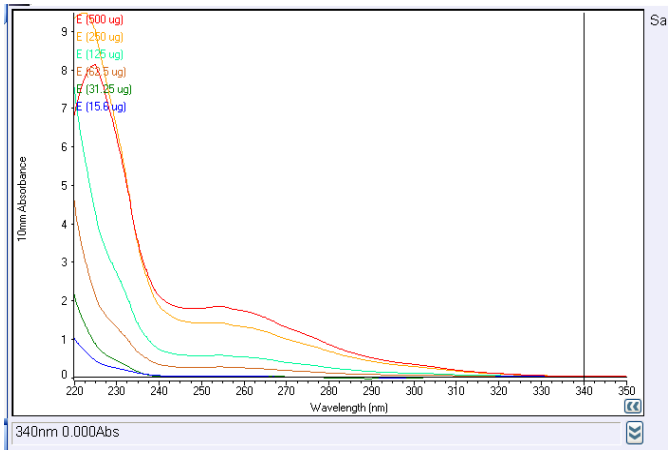
8	E (31.25 ug) + A. indica	Student	27/08/2016 15:44	0.913	mg/ml	0.913
9	E (62.5 ug) + A. indica	Student	27/08/2016 15:44	1.072	mg/ml	1.072
10	E (125 ug) + A. indica	Student	27/08/2016 15:46	1.234	mg/ml	1.234
11	E (250 ug) + A. indica	Student	27/08/2016 15:58	2.041	mg/ml	2.041
12	E (250 ug) + A. indica	Student	27/08/2016 15:59	2.974	mg/ml	2.974



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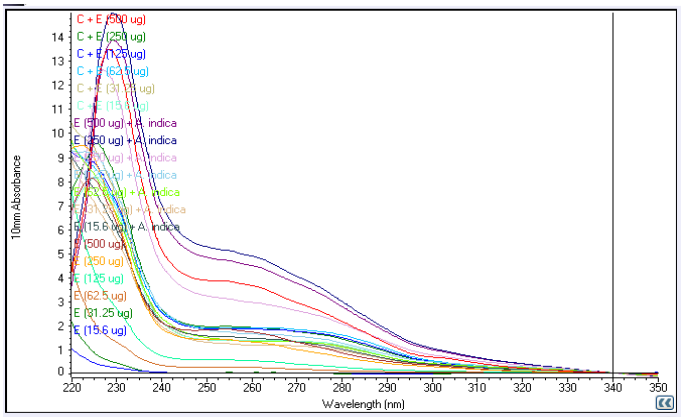
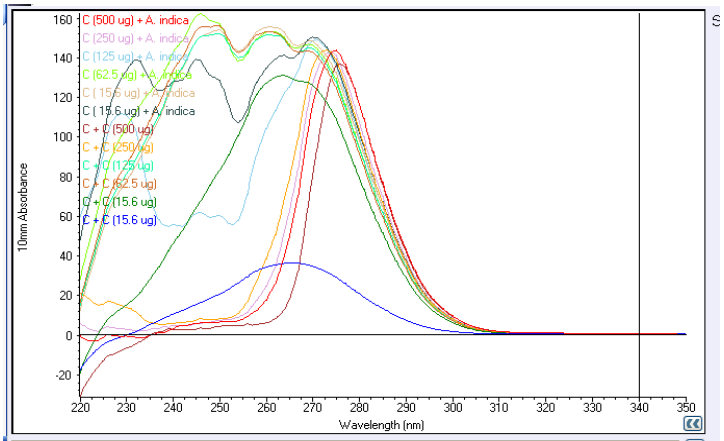
2	E (31.25 ug)	Student	27/08/2016 15:36	-0.042	mg/ml	-0.042	-0.18
3	E (62.5 ug)	Student	27/08/2016 15:37	0.082	mg/ml	0.082	2.65
4	E (125 ug)	Student	27/08/2016 15:38	0.224	mg/ml	0.224	2.23
5	E (250 ug)	Student	27/08/2016 15:39	0.651	mg/ml	0.651	1.98
6	E (500 ug)	Student	27/08/2016 15:39	0.827	mg/ml	0.827	2.05



#	Sample ID	User name	Date and Time	Protein Conc.	Unit	A280
14	C + E (15.6 ug)	Student	27/08/2016 16:04	1.142	mg/ml	1.142
15	C + E (31.25 ug)	Student	27/08/2016 16:05	1.102	mg/ml	1.102
16	C + E (62.5 ug)	Student	27/08/2016 16:06	1.569	mg/ml	1.569
17	C + E (125 ug)	Student	27/08/2016 16:07	1.463	mg/ml	1.463
18	C + E (250 ug)	Student	27/08/2016 16:07	1.406	mg/ml	1.406
19	C + E (500 ug)	Student	27/08/2016 16:08	2.145	mg/ml	2.145

Fig.2. Effect of variable doses of vitamin E along with or without aqueous leaves extract of *Azadirachta indica*.

Lysed human whole blood (100 µl) were taken and incubate fixed concentration of aqueous leaves extract of *Azadirachta indica* (10 mg/ml, 100 µl) including vitamin E of variable concentration (6.25 - 500 µg/ml, 1 ml; serially diluted). Incubate these blood samples along with antioxidants for 2 h at 4ºC. After incubation, centrifuged these samples at high speed (10000 rpm, 4 ºC) and collect the supernatant for estimation of antioxidants and determined through Nanodrop method. After getting the readings of vitamin E of different concentration, calculate slope and intercept. Readings will be expressed in µg/ml. The difference between Vitamin E and treated samples of *Azadirachta indica* plus vitamin E is determined by one way ANOVA test (Bonferroni multiple comparison test). *P<0.05; **P < 0.01 and ***P < 0.001.



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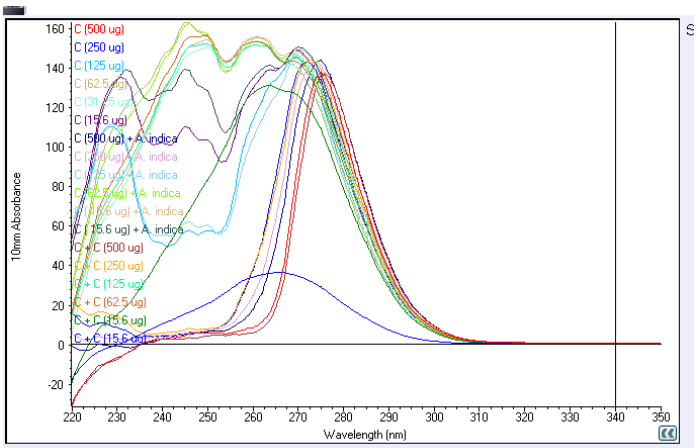
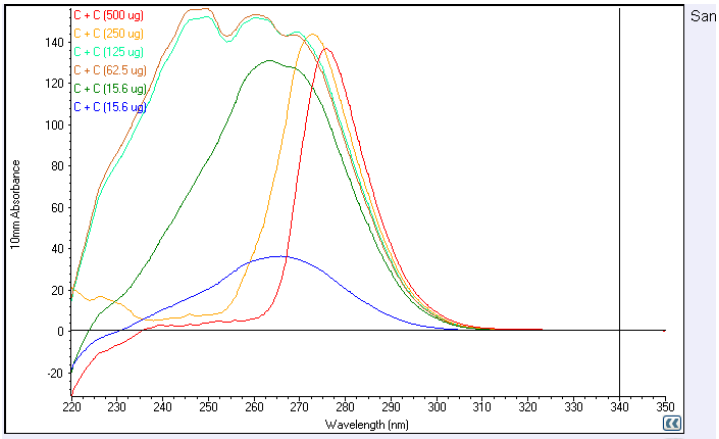
8	C (15.6 ug) + A. indica	Student	27/08/2016 16:25	102.191	mg/ml	102.191
9	C (15.6 ug) + A. indica	Student	27/08/2016 16:26	98.297	mg/ml	98.297
10	C (62.5 ug) + A. indica	Student	27/08/2016 16:27	94.730	mg/ml	94.730
11	C (125 ug) + A. indica	Student	27/08/2016 16:27	99.742	mg/ml	99.742
12	C (250 ug) + A. indica	Student	27/08/2016 16:28	107.703	mg/ml	107.703
13	C (500 ug) + A. indica	Student	27/08/2016 16:29	116.796	mg/ml	116.796



340nm 0.000Abs

#	Sample ID	User name	Date and Time	Protein Conc.	Unit	A280
14	C (15.6 ug)	Student	27/08/2016 16:34	95.451	mg/ml	95.451
15	C (31.25 ug)	Student	27/08/2016 16:34	86.416	mg/ml	86.416
16	C (62.5 ug)	Student	27/08/2016 16:35	84.069	mg/ml	84.069
17	C (125 ug)	Student	27/08/2016 16:36	87.570	mg/ml	87.570
18	C (250 ug)	Student	27/08/2016 16:37	95.325	mg/ml	95.325
19	C (500 ug)	Student	27/08/2016 16:37	109.450	mg/ml	109.450



340nm 0.000Abs

#	Sample ID	User name	Date and Time	Protein Conc.	Unit	A280	260/280
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3	C + C (15.6 ug)	Student	27/08/2016 16:18	79.102	mg/ml	79.102	1.60
4	C + C (62.5 ug)	Student	27/08/2016 16:18	91.497	mg/ml	91.497	1.67
5	C + C (125 ug)	Student	27/08/2016 16:19	94.172	mg/ml	94.172	1.61
6	C + C (250 ug)	Student	27/08/2016 16:20	103.622	mg/ml	103.622	0.37
7	C + C (500 ug)	Student	27/08/2016 16:21	116.893	mg/ml	116.893	0.05

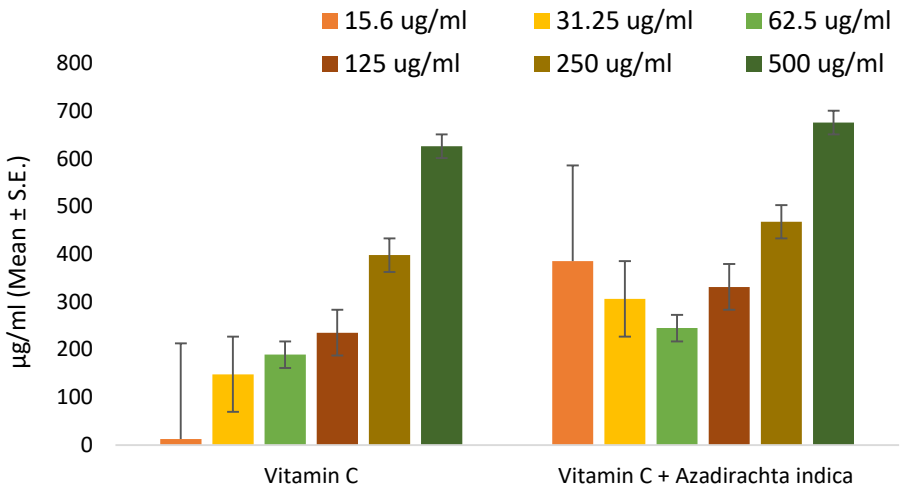
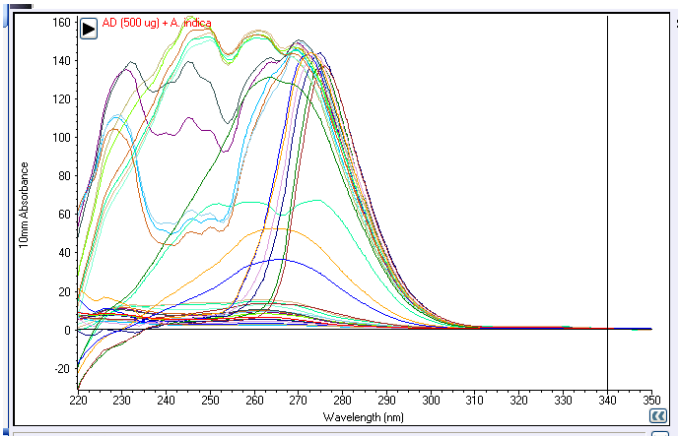


Fig.3. Effect of variable doses of vitamin C along with or without aqueous leaves extract of *Azadirachta indica*. Lysed human whole blood (100 µl) were taken and incubate fixed concentration of aqueous leaves extract of *Azadirachta indica* (10 mg/ml, 100 µl) including vitamin C of variable concentration (6.25 - 500 µg/ml, 1 ml; serially diluted). Incubate these blood samples along with antioxidants for 2 h at 4ºC. After incubation, centrifuged these samples at high speed (10000 rpm, 4 ºC) and collect the supernatant for estimation of antioxidants and determined through Nanodrop method. After getting the readings of vitamin C of different concentration, calculate slope and intercept. Readings will be expressed in µg/ml. The difference between Vitamin E and treated samples of *Azadirachta indica* plus vitamin C is determined by one way ANOVA test (Bonferroni multiple comparison test). *P<0.05; **P < 0.01 and ***P < 0.001.



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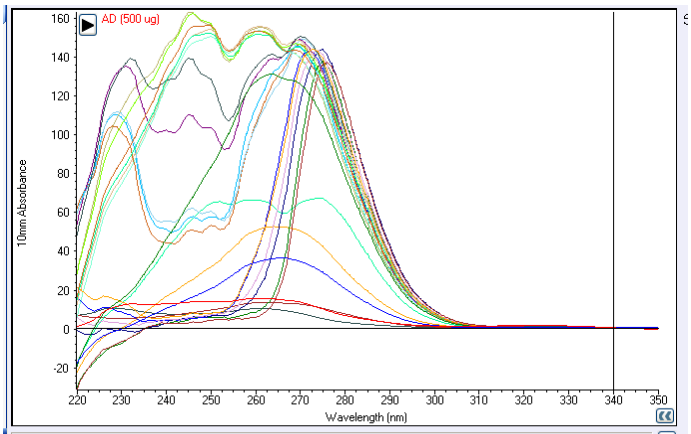
33	AD (31.25 ug) + A. indica	Student	27/08/2016 16:55	0.921	mg/ml	0.921
34	AD (62.5 ug) + A. indica	Student	27/08/2016 16:56	1.156	mg/ml	1.156
35	AD (125 ug) + A. indica	Student	27/08/2016 16:56	1.728	mg/ml	1.728
36	AD (250 ug) + A. indica	Student	27/08/2016 16:57	3.141	mg/ml	3.141
37	AD (500 ug) + A. indica	Student	27/08/2016 16:58	5.442	mg/ml	5.442



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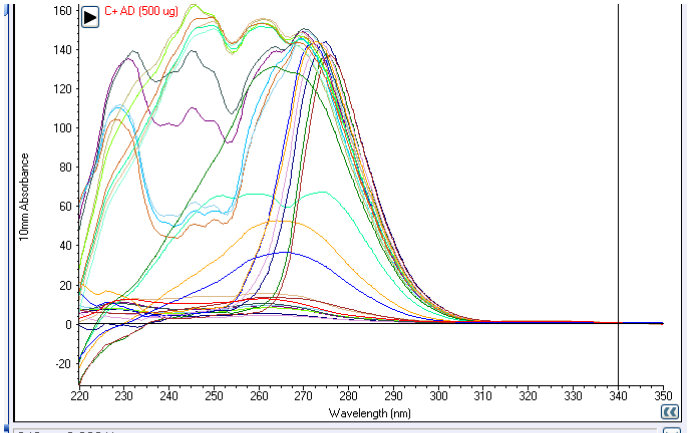
21	AD (31.25 ug)	Student	27/08/2016 16:44	55.831	mg/ml	55.831
22	AD (62.5 ug)	Student	27/08/2016 16:45	29.803	mg/ml	29.803
23	AD (125 ug)	Student	27/08/2016 16:46	7.143	mg/ml	7.143
24	AD (250 ug)	Student	27/08/2016 16:46	2.749	mg/ml	2.749
25	AD (500 ug)	Student	27/08/2016 16:47	6.344	mg/ml	6.344



#	Sample ID	User name	Date and Time	Protein Conc.	Unit	A280
26	C+ AD (15.6 ug)	Student	27/08/2016 16:49	3.063	mg/ml	3.063
27	C+ AD (31.25 ug)	Student	27/08/2016 16:49	3.999	mg/ml	3.999
28	C+ AD (62.5 ug)	Student	27/08/2016 16:50	1.469	mg/ml	1.469
29	C+ AD (125 ug)	Student	27/08/2016 16:51	1.589	mg/ml	1.589
30	C+ AD (250 ug)	Student	27/08/2016 16:51	2.729	mg/ml	2.729
31	C+ AD (500 ug)	Student	27/08/2016 16:52	3.995	mg/ml	3.995

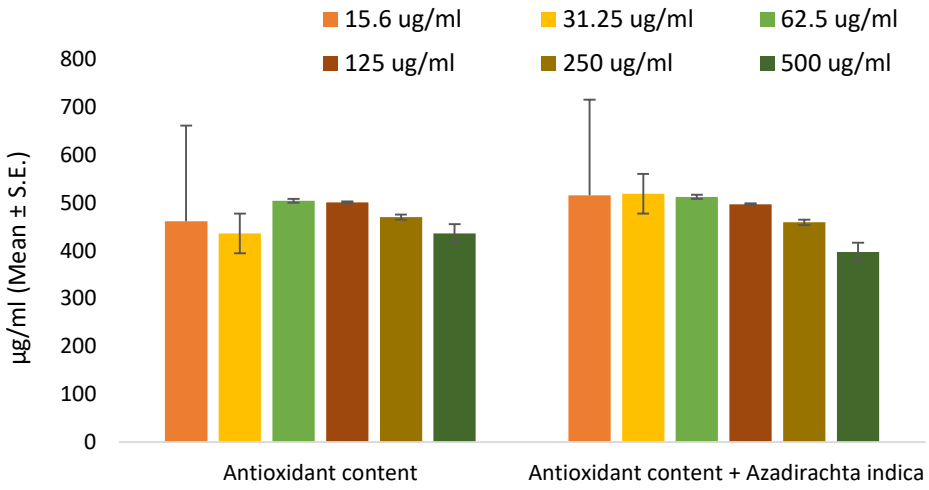


Fig.4. Effect of variable doses of antioxidant content along with or without aqueous leaves extract of *Azadirachta indica*. Lysed human whole blood (100 µl) were taken and incubate fixed concentration of aqueous leaves extract of *Azadirachta indica* (10 mg/ml, 100 µl) including antioxidant content of variable concentration (6.25 - 500 µg/ml, 1 ml; serially diluted). Incubate these blood samples along with antioxidants for 2 h at 4ºC. After incubation, centrifuged these samples at high speed (10000 rpm, 4 ºC) and collect the supernatant for estimation of antioxidants and determined through Nanodrop method. After getting the readings of antioxidant content of different concentration, calculate slope and intercept. Readings will be expressed in µg/ml. The difference between Vitamin E and treated samples of *Azadirachta indica* plus vitamin C is determined by one way ANOVA test (Bonferroni multiple comparison test). *P<0.05; **P < 0.01 and ***P < 0.001.