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In Vitro Assessment of Antioxidant Effect of Aqueous Extract of *Vernonia amygdalina* (Bitter Leaf): A Quasi-experimental Study

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Abstract: ***Background:*** Medicinal plants are widely used in many countries for different ailments due to cost effectiveness and less side effects. *Vernonia amygdalina* (*V.amygdalina*) (Bitter leaf) is one of the important medicinal plant which has many pharmacological properties. ***Methods****:* This quasi-experimental study was carried out in the Department of Pharmacology and Therapeutics, Rajshahi Medical College, Rajshahi from January 2021 to December 2021. This study was conducted in two steps: Firstly, preparation of aqueous extract of leaves of *V. amygdalina.* Secondly, assessment of its antioxidant effect through DPPH free radical scavenging activity. ***Results:*** The results of the study showed that the antioxidant activity of aqueous extract of leaves of *V. amygdalina at* the concentration of 0.0625 mg/ml, 0.125 mg/ml, 0.25mg/ml, 0.5mg/ml and 1 mg/ml were 34.9±1.14, 63.3±0.62, 76±0.83, 77.9±1.37 and 84.9±1.86 respectively. The aqueous extract of *Vernonia amygdalina* gave the highest inhibition of 84.9±1.86% at 1mg/ml and lowest inhibition of 34.9±1.14% at 0.0625 mg/ml. The antioxidant activity of standard ascorbic acid at the concentration of 0.0625 mg/ml, 0.125 mg/ml, 0.25mg/ml, 0.5mg/ml and 1 mg/ml were also being evaluated which were 80.1±1.68, 86.5±2.47, 91.6±0.65, 93.2±0.44 and 94.5±0.62 respectively. Ascorbic acid gave the highest inhibition of 94.5±0.62% at 1 mg/ml and lowest inhibition of 80.1±1.68% at 0.0625 mg/ml. Comparing the antioxidant effect of Bitter leaf & Ascorbic acid difference was found statistically significant ($p<0.05$). ***Conclusions:*** These results revealed that antioxidant activity of different concentration of aqueous extract were found but it was less when compared to standard antioxidant effect of ascorbic acid. Hence it is concluded that aqueous leaves extract of *V. amygdalina* showed antioxidant effect.

Keywords: *Vernonia amygdalina,* aqueous extract, antioxidant effect, DPPH.

Original Research Article

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Article at a glance:

Study Purpose: *Assessment of antioxidant effect of aqueous leaves extract of Vernonia amygdalina (Bitter leaf).*
Key findings: *Aqueous extract of Bitter leaf had antioxidant effect but less than standard Ascorbic acid.*
Newer findings: *Aqueous extract of leaves of V.amygdalina showed antioxidant activity in a concentration dependent manner.*
Abbreviations: *V.amygdalina: Vernonia amygdalina, DPPH: 2,2, Diphenyl, 1 picryl hydrazil.*

INTRODUCTION

The universe is enriched with a lot of medicinal plants. The parts of medicinal plants such as roots, leaves, flowers, barks, seeds and fruits have been forming the basis of effecting drugs which are used to cure diseases and to improve health since the immemorial time.[1,2] Special interest has been attracted by researchers to find out new

plant derived drugs that can combat the threat of drug resistant pathogenic microorganism, antitumor and anticancer agents. *V.amygdalina* belongs to astereceae family commonly known as "bitter leaf" because of the impastation of bitter taste of the leaves. *V. amygdalina* leaves are rich in proteins, amino acids, carbohydrates, fat, vitamins and minerals such as calcium, iron, potassium, phosphorus, manganese, copper and cobalt.[3] The aqueous extract of leaves of *V amygdalina* could be taken as appetizer or digestive tonic. The extract is also helpful in amoebic dysentery, gastrointestinal problem, stomach discomfort etc. The root and leaves are also used to treat fever, hiccups, kidney problems, diabetes.[4]

Phytochemical screening of this plant leaves extracts showed the presence of polyphenol, flavonoids, saponins and alkaloids, riboflavin, edotides, sesquiterpene, coumarins, lignans, xanthones and anthraquinones. These phytochemical might be responsible for its antioxidative, antidiabetic, anticancer, antimicrobial, antimalarial, antiparasitic, antihelminthic, antiallergic, anti-inflammatory, antipyretic, analgesic, anxiolytic, sedative, hepatoprotective and hypolipidemic physiological effects.[5,6] The antioxidant activity of *V.amygdalina* leaves is associated with biologically active compounds such as polyphenol and flavonoid levels. These compounds are also known as protective factor against tumor.[7] It has been reported that flavonoids extracted from *V.amygdalina* have cytotoxic effects in human nasopharyngeal carcinoma cell and its saponins like vernodaline and vernolide have antitumoral activities in leukemia cells. In addition, peptides from *V.amygdalina* are known to be potent inhibitor of mitogen activated protein kinases (MAPKs) which are responsible for growth and development of breast tumour.[8,9] Antioxidants are chemical substances that act as scavenger of free radicals. Oxidative stress occurs in our body when there is imbalance between free radicals and antioxidant. Oxidative stress is responsible for several pathological conditions such as cardiovascular diseases (coronary heart diseases, atherosclerosis, arterial hypertension, heart failure), diabetes, cancer, cataract, hepatotoxicity, arthritis, neurodegenerative diseases (Parkinson's diseases, Alzheimer's disease) and acceleration of the ageing process.[4,10]

Antioxidant can delay, prevent or stabilized oxidative cellular damage by inhibiting the process of oxidation even at a very low concentration and prevent the onset of diseases caused by oxidative stress. A good number of plants are the source of antioxidant agent. Natural antioxidants inhibit oxidation in food, stop oxidation chains in-vivo and quench dreaded free radicals.[11] The extract from plants not only contain primary metabolites and minerals but also secondary metabolites like flavonoids, alkaloids, saponins, plenolics etc which possess antioxidant property. The medicinal and pharmacological activities of these plants are due to the presence of these bioactive chemical compounds that produce definite physiological action in the human body.[5,12] Flavonoids and phenolic compounds of plant origin have free radical scavenging, metal ion chelating and antioxidant activities.[13] Due to safety and high efficacy of natural antioxidant scientist are interested to uncover a new source of antioxidant of plant origin which has less impact on human health.[14,15] Therefore, present study was designed to prepare aqueous leaves extract of *Vernonia amygdalina* and to assess its antioxidative effects through DPPH method..

METHODOLOGY

This quasi-experimental study was conducted at the Department of Pharmacology and Therapeutics, Rajshahi Medical College, Rajshahi. The study was carried out in one year from January 2021 to December 2021. Prior study of the experiment, permission was taken from the Ethical Review Committee in the Rajshahi Medical College, Rajshahi. In the antioxidant study five strength (1mg/ml- 0.0625mg/ml) of two solutions were taken and did the experiment six times. Present investigation was focused on the preparation of aqueous extract leaves of *V.amygdalina*. Aqueous extract of *V.amygdalina* was prepared according to the method described by Okwuzu et al., 2017 with slight modification.[16] 500gm of fresh leaves of *V.amygdalina* were collected from the garden of Rajshasi Medical College. After cleaning with fresh water, they were air-dried at room temperature by spreading them on a laboratory table for 7 days. Thereafter, they

were grounded to course powder with blender. 50gm of powdered material was extracted with 500ml of deionized water. And kept it for 72 hours the resultant formulation was then filtered with whatman No. 1 filter paper and evaporated to dryness with the aid of hot air oven at 40ºC. The powerd form of leaves of bitter leaf was made. The working solution of extract was prepared by weighing out 200mg of powdered form accurately and dissolved it in 200ml distilled water to give an effective concentration of 1mg/ml.

Determination of antioxidant activity of aqueous extract of *V.amygdalina*:

The antioxidant activity of aqueous extract of bitter leaf was determined by means of the test using the DPPH (2,2-diphenyl-1 picryl-hydrazil) radicles, which was reflected by the reduction of the absorbance of the DPPH methanol solution during the reaction with the tested solution . At first 0.1mM solution of DPPH was prepared using 2 mg of DPPH dissolved in 50 ml of absolute Methanol and thus the stock solution was prepared. Then numerous dilutions of the tested solutions were prepared in the range of 0.0625 mg/ml- 1mg/ml. Changed in the absorbance intensity was measured using the spectrophotometer (Model 340). In the test tubes (protected from light) 1ml of tested solution and 1ml of the DPPH reagent at the concentration of 0.1mM were added. After shaking the solutions in the test tubes, these were kept in the dark for 30 minutes. Then the absorbance was read, at the wave length of 517nm. Before the measurement of the absorbance of samples, the absorbance of DPPH solution was measured. The measurement was made by measuring the absorbance of 1 ml of deionized water and 1 ml of DPPH solution.

The ability to reduce free DPPH radicles was calculated based on the formula:

$$Aa=(^{A_o - Ai}/_{A_o}) \times 100\% ,$$

Where Aa means antioxidant activity (%) , Ai means average absorbance of the tested solution and A_o means average absorbance of the DPPH solution.

As ascorbic acid is a known antioxidant, ascorbic acid was used as control in this study. In addition, stock solution of ascorbic acid of 1mg/ml was prepared. Serial dilutions of ascorbic acid were prepared and mixed with DPPH solution in the above-mentioned way and then the absorbance was measured. Data processing and analysis was done using SPSS (statistical package for social science) version 24. The results was calculated as mean±standard deviation (SD). and the test statistics to be used to analyze the data were descriptive statistics, and unpaired t-test where P<0.05 was considerd as significant.

RESULTS AND OBSERVATIONS

The antioxidant activity of aqueous extract of leaves of *V.amygdalina*(bitter leaf)

500gm of fresh leaves of bitter leaf were air dried after washing with clean water for 7 days. 200mg dry powder of bitter leaf was mixed with same amount of deionized water to prepare the effective concentration of 1mg/ml aqueous extract. After that serial dilution 0.5mg/ml, 0.25 mg/ml, 0.125mg/ml and 0.0625mg/ml of aqueous extract of bitter leaf were prepared. When gradual dilution of bitter leaf extract was prepared the colour of the solution was changed from dark yellow to pale. The antioxidant activity of *V.amygdalina* (bitter leaf) was determined by DPPH method. In this method the stable nitrogen radical of DPPH was reduced by the antioxidant present in the bitter leaf and causing decreased in the absorbance which was measured at the wave length 517 nm by spectrophotometer (Model 340).

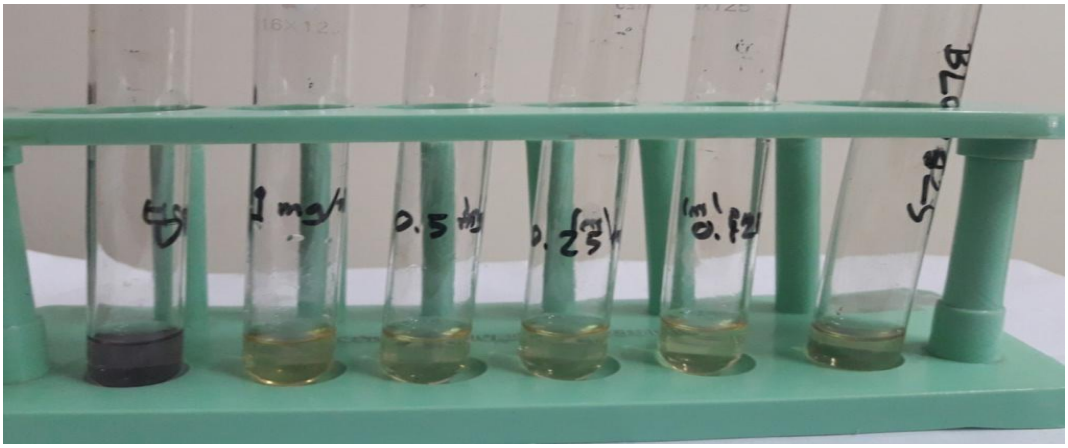

Figure 1: Changes of colour of DPPH after adding different concentration of aqueous extract of *V.amygdalina* (bitter leaf)

Figure 1 showed the colour changes of DPPH solution after adding aqueous extract of leaves of *V.amygdalina* (bitter leaf). The colour of DPPH solution changed from deep purple to yellow when aqueous extract of bitter leaf was added to the solution due to reduction of stable DPPH free radical.

Table 1: The antioxidant activity of different concentration of aqueous extract of leaves of *Vernonia amygdalina* (DPPH free radical scavenging activity)

Concentration of Bitter leaf (mg/ml)	Percentage (%) of inhibition of DPPH (Mean±SD)
0.0625	34.9±1.14
0.125	63.3±0.62
0.25	76±0.83
0.5	77.9±1.37
1.0	84.9±1.86

Table 1 showed mean antioxidant activity of bitter leaf at five different concentration. Here, *V.amygdalina* at the concentration of 0.0625 mg/ml, 0.125 mg/ml, 0.25mg/ml, 0.5mg/ml and 1 mg/ml showed mean percentage of DPPH scavenging activity 34.9±1.14, 63.3±0.62, 76±0.83, 77.9±1.37 and 84.9±1.86 respectively. The aqueous extract of bitter leaf gave the highest inhibition of 84.9±1.86% at 1mg/ml and lowest inhibition of 34.9±1.14% at 0.0625 mg/ml that indicates the antioxidant activity of aqueous extract of bitter leaf was concentration dependent i.e increase the concentration of the extract increase the antioxidant activity.

Table 2: The antioxidant activity of different concentration of standard

Concentration of Ascorbic acid (mg/ml)	Percentage (%) of inhibition of DPPH (Mean±SD)
0.0625	80.1±1.68
0.125	86.5±2.47
0.25	91.6±0.65
0.5	93.2±0.44
1.0	94.5±0.62

Ascorbic acid (DPPH free radical scavenging activity)

Table 2 showed mean antioxidant activity of Ascorbic acid at five different concentrations. Here, Ascorbic acid at the concentration of 0.0625 mg/ml, 0.125 mg/ml, 0.25mg/ml, 0.5mg/ml and 1 mg/ml showed mean percentage of DPPH scavenging activity 80.1±1.68, 86.5±2.47, 91.6±0.65, 93.2±0.44 and 94.5±0.62 respectively. Ascorbic acid gave the highest inhibition of 94.5±0.62% at 1 mg/ml and lowest inhibition of 80.1±1.68% at 0.0625 mg/ml that indicates the antioxidant activity of ascorbic acid was concentration dependent i.e increase the concentration of the ascorbic acid increase the antioxidant activity.

Table 3: Comparison of antioxidant activity between different concentration of Ascorbic acid and aqueous extract of leaves of *V.amygdalina*

Comparisons between Ascorbic acid and V.amygdalina	Mean differences	95% Confidence interval of the difference		t	df	P value
		Lower	Upper			
0.0625 mg/ml	45.15	46.99	43.3	54.49	10	<0.001
0.125 mg/ml	23.2	25.52	20.88	22.28	10	<0.001
0.25 mg/ml	15.67	16.63	14.71	36.38	10	<0.001
0.5 mg/ml	15.3	16.61	13.99	26.03	6	<0.001
1.0 mg/ml	9.55	11.50	7.6	11.97	6	<0.001

Table 3 showed comparison test between Ascorbic acid and aqueous extract of *V.amygdalina* at different concentration. At 0.0625mg/ml Ascorbic acid vs aqueous extract of *V.amygdalina* showed Mean difference = 45.15, 95% CI of difference = 46.99 to 43.3, t =54.49, df=10, P value < 0.001.So there was significant difference between aqueous extract of leaves of *V.amygdalina* and Ascorbic acid. At 0.125mg/ml Ascorbic acid vs aqueous extract of *V.amygdalina* showed Mean difference = 23.2, 95% CI of difference = 25.52 to 20.88, t=22.28, df=10, P value < 0.001.So there was significant difference between aqueous extract of leaves of *V.amygdalina* and Ascorbic acid. At 0.25mg/ml Ascorbic acid vs aqueous extract of *V.amygdalina* showed Mean difference = 15.67, 95% CI of difference = 16.63 to 14.71, t=36.38, df=10, P value < 0.001.So there was significant difference between aqueous extract of leaves of *V.amygdalina* and Ascorbic acid. At 0.5mg/ml Ascorbic acid vs aqueous extract of *V.amygdalina* showed Mean difference = 15.3, 95% CI of difference = 16.61 to 13.99, t=26.03, df=6, P value < 0.001. So there was significant difference between aqueous extract of leaves of *V.amygdalina* and Ascorbic acid. At 1mg/ml Ascorbic acid vs aqueous extract of *V.amygdalina* showed Mean difference = 9.55, 95% CI of difference = 11.50 to 7.6, t=11.97, df=6, P value < 0.001. So aqueous extract of leaves of *Vernonia amygdalina* showed antioxidant activity in all concentration. But in comparison to ascorbic acid, aqueous extract of *V. amygdalina* showed less antioxidant effect than ascorbic acid.

DISCUSSION

In the present study aqueous extract of *V.amygdalina* were prepared using fresh *V.amygdalina* leaves and distilled water. In a similar study Anyasor et al. 2010 compare the phytochemical and antioxidant activity of aqueous and methanolic extract of leaf of *V.amygdalina*. The researchers found higher phenolic content in the aqueous extract of leaves of *V.amygdalina*. They also mentioned that phenolics are major group of compounds acting as primary antioxidants and free radical scavenger. In the current study the antioxidant activity of bitter leaf was determined by using DPPH method and ascorbic acid was taken as a control. DPPH free radical scavenging assay has been widely used for the assessment of antioxidant activity due to relatively short time required for analysis.This method is based on the reduction of stable radical DPPH to the non-radical form diphenyl-picrylhydrazine (DPPH-H) by a hydrogen donating antioxidant. As a consequence, the solution of DPPH loses its deep purple colour and becomes yellowish which is measured from the changes in absorbance at wave length of 517 nm. After adding the solution with antioxidant, the colour change of the solution of DPPH indicate the

free radical scavenging properties of the antioxidant .[17, 18]

In our study five dilutions (1mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml) of *V.amygdalina* and ascorbic acid were prepared to observe the antioxidant activity. DPPH free radical scavenging activity of aqueous extract of bitter leaf was 34.9±1.14, 63.3±0.62, 76±0.83, 77.9±1.37 and 84.9±1.86 respectively at the concentration of 0.0625 mg/ml, 0.125 mg/ml, 0.25mg/ml, 0.5mg/ml and 1 mg/ml respectively. The aqueous extract of bitter leaf gave the highest inhibition of 84.9±1.86% at 1mg/ml and lowest inhibition of 34.9±1.14% at 0.0625 mg/ml. The percentage of DPPH scavenging activity of ascorbic acid was 80.1±1.68, 86.5±2.47, 91.6±0.65, 93.2±0.44 and 94.5±0.62 respectively at the concentration of 0.0625 mg/ml, 0.125 mg/ml, 0.25mg/ml, 0.5mg/ml and 1 mg/ml respectively. Ascorbic acid gave the highest inhibition of 94.5±0.62% at 1 mg/ml and lowest inhibition of 80.1±1.68% at 0.0625 mg/ml. Omede et al, 2018 investigated the antioxidant activities of aqueous extract of leaves of *V.amygdalina* by DPPH method and found the highest inhibition of 74.55±1.07% at 0.5 mg/ml and lowest inhibition of 6.55±2.06% at 0.003125 mg/ml. The results justified the scavenging activity of aqueous extract which is consistent with the findings of the present study.[09]

Adesanoye and Farombi, 2014, conducted a study where the antioxidant ability of methanolic extract of *Vernonia amygdalina* was examined using different method. The result of this previous study showed maximum radical scavenging activity (RSA) of 29.6% on DPPH radical at 1000µg. Methanolic extract of *V.amygdalina* showed significant free radical scavenging and antioxidant activities.[4] In a study by Erasto, et al 2007, found that the radical scavenging activity of extract were concentration dependent i.e increase the concentration of the extract increased the radical scavenging activity and the IC_{50} value of methanol extract was <0.025 mg/ml.[19] In a previous study conducted by Raimi, et al., 2020 the phytochemical constituents of aqueous extract of bitter leaf were found and revealed the presence of alkaloids, saponin, tarpenoids, flavonoids, cardiac glycosides and tannins in the extract.The result of this previous study showed the percent of DPPH radical scavenged by aqueous extract of bitter leaf increased with increasing concentrations.The highest concentration of the extract 400µg/ml had the highest scavenging activity 85.42±0.25%.[2] From the above discussion it could come to conclude that the aqueous extracts of leaves of *V.amygdalina* have antioxidant activity.

CONCLUSION

In the current study different concentrations of aqueous extract of *Vernonia amygdalina* showed strong ability to scavenge free radicals by DPPH method. Aqueous extract of *Vernonia amygdalina* also showed antioxidant activity in a concentration dependent manner. In comparison to anti-oxidant activity of standard ascorbic acid, bitter leaf extract showed lower antioxidant activity at all strength. So from the overall findings of our study, it can be concluded that the aqueous extracts of *Vernonia amygdalina* has antioxidant activity.

Limitations of the study

The present study used only one method (DPPH) to assess the antioxidant effect of aqueous extract of *Vernonia amygdalina.*

Recommendations

Vernonia amygdalina is one of the important medicinal plants which is a great source of natural antioxidants. Further study are required to determine phytochemical characterization and explore the molecular mechanism underlying the effects of the aqueous extract of *V. amygdalina* as an antioxidant.

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Authors' contributions

Professor Dr. Sabiha Yasmin Moni along with Associate Professor Dr. Md. Iqbal Hossain helped me (Assistant Professor Dr. Arika Jannat) to select the study topic & formulated the study

design of the research work. Associate Professor Dr. Md. Awlad Hossain helped me to fix the method of the study. Assistant Professor Dr. Md. Murshid-Ur-Rahman & Dr. Pervin Akter helped me to formulate the table & figures for the data analysis. Assistant Professor Dr. Lotifa Hoque helped me to write the manuscript of the article. Professor Dr. Sabiha Yasmin Moni & Associate Professor Dr. Md. Iqbal Hossain supervised & directed the whole process of the research & manuscript of the article writing.

Declarations

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Conflict of interest

There is no conflict of interest.

Ethical approval

Approval from the ethical committee of the Rajshahi Medical College had been before the starting of data collection.

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