PHYTOCHEMICAL ANALYSIS, HPTLC FINGERPRINTING, ANTIOXIDANT PROFILE AND ANTI-ANEMIC EFFECT OF *AMARANTHUS BLITUM L* LEAF AQUEOUS EXTRACT

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ABSTRACT:

Background: In human body, oxidative stress plays an important role in generation of free radicals which intern contributes in generation of many disease conditions. Plant extracts are rich in various antioxidants which are having free radical scavenging activity. The aim of present study was to estimate the phytochemical constituents, to explore high performance thin layer chromatography (HPTLC) fingerprinting, to determine antioxidant potential, and to evaluate anti-anemic effect of an *Amaranthus blitum L.* leaf aqueous extract.

Results: An aqueous extract of *Amaranthus blitum L.* leaves revealed the presence of flavonoids, glycosides, phenolics, tannins and triterpenoids. The extract was found to have DPPH (53.78 ± 0.66), ABTS (38.53 ± 5.7) and Superoxide scavenging activity (65.73 ± 0.9) and the results were similar and statistically significant as like standard antioxidant ascorbic acid (1000 µg/ml). The total phenolic content of the extract was 1696.06µg gallic acid equivalent/g; and the total flavonoid content was 1529.88±10.38µg quercetin equivalent/g. The HPTLC results revealed multi-colored bands of the varying intensities, which confirmed the presence of the polyphenols and flavonoids like active constituents in this extract. Treatment with Phenylhydrazine decreased Red blood cell count, Hemoglobin content and Hematocrit %. However, administration of this aqueous extract reversed the hematological changes induced by phenyl hydrazine.

Conclusion: This study confirmed that, *Amaranthus blitum L.* leaves aqueous extract possess multidirectional, antioxidant and free radicals scavenging activity which is because of presence of the polyphenolic and flavonoid content into it. These findings provide evidence that the aqueous leaf extract of *Amaranthus blitum L* is a potential source of natural antioxidants and free radicals scavenging compounds. Also antioxidant properties of *Amaranthus blitum L.* leaves aqueous extract have positive impact on inhibition of hemolytic anemia induced by phenylhydrazine.

Keywords: *Amaranthus blitum L*; Antioxidant activity; Flavonoids; Free radicals; Oxidative stress; Anti-anemic.

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1. Introduction

In human body free radicals are continuously generated by various mechanisms. These free radicals, which are unstable, have an attraction for biomolecules; they produce oxidative stress which plays an important role in the development of different diseases. To reduce this oxidative stress, the human body naturally produces antioxidants or they are externally supplied through food supplements. [1]

The destruction of red blood cells leads to the appearance of anemia. [2] Globally two billion people are suffering from anemia. [3] Phenylhydrazine (PHZ) generates reactive oxygen species (ROS), which creates oxidative stress and induces hemolytic anemia. [4] In the present study, we used PHZ induced anemia to demonstrate anti-anemic activity of the *ABL* extract.

Now a days food, pharmaceutical and nutraceutical industries add few synthetic antioxidant compounds which produces unwanted effects or toxic effects on human health. [5] Natural antioxidants from plant source are having therapeutic efficacy against ROS. [6] Therefore they are being used in the prevention of oxidative stress-related diseases. The earlier study also demonstrated that phytoconstituents widely distributed in the medicinal plants, vegetables, and dietary fruits and are having radical scavenger activity. Hence, researchers are focusing noticeably in finding naturally occurring antioxidant compounds. [7] So, antioxidants from natural origin with bioactive compounds such as phenolic compounds, flavonoids, tannins, terpenoids, and iridoids are administered as complementary medicine in the prevention, healing or cure of chronic diseases [7, 8].

Genus *Amaranthus* belongs to the Order-Caryophyllales, Family-Amaranthaceae and sub family-Amaranthoideae. Around 70 species of *Amaranthus* in the world are known, out of which seventeen are edible. In ancient Indian, Nepalese, Chinese and Thai medicine, *Amaranthus* plant extracts were used in the treatment of infections of gonads, urinary and respiratory tracts. It was also employed as antidiarrhoeal, analgesic, antidiabetic and diuretic. Aerial parts of *Amaranthus* species has distinguished occurrence of phytoconstituents like alkaloids, flavonoids, glycosides, phenolic acids, steroids, saponins, amino acids, vitamins, minerals, terpenoids, lipids, betaine, catechuic tannins and carotenoids. *Amaranthus* species consists of abundance of Antioxidants, molecules that reduce effect of free radicals; important for protection against cancer and degenerative disorders. Extract of leaves and flowers of *Amaranthus* species was shown to have maximum antioxidant action [9].

So far, no study has explored the phytoconstituents, antioxidant and anti-anemic actions of *Amaranthus Blitum L. (ABL)* Therefore; this study was aimed to investigate the photochemical, antioxidant potential, HPTLC fingerprinting and anti-anemic effect of *ABL* leaf extract.

2. Methodology

Drugs and chemicals

Analytical grade chemicals such as 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), quercetin, 2-2-diphenyl-1-picrylhydrazyl (DPPH), Trolox, ascorbic acid, Folin Ciocalteu phenol, gallic acid, ABTS working solution, PHZ were obtained from Sigma-Aldrich. All high quality chemical and reagents were used in the study.

Collection and authentication of plant

Fresh leaves of the *ABL* were collected from agriculture land located at Ichalkaranji, Kolhapur District, Maharashtra, India. The plant was authenticated by botanist, Dr. Vikas B. Awale, Bharati Vidyapeeth's Dr. Patangrao Kadam Mahavidyalaya, Sangliwadi, Sangli, Maharashtra. The leaves were separated and subsequently washed with water to remove adhered dust. Leaves were further shade dried at room temperature, coarsely powdered and stored in air tight amber glass container throughout the study.

Extract preparation

Total of 50 gm of *ABL* coarsely powdered leaves were extracted with 500 ml of distilled water by maceration method. After 48 hours the resulting extract was filtered through the Whatman No.1 filter paper and filtrate was concentrated on water bath at 60°C. The extract so prepared was stored at cool place (temperature ranging between 4-8°C) during the period of study. The percentage yield of the extract was calculated.

Preliminary phytochemical analysis

ABL aqueous leaf extract was screened for the presence of secondary metabolites such as alkaloids, glycosides, tannins, flavonoids, and phenolics using conventional raboratory reagents.

In-vitro measurement of an antioxidant property

a) DPPH (Diphenyl-1-picrylhydrazyl and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity:

Antioxidant activity of an extract was estimated by its free radical scavenging ability against DPPH (1, 1-Diphenyl-2, Picryl-Hydrazyl) free radicals. A total of 100µL of *ABL* leaf extract (1mg/ml) was taken in the micro titer plate and 100µL of 0.1% methanolic DPPH was added over it and incubated for 30 minutes in dark condition. The mixture was then observed for color change from purple to yellow and purple to pale pink; considering strong and weak positive, respectively. Plates were evaluated using Elisa plate reader at 490nm. [10, 11]

Radical scavenging activity was calculated by the following equation:

DPPH radical scavenging activity (%) = [(Absorbance of control - Absorbance of test sample) /(Absorbance of control)] X100

b) ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) Assay

In a 96-well assey well plate, either Trolox (standard) 10 µL, an extract (test) 10 µL or a solvent 10 µL (control) were thoroughly mixed with ABTS working solution (290 µL). The microplate was subsequently incubated in the dark at 37°C for 6 minutes. All experiments were performed in triplicate (n = 3). An absorbance of a blank (distilled water) was set to zero and an absorbance of the solution in each assay well was determined using a microplate reader at a wavelength of 734nm. Working solution was a diluted solution (1/50 in Phosphate buffer saline) of preformed ABTS radical from 1:1 chemical reaction of 7 mM ABTS and 2.45 mM potassium persulfate. [12]

c) Superoxide scavenging activity (SOD)

Superoxide scavenging activity was estimated by using a validated method [13,14,15]. To the reaction mixture containing 0.2ml of NBT (1mg/ml of solution in DMSO) and 0.6ml of the extract in DMSO; 2ml of alkaline DMSO (1ml DMSO containing 5mM NAOH in 0.1ml H2O) was added to get final volume of 2.8 ml. The absorbance of this mixture was measured at 560 nm (Hyland K et al., 1983). Superoxide free radicals were formed by alkaline DMSO which reacted with NBT to produce colored diformazan. The blank consisted of pure DMSO instead of alkaline DMSO. The absorbance was measured at 560 nm using a spectrophotometer. Radical scavenging activity was expressed as a percentage and was calculated using the following formula.

% Scavenging = $(A_{sample} - A_{control})/A_{sample}$ X 100

Where, A_{sample} = absorbance of the test sample; $A_{control}$ = absorbance of the control. [13,14,15]

d) Estimation of total phenolic contents

The estimation of total phenolic contents in *ABL* leaf extract was determined with Folin-Ciocalteu method using UV-visible spectrophotometer [13]. Ethanolic solution of the extract in the concentration of 1 mg/ml was used in the analysis. The reaction mixture consists of 1 ml of extract and 9 ml of distilled water in a 25 ml volumetric flask. 1 ml of Folin-Ciocalteu phenol reagent was added to the mixture and shaken vigorously. After 5 minutes, 10 ml of 7% sodium carbonate solution was added to the mixture. Finally volume was adjusted to 25 ml using distilled water.

Similarly, a set of standard solutions of Gallic acid (200, 400, 600, 800 and 1000 µg/ml) were prepared in the similar manner. Absorbances of test and standard solutions were determined against the reagent blank at 550 nm using spectrophotometer after the incubation of samples for 90 minutes at room temperature. The total phenolic contents were expressed as, 'mg of Gallic acid equivalents (GAE) per g of an extract'. The absorbance of test sample was conducted in triplicate. [16]

e) Estimation of total flavonoid content

Total flavonoid content was measured by the aluminium chloride colorimetric assay. The reaction mixture was containing 1 ml of extract and 4 ml of distilled water, in a 10 ml volumetric flask. To this flask 0.30 ml of 5% sodium nitrite was added. After 5 minutes, 0.3 ml of 10% aluminium chloride was mixed. Again after 5 minutes, 2 ml of 1M sodium hydroxide was added and mixture was diluted to 10ml with distilled water. A set of reference standard solutions of quercetin (200, 400, 600, 800 and 1000 µg/ml) were prepared in the similar manner. The absorbances for test and standard solutions were measured against the reagent blank at 510 nm using spectrophotometer. The total flavonoid content was expressed, 'as mg of quercetin equivalents (QE) per g of an extract'. The absorbances of test sample were recored in triplicate. [16]

f) HPTLC Fingerprinting

HPTLC finger printing studies were carried out according to the method of Wagner, Baldt and Harbone. About 100mg of an aqueous extract of *ABL* leaves was dissolved in 1mL of distilled water and it was centrifuged at 3000 rpm for 5min. This solution was used as test solution for

HPTLC analysis. A Camag HPTLC system, comprising a Linomat 5 automatic applicator with a 100uL syringe, a twin trough plate development chamber, Camag TLC scanner 3, and Server DESKTOP-5IHGUM1, version 3.1.21109.3 software was used. Suitable volume of solutions (about 7 uL) were spotted in the form of bands having band width of 5mm on precoated silica gel 60 F254 HPTLC plate (10 × 10 cm, 250 um thickness) (E. Merck, Mumbai, India). Spots were located 8mm from the bottom, and 15mm from the side edges and were allowed to dry for 5min . Densitometric scanning was performed with a TLC scanner equipped with DESKTOP-5IHGUM1, version 3.1.21109.3 software (Camag) in reflectance absorbance. The slit dimensions were 6mm × 0.45 mm, scanning speed was 20mms−1, data resolution 100 um/step. Plates were scanned at 254 nm which was selected experimentally on the basis of distinctive absorption spectra of the compounds between 200 and 400 nm. Each plate was kept in photo-documentation chamber (CAMAG) and captured the images at visible light and UV 366 nm and 254 nm. The peak numbers with its height and area, peak display, and peak densitogram were identified. The retention factor (Rf) values at fingerprint data were recorded by DESKTOP-5IHGUM1, version 3.1.21109.3 software. [17]

g) Pharmacological evaluation:

1) Approval of research protocol:

The animal experiment was conducted as per the experimental protocol approved by the Institutional Animal Ethical Committee (IAEC) which was constituted as per guidelines of the CPCSEA (Committee for Purpose of Control and Supervision of Experimental Animals), India.

2) Experimental Animals:

Female Wistar rats required for the study were obtained from CPCSEA certified vendor. Rats were kept for one week in the quarantine for acclimatization to the laboratory conditions before study. Rats with weight ranging between 200gm ± 20% and age between 8-12 weeks were selected for the study. Animals were kept in spacious cages supplied with standard diet. Animals were having full access to food and water throughout the study. These cages were housed in a laboratory maintained at temperature 24^0C ± 1^0C, relative humidity 45-55%, and 12:12 hour's light-dark cycle. Food was withdrawn 3 hr before the commencement of the experiment.

3) Acute toxicity study:

The acute toxicity study of the extract was conducted as per OECD 420 guideline at maximum single dose 2000 mg/kg. A group of six Female Wistar rat was taken for the study. Rats with weight ranging between 200gm ± 20% and age between 8-12 weeks were selected for the study. The single maximum dose was administered by oral gastric intubation. After administration of the dose, each animal was observed for 30 min, intermittently for the next 24 h, and thereafter every day for total of 14 days. The sign of toxicity and/or mortality was recorded during this period. [18]

4) Phenyl-hydrazine induced Anemia: [19]

Female Wistar rats were divided into five groups each containing 6 rats. [Table1] All groups received food and water *ad-libitum* during the period of study. The negative control was administered with vehicle, while other groups were injected with PHZ (60mg/kg body weight) intraperitoneally, in divided doses 20mg/Kg for three days. Standard group was administered with Orofer-XT 10mg/Kg, daily for two weeks orally. Dose of Orofer XT was calculated by extrapolation method from the human dose (100 mg of Iron, twice daily). The test groups were administered with 200mg/Kg and 400mg/Kg *ABL* extract orally, respectively. All oral administration was done using soft rubber tube gastric intubation without anesthesia. The treatment was continued for two weeks. The blood samples were collected under mild pet ether anesthesia on the 1^{st}, 3^{rd}, 7^{th}, and 14^{th} day by the retro-orbital method into ethylenediaminetetracetate (EDTA) coated vials. Blood samples were analyzed for red blood cells (RBC) count, hemoglobin content (HB) and hematocrit (HCT) levels.

Statistical Analysis:

The experimental results were expressed as mean ± standard deviation (SD). All the measurements were performed in triplicates (n = 6). All the data were analyzed using analysis of variance (ANOVA) with the statistics software Prism graph pad.

Figure 1. DPPH scavenging activities of *Amaranthus blitum L* aqueous extract and ascorbic acid.

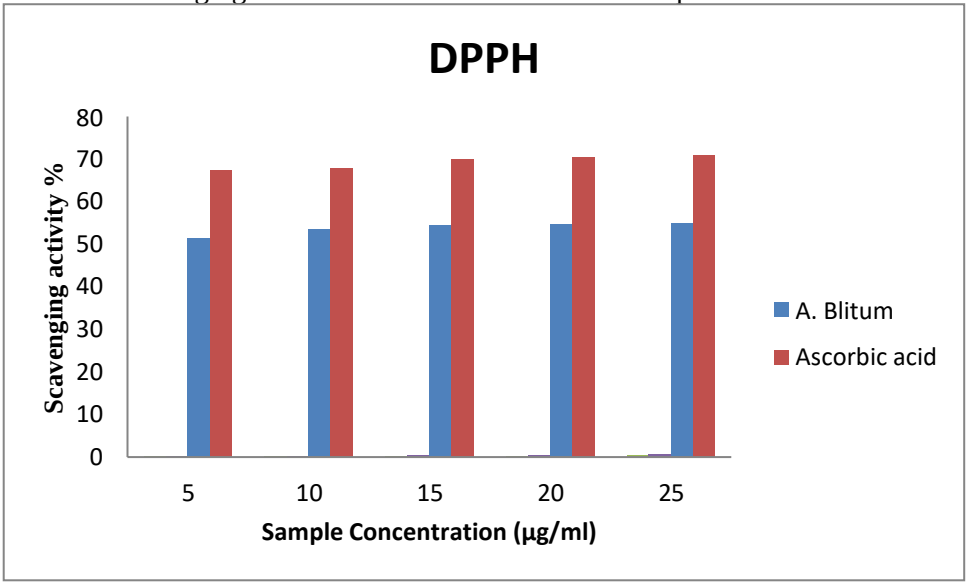

Figure 2. ABTS scavenging activities of *Amaranthus blitum L* aqueous extract and ascorbic acid.

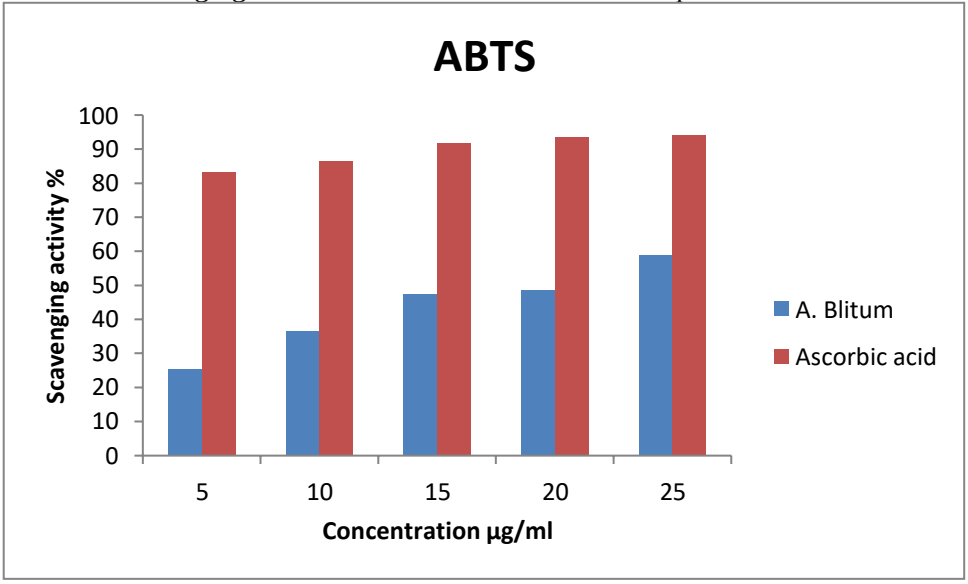

Figure 3 Superoxide radical scavenging activities of *Amaranthus blitum L* aqueous extract and ascorbic acid.

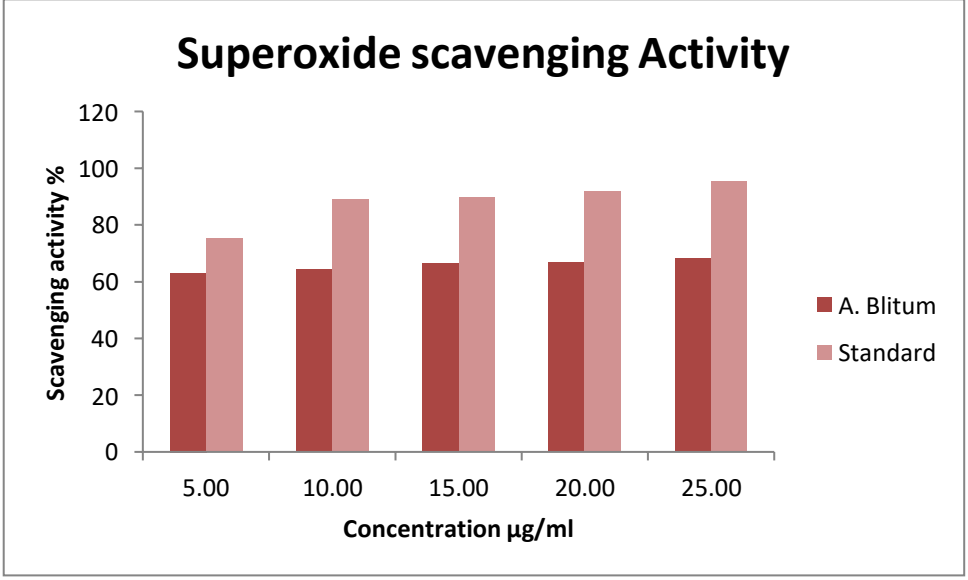

Figure 4. HPTLC chromatogram of Aqueous extract of *Amaranthus blitum L* of Phenolic compounds

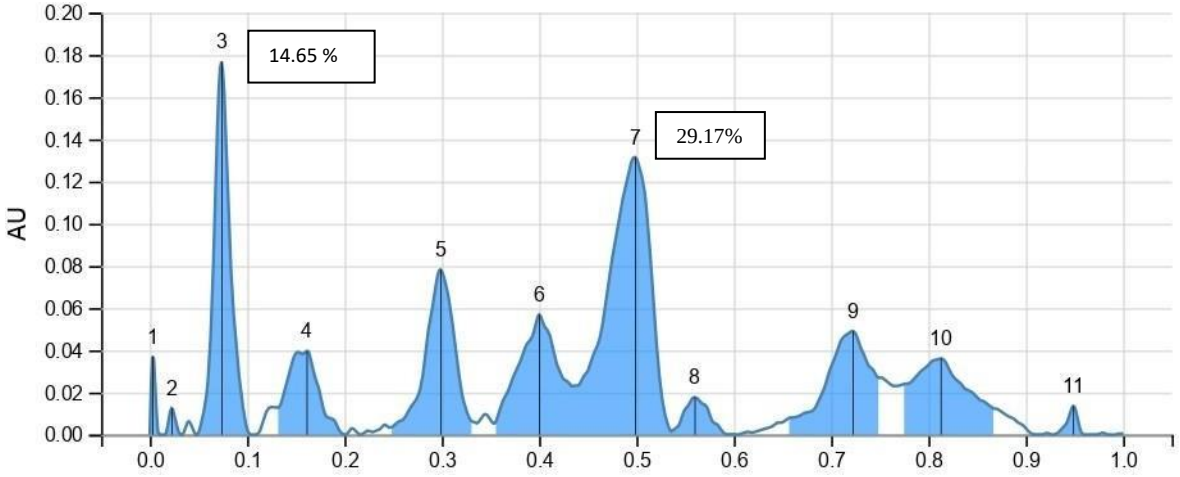

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Figure 5. HPTLC chromatogram of Aqueous extract of *Amaranthus blitum L* for Flavonoid compounds

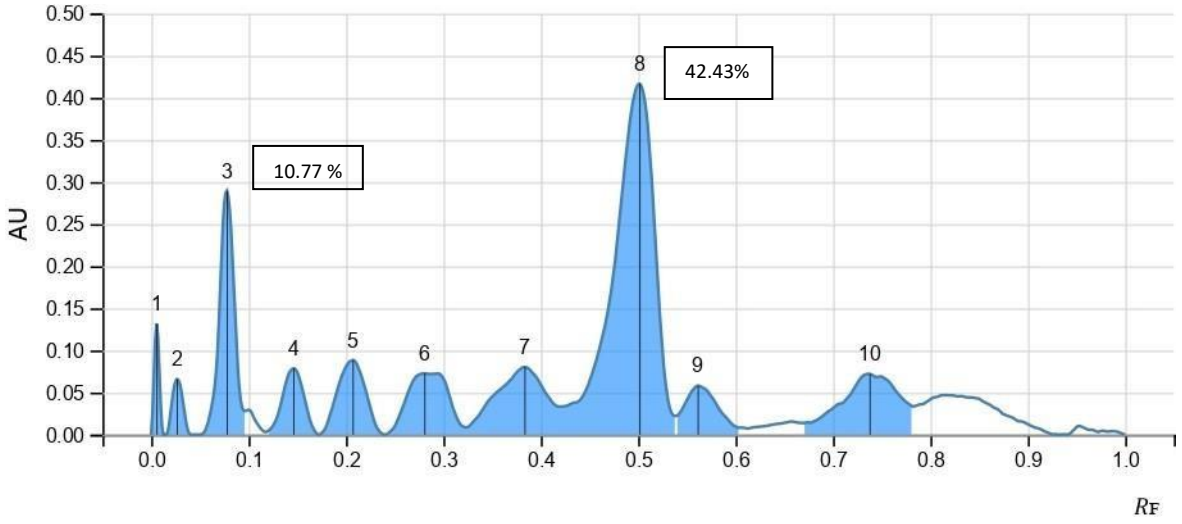

Figure 6. Photodocumentation of aqueous extract of *Amaranthus blitum L* for presence of
A. Phenolic compounds
B. Flavonoids at 366nm
C. Remission at 254 nm.

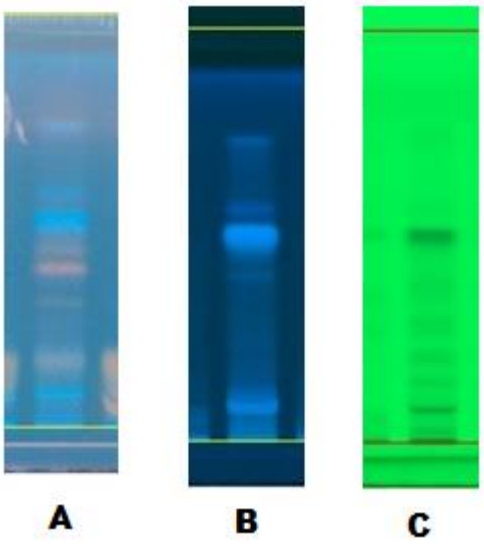

Table 1. Phenyl Hydrazine Induced Anaemia: (n=6)

Sr No.	Name of Group	Treatment	Oral Doses, frequency	Blood withdrawal
1	- Ve Control	Vehicle	0.5ml, daily	Retro orbital sinus, 0.5-1ml on 1st, 3rd,7th and 14th day using Pet. Ether.
2	+ Ve Control	Vehicle + PHZ	60mg/Kg, 20mg 3 days	
3	Standard	Orofer-XT+ PHZ	10mg/kg, daily	
4	Test 1	*ABL* + PHZ	200 mg/Kg, once daily	
5	Test 2	*ABL* + PHZ	400 mg/Kg, once daily	

Note: Except –ve control, all groups were administered with PHZ 20mg /Kg intraperitoneally as inducer for 3 days.

Table 2. Effect of ABL aqueous extract on Red blood cell count, Hemoglobin count and Hematocrit % in phenylhydrazine induced anemia.

Group	RBC count (lacs/mm^3)				Hemoglobin count g/dl				HCT count %			
	Day 1	Day 3	Day 7	Day 14	Day 1	Day 3	Day 7	Day 14	Day 1	Day 3	Day 7	Day 14
Negative control	64.0 ±5.0	63.4±6.7	62.3±2.8	64.6±4.2	13.31± 0.34	12.68± 0.42	12.89± 0.12	13.27± 0.18	38.12± 1.21	41.42± 1.67	39.33± 1.78	40.79± 1.34
Positive control	65.8±4.3	31.8±8.7	39.5±4.5	41.9±8.3	13.08± 0.46	6.35± 0.28	6.92± 0.34	7.23± 0.35	39.89± 2.63	24.31± 2.04	27.24± 1.56	30.29± 1.45
Standard	68.0±6.7	39.1±5.6	58.9±6.7	69±3.5**	12.93± 0.35	8.14± 0.31	11.46± 0.41	14.23± 0.18**	40.18± 1.67	23.48± 1.45	33.67± 2.03	40.23± 1.56**
ABL 200 mg/kg	61.2±3.6	34.5±8.7	48.4±2.3	58±1.4*	13.08± 0.45	7.47± 0.41	11.03± 0.35	12.56± 0.28*	40.56± 2.45	23.41± 2.23	31.34± 1.35	36.56± 1.45*
ABL 400 mg/kg	66.0±5.7	31.5±6.2	59.3±4.6*	68±6.3**	13.65± 0.56	8.15± 0.34	11.78± 0.36*	13.89± 0.37**	43.24± 1.56	22.34± 2.41	35.12± 1.46*	40.14± 1.56**

Values are expressed as mean ± SD followed by one-way ANOVA *P<0.05, ** P<0.01, *ABL: Amaranthus blitum L,* RBC: Red blood cell, HCT: Hematocrit.

3. Results:

The preliminary qualitative phytochemical analysis of the aqueous extract of *ABL* showed presence of flavonoids, glycosides, phenolics, tannins and triterpenoids. The percentage yield of the extract was 11.8%.

In-vitro antioxidant activities:

Diphenyl-1-picrylhydrazyl radical scavenging activity:

The results of free radical scavenging activities of *ABL* leaf extract and standard drug are shown in Figure 1. Increasing concentration of extract significantly increased the DPPH scavenging effect. Standard ascorbic acid showed 69.27 ± 0.7 %, while aqueous extract showed 53.78 ± 0.6 % of DPPH radical scavenging.

ABTS (2,2-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolourization assay

A comparable scavenging activity was observed between the *ABL* extract and the standard drug ascorbic acid. Percentage of ABTS scavenging for standard was found to be 89.77 ± 2.103 % while that of aqueous extract was 38.53 ± 5.7 % (Figure 2).

Superoxide scavenging activity (SOD)

Superoxide scavenging of aqueous extract was measured at different concentrations and compared with standard. The radical scavenging exhibited by standard and *ABL* extract was 88.17± 3.4 %, and 65.73 ± 0.9 %, respectively (Figure3).

Total phenolic contents:

The estimation of total phenolic contents in plant extract was determined with Folin-Ciocalteu method using spectrophotometer. The total phenolic content was expressed as milligram (mg) of Gallic acid equivalent (GA) per gram (g) of extract. The total phenolic content of aqueous *ABL* extract was found to be 1696.06 µg GA/g (0.169 ±0.046 mg GA/g) of phenolic content.

Total flavonoid contents:

Total flavonoid content was measured by the aluminium chloride colorimetric assay. The total flavonoid content was expressed as milligram (mg) of quercetin equivalents (QC) per gram (g) of extract. *ABL* Aqueous extract was found to have 1529.88±10.38 µg QC /g of dry material (0.15 ±04.19 mg QC/g dry material)

HPTLC Fingerprinting

Flavonoids & Phenolic compounds:

Chromatogram was developed for *ABL* aqueous extract under mobile phase saturated condition using mobile phase Ethyl Acetate: Water : Ethanol : Formic Acid (2 : 7 : 1 : 3 : 0.5 v/v/ v/v/v). The HPTLC fingerprinting of aqueous extract showed presence of 7 peaks with RF values in the ascending order of 0.007, to 0.74. The largest area (%) of a phytoconstituent was found to be 64.80% and its corresponding RF value was 0.537.

The HPTLC fingerprinting of *ABL* aqueous extract showed presence of 11 peaks with RF values for phenolic compounds in the ascending order of 0.010, to 0.960 end values. The highest area (%) of the phytoconstituents was found to be 29.17% and its corresponding RF value was 0.536.

Also the HPTLC fingerprinting of *ABL* aqueous extract showed presence of 10 peaks with RF values for flavonoid compounds in the ascending order of 0.013, to 0.782 end values. The highest area (%) of the phytoconstituents was found to be 42.34% and its corresponding RF value was 0.537. Exposure of spotted and developed HPTLC plate at UV 254 nm showed presence of light and dark bands. At UV 366 nm exposure showed multi-colored bands of different intensities. This revealed presence of polar and non-polar constituents in the aqueous extract. In the developed HPTLC fingerprinting, blue colored bands indicated presence of phenolic compounds and yellow colored zones indicated presence of flavonoids in *ABL* aqueous extract.

Anti-anemic activity

Experimental protocol was approved by IAEC of Biocyte Institute of Research and Development, Sangli. (IAEC/Sangli/2022-23/01).

Acute toxicity study:

The acute toxicity test of *ABL* extract was performed as per OECD 420 guideline at fixed dose 2000 mg/kg. During the two weeks study the toxicity signs were not observed and there were no mortality recorded. As the doses used in the later study were 5-10 times smaller than the fixed dose used in the acute toxicity study, we can consider that later study was conducted using safe doses.

Induction of anemia in participated group was done using PHZ method. [19] There was progressive anemia induction carried out in first three days by administering divided intraperitoneally doses. From the 4th day different daily treatments were administered to different groups orally.

RBC count was performed in the rat blood sample collected in EDTA containing vials using MISPA VIVA (KT21092350) semi-autoanalyser. The results were expressed in lacs/mm^3 in order to have sufficient resolution of the mesurement. ANOVA was conducted to prove the difference amongst the groups. Except Negative control group all other groups participated in ANOVA. Dunnett's "t" test was used as post ANOVA test and it was employed to measure significance difference of three treated groups from positive control. [Table3] On seventh day, test 2 group (400mg/kg) showed significant antianaemic activity ($p<0.05$), while test 1 group (200mg/Kg) showed similar activity on 14th day ($p<0.05$). Test 2 group (400mg/kg) and standard group (Orofer XT 10mg) showed significant activity ($P < 0.01$) against positive control group on 14th day. [Table3]

Hb content was determined in the rat blood sample collected in EDTA containing vials using MISPA VIVA (KT21092350) semi-autoanalyser. The results were expressed in mg/dl. ANOVA was conducted to prove the difference amongst the groups. Except Negative control group, all other groups participated in ANOVA. Dunnett's "t" was used as post ANOVA test and it was employed to measure significance difference of three treated groups against positive control. On seventh day, test 2 group (400mg/kg) showed significant antianaemic activity ($p<0.05$), while test 1 group(200mg/Kg) showed similar activity on 14th day ($p<0.05$). Test 2 group (400mg/kg) and standard group (Orofer XT 10mg) showed significant activity ($P < 0.01$) against positive control group on 14th day. [Table3]

Hematocrit % was determined in the rat blood sample collected in EDTA containing vials using MISPA VIVA (KT21092350) semi-autoanalyser. The results were expressed in hematocrit %. ANOVA was conducted to prove the difference amongst the groups. Except Negative control group, all other groups participated in ANOVA. Dunnett's "t" was used as post ANOVA test and it was employed to measure significance difference of three treated groups against positive control. On seventh day test 2 group (400mg/Kg) showed

significant antianaemic activity ($p<0.05$), while test 1 group(200mg/Kg) showed similar activity on 14th day ($p<0.05$). Test 2 group (400mg/kg) and standard group (Orofer XT 10mg) showed significant activity ($P < 0.01$) against positive control group on 14th day. [Table3]

4. Discussion:

During metabolism, free radicals get generated as byproducts. They impart injury to different tissues that later contributes to various chronic diseases. [20] In the preliminary qualitative phytochemical analysis, aqueous extract of *ABL* showed the presence of various bioactive principles as depicted in table 2. Phenolic compounds, flavonoids belonging to polyphenolic compounds play an important role in antioxidant activity. Antioxidants present in plant extract reduces free radical induced tissue damage. Polyphenolic compounds present in aqueous extract of *ABL* prompted us to evaluate antioxidant activity. [21]. DPPH is one of the rapid, easy and sensitive methods for evaluating radical scavenging activity of plant extracts. DPPH radical scavenging activity of aqueous extract of *ABL* showed similar antioxidant potential as compare to standard ascorbic acid. Quantity of phenolic compounds present in any plant extract has corelation with antioxidant activity of that extract. [22]

In ABTS assay, hydrogen-donating and chain breaking ability of antioxidants present in plant extract is expressed through ABTS radical cationic decolorization. The aqueous extract of *ABL* decolorize blue and green colored ABTS radical cations. This scavenging ability was measured at 734nm. [23].

Oxidative enzymes and nonenzymatic reactions, generate superoxide radicals which induce oxidative damage of biomolecules. [24] Superoxide scavenging activity of ascorbic acid and *ABL* extract that reduces NBT to blue colored radical formation was measured at 560 nm and compared. Results showed that the percentage inhibition increases with increase in superoxide scavenging molecules.

Flavonoids belonging to polyphenol group were responsible for antioxidant activity of an extract. Total phenolic content and flavonoids content in plant extract was correlating with free radical scavenging activity. [25,26] Antioxidant activity of *ABL* extract was quite similar and comparable with activity of standard ascorbic acid.

HPTLC fingerprinting is qualitative assessment tool for detection of presence of phytoconstituents present in plant extracts. Results of preliminary phytoconstituent study revealed presence of phenolic compounds and flavonoids which are confirmed by HPTLC fingerprinting that shows multicolored bands of different intensities. For achieving high resolution and reproducible peaks different solvent composition of mobile phase were used. HPTLC fingerprinting of aqueous *ABL* extract showed different chromatograms at 254 nm & 266 nm. The chromatogram confirmed the presence of polar and non-polar constituents with different color bands. Blue zones detected in chromatogram indicate presence of phenolic compounds [27] while yellow bands revealed presence of flavonoids. [28].

PHZ induced animal model of anemia is reliable, rapid and widely used method.[29] PHZ induces hemolytic anemia by destroying red blood cells through overproduction of ROS, which denaturalize RBCs, causes oxidative denaturation of hemoglobin and hemolysis that results in decrease HCT %, membrane phospholipids and key enzymes involved in erythrocyte metabolism.[30, 31, 32] In this study PHZ causes decline in RBC, Hemoglobin and hematocrit count. These hematological parameters were improved by treatment with 200mg/kg and 400mg/kg of *ABL* extract as that of standard drug. *Amaranthus blitum L* aqueous extract reverses these three parameters because of its protective effect against PHZ induced anemia.

5. Conclusion:

Aqueous extract of *ABL* showed antioxidant potential on ABTS, DPPH, SOD models. Preliminary phytoconstituent evaluation indicated presence of phenolic compounds and flavonoids; which was later confirmed by HPTLC fingerprinting. This *in-vitro* antioxidant activity serve primary basis for future pharmacological studies on *ABL*. Extract of *ABL* can be used as natural antioxidant. Also aqueous extract of *ABL* improved PHZ induced decline in Red blood cell count, Hemoglobin content and Hematocrit %. Thus aqueous extract of *ABL* showed beneficial effect against hemolytic anemia. Further molecular level research is necessary to understand the mechanism involved in anti-anemic effect of *Amaranthus blitum L.*

Declaration:

Ethics approval and consent to participate

This study did not include any human subjects. Institutional Animal ethics Committee approval letter is attached in annexure. (Annexure 1)

Consent for publication

The authors declare no conflict of interest. We hereby give consent for publication.

Availability of data and material

All data generated or analyzed during this study are included in this published article. The datasets generated and/or analyzed during the current study are not publicly available because this is original data. It can be made available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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List of abbreviations:

ABL = Amaranthus blitum L. , GA = Gallic acid, QC = Quercetin, ABTS = 2,2-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid, DPPH = Diphenyl-1-picrylhydrazyl and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid, SOD = Superoxide scavenging activity Superoxide scavenging activity.

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