Physico-Chemical, Phytochemical and Antioxidant Evaluation of *Zizyphus nummularia* (Burm. F.) Stem Bark

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Abstract: *Zizyphus nummularia* (Burm. F.) (Family: Rhamnaceae) used as a folk medicine. The study was designed to lay down the various physicochemical and phytochemical standards which will be helpful to ensure the purity, safety, and efficacy of this medicinal plant. In the lime light of fewer and less potent antioxidant extracts, it is screened for antioxidant potency also. All physico-chemical properties were found within the measurable amount. Preliminary phytochemical analysis confirmed the presence of alkaloids, flavonoids, tannins and steroids. Total phenolic content and flavonoid content was found to be 10.7±0.1 and 2.57±0.1 respectively. Extract also found to posses significant antioxidant activity which can be correlated with phenolic content. IC_{50} of 0.042 mg/mL was found during DPPH radical scavenging activity. 2.75 mmol AA/ mg extract was found to be as total antioxidant capacity along with a concentration of 383.4 µg/ml during reducing power activity. The study would help in the authentication stem bark useful for laying down pharmacopoeial standards and help in finding improved antioxidant agents from the extract. It is found to be posse's significant antioxidant property, possibly by adsorbing and neutralizing free radicals.

Keywords: Anti oxidant, Physicochemical, Phytochemical, Standardization, *Zizyphus nummularia.*

INTRODUCTION

In Ayurveda, drugs from natural origin are widely used due to their safety and efficacy in treating the disease and about 25% of drugs are obtained from the higher plants. Therefore, standardization of crude drugs or polyherbal formulation is the prime requirement to ensure the safety, quality and efficacy of the drug [1, 2]. These standards include pharmacognostical, physicochemical, phytochemical, and other biological parameters.

Zizyphus nummularia (Burm. F.), a member of family Rhamnaceae, locally known as chhoti ber or Jhar Beri, is a small, thorny bush distributed in North Western India, majorly grows in dry and arid regions [3, 4]. Plant may attain a height of 1-2 meters and valued for nutritious edible fruits and as folk medicine [5]. Traditionally plant is used as astringent, and effective in the treatment of bilious sickness, scabies and boils. Plant is reputed for its nutritious fruit which is used as cooling, astringent, tonic, digestible, laxative, aphrodisiac, and anti-emetic; remove biliousness, thirst and burning sensation [3]. Bark decoction is used in joint pains, gargle for sore throat and bleeding gums [6], as effective remedy against asthma, bronchitis and fever [7]. Bark is good for the treatment of diarrhea, dysentery and colic [6]. *Zizyphus* mainly consist of cyclopeptide alkaloids with 13, 14 and 15 member ring system. The cyclopeptide alkaloids are highly modified polyamide plant bases composed of amino acid residues [8].

As *Z. nummularia* is used as a folk medicine and its extensive literature survey did not reveal any information on studies of standardization, so, we designed to lay down the standards which will be helpful to ensure the purity, safety, and efficacy of this medicinal plant with the help of following-

1. Physico-Chemical Properties along with Florescence Analysis

- Foreign matter
- Loss on drying
- Ash Values
  - Total Ash
  - Acid insoluble ash
  - Water soluble ash
- Cold extractive value
  - Petroleum ether extractive
  - Chloroform extractive
  - Alcohol extractive
  - Water extractive
  - Hydroalcoholic extractive

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ISSN: 2223-3806 / E-ISSN: 1927-5951/18

- Hot extractive value

  Petroleum ether extractive

  Chloroform extractive

  Alcohol extractive

  Water extractive

  Hydroalcoholic extractive

  Foaming Index

2. Phytochemical Analysis Qualitative

- Alkaloids
- Flavonoids
- Tannins
- Glycosides
- Steroids
- Carbohydrates
- Proteins

Quantitative

- Total phenolic content
- Total flavonoid content

Further the drug was also screened for anti oxidant potency to combat the oxidative stress with the help of following-

1. DPPH radical scavenging activity
2. Reducing power assay
3. Total antioxidant capacity

MATERIAL AND METHODS

Standards and Reagents

Quercetin, Gallic acid, and Ascorbic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and solvents were purchased from Merck Chemicals (Mumbai, India). Whatman (Florham Park, NJ, USA) No. 1 filter paper was used for filtration of the samples.

Plant Material and Extraction

The stem bark of *Zizyphus nummularia* was collected from Sikandarabad, UP, India. The plant material was authenticated by Principal Scientist, NBPGR, Pusa, Delhi. The voucher specimen (NHCP/NBPGR/2016-4/5633) was preserved in NBPGR and the crude drug sample was deposited in the Department of Pharmacognosy, School of Pharmaceutical Sciences, Jaipur National University, Jaipur, Rajasthan, India for future reference. Stem bark was dried under shade, reduced to moderate coarse powder and extracted with ethanol (95%). The extract was decanted, filtered with Whatman No. 1 filter paper and concentrated at reduced pressure below 40°C using rotary evaporator to obtain dry extract. This alcoholic extract were designated as AZN and taken up for biological screening. Extracts with other solvents such as petroleum ether, chloroform and water were prepared in the similar way for preliminary phytochemical screening.

Physico-Chemical Evaluation

Physico-chemical parameters such as the percentage of loss on drying (LOD), Total ash, acid insoluble ash, water soluble ash, cold extractive, and hot extractive value were determined according to the method prescribed by WHO [9]. All the parameters were taken in triplicate and the result which was obtained presented as mean ± standard error of the mean (SEM). Florescence analysis was also done [10, 11].

Preliminary Phytochemical Investigation

Preliminary phytochemical screening was performed using standard procedures. The extracts obtained from different solvents were subjected to identification tests for the detection of various organic phytoconstituents such as alkaloids, glycosides, saponins, flavonoids, tannins, and steroids [12, 13].

Quantification of Secondary Metabolites

Estimation of important secondary metabolites such as total phenolic content and flavonoids was done.

Total Phenolic Content

Total phenolic content (TPC) in alcoholic extract was determined by the Folin–Ciocalteu method [14, 15] and gallic acid as standard. Briefly, 0.5mL of crude extract (1mg/mL) were made up to 10 mL with distilled water, mixed thoroughly with 1.5 mL of Folin–Ciocalteu reagent for 5 min, followed by the addition of 4 mL 20% (w/v) sodium carbonate. The mixture was allowed to stand for further 30 minutes at room temperature. The absorbance was measured at 765nm using a UV-VIS

spectrophotometer. The total phenolic content is expressed as milligrams of gallic acid equivalent (GAE) per gram of dry extract.

Total Flavonoid Content

Flavonoid content in alcoholic extract of stem bark was determined using spectrophotometric method using Quercetin as standard [16]. The sample contained 0.5 mL methanolic solution of the extracts in the concentration of 1 mg/mL and 0.5 mL of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at λmax = 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of quercetin and the calibration line was constructed. The content of flavonoids in extracts was expressed in terms of quercetin equivalent (mg of QU/g of extract).

Anti Oxidant Activity by DPPH Assay

The ability of the plant extract to scavenge 1, 1-diphenyl-2-picryhydrazyl (DPPH) free radicals was assessed by the standard method [17]. The stock solution of extracts was prepared in ethanol to achieve the concentration of 1 mg/mL. Different concentrations of extracts were mixed with 3 ml of ethanolic solution of DPPH (DPPH, 0.004%). After 30 min of incubation at room temperature the reduction of the DPPH free radical was measured by reading the absorbance at 517nm using UV-Visible Spectrophotometer. Initially, absorption of blank sample containing the same amount of ethanol and DPPH solution was prepared and measured as control. Gallic acid was used as standard. The experiment was carried out in triplicate. Percentage inhibition was calculated using equation (1), whilst IC_{50} values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm.

$$\%\ Inhibition = \left(\frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \right) \times 100 \quad (1)$$

Reducing Power Assay

The reducing powers of the extracts were determined by the spectroscopic method [18]. Various concentration of extracts were prepared in 1mL of distilled water was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 RPM for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. Gallic acid was used as the reference material. All the tests were performed in triplicate and the graph was plotted with the average of three observations.

Total Antioxidant Capacity

The total antioxidant capacity of extract was evaluated by the phosphomolybdenum reduction assay method according to the procedure described by Prieto [19]. 1mL of various concentrations of extract were combined with 1 mL of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. The absorbance of the reaction mixture was measured at 695 nm against reagent blank using a spectrophotometer. The total antioxidant capacity was expressed as the number of equivalent of ascorbic acid.

Statistical Analysis

All the experiments were done in triplicates. The experimental results are expressed as mean±SEM

RESULTS

Physicochemical Evaluation

Various physicochemical parameters such as moisture content, ash values, extractive values and foaming index of *Z. nummularia* stem bark were established. The results for physico-chemical parameters such as moisture content, total ash value, cold and hot extractive values are summarized in Table **1**.

Florescence Analysis

Florescence analysis of powdered drug sample was carried after treating with different reagents / solvents. Florescence was observed at 254 and 365 nm comparing its change of color in visible light. The result for florescence analysis was given in Table **2**.

Phytochemical Analysis

Identification of the different classes of phytochemical constituents of the plant is an important parameter, which gives an indication of the

Table 1: Physicochemical Parameters of *Zizyphus nummularia* Stem Bark

Parameters	Results (%w/w)
Foreign matter	0.2±0.01
Loss on drying	9.409±0.091
Ash Values	
Total Ash	2.27±0.061
Acid insoluble ash	1.82±0.039
Water soluble ash	0.91±0.039
Cold extractive value	
Petroleum ether extractive	0.284±0.002
Chloroform extractive	0.533±0.025
Alcohol extractive	2.319±0.008
Water extractive	4.103±0.038
Hydroalcoholic extractive	4.395±0.083
Hot extractive value	
Petroleum ether extractive	0.322±0.019
Chloroform extractive	0.886±0.028
Alcohol extractive	2.560±0.029
Water extractive	4.844±0.023
Hydroalcoholic extractive	4.952±0.057
Foaming Index	<100

Each value represents the mean ± SEM, N=3.

pharmacologically active metabolites present in the plant [20]. Preliminary phytochemical screening showed the presence of alkaloids, flavonoids, steroids and tannins in the various extracts of the stem bark (Table **3**).

Total Phenolic and Flavonoid Content

The linear regression line of gallic acid (y=0.173x – 0.002, R^2 = 0.995) was used to calculate the TPC and expressed as gallic acid equivalents. TPC in alcoholic extract of *Z. nummaria* stem bark was found to be 10.73±0.1 mg GAE/g extract.

TFC was determined using quercetin as standard and expressed as quercetin equivalent, using regression equation of quercetin (y = 37.3x – 0.073, R^2 = 0.993). The TFC in AZN was found to be 2.57±0.1 mg QE/g extract (Table **4**).

Antioxidant Activity by DPPH Assay

AZN extract strongly scavenged DPPH radicals with the IC_{50} being 0.0042 mg/mL when compared to 0.00195 mg/mL for gallic acid used as standard. The scavenging was found to be concentration dependent (R^2>0.97) as depicted in Figure **1**. AZN showed 94.11% activity at a concentration of 100 μg/mL which is comparable to Standard gallic acid activity (93.74%) produced at 4 μg/mL concentration.

Reducing Power Assay

The presence of antioxidants in the extracts resulted into reduction of the ferric cyanide complex (Fe^{3+}) to the ferrous cyanide form (Fe^{2+}). Strong reducing agents, however, formed Perl's Prussian blue color and absorbed at 700 nm. Concentration dependent reducing power of AZN is illustrated in Figure **2**. The higher the absorbance of the reaction mixture, the higher would be the reducing power. Many

Table 2: Fluorescence Characteristic of Powdered *Z. nummularia* Stem Bark

Chemical treatment	Observation under UV Cabinet		
	Ordinary light	UV short WL (254nm)	UV long WL (365nm)
Powdered drug	Light brown	Florescent greenish yellow	Green
Powdered Drug + distilled water	Light brown	Florescent greenish yellow	Florescent blue spots
Powdered Drug + 5% NaOH	Light brown	Florescent greenish yellow	Florescent blue spots
Powdered Drug + NH_3	Light brown	Florescent greenish yellow	Blue spots
Powdered Drug + conc. H_2SO_4	Black	Light florescent greenish yellow	Black
Powdered Drug +conc. HCl	Brown	Florescent green	----
Powdered Drug + conc. HNO_3	Brown	Florescent greenish yellow	Dark brown
Powdered Drug + 5% Iodine	Brown	Florescent greenish yellow	Dark green
Powdered Drug + 5% $Fecl_3$	Brownish yellow	Florescent greenish yellow	Yellow
Powdered Drug + Picric acid	Brown	Florescent green (Prominent)	Green

Table 3: Qualitative Phytochemical Investigation of *Z. nummularia* Stem Bark

Chemical group	Name of extract				
	Petroleum ether	Chloroform	Alcohol	Water	Hydroalcohol
Alkaloids	-	-	+	+	+
Flavonoids	-	-	+	+	-
Tannins	-	-	+	+	+
Glycosides	-	-	-	-	+
Steroids	-	+	+	+	-
Carbohydrates	-	-	+	+	+
Proteins	-	+	-	+	-

reports have revealed that there is a direct correlation between antioxidant activities and reducing power of certain plant extracts [21].

Table 4: Quantitative Estimation of Secondary Metabolites

Parameter	Alcoholic extract of stem bark (mg/g extract)
Total Phenolic content (Gallic acid equivalent)	10.73±0.1
Total flavonoid content (Quercetin equivalent)	2.57±0.1

Each value represents the mean ± SEM, N=3.

Total Antioxidant Capacity

The total antioxidant capacity of the extract was calculated based on the formation of the phosphomolybdenum complex which was measured spectrophotometrically at 695nm. A direct correlation was found to exist between the concentration of the extract used and the spectrophotometrically measured phosphomolybdenum complex (R^2>0.98) (Figure **3**). The total antioxidant capacity is expressed as number equivalent of ascorbic acid per gram of extract. A calibration curve was also plotted for ascorbic acid (y=4.835x+0.017; R^2>0.97). The total antioxidant capacity AZN extract was found to be 2.75 mmol ascorbic acid equivalent/mg extract. Results for antioxidant profile of AZN have been summarized in Table **5**.

DISCUSSION

In the present study, various physicochemical parameters such as moisture content, ash values, extractive values and foaming index of *Z. nummularia* stem bark were established. The quality control of crude drugs and herbal formulation is of great importance and act as a tool for determination of identity, quality and purity of the drug. Moisture content is a foremost parameter, as in conjunction with a suitable temperature; it leads to activation of many enzymes and provides favorable environment for the infestation of micro-organisms and hence proves to be major cause for deterioration of herbal drug or formulation [19]. Inorganic matter present in the drug is indicated by total ash value. Acid insoluble ash is obtained when the total ash is boiled with HCl and insoluble residue is ignited. Acid insoluble ash gives a measure of sand and other siliceous matter. Extractive

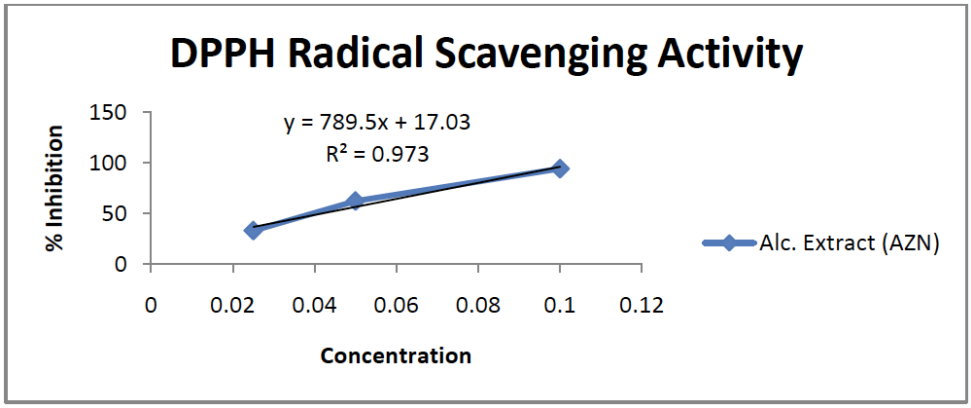

Figure 1: DPPH radical scavenging activity of alcoholic extract of stem bark (AZN).

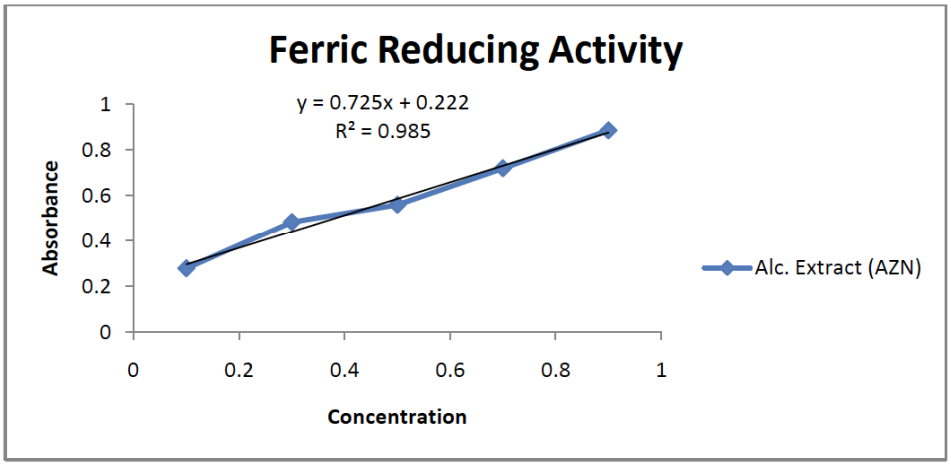

Figure 2: Ferric reducing power of AZN.

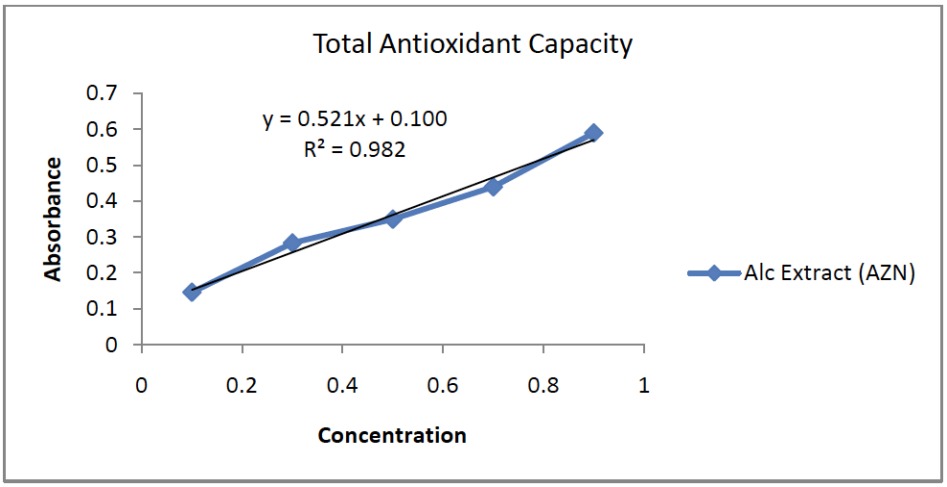

Figure 3: Total antioxidant capacity of AZN.

Table 5: Antioxidant Profile of AZN

Methods \ Results	IC_{50} (µg/mL)		mmol AA/ mg extract		Concentration µg/mL (Absorbance 0.5)	
	Std.	Test	Std.	Test	Std.	Test
DPPH assay	1.95	42.0	-	-	-	-
Total antioxidant capacity	-	-	-	2.83	-	-
Reducing Power	-	-	-	-	56.69	383.4

value represents the amount of active constituent dissolved in the specified solvent. Some constituents show fluorescence in the visible range in daylight. The ultra violet light produces fluorescence in many natural products which do not visibly fluoresce in daylight. If substance themselves are not fluorescent, they may often be converted into fluorescent derivatives or decomposition products by applying different reagents. Hence crude drugs are often assessed qualitatively in this way and it is an important parameter for pharmacognostic evaluation of crude drugs [22].

Reactions propagated by free radicals may cause extensive tissue damage, lipids, proteins and DNA are all susceptible to free radical attack. Oxidative stress or tissue damage by free radicals may be inhibited by scavenging the free radicals, inhibiting lipid peroxidation etc. Thus antioxidants may offer resistance against oxidative stress [23].

Preliminary phytochemical investigation revealed the presence of alkaloids, steroids, flavonoids and phenolics. Flavonoids and phenolics are widely

distributed secondary metabolites in plants, known for diverse properties, which include free radical scavenging inhibition of hydrolytic and oxidative enzymes and anti- inflammatory action. A number of reports also suggested the role of phenolics in foods for their oxidative stability and antimicrobial protection [24]. On the basis of previous findings plant was explored for determination of total phenolic and flavonoid content and hence for antioxidant potential in stem bark. The antioxidant efficacy was assessed by investigating the plant using three *in vitro* models namely, DPPH radical scavenging assay, ferric reducing power and total antioxidant capacity.

DPPH radical scavenging assay is considered to be uncomplicated, reliable and most convenient method. Radical scavenging activity of AZN was found to be concentrated dependent (R^2>0.97). % inhibition increased with increase in concentration of AZN and found to be maximum at concentration 100 μ g/mL i.e. 94.11% activity was observed.

The reducing power is associated with the ability of an antioxidant to donate an electron [25]. In reducing power assay, antioxidants cause the reduction of the Fe^{3+} into Fe^{2+}, thereby changing the yellow colored reaction mixture into various shades from green to blue, depending on the reducing power of the compounds. Strong reducing agents, however, formed Perl's Prussian blue color and absorbed at 700 nm [26]. Dose dependent reduction ability of AZN was indicated by increase in absorbance indicating the potential antioxidant activity.

Total antioxidant capacity was investigated by phosphomolybdate assay. This assay relies on the ability of an extract containing antioxidant(s) to donate an electron to Mo (VI) and reducing same to Mo (V) with the corresponding formation of green colored phosphomolybdenum V complex which displays maximum absorbance at 695 nm. A linear relationship was existed in concentration and total antioxidant capacity of AZN (R^2>0.98). These results indicated the usefulness of AZN as a potent antioxidant and will find its efficacy in reducing the oxidative stress and other disorders associated with oxidative stress.

The deluged data obtained and results found during this study would help in the authentication of *Z. nummularia*, stem bark, a very important constituent of various herbal drug formulations. This may lead to easier authentication of herbal drugs procured from markets for the correct identification of the medicinal plant ingredients and improved / better antioxidant agents may be extracted. Phenolic substances and flavonoids are associated with antioxidant activity. Plants may serve as a good source of antioxidants and vital for preventing *in-vivo* oxidative damages. The present study indicated the presence of alkaloids, flavonoids, tannins and steroids during the phytochemical investigation. The plant was used to evaluate the antioxidant potential using DPPH free radical scavenging assay, Ferric (Fe^{3+}) reducing power assay and Total antioxidant capacity (Phosphomolybdenum reduction assay) methods which were found to be posses significant antioxidant property. Possible mechanism of action of extract for producing their protective effect may be by adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides, however, further clinical studies need to be carried to clinically re-confirm the findings.

CONFLICTS OF INTEREST

No conflict of interest.

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Received on 28-03-2018 Accepted on 10-04-2018 Published on 25-06-2018

DOI: https://doi.org/10.6000/1927-5951.2018.08.03.5