Phytochemical analysis, antioxidant and antimicrobial activities of *Ammannia baccifera* L.

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Abstract: *Ammannia baccifera* has wide application in ethnomedicine around the globe and it has been investigated for various pharmacological activities. However, *A. baccifera* from Nepal is not investigated phytochemically and biologically. Here we report about the screening of extracts for phytochemicals, estimation of phenolics, flavonoids, tannins and sugars as well as antioxidant and antibacterial activities of different extracts. In phytochemical screening, phenolics, flavonoids, glycosides, saponins, quinones, reducing sugar, tannins, steroids and coumarin were detected in methanol and 50% aqueous methanol extracts so these extracts were further investigated for phenolics, flavonoids, tannins and sugars contents. Methanol extract showed the presence of higher amounts of phenolics (178.65 ± 1.16 mg GAE/g), flavonoids (60.05 ± 2.24 mg CE/g) and tannins (194.29 ± 2.96 mg TAE/g) than the 50% aqueous methanol extract (107.66 ± 2.04 mg GAE/g, 23.95 ± 2.07 mg CE/g, 120.28 ± 2.25 mg TAE/g). Similarly, 50% aqueous methanol extract showed the presence of higher amounts of sugars than (206.86 ± 4.21 mg GE/g) methanol extract (153.03 ± 3.80 mg GE/g). In DPPH free radical scavenging assay, methanol extract showed lower IC_{50} values (95.52±6.27µg/mL) than 50% aqueous methanol extract (114.66±8.49 µg/mL). In antibacterial assay, methanol extract showed activity against Gram positive bacteria, *B. subtilis, S. aureus* and *S. epidermidis* with inhibition zone ranged from 12-20 mm but the extract was inactive against Gram negative bacteria and fungi. The minimal inhibitory concentration of the extract against all three Gram positive bacteria was found to be between 1.56 to 0.78 mg/mL. The findings of this study support the traditional use of this plant to treat skin diseases.

Key words: *Ammannia baccifera*; Antioxidant**;** Antimicrobial**;** Phytochemicals**.**

Introduction

A baccifera L. commonly known as Blistering Ammannia, Acrid weed, or Monarch red stem and locally known as Agnikhar or Ambar belongs to family Lythraceae, is an annual erect, branched, and glabrous reddish herb distributed throughout the tropical region of America, Asia, and Africa. Generally, it grows in low-elevation marshes, swamps, rice fields, and watercourses[1,2]. Traditionally, *A. baccifera* is used in the treatment of different ailments. The leaf paste is applied in rheumatic pain, urinary calculi and skin condition such as rubefacient, eczema[3], parasitic skin infection[4].The ashes of this plant are mixed with oils to cure herpetic eruptions[5]. Likewise, the oral administration of the plant powder is used against snakebite and scorpion sting[6]. It is also widely used in traditional Chinese medicine. Many herbal formulations containing *A. baccifera* have been patented for treatment of serious diseases like cancer, spinal diseases ,inflammatory diseases, infertility, bladder stones,urinary tract infection and dermatitis[7].

Several pharmacological potentials of *A. baccifera* have been reported such as antioxidant, anti-inflammatory, Antinociceptive[8,9,10],Anti-urolithic[11], woundhealing[12], gas-

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troprotection[13], Anti-tubercular[14], anti-fungal[15,16], anti-diabetic[17], Antitumor[18] larvicidal[19], antihyperlipidemic[20]. Some phytochemicals like β-4C1-(6″-O-galloyl-glucopyranoside)-7-O-β-4C1-glucopyranoside, kaempferol 3-O-rutinoside, quercetin 3-O-rutinoside, tellimagranidine-I and 2,3-α,β- digalloyl glucose have been identified from *A. baccifera* subspecies *aegyptiaca*. In addition, tetralone derivatives, (-)-(4R)-hydroxy-1-tetralone, (-)-(4S)- acetoxy -1-tetralone, (-)-(4S)-hydroxy-1-tetralone-4- O-β-D-gluco--side, β-sitosterol and β-sitosterol-β-D- glucoside have been reported[17,21]. GC-MS analysis of methanol extract showed 34 bioactive phytochemicals including fatty acids, terpenes, hydrocarbons and phenolics[22]. The total content of phenolics and flavonoids has also been reported in ethanol extract[17].

It is well known that traditional medicine is always a good source of pharmacologically active molecules that can be the source of modern drugs. Extensive literature review revealed that *A. baccifera* of Nepalese origin has not been investigated so far. The value of such plant can be explored by conducting phytochemical analysis as well as bioassay. Hence, the present study was carried out with the aim to screen the different extracts of *A. baccifera* for phytochemicals, estimate the content of phenolics, flavonoids, tannins and sugars and evaluation of antioxidant as well as antibacterial activities.

Materials and method

Plant materials

The aerial part of the plant, *A. baccifera* was collected from the Sunsari district in August 2019. The plant was authenticated by comparison with the herbarium species deposited at National Herbarium and Plant Laboratories, Department of Plant Resources, Ministry of Forest and Environment, Kathmandu, Nepal. The voucher specimen (#UC-019) was deposited at Research Center for Applied Science and Technology, RECAST, TU. The collected material was cleaned, shade dried and ground to powder.

Extraction

The powdered plant material (35 g) was first extracted with 200 mL of n-hexane in a Soxhlet extractor for 5-6 hours until the last extract was colorless. The residue was dried and then extracted successively with 200 mL of dichloromethane, 150 mL of ethylacetate and 150 mL methanol (150 mL) by using Soxhlet extractor for 5-6 hours. The dried residue was refluxed with 100 mL of 50% aqueous methanol for 2 hours. The extracts were filtered and the solvents were evaporated separately under reduced pressure using rotary evaporator. The concentrated solid or semi-solid extracts were kept in freeze for further use.

Test for phyto constituents

The different extracts were analyzed qualitatively by using different specific reagents to identify the presence of different phytochemicals like phenolics, flavonoids, terpenoids, alkaloids, glycosides following the standard procedure of Culie[23].

Estimation of total phenolic content

The total phenolic content in different extracts was estimated by colorimetric method using Folin-Ciocalteu reagent[24]. The diluted extract was mixed with Folin-Ciocalteu reagent and Na_2CO_3 and incubated for 30 min as described before[25]. Then the absorbance was measured at 760 nm. All extracts were analyzed in triplicate. Total phenolic content was calculated using the formula C = cv/m, where C-total phenolic content in mg GAE/g dry extract, c-concentration of gallic acid obtained from calibration curve in mg/mL, v-volume of extract in mL, m-mass of extract in gram and it is expressed as gallic acid equivalents (GAE) in milligrams per gram extract (mg GAE/g extract).

Estimation of total flavonoid content

The total flavonoid content in different extract was estimated by aluminium chloride colorimetric assay[26]. In the diluted extract, $NaNO_2$, $AlCl_3$ and NaOH was added, mixed thoroughly and the absorbance was determined at 510 nm as described before[25]. All extracts were analyzed in triplicate. Total flavonoids content was calculated as in the case of phenolics and expressed as catechin equivalents (CE) in milligrams per gram extract (mg CE/g extract).

Estimation of total tannin content

The total tannin content in different extracts was estimated by using Folin-Ciocalteu method[27]. The extract was mixed with Folin reagent and Na_2CO_3 and incubated for 30 minutes and the absorbance was taken at 700 nm as described before[25]. All extracts were analyzed in triplicate. Total tannins content of the extracts was calculated using the formula C=cv/m expressed as mg tannic acid equivalents (TAE) per gram of dry extract (mg/g).

Estimation total sugars content

The total carbohydrate/sugar content in different extracts was estimated using anthrone reagents[28]. The hydrolysed extracts were treated with freshly prepared anthrone reagent, heated and the absorbance was measured at 630 nm as described before[25]. All extracts were analyzed in triplicate. Total sugar content was calculated and expressed as mg glucose equivalents (GE) per gram of dry extract (mg/g).

Determination of antioxidant activity

Antioxidant activity of extracts was determined using DPPH free radical[29]. Absorbance of control (Ac) and sample (As) were measured at 517 nm as described before[25]. DPPH radical scavenging activity (%) was calculated using the formula [I]. The percentage scavenging was then plotted against concentration and IC_{50} value of the extracts determined graphically.

$$\%\ \text{of radical scavenging} = \frac{\text{Ac}-\text{As}}{\text{Ac}} \times 100 \ldots\ldots\ldots\ldots [\text{I}]$$

Determination of antimicrobial activity

The antimicrobial activity of methanol extract was determined by agar well diffusion method[30] against three gram positive bacteria, *Bacillus subtilis* (ATCC 6059), *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 1228), and six gram negative bacteria *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 700603), *Proteus vulgaris* (ATCC 6380), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella typhii* (Clinical sample), *Shigella dysenteriae* (Clinical sample) and two fungi *Candida albicans* (ATCC 2091) and *Saccharomyces cerevisiae* (ATCC 18824). An aliquot of 50 µl sample of each concentration (100 mg/ml prepared in 50% DMSO) was introduced into each well (6 mm diameter) in a Petri plate seeded with respective microorganism so that the exact amount of extracts in each well was 5 mg. Negative control experiments were performed using equivalent volume of 50% DMSO and positive control experiments were performed by use of a standard antibiotic, ciprofloxacin for bacteria and clotrimazole for fungi (1mg/mL). At the end of the incubation period, the clear inhibition zones of bacterial growth around the wells were observed in the presence of different extracts. Inhibition of the bacterial growth in the presence of extracts was measured in the form of zone of inhibition (ZOI).

Determination of minimum inhibitory concentration (MIC)

MIC was determined by two-fold serial broth dilution method[31]. The stock solution of extract was prepared at a concentration of 50 mg/mL and mixed with 1 ml of broth. Then it was subjected to two fold serial dilutions such as 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390 and 0.195 mg/mL. Then 20 µL of inoculum (1:100 dilution of a suspension of turbidity equal volume of McFarland Standard 0.5 supposed to have organism 1.5x106 CFU/mL) was added to each diluted tube. Positive and negative controls were also included and all the tubes were incubated overnight at 37 °C. Bacterial growth was observed by visual inspection of the tubes. The MIC is the lowest concentration of antimicrobial agent that inhibits the growth of bacteria as determined by the lack of visual turbidity of tubes containing two-fold serial dilutions.

3. Results and Discussion

Extractive values and phytochemicals screening

For the extraction of phytochemicals from herbal drugs, it is necessary to standardized the extraction procedure to get accurate information about chemical profile and biological activity[32]. In the present study, we have used the polarity based Soxhlet extraction method starting from hexane, dichloromethane, ethyl acetate and methanol for the

extraction of bioactive phytochemicals from *A. baccifera*. Again, 50% aq. methanol extract was prepared by reflux method. Depending on the solvent polarities, the yield was found to be different. The highest amount of extract was obtained with 50% aq. methanol and the lowest amount was obtained with methanol extract. This indicated that *A. baccifera* contains more polar compounds such as glycosides that are soluble in 50% aq. methanol than methanol. The extractive value is one of the key components in quality evaluation and standardization of herbal drugs. The yields of various extracts are presented in Table 1.

Table 1: Extractive values and phytochemicals present in different extracts .

Extracts	Hex	DCM	EtOAc	MeOH	50% aq. MeOH
Percentage yield	1.28	1.24	1.36	2.11	4.21
Volatile Oil	-	-	-	-	-
Phenolics	-	-	-	+	+
Flavonoids	-	-	-	+	+
Alkaloids	-	-	-	-	-
Terpenoids	-	-	-	-	+
Glycosides	-	-	-	+	+
Saponins	-	-	-	-	+
Quinones	+	+	+	+	+
Reducing sugars	-	-	-	+	+
Tannins	-	-	-	+	+
Steroids	+	+	+	+	+
Coumarin	-	-	+	+	+

The phytochemical screening of different extracts of *A. baccifera* were carried out to get general information about the classes of phytochemicals present in the plant. The results are presented in Table 1. In hexane and dichloromethane extracts, only quinones and steroids were present. In ethyl acetate extract, quinones, steroids and coumarins were present. Most of the polar phytochemicals like phenolics, flavonoids, glycosides, reducing sugars, tannins, steroids and coumarins were present in the methanol and 50% aqueous methanol extract. This result confirmed that polar solvents are suitable for the extraction of phytochemicals from *A. baccifera*. However, alkaloids were absent in all extracts.

Total phenolic, flavonoid tannin and sugar content

Polyphenols are widespread in plants and they include phenolic acids, flavonoids and tannins. In addition, stilbenes and lignans are also common in plants. They have role in the prevention of various oxidative stress associated diseases. This has attracted the attention of researchers on plant phenolics[33]. As the phytochemical content is directly related to the biological activities, it is always interesting to quantify their contents. So we have estimated the phenolics, flavonoids, tannins and sugars content in methanol and 50% aq. methanol extracts of *A. baccifera* on the basis of phytochemical screening (Table 1). In our investigation, it was observed that the higher amounts of phenolics (178.65 ± 1.16 mg GAE/g dry extract), flavonoids (60.05 ± 2.24 mg CE/g dry extract) and tannins (194.29 ± 2.96 mg TAE/g dry extract) were observed in methanol extract and somewhat lesser amounts of phenolics (107.66 ± 2.04 mg GAE/g dry extract), flavonoids (23.95 ± 2.07 mg GAE/g dry extract) and tannins (120.28 ± 2.25 mg GAE/g dry extract) were observed in 50% aq. methanol extract. The higher amounts of sugars (206.86 ± 4.21 mg GE/g dry extract) were observed in 50% aq. methanol extract than methanol extract (153.03 ± 3.80 mg GE/g dry extract). The results are presented in Table 2. In the ethanolic extract of *A. baccifera* from Egypt, the total phenolic content, TPC (380.50 ± 3.88 mg GAE/g) and total flavonoid content, TFC (190.00 ± 2.31 mg CE/g) have been reported[17]. Similarly, in the methanol extract of *A. baccifera* collected from Indian, TPC (95.70 ± 1.60 g/100 g), TFC (43.40 ± 0.10 g/100 g) and total tannin content, TTC (6.20 ± 4.20 g/100 g) have been reported[18]. When comparing the data, *A. baccifera* from Nepal contains lower amounts of phenolics and flavonoids than the plant grown in Egypt. Again, the plant from India contains lower amounts of phenolics, flavonoids and tannins than the plant from Nepal. Plants that grow in particular ecosystem or geographic region have different quantity/quality of phytochemicals. This diversity of natural products is really interesting in the search of biologically active compounds. Thus, the quantification of chemical groups is a method to standardize and quality evaluation of the extracts. However, the report on the quantitative analysis of sugar content was still not available for *A. baccifera.*

Table 2: Total phenolic, flavonoid, tannin, sugar content and DPPH free radical scavenging activity .

Extracts	MeOH	50% aq. MeOH
TPC (mg GAE/g dry extract (Mean ± S.D) (n=3)	178.65±1.16	107.66±2.04
TFC (mg CE/g dry extract) (Mean ± S.D) (n=3)	60.05±2.24	23.95±2.07
TTC (mg TAE/g dry extract) (Mean ± S.D) (n=3)	194.29±2.96	120.28±2.25
TSC (mg GE/g dry extract) (Mean ± S.D) (n=3)	153.03±3.80	206.86±4.21
IC_{50} µg/mL against DPPH (Mean ±S.D) (n=3)	95.52±6.27	114.66±8.49

DPPH free radical scavenging activity

The DPPH radical scavenging test is one of the most widely used method and it is the initial method for determining antioxidant activity. For DPPH radical scavenging assay, methanol and 50% aq. methanol extracts were selected. Methanol extract showed lower IC_{50} values (95.52±6.27µg/mL) than 50% aqueous methanol extract (114.66±8.49 µg/mL). However, both extracts showed weak antioxidant activity in comparison to standard antioxidant, ascorbic acid (IC_{50} 20.75 µg/mL). The results are listed in Table 2. It was reported that in DPPH radical scavenging assay, the methanol extract of *A. baccifera* from Indian showed the IC_{50} value of of 8.3 µg/mL[18]. In addition, the ethanolic extract showed *in vivo* antioxidant properties in rats[34]. Although the Indian plant contains lower amounts of phenolics, flavonoids and tannins, it showed greater antioxidant activity. This could be due to the different chemical composition of Indian sample.

3.3 Antibacterial activity

At present, antimicrobial drug resistance is the serious threat to our society and new antibiotics are still lacking to tackle such problem. Plants are the sources of different phytochemicals that could be the source of new antibiotics[35]. In this respect, the extracts of *A. baccifera* were tested for their antibacterial effect. In our findings, only the methanol extract of *A. baccifera* showed activity against Gram positive bacteria *B. subtilis, S. aureus* and *S. epidermidis,* with the zone of inhibition, ZOI ranged from 12-20 mm. It was reported that the hydroalcoholic extract of *A. baccifera* collected from Chennai, India showed activity against both Gram positive and Gram negative bacteria with inhibition zone ranged between 8-29 mm[15]. However, in our case, no activity was observed against Gram negative bacteria, *K. pneumonia, E. coli, P. vulgaris, P. aeruginosa, S. dysenteriae, S. typhii* and fungi, *C. albicans*, *S. cerevisiae*. This indicated that the plant samples collected from different regions showed different biological activities due to the difference in quality and quantity of phytochemicals. The methanol extract which showed antibacterial activities was further subjected to minimal inhibitory concentration, MIC determination. The MIC of methanol extract of *A. baccifera* was found to be 0.78-1.56 mg/mL for all three Gram positive bacteria, *B. subtilis*, *S. aureus* and *S. epidermidis*. The results are presented in Table 3.

Table 3: Antibacterial activities of *A. baccifera.*

Sample	Bacteria	ZOI in mm	MIC mg/mL
MeOH extract	*B. subtilis*	12	0.78-1.56
	S.aureus	15	0.78-1.56
	S. epidermidis	20	0.78-1.56
Ciprofloxacin 10 µg/well	*B. subtilis*	32	-
	S.aureus	34	
	S. epidermidis	34	

Conclusions

A. baccifera was investigated phytochemically and biologically. The polar extracts of this plant are the good sources of bioactive phenolics, flavonoids, tannins, carbohydrates

with antibacterial and antioxidant properties. The findings of this study validate the traditional use of this plant to treat skin diseases.

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