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EVALUATION OF *ARISTOLOCHIA ASSAMICA* D. BORAH & T.V. DO; A NOVEL BIORESOURCE OF NORTH EAST INDIA AS RESERVOIR OF SECONDARY METABOLITES AND ANTIOXIDANT POTENTIALITIES

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ABSTRACT

Aristolochia assamica D. Borah & T.V. Do of family Aristolochiaceae, is a newly recorded species for flora of India from the Eastern Himalayan region. It is reported to be utilized as an ingredient of herbal medicine by different traditional healers from North-East India to cure different ailments. Quantification of phytoconstituents behind its medicinal value is the point of interest in this study. Presence of total contents of phenol, flavonoid, alkaloid, tannin and saponins of polarity based different leaf extracts were determined and expressed as µg of standard equivalent/g. In vitro antioxidant activity of this plant was revealed through studying by DPPH and ABTS radical scavenging assay method. The greatest antioxidant activity was recorded for aqueous extracts in both assays. The results of the study established that the *A. assamica* could be a promising source of antioxidant agents.
Keywords **:** Antioxidants, Flavonoid, Medicinal plants, Phenol, Phytoconstituents

Introduction

Being versatile, safe, cost-effectual with lesser side effects; the herbal remedies accomplish spectacular performance at present circumstances. Herbal remedies constitute the sole part of traditional medicine which have been used since historical ages in preventing illness and promoting health across the globe (Makhubele *et al.*, 2024). These are the synergistic effects of secondary metabolites behind the therapeutic potentialities. Secondary metabolites are certain bioactive chemical compounds synthases by the plants cellular metabolisms derived from primary metabolic pathways. The major groups of secondary metabolites classified according to their structure, composition, solubility on solvents and biosynthetic pathways are terpenoids, alkaloids and phenolics with subclasses of each one (Hussein and El-Anssary, 2019). Apart from defending the biotic and abiotic stress condition of plants caused by physiological, environment and climatic factors; the secondary metabolites account for numerous pharmacological properties like antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, antidiabetic, anticancer, antiallergic, lipid lowering in human health (Kaushik *et al.*, 2021; Twaij and Basan, 2022). During metabolic reactions, human body rapidly produces highly unstable reactive oxygen species or free radicals with one and more unpaired electrons. Overproduction of the free radicals creates oxidative stress leads to damage in DNA, protein crosslinking resulted in ageing, hypertension, diabetes, arthritis, cancer, neurodegenerative, cardiovascular diseases (Sunitha *et al.*, 2018). Antioxidants counter react with the free radicals by donating an electron and neutralize them. Supplemented plant remedies rich in phenolic, flavonoids, alkaloids, tannins, saponins etc. possess strong antioxidant activity are better safeguards than the synthesized antioxidants, thus protecting the body from various degenerative diseases (Lobo *et al.*, 2010).

Aristolochia, the notable genus of family Aristolochiaceae famed for containing Aristolochic acids which has been used in treating arthritis, gout, obstetrics, rheumatism, and festering wounds (Chen 2007). Species of *Aristolochia* are blessed with anti-snake venom, abortifacient, antimicrobial, antioxidant, anti-helminthic, antiulcer, anti-plasmodial, antiperiodic, emmenagogue and purgative properties (Nandhini *et al.*, 2017; Dey *et al.*, 2021). *A. assamica* D. Borah & T.V. Do, sp. nova is a newly illustrated plant resource from the Eastern Himalayan region of India reported to be blooming on the wilderness of the Arunachal Pradesh and Assam (Borah *et al.*, 2019) as shown in Plate 1. It is a climbing herb with purplish to greenish slender stem, cordate leaves and creamy-white to greenish-yellow tubular perianth. Investigation on the traditional utilization of this resource exhibit therapeutical work on healing of fever, malaria, pneumonia, stomach disorders, body pain, snake bite, mad dog bite, high blood pressure, diabetes, urinary tract infections by the ethnic folks of Assam.

Plate 1 : Plant body of *A. assamica*

Previous investigations of author have established the presence of alkaloids, glycosides, flavonoids, phenolic compounds, terpenoids and tannin in various extracts of this ethnomedicinal plant along with GC-MS chromatograph exposed the presence of several compounds mainly 2-Octylcyclopropene-1-heptanol, 1-Undecyne, Neoisolongifolene, 8-bromo-, 1-Naphthalenemethanol, 1,4,4a,5,6,7,8,8a-octahydro-2,5,5,8a-tetramethyl-, 3-Hexadecyne, 17-Oxo-4-nor-3,5-seco-5-androsten-3-oic acid, methyl ester, Fenretinide, 4-Tetradecyne, Butanal, 3-hydroxy-2-methyl-4-[4-t-butyl]-, alpha-ylangene, 1,1'-Butadiynylenedicyclohexanol, Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester, Levomenthol, Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1.alpha.,2.alpha.,5.beta.)]-, Eudesma-5,11(13)-dien-8,12-olide, 7-Hexadecenal, (Z)-, 6,10,14,18,22-Tetracosapentaen-2-ol, 3-bromo-2,6,10,15,19,23-hexamethyl-, (allE)- etc (Borah and Sarma, 2022). Assessment of phytoconstituents as well as antioxidant activity in any medicinal plant is requisite for the quality, efficiency, and standardization of herbal remedies. The present study is a first scientific report designed to estimate the quantity of phenol, flavonoid, alkaloid, tannin saponin and antioxidant activity of various leaf extracts of *A. assamica.*

Materials and Methods

Collection and authentication of plant material

Fresh and healthy leaves were collected from the Behali Reserve Forest, Assam, India after receiving proper permission from government officials and identified by the author himself of this new species. Specimen was preserved in the form of mounted herbarium sheet (Accession no. 98880) submitted to the ASSAM herbarium, Botanical Survey of India, Eastern Regional Centre.

Preparation of extracts

The collected leaves were washed properly, shade dried and ground in a mixer grinder to a coarse powder form. Powdered sample of 100g was extracted with 1000ml of methanol, aqueous, acetone, petroleum ether using a Soxhlet apparatus for 24 hours with a lower boiling point of the solvents. Solvents are evaporated using hot water bath in 30 to 40^{0}C and concentrated dried crude extracts were stored in the refrigerator for further phytochemical tests.

Determination of Total Phenolic Content

Total phenolic content in the plant extracts were determined by Folin-Ciocalteu assay method (Senguttuvan *et al.*, 2014). Each extract of 1mg/ml concentration was mixed with 45ml of distilled water. Folin-Ciocalteu reagent of 1ml was added to the mixture and shaken properly. After three minutes, 3ml of 20% sodium carbonate was added to the mixture and it was allowed to stand for two hours with occasional shaking. A set of reference standard Gallic acid (50, 100, 150, 200, 250 µg/ml) was prepared in the same described manner. The absorbance of the blue colour developed from the resulted solution was measured at 760nm in UV-vis spectrophotometer to determine the standard and extract samples.

Determination of Total Flavonoid Content

Total flavonoids content in the extracts were estimated by Aluminium chloride calorimetric method (Iqbal *et al.*, 2015). With 2ml of distilled water and 1mg/ml of extract, a mixture was prepared. Another mixture consisted of 3ml of 5% sodium nitrite and 0.3ml of 10% aluminium chloride were added to the former after five minutes. To the tested solution, 2ml of 1M sodium hydroxide was added after 6 minutes and volume was made to 10mL by adding distilled water. The absorbance of tested solutions and standard was measured with a UV-vis spectrophotometer against the blank reagent at 510nm. Standard curve was made with Quercetin at a series of 10, 50, 100, 200, 250 µg/ml.

Determination of Total Alkaloid Content

Dragendroff's reagent method was used to determine the total alkaloid content through a UV-vis spectrophotometer at 435nm. Briefly, 2ml of Dragendroff's reagent was added to the mixture of 5ml extract and dilute HCl. For this mixture, pH was maintained to 2-2.5. Centrifugation of this mixture resulted precipitate which was washed by alcohol. To add 2ml of Disodium sulphide solution to the residue and centrifuge again. Residue was heated after mixed in 2ml of concentrate nitric acid and diluted with 10ml of distilled water. Thiourea solution of 5ml was added to the 1ml pipetted out solution. Concentration of Berberine in the range of 20, 40, 60, 80, 100 µg/ml was taken as standard (Lala, 2019).

Determination of Total Tannin Content

Quantification of total condensed tannin of the samples was measured by the acid condensation of vanillin method (Medini *et al.*, 2014). Vanillin reagent of 3ml was added to 0.5 ml of each sample dissolved in 4% methanol (w/v). Concentrated HCl (37%) of 1.5 ml of was added to the mixture incubated at 20°C for 15min. Standard solutions of Catechin at 200, 400, 600, 800, 1000 µg/ml concentration was carried to prepared as described manner. Absorbance for standard and tested solutions was taken at 500nm against reagent blank by UV-vis spectrophotometer.

Determination of Total Saponin Content

Anisaldehyde reagent (500µl) of 0.5% of was added to the 500µL sample of each extract. The mixture was kept in the water bath for 10min constantly at 60º C after adding 2ml of 50% H_2SO_4 and cooled. Standard saponin of 20, 40, 60, 80, 100 µg/ml was taken to determine the calibration curve at 435nm of absorbances (Vador *et al.*, 2012).

Determination of *In vitro* Antioxidant Activity

The antioxidant activities of each extract were determined by DPPH free radical scavenging assay and ABTS decolorization assay method (Suseela *et al.*, 2010, Proestos *et al.*, 2013).

DPPH free radical scavenging assay

Two mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent of 0.15mM was prepared in methanol and used to estimate the free radical scavenging activity of plant extracts. This content was then mixed with 2ml of extracts (100, 200, 300, 400, 500 µl) and incubated in the dark at room temperature for 30min. Absorbance of the reaction mixture was measured in 517 nm using a spectrophotometer. A set of reference standard was taken for Ascorbic acid with same concentration ranges of sample. The percentage inhibition of DPPH radical was calculated using the following equation.

$$\text{DPPH inhibition } (\%) = ((A_B - A_A)/ A_B) \times 100),$$

where, A_B is absorbance of control; A_A is absorbance of sample/standard

ABTS decolorization assay

The stock solution of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was prepared by mixing 7 mM ABTS in water and 2.45 mM ammonium persulfate (1:1) and incubated in the dark at room temperature for 12-16 h. After that, the ABTS solution was diluted in methanol to obtain an absorbance at 734 nm in a spectrophotometer. Two ml of diluted ABTS solution was added to the extracts (100, 200, 300, 400, 500 µl) and standard Ascorbic acid as same manner. Percentage inhibition of $ABTS^+$ was calculated using the following equation:

$$\text{ABTS radical scavenging effect}(\%)=((A_B - A_A)/A_B) \times 100)$$

where, A_B is absorbance of control; A_A is absorbance of sample/standard

Statistical Analysis

The data for statistical analysis was taken by performing the experiments in triplicate (n=3). The data was represented by mean values standard deviation. One- and two-way analysis of variance (ANOVA) was used to analyse the significant difference of phytochemical contents, antioxidant activities between different extracts, where a probability value of ($P<0.05$) is considered as statistically significant.

Results and Discussion

Quantification of Phytochemicals

The total flavonoid, alkaloid, tannin and saponin contents in different extracts of *A. assamica* leaf are expressed as µg of standard equivalent/g is represented in Figure 1. The aqueous extract exhibited significantly ($P<0.05$) the highest quantity of total phenol content (98.48 ± 0.44 µg of gallic acid equivalent/g). The highest alkaloids and tannin content were depicted by methanolic extract that are 33.71±1.49 µg of berberine equivalent/g and 3.39±0.02 µg of catechin equivalent/g. Total flavonoid content were maximum for aqueous extract (96.45±0.90 quercetin equivalent/g) whereas highest saponin content was occupied by aqueous extract (57.66±1.22 µg of standard saponin equivalent/g).

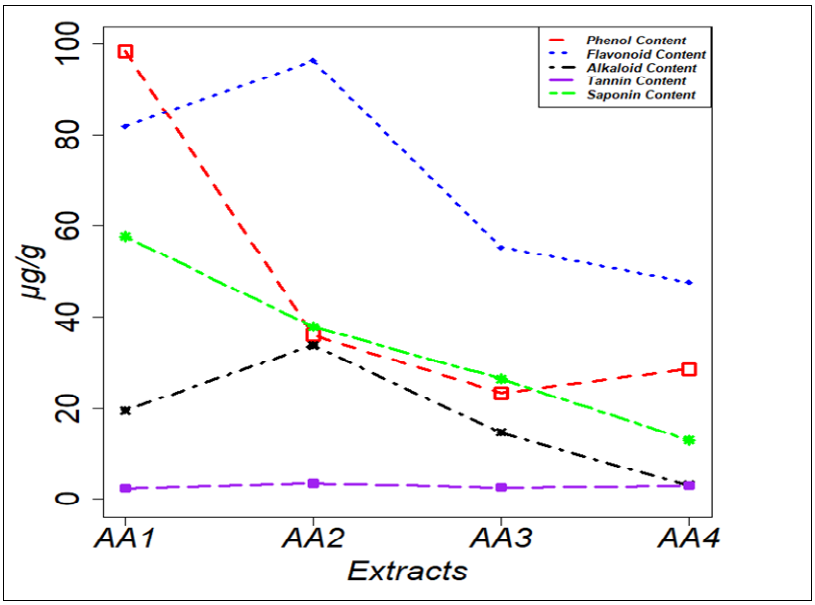

Fig. 1 : Phytochemical contents of *A. assamica* leaf extracts

Determination of Anti-oxidant activity

The free radical scavenging activity of different extracts was assessed by DPPH and ABTS assay method. The regression curve was made by using ascorbic acid as positive control ($y = 0.1441x + 19.849$; $R^2 = 0.9563$) for DPPH scavenging activity and ($y = 0.1441x + 19.849$; $R^2 = 0.9835$) for ABTS scavenging activity. In both methods, it was found that the extracts showed increasing inhibition of scavenging dependent to increasing concentration. Aqueous extracts in different concentrations were found to possess higher DPPH scavenging effect compare to the other extracts (Table-1, Figure-2).

Table 1 : Inhibition of DPPH by different extracts

Sl. No.	Concentration (µl)	DPPH percentage inhibition (%) activity			
		Aqueous Extract	Methanol Extract	Acetone Extract	Petroleum Ether Extract
1	100	20.97±0.78	37.29±0.50	3.84±0.95	18.09±0.90
2	200	35.13±0.66	42.94±0.45	6.64±0.42	22.29±0.37
3	300	48.54±0.84	48.14±0.43	10.12±0.48	28.13±0.49
4	400	56.54±0.79	52.62±0.42	28.77±0.68	40.86±0.48
5	500	71.07±0.67	61.90±0.82	27.25±1.05	43.66±0.37

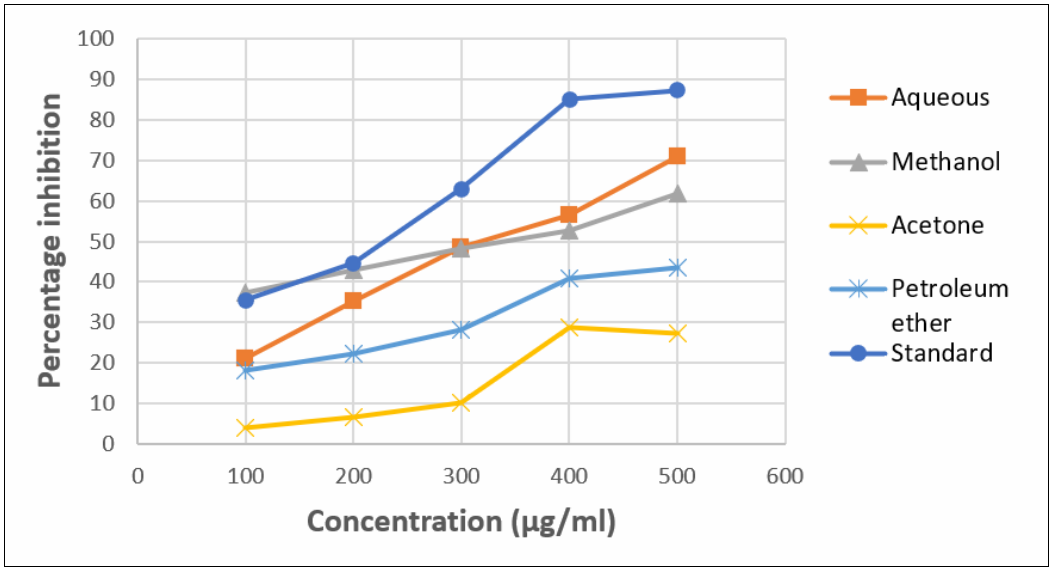

Fig. 2 : DPPH radical scavenging activity of different leaf extracts of *A. assamica*

Table 2 : Inhibition of ABTS by different extracts

Sl. No.	Concentration (µl)	ABTS percentage inhibition (%) activity			
		Aqueous Extract	Methanol Extract	Acetone Extract	Petroleum Ether Extract
1	100	76.40±0.11	25.22±0.28	8.3±0.46	2.06±0.65
2	200	77.09±0.35	27.48±0.37	7.61±0.32	4.33±0.88
3	300	80.38±0.20	30.05±0.19	17.05±0.17	16.89±0.63
4	400	82.77±0.32	34.61±0.36	24.95±0.37	20.07±0.28
5	500	88.17±0.37	38.78±0.32	32.36±0.29	19.12±0.61

The lowest half maximal inhibitory concentration (IC_{50}) of 3.24±0.03 was inhibited by methanolic extracts in DPPH scavenging assay method while ascorbic acid obtained an IC_{50} of 2.09±0.02 indicates the significant potency of antioxidant effect in this plant. The highest percentage inhibition in ABTS assay method as shown in the Table-2 and Figure-3 again occupied by the aqueous extracts with an IC_{50} of 7.61±0.49.

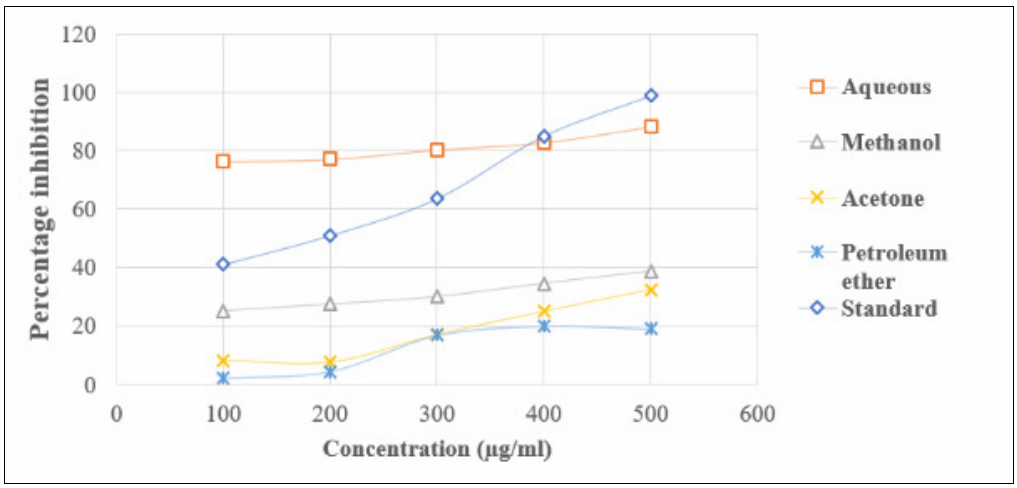

Fig. 3 : ABTS radical scavenging activity of different leaf extracts of *A. assamica*

Conclusion

The level of phenol and flavonoid contents are responsible in the contribution of scavenging free radicals in medicinal plants. The quantitative evaluation of secondary metabolites in this study has revealed the significant presence phenolic compounds as well as flavonoids in all extracts which may lead the potent antioxidant activity of the plant. Antioxidant activity of this plant may be correlate to its saponin, tannin and alkaloid contents also. Further exploration of pharmacological activities of these secondary metabolites may offer various scope in discovering novel therapeutic drugs.

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Conflicts of Interests

The authors declare there is no any conflicts of interests.

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