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Amrit Research Journal December 2022, Vol. 3, Issue 1, 84-93
ISSN: 2738-9553
RESEARCH ARTICLE

Phytochemical Analysis, Cytotoxicity, Antibacterial and Antioxidant Activities of Extracts of Leaf of *Ageratina adenophora* (Spreng.)

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(Received: August 22, 2022, Received in revised form: November 20, Accepted: December 6, 2022, Available Online)

Highlights.

- The leaf part of *Ageratina adenophora* was extracted in methanol, hexane, chloroform and ethyl acetate.
- GC-MS analysis of chloroform extract showed the presence of 10 different compounds.
- The antibacterial activity showed ZOI of 12 mm against *Escherichia coli* and *Proteus vulgaris.*
- IC_{50} value was calculated as 1460 µg/mL and LC_{50} value as 174.78 µg/mL.
- TPC (89.75 mg gallic acid equivalent/g) and TFC (49.25 mg quercetin equivalent/g).

Abstract

The powdered leaf of Ageratina adenophora was subjected to extraction using a cold percolation method with methanol. After suspending the crude methanol extract in 1% HCl and neutralizing with NH_4OH, the obtained solution was extracted with hexane, chloroform, and ethyl acetate solvent, respectively. Qualitative phytochemical analysis of methanol, hexane, chloroform, and ethyl acetate extracts of A. adenophora plant showed the presence of alkaloids, flavonoids, phenols, steroids, quinones, saponins, tannins, cardiac glycosides, carbohydrate, terpenoids, proteins, and amino acids. GC-MS analysis of chloroform extract showed 10 different major compounds in which α-Muurolol (24.33%) was found most abundant. The IC_{50} value of chloroform extract was found to be 1460 µg/mL from DPPH scavenging antioxidant assay, and the LC_{50} value was found to be 174.78 µg/mL from the brine shrimp lethality assay. Antibacterial activity was shown the highest against Escherichia coli and Proteus vulgaris with ZOI of 12 mm on each in chloroform extract. It was determined that 89.75 mg of gallic acid equivalent/g of dry extract accounted up the total phenolic content and 49.25 mg of quercetin equivalent/g of dry extract was observed to be the total flavonoid content in the chloroform extract.

Keywords: *Ageratina adenophora, GC-MS, TPC, TFC, bioactivities*

Introduction

Ageratina adenophora (Spreng.) R.M. King and H. Rob. (*A. adenophora*) is a perennial herb, usually called the sticky snakeroot

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and commonly as banmara or kalimunte (killer of the forests) [1–4] . It invades disturbed regions and limits the self-seeding of planted trees, making it a controversial weed in forest plantations [5–7]. Its low susceptibility to bacterial, fungal, and insects attack points to the presence of abundant secondary metabolites in this plant that may be defense-related [8–10]. In the past, this species has been linked to a variety of structurally varied chemicals, some of which have been proven to have allelopathic, phytotoxic, and antifeedant properties. These chemicals include terpenoids, phenylpropanoids, flavonoids, coumarins, sterols, and alkaloids [11,12]. *A. adenophora* has been used traditionally as folklore medicine across the world. It is licensed for numerous healthful properties and finds therapeutic applications in ancient medicines as an anti-inflammatory, antimicrobial, antiseptic, analgesic, antipyretic, and coagulant and phenol barbitone induced sleep enhancer. *A. adenophora* leaf is accustomed to treating various diseases like wound healing, sleeping disorder, fever, infection of gum, toothache, skin sores, dysentery, jaundice, diabetes and ulcers [2,13,14].

Materials and Methods

Collection of the Plant Part

A. adenophora leaves were collected from Ribdikot-7, Palpa, Nepal, at about 2000 m altitude in October 2019. The plant was identified with the help of Department of Botany, Amrit Science Campus, Lainchour, Kathmandu. The gathered leaves were cleaned, shade dried and ground into powder. About 4 kg of powdered plant leaves were taken for extraction.

Extraction

A cold percolation extraction using methanol solvent was performed on the dried and powdered leaves of *A. adenophora*. The resulting extract was filtered and concentrated, using a rota evaporator. After being further suspended with 1% HCl and neutralized with NH_4OH, the obtained solution was then extracted with hexane in a separating funnel to wash away chlorophyll and waxy compounds. The aqueous layers were collected for further chloroform extraction. The chloroform extract was extracted from the aqueous layer after the mixture was vigorously shaken in a separating funnel. Chloroform extract was taken as the main extract for further analysis. Rota evaporator was used to concentrate the resultant chloroform extract before it was further examined. The aqueous layer was then extracted using ethyl acetate as the solvent in the separating funnel and further concentrated over Rota evaporator to obtain crude ethyl acetate extract. Due to very less amount of the ethyl acetate extract, it couldn't be taken for further analysis.

Phytochemical Screening

Methanol, hexane, chloroform, and ethyl acetate extracts were subjected to normal phytochemical screening procedures. Several tests including alkaloids, flavonoids, phenols, steroids, quinones, saponins, tannins, cardiac glycosides, carbohydrates, terpenoids, proteins and amino acids were carried out using previously described methods [15–19].

Gas Chromatography-Mass Spectrometry

The chloroform extract was analyzed by GC-MS with the help of the Department of Food and Technology and Quality Control, Babarmahal, Kathmandu, Nepal. Gas chromatography-mass spectrometry analysis was carried out using a GCMS-QP 2010 under the following circumstances: Helium was used as the carrier gas in an Rtx-5MS column with dimensions of 30 m by 0.25 mm by 0.25 μm, which was temperature-programmed at 80 °C and 300 °C withhold times of 2.0 and 5.0 min, respectively, while the ion source temperature and interface temperature were maintained at 200 °C and 250 °C. Identification was done along with an MS comparison.

Antibacterial Activity

The agar well diffusion method was used to measure the antibacterial activity in Muller-Hinton Agar. A sterile cork borer (4 mm) was used to create wells in the incubated media plates, and each well was appropriately labelled. With the aid of a micropipette, 15 μL of the plant extract working solution was then added to each well. In a different well, the solvent (methanol) was also tested for activity at the same time as a control. Extracts were then diffused throughout the media by leaving the plates with the lid closed for 30 minutes. At 37 °C, the plates were incubated for 6 hours. The plates were examined after the incubation for the zone of inhibition around the well, which is indicated by a clear zone without growth, and this zone was noted. With the aid of the ruler, the ZOI was measured, and the mean value was recorded to determine the antibacterial efficacy [6].

Antioxidant Activity

Against DPPH, an antioxidant assay was run. The initial stock solution was made by dissolving 1 mg sample in 1 mL of methanol, yielding solution with concentration of 1 mg/mL. Similar to this, several concentrations of the 1000 µL (1 mL) extracts were made using the stock solution using a two-fold dilution procedure, including 1500, 1000, 500, 250, and 125 µg/mL. Differently, 1500 µL (1.5 mL) of 0.1 mM DPPH (4 mg DPPH in 100 mL methanol) were mixed with 500 µL (0.5 mL) of each of these solutions. Aluminum foil was used to cover the solutions to keep them from igniting, and they underwent a rigorous 2-minute shaking process. The solutions were kept at room temperature for 30 minutes in a darkened place. Their absorbance was measured at 517 nm against methanol as a blank after 30 minutes. The mixture of 1.5 mL of DPPH solution and 0.5 mL of methanol was used as a control to test the mixture's absorbance at 517 nm [10,20]. Additionally, a calibration curve was made.

The percentage of radical scavenging activity was calculated using the following formula:

$$\text{Percentage scavenging} = \frac{(A_0 - A_T)}{A_0} \times 100\% \quad (1)$$

Where, A_0 = Absorbance of the DPPH

A_T = Absorbance of the DPPH free radical solution containing the sample extract.

The effective sample concentration that must be present in order for the sample to scavenge 50% of the DPPH free radicals is known as the IC_{50} value (Inhibitory concentration of 50%). Plotting the extract concentration vs the corresponding scavenging action allowed us to get the IC_{50} values using the dosage inhibition curve in the logarithm range.

Total Phenol Content Assay

By using the Folin-Ciocalteu method, the total amount of phenolic compounds in chloroform extracts was measured. Folin-Ciocalteu reagent was diluted 10 times with distilled water after being placed in a beaker with 1 mL of the reagent. Since 1 mL of distilled water was used to dissolve 1 mg of gallic acid, the solution's concentration is 1 mg/mL, or 1000 µg/mL. 1 mL of Folin-Ciocalteu phenol reagent (1:10 dilution with water), 1 mL of sample (1 mg/mL in methanol), and 0.8 mL of an aqueous 1 M Na_2CO_3 solution were combined. The reaction mixture was left to stand for approximately 15 minutes in complete darkness before absorbance of reactants was measured at 765 nm in comparison to a control (methanol). Gallic acid was used as standard. Gallic acid equivalents per gram of dried extract are used to measure total phenolic content [21].

The following relation was used to calculate the amount of total phenolic content in the sample as milligrams of gallic acid equivalent:

$$\text{TPC} = \frac{(C \times V)}{m} \quad (2)$$

Where, C= concentration of gallic acid from curve (mg/mL)

V= volume of extract (mL)

m= weight of plant extract (g)

Total Flavonoid Content Assay

The total flavonoid content in the chloroform extract of *A. adenophora* was determined using aluminium chloride colorimetric method. 1 mL of sample (0.1 mg/mL in methanol) was mixed with 1 mL $AlCl_3$ (dissolved in methanol) for 1 hour, absorbance was measured at 415 nm in comparison to the control (methanol). Quercetin was used as standard. The total flavonoid concentration is expressed as mg of quercetin equivalents per gram of dried sample [22]. Using the following equation, the amount of total flavonoid content in the sample was calculated as milligrams of quercetin equivalent:

$$\text{TFC} = \frac{C \times V}{m} \quad (3)$$

Where, C= concentration of quercetin from curve (µg/mL)

V= volume of extract (mL)

m = weight of plant extract (g)

Brine Shrimp Lethality Assay

Lethality assays on brine shrimp (*Artemia salina*, fairy shrimp, or sea monkeys) are frequently performed to evaluate the cytotoxic potential of bioactive compounds. It is a quick (24 hours), low-cost, and simple test because aseptic methods are not used. For 24 hours, the nauplii of brine shrimp were treated with solutions containing chloroform extracts at various concentrations. The efficacy of the chloroform extracts was measured by the number of motile nauplii. The Brine shrimp lethality assay is used to evaluate the toxicity of extracts, and it considers them to be bioactive if their LC_{50} value is less than 1 mg/mL [23–29].

Results and Discussion

Phytochemical Screening Analysis

All four extracts of *A. adenophora*—methanol (ME), hexane (HE), chloroform (CE), and ethyl acetate (EAE) - were subjected to phytochemical screening. The extracts of the *A. adenophora* plant contained alkaloids, flavonoids, phenols, steroids, quinones, saponins, tannins, cardiac glycosides, carbohydrates, terpenoids, proteins, and amino acids, as per the qualitative phytochemical analysis.

Table 1. Phytochemical Screening of Methanol; Hexane; Chloroform; and Ethyl acetate extracts

S. N.	Detection	Test name	Observation	Results			
				ME	HE	CE	EAE
1	Alkaloids	Mayer's Test	Yellow ppt.	+	+	+	+
		Dragendorff's Test	Orange-brown ppt.	+	+	+	+
		Wagner's test	Reddish-brown ppt.	+	+	+	+
2	Flavonoids	Sodium hydroxide	Yellow ppt	+	+	+	+
		Lead acetate test	Yellow ppt	+	+	+	+
		Shinoda test	Green colour	+	+	+	+
3	Phenols	Ferric chloride test	Greenish-yellow colour	+	+	+	+
4	Steroids	Salkowski test	Golden colour	+	+	+	+
5	Quinones	Sodium hydroxide and H_2SO_4	Green colour	+	+	+	+
6	Saponins	Froth test	Frothing present	+	+	+	+
7	Tannins	Gelatin test	White ppt.	+	+	+	+
		5 % $FeCl_3$	No appear	-	-	-	-
8	Cardiac Glycosides	Keller-Kiliani test	Ring formation	+	+	+	+
		Molisch's test	Colour change	+	+	+	+
9	Carbohydrates	Fehling's test	Red ppt	+	+	+	+
		Molisch's test	Ring formation	+	+	+	+
		Benedicts test	Red colour	+	+	+	+
10	Terpenoids	Chloroform and conc. H_2SO_4	Reddish-brown colour formation	+	+	+	+
11	Proteins and Amino acid	Xanthoproteic test	Yellow colour	+	+	+	+
		Ninhydrin test	Blue colour (Amino acids)	+	+	+	+
12	Resin	Acetone-water test	No turbidity	-	-	-	-

'+'indicates the presence and '-' indicates the absence.

GC-MS Spectra Analysis

The GC-MS chromatogram of chloroform extract of *A. adenophora* is presented below:

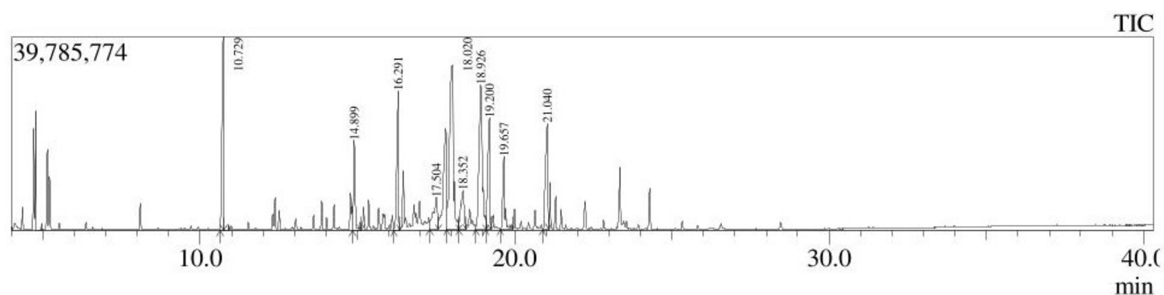

Fig 1. GC-MS Chromatogram of chloroform extract of *A. adenophora*

Chloroform extract of *A. adenophora* included 10 significant chemicals, the most prevalent of which were α-Muurolol (24.33%), α–Bisabolol (18.01%), Bornyl acetate (10.21%), in accordance with the GC-MS chromatogram.

Table 2. List of Compounds detected in the chloroform extract

S. N	Name of the compound	Retention time	Mol. formula	Mol. wt.	Area (%)
1.	Bornyl acetate	10.729	$C_{12}H_{20}O_2$	196	10.21
2.	γ-Cadinene	14.899	$C_{15}H_{24}$	204	5.22
3.	Elemol	16.291	$C_{15}H_{26}O$	222	8.53
4.	Nerolidol	17.504	$C_{15}H_{26}O$	222	5.24
5.	α-Muurolol	18.020	$C_{15}H_{26}O$	222	24.33
6.	Spathulenol	18.352	$C_{15}H_{24}O$	220	4.93
7.	α–Bisabolol	18.926	$C_{15}H_{26}O$	222	18.01
8.	4,4,6b-trimethyl-2-prop-1-en-2-yl-5,5a,6,6a-tetrahydro-2H-cyclopropa[g][1]benzofuran	19.200	$C_{15}H_{22}O$	218	8.90
9.	Clovene	19.657	$C_{15}H_{24}$	204	5.08
10.	1,2-dimethyl-3,5-bis(prop-1-en-2-yl)cyclohexane	21.040	$C_{14}H_{24}$	192	9.55

Antibacterial Screening Analysis.

Against many microorganisms, chloroform extract exhibited antibacterial action. However, the antibacterial activity differed depending on the type of microorganisms. The highest ZOI (12 mm) was shown in *Escherichia coli* and *Proteus vulgaris.*

Table 3. The diameter (mm) of the inhibitory zone of the A. adenophora chloroform extract's antimicrobial activity

Test Organism	**Zone of inhibition**	
Bacteria	**Negative Control (DMSO)**	**Activity of *A. adenophora***
Klebsiella pneumoniae ATCC 700603	No activity	8 mm
Bacillus subtilis ATCC 6051	No activity	8 mm
Pseudomonas aeruginosa ATCC 9027	No activity	6 mm
Staphylococcus aureus ATCC 6538P	No activity	8 mm
Escherichia coli ATCC 8739	No activity	12 mm
Enterococcus faecalis ATCC 29212	No activity	No activity
Proteus vulgaris ATCC 6380	No activity	12 mm
Shigella dysenteriae ATCC 13313	No activity	4 mm
Salmonella enterica Subsp.enterica pv Typhi ATCC 29630	No activity	8 mm
Staphylococcus epidermidis ATCC 1228	No activity	No activity
Fungi	**Negative control (DMSO)**	**Activity of A. adenophora**
Candida albicans ATCC 2091	No activity	No activity
Saccharomyces cerevisiae ATCC 18824	No activity	No activity

Antioxidant Screening Analysis

The logarithmic regression of the percent inhibition vs antioxidant activity can be used to calculate the inverse relationship between the antioxidant potential and the IC_{50} value. The greater the antioxidant activity, the lower the IC_{50} value. All the calculations are based on the standard method. At 517 nm, absorbance was measured. The absorbance of each solution was measured and recorded as follows:

Table 4. Antioxidant activity of Chloroform Extract of *A. adenophora* by DPPH method

Concentration (µg/mL)	Percentage Scavenged
2000	62.838
1500	46.912
1000	41.495
500	24.918
250	15.709

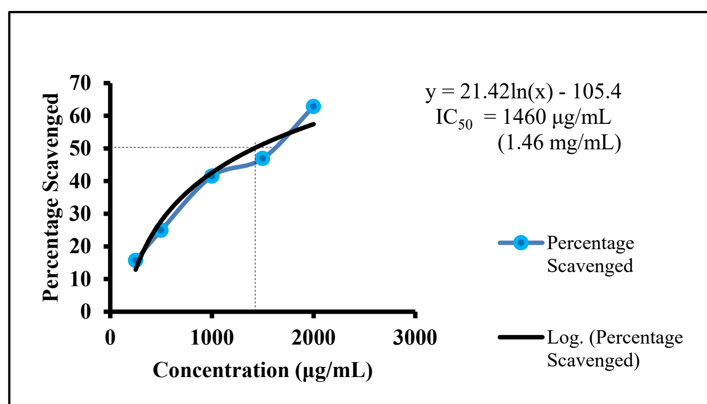

Fig 2. Graphical representation of the DPPH assay of the chloroform extract

The Food and Drug Administration (FDA) defines IC_{50} as the drug concentration necessary for 50% inhibition *in vitro*. The chloroform extract of *A. adenophora* was found to have an IC_{50} value of 1460 µg/mL.

Total Phenolic Content Analysis

Using the gallic acid calibration curve, the total phenolic content was calculated as a milligram of gallic acid equivalent.

Each solution's absorbance was measured and noted as follows:

Table 5. Absorbance of gallic acid

Concentration (µg/mL)	Average Absorbance (nm)
10	0.03
20	0.12
40	0.27
60	0.43
80	0.60
100	0.79

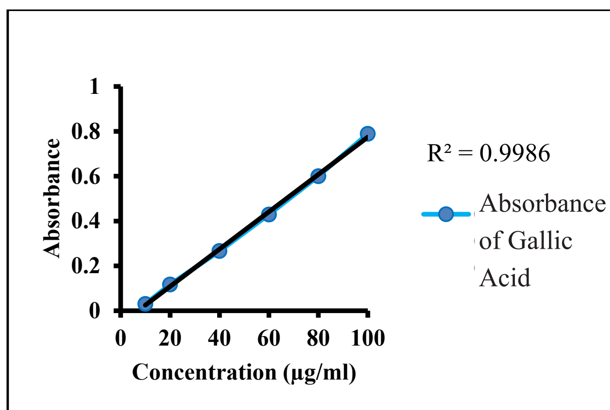

Fig 3. Calibration curve of gallic acid for total phenolic content determination

The total phenolic content with necessary data is shown in the following table.

Table 6. Total phenolic content in chloroform extract of *A. adenophora*

Sample Concentration (mg/mL)	Weight of extract per mL (g)	Absorbance	GAC (mg/mL)	TPC as GAE (mg/g)
1	0.001	0.68	0.08975	89.75

The total phenolic content of chloroform extract was evaluated as the gallic acid equivalent (GAE) by reference to a standard curve ($y = 0.0083x - 0.0584$, $R^2 = 0.9986$) using table 6 and figure 3 as a basis. Chloroform extract of *A. adenophora* was discovered to have a total phenolic content of 89.75 mg gallic acid equivalent/g of dry extract.

Total Flavonoid Content Analysis

The amount of total flavonoids per gram of dried material was represented as mg of quercetin equivalents [30].

Each solution's absorbance was measured and noted as follows:

Table 7. Absorbance of quercetin

Concentration (µg/mL)	Average Absorbance (nm)
5	0.06
10	0.12
20	0.43
40	0.96
80	1.90

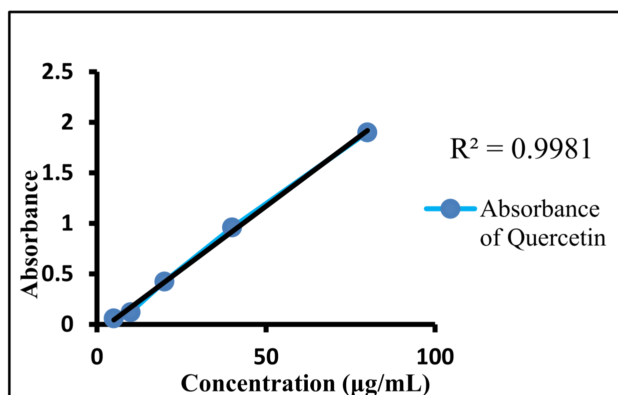

Fig 4. Calibration curve of Quercetin; for total flavonoid content determination

The total flavonoid content with necessary data is shown in the following table:

Table 8. Total flavonoid content in chloroform extract of *A. adenophora*

Sample Concentration (mg/mL)	Weight of extract per mL (g)	Absorbance	QC (mg/mL)	TFC as QE (mg/g)
1	0.001	1.109	0.049252	49.25

The total flavonoid content of chloroform extract was evaluated as quercetin equivalent (QE) by reference to a standard curve ($y = 0.025x – 0.0813$, $R^2 = 0.9981$) using table 8 and figure 4 as a basis ($y = 0.025x - 0.0813$, $R^2 = 0.9981$). Chloroform extract of *A. adenophora* was discovered to have a total flavonoid content of 49.25 mg quercetin equivalent/g of dry extract.

Brine Shrimp Lethality Analysis

The degree of mortality of *A. adenophora*-produced brine shrimp nauplii that increases as the concentration increases suggests that the chloroform extract contains cytotoxic agents.

The results of the Brine Shrimp Lethality assay are given in the table below:

Table 9. Effect of chloroform extract of *A. adenophora* in brine shrimp

Concentration (μg/mL)	Percentage Mortality
800	100
400	93.19
200	82.98
100	28.57
50	14.95

A. adenophora chloroform extracts were found to have an LC_{50} value of 174.78 μg/mL.

Conclusions

In methanol, hexane, chloroform, and ethyl acetate, the phytochemical examination of the extracts revealed the presence of alkaloids, flavonoids, phenols, steroids, quinones, saponins, tannins, cardiac glycosides, carbohydrates, terpenoids, proteins, and amino acids. 10 possible compounds were identified by GC-MS analysis of the chloroform extract, with α-Muurolol (24.33%), α–Bisabolol (18.01%), Bornyl acetate (10.21%) being the most prevalent. Chloroform extract showed antibacterial activity against *Escherichia coli, Proteus vulgaris* showing a maximum zone of inhibition of 12 mm. The chloroform extract was found to have an IC_{50} value of 1460 μg/mL. Folin-Ciocalteu was used to calculate the total phenolic content (TPC), which was calculated 89.75 mg/g gallic acid/g of equivalent. Using aluminum chloride colorimetric techniques, the total flavonoid content (TFC) was calculated and determined to be 49.25 mg/g quercetin equivalent/g of dry extract. With LC_{50} values of 174.78 μg/mL, *A. adenophora* chloroform extracts showed harmful action toward brine shrimp.

Acknowledgements

The authors appreciate the laboratory facilities provided by the Department of Chemistry, Amrit Campus, and Kathmandu Valley College, as well as the Department of Food and Technology, Quality Control for GC-MS, the Department of Plant Resources, and Research Institute of Bioscience and Biotechnology for biological assays.

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