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RESEARCH ARTICLE

Chemical Composition, Antibacterial, Antioxidant, and Cytotoxicity Activities of Essential Oil of Leaf of *Ageratina adenophora* (Spreng.) R.M. King and H. Rob.

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Abstract

Modern medicine recognizes the therapeutic applications of medicinal plants, which are utilized in indigenous therapies. *Ageratina adenophora* (Banmara) is an invasive plant growing in tropical and subtropical regions, traditionally used for treating wounds, sleeping disorders, jaundice, ulcers, etc. Using Clevenger apparatus for hydro-distillation, the essential oil was extracted, and gas chromatography-mass spectrometry (GC-MS) was used to determine its chemical constituents. GC-MS analysis of essential oil showed 14 possible compounds in which α-Muurolol (24.56%) was found to be most abundant. The essential oil exhibited a total phenolic content of 53.42 mg of gallic acid equivalent per gram of dry extract, while the total flavonoid content was determined to be 3.37 mg of quercetin equivalent per gram of dry extract. Essential oil of *A. adhenophora* showed the high antibacterial action against *Staphylococcus aureus* of ZOI of 12 mm. The antioxidant assay revealed weak activity of essential oil of IC_{50} 17.21 mg/mL, while the brine shrimp lethality assay revealed its LC_{50} value to be 64.56 µg/mL.

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

Introduction

Indigenous treatments and ethnopharmacological applications are becoming accepted tools in therapeutic modern medicine. As a rich source of new pharmaceuticals, medicinal plants are regarded as a priceless natural reservoir. They have been

consistently explored for their pharmacological actions against various diseases. *Ageratina adenophora* (Spreng.) R.M. King and H. Rob. (*A. adenophora)*, popularly known as Banmara or Kalimunte (killer of the forest), is a perennial herb typically referred to as sticky snakeroot [1–4]. It is a challenging weed to control in forest plantations because it invades damaged regions and hinders planted trees from self-seeding [5,6]. This plant has numerous secondary metabolites, potentially linked to its defense mechanisms, as indicated by its limited susceptibility to bacterial, fungal, and insect attacks [7–9]. This particular species has been previously linked to various chemical compounds, several of which have demonstrated allelopathic, phytotoxic, and antifeedant properties. Terpenoids, phenylpropanoids, flavonoids, coumarins, sterols, and alkaloids are among the chemicals identified in earlier studies [10,11]. Essential oils of *A. adenophora* have been found composed of compounds like cymene, phellandrene, camphene, bornyl acetate, bisabolol, and azulenone [12–14]. *A. adenophora* has been historically employed as a traditional treatment in several regions across the globe. It has been scientifically validated for various health benefits and has historically been employed in traditional medicinal practices for its anti-inflammatory, antibacterial, antiseptic, analgesic, antipyretic, and coagulant properties. Additionally, it has been recognized for its ability to promote sleep through the induction of phenol barbitone. The leaf of *A. adenophora* has been traditionally employed to treat several ailments, such as ulcers, diabetes, dysentery, jaundice, gum infections, fever, toothaches, and skin sores [2,15,16].

Materials and Methods

Plant Collection

The leaves of *A. adenophora* were collected in October 2019 at Ribdikot-7, Palpa, Nepal, at an approximate altitude of 2000 m and identified by Prof. Dr. Bipana Devi Acharya, Department of Botany, Amrit Campus, Lainchaur, Kathmandu, Nepal. The leaves were collected and then immediately cleaned to remove dirt and debris.

Extraction of Essential oil

The essential oil was extracted from the *A. adenophora* fresh leaves using hydro-distillation of the Clevenger type. The plant material was cut into small pieces and after that introduced into a round-bottom flask with a capacity of 5000 mL. Distilled water, measuring 3000 mL, was added to the flask. The setup was then equipped with hydro-distillation apparatus of the Clevenger type. The contents of the flask were subjected to heating at the boiling point for two hours using a heating mantle. The isolated essential oil was stored in a glass vial for later analysis.

Total Phenol Content Assay

The Folin-Ciocalteu technique was employed to determine the total concentration of phenolic components in the essential oil. The Folin-Ciocalteu reagent was introduced into a beaker

containing 1 mL of the reagent, and subsequently diluted by a factor of 10 using distilled water. The dissolution of Gallic acid was achieved by adding it to 1 mL of distilled water, resulting in a solution with a concentration of 1 mg/mL equivalent to 1000 µg/mL. A mixture was prepared by combining 1 mL of the sample with a concentration of 1 mg/mL in methanol and 1 mL of the Folin-Ciocalteu phenol reagent, which was diluted 1:10 with water. Additionally, 0.8 mL of a 1 M Na_2CO_3 solution in aqueous form was added to the mixture. Before measuring the absorbance of the reactant at a wavelength of 765 nm compared to the control, the reaction mixture was subjected to a resting period of approximately 15 minutes under conditions of complete absence of light (methanol). Gallic acid served as the standard. Total phenolic content is quantified by measuring the amount of gallic acid equivalents per gram of dried extract [17].

The amount of total phenolic content in the sample was calculated as milligrams of gallic acid equivalent using the relationship shown below:

$$\text{Total Phenolic Content (TPC)} = \frac{(C \times V)}{m}$$

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 aluminium chloride colorimetric method was applied to quantify the total flavonoid content present in *A. adenophora* essential oil. Absorbance was measured at 415 nm after 1 hour of mixing 1 mL of sample (0.1 mg/mL in methanol) with 1 mL 10% $AlCl_3$ (dissolved in methanol). Methanol was chosen as the control, while quercetin remained as the standard. Total flavonoid concentration is expressed as mg of equivalent quercetin per gram of dried sample [18]. The total flavonoid content of the sample was calculated as milligrams of quercetin equivalent using the following equation:

$$\text{Total Flavonoid Content (TFC)} = \frac{(C \times V)}{m}$$

Where,

C= concentration of quercetin from curve (mg/mL)

V= volume of extract (mL)

m= weight of plant extract (g)

Gas Chromatography-Mass Spectrometry

The essential oil was subjected to GC-MS analysis with the collaboration of the Department of Food and Technology and Quality Control in Babarmahal, Kathmandu, Nepal. Gas chromatography-mass spectrometry (GC-MS) analysis was conducted operating a GCMS-QP 2010 instrument, operating under the specified conditions: The Rtx-5MS column, with dimensions of 30 meters in length, 0.25 mm in inner diameter, and 0.25 mm in film thickness, employed helium as the carrier gas. The column experienced temperature fluctuations ranging

from 80°C to 300°C, with consecutive holding durations of 2.0 and 5.0 minutes. Meanwhile, the ion source and interface temperatures were maintained at 200°C and 250°C, respectively. The identification process was conducted with a comparison using mass spectrometry (MS) with the NIST library.

Antimicrobial Activity

The agar well diffusion method was employed in determining the antibacterial activity [19]. The media plates that had been incubated were separated into individual wells using a sterile cork borer with a diameter of 4 mm. Each well was appropriately labeled. Subsequently, a micropipette introduced 15 µL of the essential oil working solution into each well. Simultaneously, a control experiment was conducted to evaluate the solvent's (methanol) activity in a separate well. Afterward, the oil was evenly dispersed throughout the substance by maintaining the plates in a covered state for 30 minutes. Following this, the plates were put in an incubation chamber set at a temperature of 37°C for 6 hours. After the designated incubation period, the plates were examined to visually assess the region surrounding the well for the presence of a zone where bacterial growth was suppressed, which was characterized by a distinguishable transparent area. This particular region was recorded correctly. The zone of inhibition (ZOI) measurement was performed using a ruler, and the resulting mean value was recorded to assess the antibacterial efficacy.

Antioxidant Activity

An antioxidant assay was conducted using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method [20]. The initial stock solution was prepared by dissolving 1 mg of the sample in 1 mL of methanol, resulting in a 1 mg/mL concentration. Similarly, several concentrations of the 1000 µL (1 mL) extracts were generated using a two-fold dilution procedure from the stock solution. These concentrations included 1500, 1000, 500, 250, and 125 µg/ mL. In an alternative approach, 500 µL (0.5 mL) of each of the solutions mentioned above was mixed with 1500 µL (1.5 mL) of a 0.1 mM concentration of DPPH (4 mg of DPPH dissolved in 100 mL of methanol). The solutions were vigorously shaken for 2 minutes and thereafter covered with aluminium foil to mitigate the risk of ignition. The solutions were incubated at room temperature in a dark setting for 30 minutes. Following a 30-minute interval, the absorbance of the sample was evaluated at a wavelength of 517 nm, with methanol serving as the reference. To evaluate the absorbance of the mixture at a wavelength of 517 nm, a combination of 1.5 mL of DPPH solution and 0.5 mL of methanol was prepared as a control. A calibration curve was also created. The positive control taken during the test was standard ascorbic acid.

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

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

Where,

A_0 = Absorbance of the DPPH

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

The IC_{50} value, also known as the inhibitory concentration of 50%, refers to the minimum concentration of a sample required to scavenge 50% of the DPPH free radicals. The IC_{50} values were determined by constructing a dose inhibition curve in the logarithmic range, where the extract concentration was plotted against the corresponding scavenging action.

Brine Shrimp Lethality Assay

Brine shrimp (*Artemia salina*) lethality tests are conducted to determine the cytotoxic potential of bioactive compounds [21]. Due to the absence of aseptic procedures, the test can be conducted cheaply in less than twenty-four hours. The brine shrimp nauplii were exposed to solutions containing essential oil at varying concentrations for 24 hours. The number of motile nauplii was used to determine the efficacy of the essential oil. A substance is considered bioactive if its LC_{50} is less than 1 mg/mL.

Results and Discussion

Total Phenolic and Flavonoid Content

The total phenolic content (TPC) was quantified by measuring the amount of gallic acid equivalent in milligrams, employing the gallic acid calibration curve. This determination was made by calculating the gallic acid equivalent (GAE) by comparison to a standard curve ($y = 0.0083x - 0.0584$, $R^2 = 0.9986$). The determined TPC of the essential oil derived from *A. adenophora* was measured to be 53.42 mg GAE/g of dry extract.

Gas Chromatography Mass Spectrometry Analysis

S.N.	Name of the compound	Retention time	Molecular formula	Molecular weight	Area (%)
1.	Borneol	7.932	$C_{10}H_{18}O$	136	2.84
2.	Bornyl acetate	10.604	$C_{12}H_{20}O_2$	152	10.89
3.	β-Farnesene	14.204	$C_{15}H_{24}$	152	6.59
4.	Epizonarene	14.787	$C_{15}H_{24}$	152	8.61
5.	β-Bisabolene	15.319	$C_{15}H_{24}$	154	7.81
6.	4-Thujen-2α-yl acetate	15.754	$C_{12}H_{18}O_2$	196	3.45
7.	Caryophyllene oxide	16.895	$C_{15}H_{24}O$	204	2.49
8.	Nerolidol	17.418	$C_{15}H_{26}O$	204	2.29
9.	α-Muurolol	17.938	$C_{15}H_{26}O$	294	24.56
10.	α-Bisabolol	18.816	$C_{15}H_{26}O$	204	14.74
11.	9H-Cycloisolongifolene, 8-oxo-	19.096	$C_{15}H_{22}O$	204	8.56
12.	8,8-Dimethyl-4,6-bis(1-methylethylidene)bicyclo[5.1.0]octan-2-one	22.151	$C_{16}H_{24}O$	204	2.29
13.	6-(1-hydroxymethylvinyl)-4,8a-dimethyl-3,5,6,7,8,8a-hexahydro-1H-napthalene-2-one	24.223	$C_{12}H_{22}O_2$	234	2.59
14.	Cycloeucalenyl acetate	28.391	$C_{32}H_{52}O_2$	468	2.29

The Gas Chromatography Mass Spectrometry Analysis of the essential oil derived from *A. adenophora* consisted of 14 major compounds, with the highest proportions being α-Muurolol (24.56%), α-Bisabolol (14.74%), and Bornyl acetate (10.89%), as indicated by the GC-MS Chromatogram.

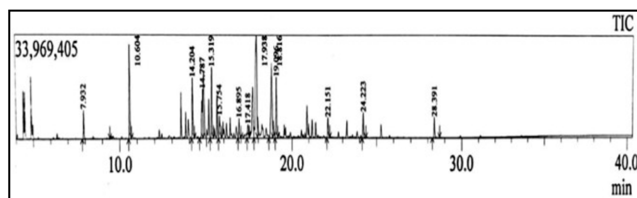

Fig 1. GC-MS Chromatogram of essential oil of *A. adenophora*

Antimicrobial Screening Analysis.

Different bacteria that were exposed to essential oil showed antimicrobial activity. The antibacterial activity did, however, differ depending on the type of microbe. *Staphylococcus aureus* displayed a greater ZOI of 12mm.

Antioxidant Screening Analysis

The presence of antioxidant chemicals, primarily phenolic compounds, carotenoids, tocopherols, ascorbic acid, and the activity of "free radical scavenging enzymes" is related to the antioxidant capacity of plants. Using logarithmic regression on the % inhibition versus antioxidant activity allows for the computation of the reciprocal correlation between the antioxidant capacity and the IC_{50} value. Antioxidant activity is higher when the IC_{50} value is lower. The standard approach is the foundation for all calculations. Absorbance was measured at wavelength 517 nm using a spectrophotometer. The definition of IC_{50}, as provided by the Food and Drug Administration (FDA), USA, pertains to the quantity of a pharmaceutical substance required to induce a 50% inhibition in vitro which was found of 17.21 mg/mL for *A. adenophora* essential oil. The higher IC_{50} signified the lower antioxidant potential of the essential oil. Ascorbic acid was used as standard compound that showed the IC_{50} value of 12 µg/m.

Table 3. The diameter (mm) of the inhibitory zone of the antimicrobial activity of the essential oil of *A.adenophora.*

Test Organism	**Zone of inhibition**		
	Positive control (Chloramphenicol)	**Negative Control (DMSO)**	**Oil Activity**
Klebsiella pneumoniae KCTC 2242	26 mm	-	8 mm
Bacillus subtilis KACC 17047	21 mm	-	7 mm
Pseudomonas aeruginosa KACC 10232	21 mm	-	-
Staphylococcus aureus KCTC 1916	22 mm	-	12 mm
Micrococcus luteus KACC 13377	17 mm	-	7 mm
Enterobacter cloacae KACC 13002	19 mm	-	-
Fungi	(Clotrimazole)		
Aspergillus flavus	25 mm	-	-
Fusarium spp.	22 mm	-	-

Brine Shrimp Lethality Analysis

The essential oil may contain cytotoxic chemicals because the mortality of brine shrimp nauplii produced by *A.*

adenophora rises with concentration. The LC_{50} value of *A. adenophora* essential oil was found to have 64.56 µg/mL value from a logarithm plot equation using Excel software. Given that the data is collected at values below 1000 µg/mL, it can be regarded cytotoxic [22].

Conclusion

The essential oil was subjected to chemical profiling by GC-MS analysis, which identified a total of 14 possible chemical compounds. Among these compounds, α-Muurolol was the most abundant, accounting for 24.56% of the total composition. The maximum zone of inhibition observed for the antibacterial activity of essential oil against *Staphylococcus subsp. aureus*. DPPH assay revealed the weak antioxidant activity of essential oil of the *A. adenophora*. The TPC value of 53.42 mg GAE/g and the TFC value of 3.372 mg QE/g were observed for the oil. The essential oil of *A. adenophora* exhibited a detrimental effect on brine shrimp, as evidenced by the LC_{50} values of 64.56 µg/mL. The results of this study could be used to evaluate other pharmaco-therapeutic properties of the plant.

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