# Phytochemical screening and in-vitro evaluation of antimicrobial activity of invasive species *Ageratina adenophora* collected from Kathmandu valley, Nepal

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**Abstract:** Antimicrobial activity of invasive alien plant species *Ageratina adenophora* (Sprengel) R. King & H. Robinson was evaluated against six human pathogenic bacteria. The aqueous (distilled water) and alcoholic (methanol) crude extracts from the leaves of the plant were tested against three gram negative bacteria: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniaea* (ATCC 15380) and *Proteus mirabilis* (ATCC 49132) and three gram positive bacteria: *Enterococcus faecalis* (ATCC29212) *Bacillus subtilis* (ATCC6633) and *Staphylococcus aureus* (ATCC 25923) using disc diffusion method. Different concentrations of plant extracts (5, 10, 15, 20 and 25%) were applied and diameter of zone of inhibition (ZOI) of bacterial growth were recorded. Plant extract in both solvent exhibited pronounced results against tested bacteria. Methanolic extract of plant exhibited good activity against tested bacteria when compared with aqueous extract. The zone of inhibition of bacterial growth increased with increasing concentrations. Phytochemical screening of plant exhibited alkaloids, saponin, tannin and flavonoids and plant sample contains higher amount of alkaloid. The demonstration of activity against all tested organisms had shown that *Ageratina adenophora* can be used to produce raw materials/substances for further development of diverse antibiotics with broad spectrum of activity.

**Keywords:** Antimicrobial; Invasive plant; Crude plant extract; Phytochemical screening.

## Introduction

Plant materials continue to play an important role in the maintenance of human health as over 50% of all modern chemical drugs originates from natural plant sources. Several plants are now being used in part or as a whole to treat many diseases. Natural products of higher plants may give a new source of antimicrobial agents [6, 7]. The effects of plant extracts on bacteria have been studied by a very large number of researchers in different parts of world [8, 9].

Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, glycosides, etc., which have been found *in vitro* to have antimicrobial properties[10,11]. In an effort to expand the spectrum of antibacterial agents from natural resources, an invasive alien species *Ageratina adenophora* (Asteraceae) has been selected.

*Ageratina adenophora* (Sprengel) R. King & H. Robinson, the Crofton weed, is a perennial sub-shrub natively found in Mexico[12]. It is invasive to more than 30 countries including China, India, Indonesia, Vietnam, Nepal and Maynmar[13]. Invasion of *A. adenophora* has become a serious threat to native ecosystem dynamics and biodiversity in its nonnative range [14]. In Nepal it has been

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invading road side, fallow lands, forest margins or disturbed areas [13].

In Nepal, Invasive Alien Species (IAS) is of a major problem and causing a negative impact on the environment, either socially or economically on biodiversity of the country. Management of such alien invasive plant is necessary for conservation of biodiversity and native species. Antimicrobial studies of invasive alien plants help to find the potentialities of effect of these plants on growth of organisms. Further phytochemical study also helps to find the chemical constituents that may be useful for pharmaceutical formulation. Invasive plants have important ecological and economic impacts world-wide and increasing attention is now being paid to eradication and management efforts[15]. So the result of present study may have great significance in the management of such invasive alien weed in Himalayan country like Nepal.

**Materials and methods**

The leaf sample of *Ageratina adenophora* was collected from Kirtipur, Kathmandu, Nepal. The leaves were washed thoroughly 2–3 times with running tap water, chopped into small pieces and then air dried on the newspaper at room temperature till they become completely dry and then powdered with the help of grinder. Powder was stored in air tight zipper bag.

**Preparation of extracts**

Dried powdered leaf samples (25 g) of *Ageratina adenophora* was soaked in 250 ml of methanol (95%) and distilled water for 72 h. Then the mixture was stirred at 24 h interval using a sterile glass rod [16]. The plant samples squeezed and then filtered with the help of 3- layered cotton cloths. Water content of distilled water filtrate was evaporated on heating mantle using water bath till the solution reduced to semisolid form[17]. The methanolic filtrate was concentrated down with rotary vaccum evaporator under negative pressure and extracts were then transferred into sterile labeled bottles and they were made into semisolid form by evaporation to water bath at 50°C. Obtained crude extracts were weighted and made the bottles air tight and stored in a refrigerator at temperature 4°C for prior to use [18].

**Plant extract concentration**

Different concentrations of the extracts (5, 10, 15, 20 and 25%) were made in both solvents i.e. methanol and distilled water separately. Methanol and distilled water solvents were used as control.

**Collection of microorganisms**

Three Gram negative bacteria: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniaea* (ATCC 15380) *and Proteus mirabilis* (ATCC 49132) and three Gram positive bacteria: *Enterococcus faecalis* (ATCC29212) *Bacillus subtilis* (ATCC6633) and *Staphylococcus aureus* (ATCC 25923) were selected for the antibacterial study. The bacterial strains used for the test were obtained from Department of Microbiology, Teaching hospital, Maharajgunj, Nepal. They were taken on slants and later cultured in test tube having nutrient broth.

**Antibacterial test**

The disc diffusion method[19] was used for the antibacterial study with slight modifications. The paper disc (5mm diameter) was made by punching the filter paper Sartorious stedium 292.The test discs were prepared by dipping and saturating sterilized punched filter paper in both control and different concentrations of the plant extracts (5,10, 15, 20 and 25%). For antibacterial test Nutrient Agar media was applied [19]. The melted nutrient agar poured almost 10–10 ml in each sterilized petriplates , divided into six chambers, labeled with the date, code name of the bacteria and the concentrations code) and was left to solidify for 15–20 minutes. The inoculums of bacteria were transferred into petriplates containing solidified media using sterile cotton swab. The sterile cotton swab was dipped into well mixed distilled water test culture and was spread on the media by moving the swab in Z –shape. One swab was used for single bacterium. Seven replicates were used for each bacterium. The culture plates were allowed to dry for 5–10 minutes. Then in each petriplate in each chamber different concentrated and controls disc were put with the help of

sterile forceps. The plates were then incubated at 37ºC for 24 h. Microbial growth was determined by measuring the diameter of zone of inhibition (ZOI).

Zone of Inhibition (mm) = diameter of zone around the growth inhibited zone

## Preliminary phytochemical screening

The qualitative phytochemical screening for *A. adenophora* leaves (aqueous and methanolic extract), was conducted based on standard methods of analysis described by several scientists[20-22]. Change in color was noted for the result.

**Test for the presence of alkaloids**: In a test tube, to about 3 ml of each extract, 2 drops of Dragendoff's reagent was added. The formation of orange red precipitation proved the presence of alkaloids.

**Test for the presence of flavonoids**: In a test tube, to about 4 ml of each extract, a piece of magnesium ribbon and a drop of concentrated HCl were added. The red to crimson color detected the presence of flavonoids.

**Test for the presence of saponins**: In a test-tube, 5 ml of each extract was shaken vigorously for 2 min. The presence of saponins was indicated by the formation of froth that lasted for some minutes.

**Test for the presence of tannins**: In a test tube, to 2 ml of each extract, 3 drops of 5% ferric chloride ($FeCl_3$) solution was added. The presence of tannis was detected by the appearance of green,black or blue colour.

**Test for the presence of terpenoids**: In a test tube, to 5 ml of each chloroform extract, 5 ml of concentrated sulphuric acid was carefully added. The formation of a red-brown layer indicated the presence of terpenoids.

### Quantitative test

Alkaloid, siphonin and flavonoid were determined in the crude form of compounds by following methods.

### Alkaloid content

Alkaloid content of sample was determined by following Harborne method[20]. 2.5 g of the sample was weighed into a 250 ml beaker and 100 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4h. This was filtered and the extract was concentrated on a water bath to one-quarter (25 ml) of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitate was completed. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue was the alkaloid, which was dried and weighed (initial and final weight of the filter paper was calculated).

### Saponin content

Saponin content of sample was determined by following Obadoni and Ochuko[23]. 10 g of grinded sample was kept into a conical flask and 50 $cm^3$ of 20 % ethanol was added. The samples were heated over a hot water bath for 2 h with continuous stirring at about 50°C. The mixture was filtered and the residue re-extracted with another 100 ml 20% ethanol. The combined extracts were reduced to 20 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separatory funnel and 10 ml diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ethyl layer was discarded. The purification process repeated. 30 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 5 ml of 5% of aqueous sodium chloride. The remaining solution was heated in a water-bath. After evaporation the sample was dried in the oven to a constant weight; the saponin content was calculated as percentage.

### Flavonoid content

Flavonoid content of sample was determined by Bohm and Kocipai-Abyazan method [24]. 10 g of leaf sample was extracted with 100 ml of 80% methanol at room temperature. The solution was filtered through Sartorius stedium, grade 292 filter paper. The filtrate was later transferred into crucible and evaporated in dryness over a water bath and weighed to a constant weight.

### Data analysis

The values were expressed as mean ± standard deviation (SD). Each value was a mean of seven replicates. The one – way Analysis of Variance (ANOVA) was used to

determine the significant differences between the parameters and the Duncan test was done to compare the differences at $p < 0.01$ using statistical package SPSS version 16.

**Results and discussion**

The antimicrobial activities of *Ageratina adenophora* was investigated against six human pathogenic bacteria. The antimicrobial capacity of crude extracts of plants at different concentrations was measured by zone of inhibition (ZOI). There were significant differences in mean value of ZOI of bacterial growth in different concentrations of plant leaf extract (Tables 1 & 2). During the study it was found that plant extract in both solvents *viz.* methanolic and distilled water extract showed different activities against an array of Gm+ve and Gm–ve bacteria.

Similarly, methanolic extract of *Ageratina adenophora* had shown the highest ZOI (11–14 mm diameter) on *Proteus mirabilis*, 10 –13 mm diameter on *Bacillus subtilis* at 5-25% concentrations respectively. At 5 –25% concentration, ZOI was 10 –12 mm diameter on *Staphylococcus aureus,* while 10–12 mm diameter on *K. pneumoniae* and *E. coli* (Table 2). In distilled water extract *Ageratina adenophora* had shown the highest ZOI (8 mm diameter) at 25% concentration on *K. pneumoniae* and *E. faecallis* (Table 1). Distilled water (DW) extract of *A. adenophora* did not show effect on *P. mirabilis* at 5 –25% concentrations (Table 1), *B. subtilis* at 5–20% concentrations, *K. pneumoniae* at 5–15.0 % concentrations and *E. coli* at 5.0 -10.0% (Table 1) concentration. With no antibacterial activities of extract of some plants against some selected bacteria may be due to the low concentration of extracts[25] and can thus only be proven by using large doses [26].

Three Gram negative and three Gram positive bacteria were tested with plant extract in the present study. In comparison, generally Gram positive bacteria seem more susceptible than Gram negative bacteria [27, 28]. But in this study mixed type of result was found. In distilled water extract, at 25 % concentration exhibited equal ZOI (8 mm diameter.) in both *E. faecalis* and *K. pneumonia.* (Table 1); while in methanolic extract at 25% concentration exhibited the highest ZOI (14 mm diameter) against *P. mirabilis* (Table 2). Almost similar type of result was reported by Biswas *et al.* [29]. They found that the neem leaves and seeds had antibacterial activity against a wide spectrum of Gram-positive and Gram-negative microorganisms.

It is revealed from the results that crude extract of plant exhibited potential antibacterial activity against at least two bacteria at 5-25% concentrations in both distilled water and methanol extract. Methanol extract was found more effective than aqueous extract. The methanol extract of plant inhibited the selected bacterial growth at all concentrations but distilled water extract was effective only at higher concentrations and it was found ineffective against *Proteus mirabilis* at all concentrations. This greater effectiveness of methanol extract compared to aqueous extract may be due to differences in constituents and amount of extraction of phytochemicals, which are toxic to targeted pathogens, present in leaf of tested p The leaf extracts of plant contain alkaloids, saponins and tannins (Table 3). Among the tested chemicals, higher amount of alkaloid (6.12%) was recorded in the plant; which was followed by saponin (5.24%) and flavonoids (3.11%) (Figure 1). Alkaloids and tannins have antibacterial and antihelminthic properties [30]. The methanol extract had antimicrobial activity against selected organisms; this may be due to the ability of the methanol to extract some of the active properties like phenolic compounds, saponin, bryophyllin and other secondary metabolites which are reported to be antimicrobial [11, 31] from the tested plants. In aqueous extract, there may be lack of solubility of active constituents or may be due to loss of some active compounds during extraction process of the plant sample [32].

Similar type of result was reported by Lalfakzuala *et al.* [33]. In their study methanolic extract of the leaves of four common weeds were investigated on *Bacillus subtilis* and *Bacillus pumilis* at different concentrations; minimum ZOI was recorded at 2.5 % and maximum at 10 %.

**Table 1. Zone of inhibition (mm) of bacteria due to distilled water leaf extract of Ageratina adenophora.**

| Bacterial strains | Plant extracts concentrations (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **5** | **10** | **15** | **20** | **25** | **Control** (DW | P | F |
| *Klebsiella pneumonia* | 0±0 a | 0±0 a | 0±0 a | 7±7 b | 8±0 .1 b | 0±0 a | 0.000 | 155.65 |
| *Enterococcus faecalis* | 6±3 b | 6±3 b | 6±.2 b | 7±.3 b | 8±.3 b | 0±0 a | 0.000 | 359.58 |
| *Escherichia coli* | 0±0 a | 0±0 a | 6±0 b | 6±0 b | 6±0 b | 0±0 a | 0.000 | 304.51 |
| *Bacillus subtilis* | 0±0 a | 0±0 a | 0±0 a | 0±0 a | 6±.2 a | 0±0 a | 0.000 | 87.50 |
| *Proteus mirabilis* | 0±0 a | 0±0 a | 0±0 a | 0±0 a | 0±3 a | 0±0 a | 0.000 | 540.37 |
| *Staphylococcus aureus* | 6±.3 a | 6±.2 a | 7±.6 a | 7±.8 a | 7±.8 a | 0±0 a | 0.000 | 32.72 |

The values were expressed as Mean ±S. D. and statistically analysis using One Way ANOVA for obtaining F and P value. Tukey HSD multiple comparison tests were done to compare the different letters at P< 0.05. n=7.

**Table 2. Zone of inhibition (mm) of bacteria due to methanol leaf extract of Ageratina adenophora**

| Bacterial strains | Concentrations (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **5** | 10 | **15** | **20** | **25** | **Control** | P | F |
| *Klebsiella pneumoniae* | 9.0±1 b | 10 ±.5 b | 10±1 b | 11±1 b | 12±1 b | 0±0a | 0.000 | 97.15 |
| *Enterococcus faecalis* | 8.0±1 b | 8±.5 b | 9.0±1 b | 10±1 b | 11±1 b | 0±0a | 0.000 | 250.6 |
| *Escherichia coli* | 8.0±1 b | 9± .5 b | 10.0±1 b | 11±1 b | 12±1 b | 0±0a | 0.000 | 179.2 |
| *Bacillus subtilis* | 10.0±.8b | 11±.6b | 12±.5b | 12±.5 b | 13±.5 b | 0±0a | 0.000 | 45.57 |
| *Proteus mirabilis* | 11.0±1 b | 11±1 b | 12±.7b | 13±.9 b | 14±.9 b | 0±0a | 0..000 | 220.09 |
| *Staphylococcus aureus* | 10.0±.4b | 11±.6b | 12±.5b | 12±.6 b | 12±.6 b | 0±0a | 0.000 | 24.72 |

The values were expressed as Mean ±S. D. and statistically analysis using One Way ANOVA for obtaining F and P value. Tukey HSD multiple comparison test was done to compare the different letters at p< 0.05, n=7.

**Table 3. Phytochemical screening of leaf extract of A. adenophora**

| **Phytochemical constituents** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Distilled water** | | | | | **Methanol** | | | | |
| Tannin | Saponins | Terpenoids | Alkaloids | Flavonoids | Tannin | Saponins | Terpenoids | Alkaloids | Flavonoids |
| +++ | ++ | +++ | +++ | - | +++ | +++ | - | +++ | +++ |

*Responses to various tests were denoted by +, ++ and +++ signs indicating weak, moderate and strong reactions respectively while - for no reaction*

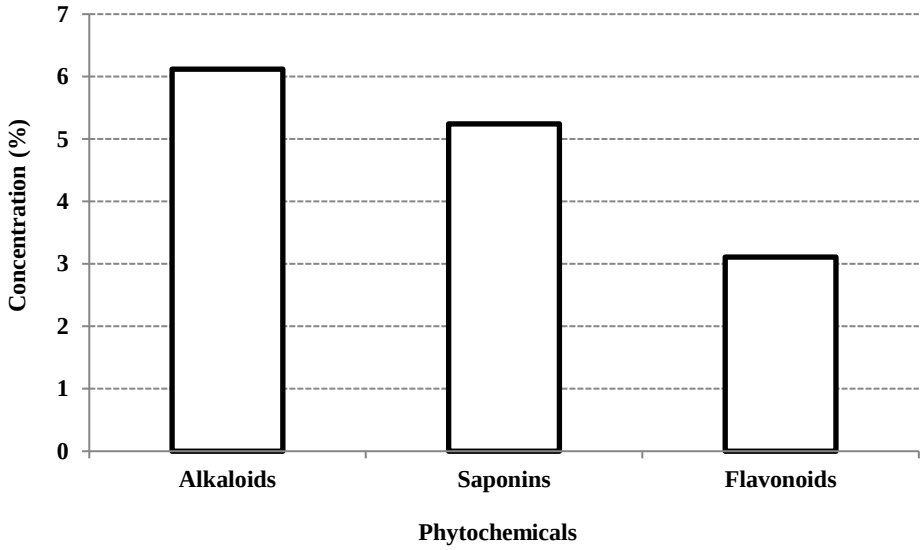


**Figure 1: Concentration of phytochemicals in leaf sample**

## Conclusion

The result of this study had shown that *Ageratina adenophora* extract had varied antimicrobial activities against the tested organisms. The demonstration of activity against all these organisms had shown that the *A. adenophora* can be used to produce raw materials/substances for further development of diverse antibiotics with broad spectrum of activity. These findings can form the basis of further studies to isolate compounds, to find new therapeutic principles. Invasive alien species are considered as one of the greatest threat to natural ecosystem of the earth. Exploitation of this rapidly growing species can be done on making the different pharmaceutical products in one hand while proper management on the other side.

## Conflict of interest

The authors declare that there is no conflict of interest.

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