



## Study of Phytoconstituents and Biological Activities of Medicinal Plants of Chitwan District

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### Abstract

Four plants species, namely; *Calotropis gigantea*, *Ageratum houstonianum*, *Catharanthus roseus*, *Thevetia peruviana* (seed), and *Thevetia peruviana* (leaves) have been collected from Chitwan, and the crude methanol extract of respective plants parts and seeds were studied for their phytoconstituents and biological activity. Phytochemical screening showed the presence of glycosides, flavonol glycosides and coumarin glycosides as rich components. The Brine-shrimp bioassay of various plants extract showed that *C. gigantea*, *A. houstonianum* and *C. roseus* exhibited high toxicity against brine shrimp nauplii at  $LC_{50}$  ( $\mu\text{g/ml}$ ) values of 23.44, 27.54 and 83.17 respectively. In addition to this, study of antimicrobial activity of respective plants extract on methanol showed that, all the four plants species were highly active for *Staphylococcus aureus* and *C. gigantea* is pharmacologically active for other bacteria such *E. coli*, *S. aureus*, *K. oxytoca* and *P. aeruginosa*. Study of anti-cancer activity on human pancreatic cancer cells such as PANC-1 revealed that the preferential cytotoxic activity of crude methanol extract of *C. gigantea* was highest at  $100\mu\text{g/ml}$  for both NDM and DMEM.

**Key words:** Brine-shrimp bioassay, Anti-microbial activity, cytotoxic activity PANC-1, NDM, DMEM

### 1. Introduction

Natural product chemistry is the study of primary and secondary metabolites synthesized by plants or animals. Natural products have been a fertile area of chemical investigation for many years, driving the development of both analytical chemistry and of new synthetic reactions and methodologies.<sup>[1,2]</sup> Nowadays several drugs are synthesized in the lab, which are copies or just modifications of naturally occurring compounds obtain from plants. These drugs are used for treatment of different disease such as antifungal, antimicrobial, anti-infective agent, analgesic, anti-tumor, and antiviral<sup>[3]</sup>. Research in chemistry of natural products has endless potential and especially important for the country like Nepal which is rich in biodiversity. The Himalayan country Nepal, has extreme range of altitude, climate, and soil within a small geographical area which has created a striking vertical zonation and diversity in flora and fauna.

Plant diversity in Nepal can be illustrated from the fact that over 1000 species of Himalayan plants have originally been discovered and described from Nepalese flora<sup>[4]</sup>. Among the 7000 species of medicinal plants recognized all over the world, more

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than 900 types of precious medicinal plants are said to be found in Nepal <sup>[5]</sup> because of extraordinary biodiversity and presence of rare and more valuable crude drugs of vegetable origin. Nepal has been regarded as natural showroom of biodiversity <sup>[6]</sup>. More than 700 species of medicinal plants grow wild in the country, majority of which are used in folk herbal remedies. However, in Nepal over fifteen thousand tons of medicinal herbs representing some 100 species are harvested from the wild for commercial and industrial purpose and large number of medicinal herbs collected from the forests and pastures, and traded for foreign country <sup>[7]</sup>. The uncontrolled commercial extraction has significantly eroded the country's medicinal plant resources and particular species have gradually become more difficult to find in a given locality once where they flourished. The present study was carried out to evaluate the phytochemicals, and biological assay of following four medicinal plants from Chitwan district of Nepal. The findings from this work may add to the overall value of the medicinal potentiality of the plants.

### 1.1 Pancreatic cancer

Cancer is characterized by proliferation of abnormal cells which multiply out of control, destroying healthy tissue. Pancreatic cancer is the fourth most common cause of cancer-related deaths in the United State. <sup>[8]</sup> and the eighth worldwide. <sup>[9]</sup> Pancreatic cancer accounts for about 220,000 death each year in Asia pacific region, the risk factors as being smoking and diabetes for pancreatic cancer. <sup>[10]</sup> Human pancreatic cancer cells such as PANC-1 are known to exhibit marked tolerance to nutrition starvation that enables them to survive for prolonged period of time even under extremely deprive condition. Thus, elimination of this high tolerance to nutrition starvation as a novel approaches in anticancer drug development. <sup>[10]</sup>

## 2. Materials and Methods

### 2.1 Collection of plants

Five different plants were collected from Chitwan, Central part of Nepal. The taxonomic identification of the plants was confirmed by Prof. Dr. R.P Chaudhary Central Department of Botany (CDB) TU, Prof. Dr. Mohan Siwakoti (CDB) TU, Mr. Bishanu Bhattra, Assistant lecture, Birendra Multiple Campus, Chitwan and Mr. Mitra Pathak, Assistant officer, National Herbarium Centre Godawari.

Table No. 1: Description of plants and their medicinal value

S.N.	Scientific name	Family	Local name	Parts taken	Biological activity	Collected area
1.	<i>Ageratum houtonianum</i>	Asteraceae	Blue gande	Flower	Antifungal, antidiabetic	Chitwan
2.	<i>Calotropis gigantean</i>	Asclepiadaceae	Aank	Aerial	Antipyretic, sedative, analgesic, hepatoprotective, antidiarrhoeal	Chitwan

3.	Catharanthus roseus	Apocynaceae	Baramase rato	Aerial	Anti-diabetic, antimalarial antioxidant, anticancer	Chitwan
4.	Thevetia peruviana	Apocynaceae	Pahelo karbir	Leaves	Antifungal, antibacterial, antitermite	Chitwan
5.	Thevetia peruviana	Apocynaceae	Pahelo karbir	Seeds	Antifungal, antibacterial, antitermite	Chitwan

## 2.2 Preparation of plants extract

The collected fresh plants materials were first washed in running tap water and finally with distilled water to remove coarse materials such as dust, sand and soils. The clean plants were air dried in shade for few days. The dried plants were ground into powder and stored in a labeled airtight bag. About 100 gm of powder of each plant were extracted by maceration in methanol (200ml) for 48 hrs with frequent agitation. The mixture was filtered through muslin cloth followed by double filtration by filter paper. The same process was repeated for 4/5 times for complete extraction and filtrate was concentrated by Rotary Evaporator.

## 2.3 Phytochemical Screening

Analysis of crude methanol extract and aqueous extract of above mentioned medicinal plants species were carried out by using standard procedure put forwarded by prof. I. Culie<sup>[1]</sup>.

## 2.4 Biological screening

### 2.4.1 Brine-Shrimp Bioassay

The brine-shrimp toxicity assay for each methanol extract was carried out according to Mayer et al. <sup>[11]</sup> Briefly, the sample solutions were prepared by dissolving 20 mg of each plant extract in 2 ml dimethyl sulphoxide (DMSO) up to the mark in 10 ml volumetric flasks. To calculated volume of the sample solution for 10, 100 and 1,000 µg/ml dose levels in three replicates was introduced into freshly hatched ten brine-shrimp nauplii in artificial sea water (total volume 10 ml). A control tube for each dose level was also prepared. After 24 h of illumination under a table lamp (60 Watt), the number of survivors was counted. No death was observed in the control tubes. The LC<sub>50</sub> (Lethal concentration for 50% mortality) values was determined using the probit analysis method <sup>[12]</sup>, as the measure of toxicity of the extract.

### 2.4.2 Antimicrobial screening

The standard cultures of four bacteria under study namely; *Staphylococcus aureus*,

*Klebsiella oxytoca*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were collected from Chitwan Medical College Bharatpur. The antimicrobial activity of crude methanol extract of plants was determined by agar well disc diffusion method. <sup>[13]</sup> A suspension of test micro organisms was spread on Muller-Hinton Agar (MHA) medium of approximately 4mm thickness. The sterile filter paper discs (6 mm in diameter) were individually impregnated with different concentration of plant extract (5%, 20% and 25%) prepared in dimethyl sulphoxide (DMSO) and then placed into the agar plates which have been previously inoculated with the test micro organisms. The plates were subsequently incubated 24 hours at 37°C. After incubation the growth inhibition zones were quantified by measuring the diameter of the zone of inhibition in mm. For each concentration, one Dimethyl sulphoxide (DMSO) well discs were used as control. The entire tests were performed in triplicate. <sup>[13]</sup>

### 2.4.3 Preferential cytotoxicity

PANC-1 cancer cell preferential cytotoxicity assay was done on standard protocol <sup>[14, 15]</sup>. PANC-1 cancer in human cell were seeded in 96- well plates (1x10<sup>4</sup>/ cell well) and incubate on fresh Dulbecco's modified Eagle's medium at 37°C under 5% CO<sub>2</sub>/95% air for 24 h. The cells were then washed with Phosphate buffer and the medium was changed to both Dulbecco Modified Eagle medium (DMEM) and nutrient- deprived medium (NDM) (absence of glucose, amino acids, and serum) separately. Then it was followed by immediate addition of serial dilution of the test samples. After 24 hours of incubation, the cells were again washed with PBS, then 100 µlit of DMEM with 10% WST-8 cell counting kit solution was added to the wells, and plates was incubated for a further 2 hours. <sup>[16]</sup> Then, the absorbance of the wells at 450 nm was calculated by following equation:

$$\text{Cell viability (\%)} = \left[ \frac{\text{Abs}_{(\text{test sample})} - \text{Abs}_{(\text{blank})}}{\text{Abs}_{(\text{control})} - \text{Abs}_{(\text{blank})}} \right] \times 100$$

The experiment for cytotoxicity was carried out at Institute of Natural Medicine, University of toyama, Sugitani, Japan.

## 3. Statistical analysis

### 3.1 Phytochemical Screening

The different phytochemicals in the crude methanol extracts were identified by the color reaction with different reagents. The results of phytochemical screening are given below in table no.2.

Table No. 2: Phytochemical Screening for Methanol Extract of four different plants

S.N.	Group of Compounds	Results				
		<i>A. houstonianum</i>	<i>C. gigantea</i>	<i>C. roseus</i>	<i>T.peruviana</i> (seed kernal)	<i>T.peruviana</i> (leaves)
1.	Polyphenols	-	+	-	-	-

2.	Alkaloids	-	+	+	-	+
3.	Glycosides	-	+	+	+	+
4.	Quinones	+	+	+	+	+
5.	Anthocyanosides	+	+	+	-	-
6.	Anthracenosides	+	-	-	+	-
7.	Flavone glycosides	+	+	+	+	+
8.	Coumarin glycosides	+	+	+	+	+
9.	Reducing compounds	-	-	-	-	+

+ Signs indicate presence of phytochemicals and – Signs indicate absence of phytochemicals.

### 3.2 Biological screening

#### Brine Shrimp Bioassay

The newly hatched brine-shrimp nauplii were exposed to the plant extracts and their biological activities were evaluated on the basis of their toxicity towards these nauplii. [11] The  $LC_{50}$  values ( $\mu\text{g/mL}$ ) for each plants extract were determined and those having  $LC_{50}$  values less than 1000 are consider being pharmacologically active. Results obtained during this study are shown in table no 3.

Table No. 3:  $LC_{50}$  value of different plant extracts on Brine Shrimp assay

S.N.	Plants name	$LC_{50}(\mu\text{g/ml})$
1.	A. houstonianum	27.54
2.	C. gigantea	23.44
3.	C. roseus	83.17
4.	T. peruviana(seed kernel)	25118
5.	T. preuviana(leaves)	16982.4

### 3.3 Antimicrobial Screening

Agar well diffusion method was used to evaluate antibacterial activity which was measured in the form of zone of inhibition (ZOI)<sup>[13]</sup> The results obtained from the study are tabulated as below in table no 4.

Table No.4: Showing ZOI by different microorganisms for four different plants methanol extract, well diameter: 6mm (0.6cm) concentration of loaded extract; 5%, 20% and 25%

Plant name	Concentration (%)	Diameter of Zone of inhibition (ZOI)				
		control	E. c	K. o	P. a	S. a
<i>A. houstonianum</i>	5	6	-	11	12	11
	20	6	-	11.5	12.5	11.5
	25	6	-	11.5	13.5	14.5
<i>C. gigantea</i>	5	6	8	11	8	10.5
	20	6	11	11.5	12.5	11
	25	6	12	15	16	12
<i>C. roseus</i>	5	6	-	8.5	-	11.5
	20	6	-	10	-	16
	25	6	-	11.5	-	18.5
<i>T. peruviana</i> (leaves)	5	6	8.5	-	-	8.5
	20	6	11	-	-	10
	25	6	12.5	-	-	13.5

*Escherichia coli* (E. c); *Klebsiella oxytoca* (K. o); *Pseudomonas aeruginosa* (P. a); *Staphylococcus aureus* (S.a)

Depending on the measured values of the complete inhibition diameter in mm, the anti-microbial activity can be classified into the following types such as >12 mm zone of inhibition =high sensitivity, 9-12 mm zone of inhibition= moderate sensitivity, 6-9 mm zone of inhibition =less sensitivity, and <6 mm zone of inhibition = resistant. [17] From the above experiment, it has been observed that methanol extract *Cathranthus roseus* at 125 mg/0.5 mL DMSO showed ZOI value 18.5 mm and showed greater sensitivity for *Staphylococcus aureus*. On the other hand, methanol extract of *Calotropis gigantea* (aerial parts) showed good values of inhibition for all the tested organism at all concentration under taken. All the four different extract showed ZOI values at all concentration for *Staphylococcus aureus*

### 3.4 Preferential cytotoxicity

Preferential cytotoxicity on methanol extract of different plants was performed by using PANC-1 cancer cell lines. The test was conducted in two medium namely; Nutrient-deprived medium (NDM) and in addition of salts, amino acids, serum so called Dulbecco Modified Eagle medium (DMEM) but without addition of sugars and other nutrients. [13] The absorbance of sample wells at 450 nm was measured and % cells survival verses concentration was drawn as follows.

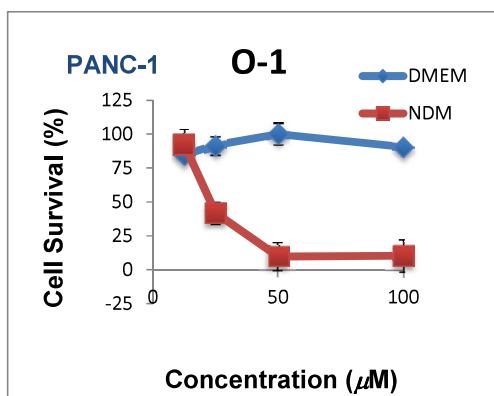


Fig1.

Preferential cytotoxicity of *A. houstonianum*

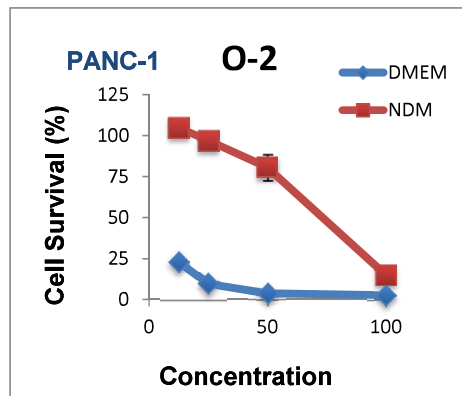


Fig2.

Preferential cytotoxicity of *C.gigantea*

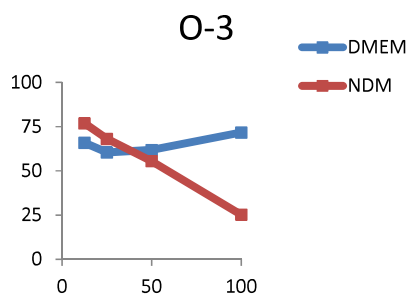


Fig3.

Preferential cytotoxicity of *C.roseus*

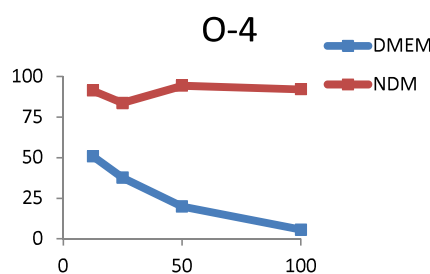


Fig4.

Preferential cytotoxicity of *T.peruviana* (seed)

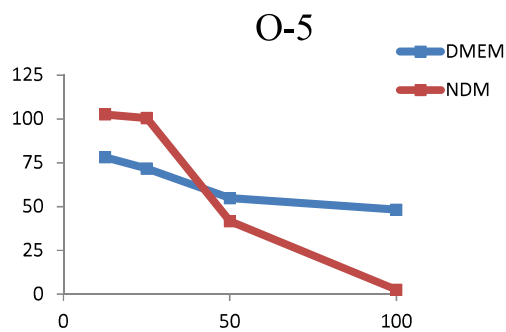


Fig5.

Preferential cytotoxicity of *T. peruviana*(leaves)

Among the five different plants, nearly 100% preferential cytotoxic activity or 0% cells survival was observed at the concentration 100 µg/mL in the cells cultured in either DMEM or NDM on *Calotropis gigantean*. In case of *Calotropis gigantea* 0%



cell survivals result also obtained at concentration 50µg/mL in cell cultured in DMEM. *Ageratum houstonianum* and *Thevetia peruviana* (seed kernels) also shows nearly 0% cell survivals at the concentration 100µg/mL in the cells cultures in DMEM. On other hand, nearly 0% cells survival was observed at concentration 100 µg/mL in the cells cultured in NDM on *Thevetia peruviana* (leaves). Other plants show milder or no activity even at high concentration.

#### 4. Result and Conclusion

Four different plants were collected from the Chitwan, central parts of Nepal. These plants were found to have high medicinal value with potential anticancer activity. Phytochemical screening report showed that, *Calotropis gigantea* contained polyphenol, flavonol glycosides, alkaloids, and other reducing compounds. Glycosides and Flavonol glycosides also reported from the plants under taken.

Brim-Shrimp bioassay result showed that *Calotropis gigantean* with LC<sub>50</sub> value 23.54 and *Ageraum houstonianum* with LC<sub>50</sub> value 27.54 were highly active. Form the antimicrobial test of crude methanol extract of five different plants it was found that *Calotropis gigantea* showed good ZOI value for all tested organism at all concentration including ZOI value 14.5mm for *Staphylococcus aureus* at concentration 125 mg/0.5 mL DMSO. Catharanthus roseus also showed high sensitivity at concentration 125 mg/0.5 mL DMSO for *Staphylococcus aureus* with ZOI value 18.5mm.

Anticancer test for PANC-1 cancer cell lines in either NDM or DMEM showed that *Calotropis gigantea* have high anticancer activity as it showed almost 0% cell survival in either Nutrient deprive medium or DMEM at the concentration 100 µg/mL.

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