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Research Article

ANTIBACTERIAL, ANTIDIABETIC AND BRINE SHRIMP LETHALITY ACTIVITIES OF SOME SELECTED MEDICINAL PLANTS FROM KAVREPALANCHOK DISTRICT OF NEPAL

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ABSTRACT

The methanol extracts of nine medicinal plants collected from Kavrepalanchok district of Nepal were subjected to their potential bioactivity viz. antibacterial, antidiabetic and toxicity tests. Antibacterial property of the extracts was evaluated against Gram-positive and Gram-negative bacteria by agar well diffusion method and antidiabetic activity was investigated by α-amylase inhibition assay. The toxicity of plant extracts was assessed by brine shrimp lethality test. All the nine different plant extracts showed antibacterial activity with the zone of inhibition (ZOI) ranging from 5 to 35 mm. Among the studied plant extracts, *Psidium guajava* showed the highest ZOI against *Salmonella typhi* (35 mm) while *Melia azedarach* was most effective against *Staphylococcus aureus* (22 mm). The percentage of α- amylase enzyme inhibition was found up to 88.56 ± 3.50 in dose dependent manner. The enzyme inhibitory concentration IC_{50} value of standard, acarbose was 166.01 µg/mL while the most effective anti-diabetic plant extract of *Utrica dioica* has 186.67 µg/mL. Moreover, various plant extracts depicted various levels of toxic activities; *Curcuma longa* demonstrated significant toxicity to *Artemia salina* with LC_{50} value 62.10 µg/mL, while *Agerantina adenophora, P. guajava* and *M. azedarach* showed moderate toxicity with 103.77, 109.37 and 383.58 µg/mL, respectively.

Keywords: Medicinal plants, Antidiabetic, Toxicity, Antibacterial, ZOI

INTRODUCTION

A great biodiversity owing to varied geographical, geo-morphological and climatic condition of Nepal has provided it many plants with medicinal and aromatic values (Baral 2005). Kavrepalanchok districts of Nepal lies between latitude 27° 21'-27° 42' N and longitude 85° 23'-85° 49' E. The altitude vary from 200-3018 m, and the average temperature ranges from 10 to 31°C (Chhetri & Gauchan 2007). About 80 % people living in rural areas of underdeveloped countries still depend on medicinal plants for their primary health care (Muhammad *et al.* 2011). Studies revealed that there was more traditional medicine provider than the allopathic practitioners especially in the rural areas (WHO 2002). In Kavrepalanchok, many communities dwell in rural areas and have no access to modern medicine. Therefore, large proportions of population especially of ethnic communities depend upon the medicinal plants to cure minor malady.

Antibacterial is an agent that kills or inhibits bacteria by directly killing them or interfere the reproduction process, thereby inhibiting bacteria proliferation. After the introduction of first antibiotic penicillin, pathogenic bacteria causing infectious diseases have been contained. However, reckless and irregular administration of commercial antibacterial drugs resulted in bacteria to develop drugs, bacteria has developed resistance against the drugs, necessitating the scientists to explore new and effective antibacterial agents that can be better alternative of current regimens (Roa *et al.* 2018, Manik *et al.* 2013). Therefore, the drug development from natural product is promising as plants show different bioactivity to cure ailments due to the presence of bioactive compounds (Joshi & Bashyal 2018, Giri & Rajbhandari 2018, Sharma *et al.* 2015a, 2015b).

Diabetes mellitus is a metabolic disease characterized by high blood sugar (glucose) levels resulting from defects in insulin secretion, or its action, or both. Such deficiency results in increased concentration of glucose in the blood which in turn damages the body's system particularly in blood vessels and nerves. Numerous medicines have been developed in order to check diabetes; however, continuous use decreases their efficacy and shows some side effects. Inhibition of α-amylase therapy is responsible for delaying the absorption of glucose after meal. Several α-amylase inhibitors have been isolated from medicinal plants for the development of new drugs with increased potentiality and lower side effects than the existing one (Arif *et al.* 2014). Plants are a potent source of antidiabetic agents. Many synthetic drugs have been synthesized to cure the diabetic mellitus. However, they are expensive and have numerous side effects. Several studies have shown that some plants used in traditional medicine have beneficial effects in diabetic patients. More than 400 plants worldwide have been documented as beneficial in the treatment of diabetes (Sathiavelu *et al.* 2013).

Toxicity studies are useful initial step in determining the potential toxicity of a substance, including plant extracts or biologically active compounds isolated from plants. Minimal to no toxicity is essential for a successful development of pharmaceutical or cosmetic preparation and in this regard, cellular toxicity studies play a crucial role (Lyndy & Jacobus 2014). For the bioactive compound of either natural or synthetic origin, lethality test is a rapid and comprehensive test. It easily utilizes a large number of organisms for statistical validation and requires no special equipment and relatively small amount of sample (2-20 mg or less) is sufficient (Sarah *et al.* 2017). The larva, nauplii about 22 mm long, are large enough to observe without high magnification and small enough for hatching in enormous amount without extensive workspace in a laboratory (Meyer *et al.* 1982).

MATERIALS AND METHODS

Collection and identification of plant samples

Different parts of nine medicinal plants *Spondias pinnata*, *Melia azedarach*, *Psidium guajava*, *Agerantina adenophora*, *Utrica dioica*, *Bauhinia variegata*, *Achyranthes aspera*, *Curcuma longa* and *Eleocarpus anjastifolius* were collected from a farmland of Panchkhal municipality, Kavrepalanchok, Nepal. The plants were collected in the summer (May/June) of 2017. The plants were identified by the Prof. Dr. Mohan Sivakoti and Prof. Dr. Sangeeta Rajbhandari, Central Department of Botany, Tribhuvan University, Kathmandu, Nepal.

Extract preparation

Cleaned parts of plants were dried under the shade at room temperature. Dried samples were chopped into pieces and powdered using a mechanical grinder. Dried powder (100 g) was mixed separately in 400 mL methanol. The flasks were sealed tightly and extraction was done for 72 hours with occasional shaking. The obtained extracts were filtered and concentrated in a rotary evaporator. The yield of each fraction was determined and all the extracts were stored at 4 ºC in a refrigerator until analyses.

Antibacterial assay

Antibacterial screening of the plant extracts was performed by agar well diffusion method. Effectiveness of antimicrobial substance was evaluated by determination of ZOI as per the protocol adopted by Cavalieri *et al.* (2005) with little modification. The bacterial strains which were used for the test were obtained from Micro Med, Thapathali, Kathmandu.

The bacterial strains were *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Salmonella typhi*. They were inoculated and cultured on nutrient broth. Sterile culture plates of Muller-Hinton agar were prepared. A McFarland 0.5 standard was prepared and the bacterial suspension was compared with it to adjust the turbidity of the inoculums for the susceptibility test. The inoculums of bacteria were transferred into Muller-Hinton agar using sterile cotton swab. The swab was used to spread the bacteria on the media in a confluent lawn. Wells were prepared by punching the bacterial culture inoculated agar plate with the help of sterile cork borer of 6 mm size. Four to six wells were made at equidistance on the plate. Then, 100 and 10 mg/mL of the working solution of the plant extract, 50 % DMSO in water as a negative control and ofloxacin (0.3 %) as a positive control were loaded into the respective wells with the help of a micropipette. The plates were then left for half an hour with the lid closed so that the extract diffused into media. The plates were incubated overnight at 37^{o} C. After 24 hours of incubation, the plates were observed for the presence of inhibition of bacterial growth indicated by clear zone around the wells. The size of the ZOI was measured. The absence of ZOI was interpreted as the absence of activity.

α-Amylase inhibition assay

α-amylase inhibition assay was performed following the standard protocol (Kusano *et al.* 2011). Starch was used as a substrate; α-amylase converts starch into sugars. But acarbose inhibits the action of α-amylase and hence decreases or stops the conversion of starch. The plant extracts whose antidiabetic activities are to be examined, must have same property as acarbose, i.e. they should retard or inhibit activity of the α-amylase enzyme. Acarbose, a drug used for treatment of type II diabetes mellitus was used as standard since it inhibits α-amylase enzyme.

The amount of 40 µL of starch solution was pre-incubated at 37^{o} C for 3 minutes with 20 µL of acarbose or plant extract at varying concentration of plant extract 40, 80, 160, 320 and 640 µg/mL followed by adding 20 µL of 3 U/mL α-amylase which were dissolved in phosphate buffer solution and incubation at 37° C for 15 minutes. Termination of the reaction was carried out by adding 80 µL of HCl (0.1 M) in pre-incubated mixture having amylase. Then, 100 µL of iodine reagent (2.5 mM) was added in each solution and the final volume was made to 5 mL by adding distilled water. Absorbance was measured at 630 nm. The assay was carried out in triplicate for reliable result. Percentage of inhibition was calculated using equation (1).

% inhibition = (1- (Abs2- Abs1/ (Abs4- Abs3)*100 (1)

Where,

Abs1 = absorbance of the incubated mixture containing plant sample, starch, and α-amylase

Abs2 = absorbance of incubated mixture of sample and starch

Abs3 = absorbance of the incubated mixture of starch and α-amylase

Abs4 = absorbance of incubated solution containing starch.

Brine shrimp toxicity bioassay

The toxicity assay was performed by using brine shrimp assay following the standard protocol (Meyer *et al.* 1982). The artificial sea water was prepared by dissolving 15 mg of sodium chloride, 0.45 g of potassium chloride, 0.55 g of calcium chloride and 1.76 g of magnesium sulphate in distilled water to make 500 mL solution. Brine shrimp nauplii hatching tank was filled with water and the eggs sprinkled in to the covered part of the tank. The bioactive compounds/extracts show toxicity towards brine shrimp larvae. This method evaluates the toxicity of extracts towards the nauplii by determining the LC_{50} (µg/mL). Compounds of LC_{50} value less than 1000 ppm are considered as potentially pharmaceutically active. LC_{50} value is the total lethal concentration dose required to kill 50 % of the shrimps. It can be determined as follows:

$$\alpha = \frac{1}{n}(\sum y - \beta \sum x) \quad (2)$$

and

$$\beta = \frac{\sum xy - \sum x \sum y / n}{\sum x^2 - (\sum x)^2 / n} \quad (3)$$

where, 'n' is the number of replicates (here three), 'x' is the log of constituents in mg/mL (log10, log100 and log1000 for three dose level respectively, 'y' is prohibit for average survivor of all replicates.

From probit regression $y = \alpha + \beta X$ (4)

$$X = \frac{(y - \alpha)}{\beta} \quad (5)$$

where, Y is a constant having value 5 for calculating LC_{50} values.

Thus, LC_{50} = Antilog X (6)

Statistical analysis

Data were recorded as mean ± standard deviation of three determinations of absorbance for each concentration from which linear correlation coefficient (R^2) value was calculated using MS Office Excel 2007. The linear regression equation for a straight line is, y = mx + c where, y = absorbance of the extract, m = slope of the calibration curve, x = concentration of the plant extract and c = intercept.

RESULTS AND DISCUSSION

Quantitative estimation of extract and percentage yield of nine different plants was studied on the methanol solvent. Different plants gave different percentage of yield. The yield percentage recovery of extract ranged from 2.94 percent to 19.38 percent in *Utrica dioica* and *Bauhinia variegate*, respectively. Table 1 presents the percentage yield of different plant extracts. The yield percentage was found in varied quantities and it might be due to the solvent polarity which affects the solubility of the constituents found in different plant parts.

Table 1. Percentage yield of different plant extracts

Name of plants	Quantity (g)	Part used	Percentage yield (%)
S. spinnata	100	Bark	13.79
M. azedarach	100	Bark	13.79
P. guajava	100	Bark	15.63
A. adenophora	100	Whole plant	10.33
U. dioica	100	Root	2.94
B. variegate	100	Bark	19.38
A. aspera	100	Whole plant	5.61
C. longa	100	Rhizome	9.67
E. anjastifolius	100	Seed	5.62

Antimicrobial activity

Antibacterial activity of the extract of different plants evaluated *in vitro* against Gram-positive and negative bacteria is shown in Table 2. The measurement of ZOI produced by different plant extracts at concentration of 10 mg/mL and 100 mg/mL against three different bacteria *Salmonella typhi*, *Escherichia coli* and *Staphylococcus aureus* are illustrated in Table 2. All the extracts exhibited some antibacterial activity against potential pathogenic bacteria. Previous study showed that high antibacterial activity was recorded in *P. guajava* extract with ZOI 35 mm and in *A. aspera* extract with 19 mm of clear inhibition zone against *S. typhi* and *E. coli* respectively. Methanolic extract of *P. guajava* showed mean ZOI 8.27 and 12.3 mm against *Bacillus cereus* and *S. aureus*, respectively and no ZOI against Gram-negative bacteria (Biswas *et al.* 2012). The zone of inhibition increased with increase in the concentration of plant extracts (Pokhrel *et al.* 2015). Standard antibiotic, ofloxacin (0.3 %) showed zone of inhibition ranging from 20 to 38 mm against all test organisms. The antibacterial activity showed by different plant extracts can be attributed to different secondary metabolites present in plants.

ZOI ranged from 12 mm to 19 mm against *E. coli* and 19 mm being ZOI of *P. guajava* at 100 mg/mL concentration and the standard, ofloxacin (0.3 %) showed ZOI of 35 mm. Similarly ZOI ranged from 12 mm to 22 mm against *S. aureus*. 22 mm being of *M. azederach* at 100 mg/mL concentration and the standard, ofloxacin (0.3 %) showed ZOI of 38 mm against *S. typhi*. The plant extract of

Psidium guajava showed ZOI 35 mm was the remarkable antibacterial potential against *Salmonella typhi* at 100 mg/mL concentration. The standard, however, showed ZOI of only 20 mm against *S. typhi* strain.

Table 2. Antibacterial activity of plant extracts on *Salmonella typhi, Escherichia coli* and *Staphylococcus aureus*

Plant extracts	ZOI mm (*Salmonella typhi*)		ZOI mm (*Escherichia coli*)		ZOI mm (*Staphylococcus aureus*)	
	10 mg/mL	100 mg/mL	10 mg/mL	100 mg/mL	10 mg/mL	100 mg/mL
Spondias pinnata	11	26	10	0	16	21
Melia azedarach	6	22	12	14	12	**22**
Psidium guajava	**20**	**35**	**17**	19	**17**	21
Agerantina adenophora	17.5	21	13	14	0	0
Bauhinia variegata	12	24	0	0	15	21
Achyranthes aspera	5	20	**17**	**20**	13	13
Eleocarpusan jastifolius	15	26	11	0	0	13
Curcuma longa	11	17	15	17	15	15
Positive control (Ofloxacin)	20		35		38	
Negative control	0		0		0	

ZOI ranged from 0 mm to 20 mm against *E. coli* and 19 mm being ZOI of *P. guajava* at 100 mg/mL concentration and the standard, ofloxacin (0.3 %) showed ZOI of 35 mm. Similarly, ZOI ranged from 12 mm to 22 mm against *S. aureus* with highest 22 mm being from *M. azederach* at 100 mg/mL concentration. The standard ofloxacin showed ZOI of 38 mm against *S. typhi*. The plant extract of *P. guajava* showed highest ZOI of 35 mm, which was the remarkable antibacterial potential against *Salmonella typhi* at 100 mg/mL concentration. The standard, however, showed ZOI of only 20 mm against *this* strain.

In-vitro α-amylase inhibition activity

α-amylase inhibitory activity of plant extracts was determined from quantitative starch-iodine method. The percentage inhibition of different plant extracts *versus* concentration and antidiabetic activity in term of IC_{50} values of different extract are shown below:

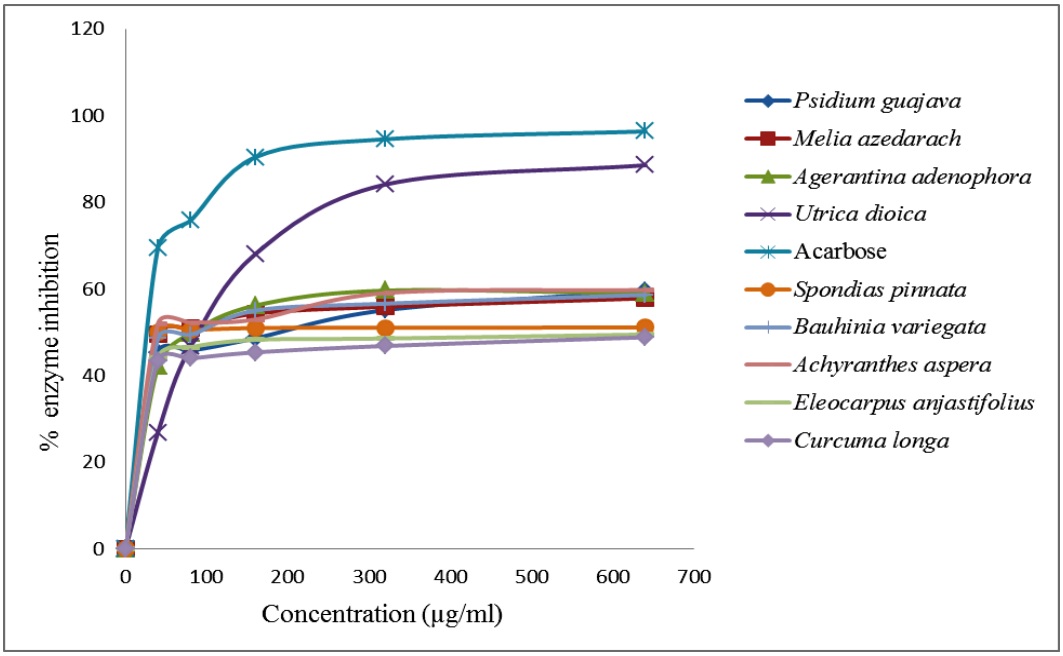

Fig. 1. α-amylase inhibition activity with different concentration of plant extracts

Table 3. α-Amylase inhibition activity

Plant extracts	IC_{50} (µg/mL)
Acarbose	166.01
Spondia spinnata	412.72
Melia azedarach	515.91
Psidium guajava	339.61
Agerantina adenophora	304.75
Utrica dioica	186.67
Bauhinia variegata	309.26
Achyranthes aspera	284.5
Eleocarpus anjastifolius	471.74
Curcuma longa	505.63

The percentage inhibition of *Utrica dioica* was found to be concentration dependent with a steep rise in percentage inhibition with concentration and its value was 88.56 ± 3.50 at 640 µg/mL concentration. The standard, acarbose displayed percentage inhibition of 90.49 ± 1.75 at the same concentration. The plant extracts except *Utrica dioica* showed the percent inhibition ranging from 40 to 60 with gradual increase in percent inhibition with increase in concentration. Again, the IC_{50} values of different plants extract along with standard were evaluated and found that the value ranged from 186.67 µg/mL to 515 µg/mL. The maximum percent inhibition by *Utrica dioica* depicted the IC_{50} value 186.67 µg/mL while standard, acarbose showed IC_{50} value of 166.01 µg/mL. Thus, among the selected medicinal plants, *U. dioica* showed the highest percentage inhibition of α-amylase.

Brine shrimp toxicity

The pure compounds/extracts from the natural source must be examined for its toxicity before it is preceded for translational studies. The extract of different medicinal plants was screened for *in vitro* cytotoxic activity by brine shrimp lethality test.

The result showed that LC_{50} of different plant extracts ranged from 62.10 to 1.20×10^7 µg/mL of *Curcuma longa* and *Achyranthes aspera*, respectively. *Curcuma longa* can be inferred to have very strong toxic effect. Likewise, *Agerantina adenophora*, *Psidium guajava* and *Melia azedarach* revealed a moderate toxicity with 103.77, 109.37 and 383.58 µg/mL, respectively. So, these plants can be suggested to be used as therapeutic agents. However, *Achyranthes aspera*, *Bauhinia variegata*, *Utrica dioica* and *Spondias pinnata* showed very low or insignificant toxicity effect. Plant extract resulting in LC_{50} less than 1 mg/mL are considered toxic to the larvae (Muhammad & Sirat 2013)

Table 4. Toxicity of plant extracts

Plant extracts	LC_{50} (µg/mL)
Spondia spinnata	146702.59
Melia azedarach	383.58
Psidium guajava	109.37
Agerantina adenophora	103.77
Utrica dioica	32944.11
Bauhinia variegata	283486.37
Achyranthes aspera	1.20×10^7
Eleocarpus anjastifolius	1391.12
Curcuma longa	62.10

CONCLUSION

This study provides some scientific support for the use of traditional medicinal plants in the management of several ailments. *P. guajava*, *M. azedarach* and *A. aspera* can be used as the potential sources for natural antibacterial agent and provides scientific support to the traditional claims of various tribes regarding curative effects. *C. longa* showed high toxic effect in comparison to plant extracts *Melia azedarach*, *Psidium guajava* and *Agerantina adenophora* with moderate cytotoxic effect. Anti-diabetic assay depicted the wide range of percent inhibition in different plants extracts. However, *U. dioica* was found potent α-amylase inhibitor and showed the inhibition near to the standard acarbose. The plant extract with lower IC_{50} value will be greatly beneficial to reduce the rate of digestion and absorption of carbohydrate and thereby contribute for effective treatment of diabetes by decreasing hyperglycemia. Thus we recommend that further bioassay guided fractionation and isolation approaches are required on the active plant extract to identify the compound responsible for the promising *in-vitro* anti-diabetic activity.

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