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Phytochemical, antimicrobial and cytotoxic evaluation of *Indigofera serpentinicola*

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Abstract

Methanol and aqueous root extracts of *Indigofera serpentinicola* were investigated for their phytochemical, antimicrobial and cytotoxic properties. Phytochemical analysis showed the presence of alkaloids, saponins, tanins, terpenoids, cardiac glycosides, phenols, reducing sugars oils and fats in both extracts. Flavanoids were only detected in the methanolic extract. Antimicrobial activity was evaluated using the Kirby-Bauer disk diffusion method. The extracts showed low activity against *Staphylococus aureus, Cornybacterium diptheriae, Pseudomonas aeruginosa* and *Salmonella enterica* and no activity against *Escherichia coli*. The diameters zones of inhibition ranged between 3-10 mm. The aqueous extract had higher activity showing zones of inhibition of 10 mm against *S. enterica*. Brine shrimp lethality test showed LC_{50} values which ranged from 0.079-0.158 mg/mL, showing that the extracts were highly toxic.

Introduction

Zimbabwe is a country with many natural resources and its people are highly dependent on vegetation as a source of many products such as food, fuel and medicine (Mavi and Shava, 1997). Out of the 6,000 plant species in the country, 500 have been reported as medicinal (Gelfand et al., 1985). Use of natural medicines has increased but many of these medicines have not been scientifically analyzed (Maroyi, 2011). "According to World Health Organization, more than 80% of the world's population relies on traditional medicine for primary healthcare needs" (Sasidharan et al., 2010). There is currently a worldwide search for phytochemicals which could be developed for the treatment of infectious diseases especially in light of the emergence of drug-resistant microorganisms and the need to produce more effective antimicrobial agents (Ncube et al., 2008)

Indigofera is a large genus of more than 700 species of flowering plants belonging to the family Fabaceae. The species occur throughout the tropical and subtropical regions of the world and in Zimbabwe approximately 100 species have been recorded. The species are shrubs, though some are herbaceous and a few can become small trees. Of the various *Indigofera* species, *Indigofera tinctoria* and *I. suffruticosum* are well known for producing the dye indigo (Leite et al., 2003). Some Indigofera species are used in anti-cancer therapy (Vieira et al., 2006) and others for toothache, ulcers and swellings or as analgesics and anti-inflammatory agents (Prabakaran et al., 2011).

I. serpentinicola is a woody perennial shrub endemic to the serpentine soils of the Great Dyke of Zimbabwe. The plants arise from underground rootstocks and bear pinnate leaves and pink brightly vivid flowers. Some local inhabitants of the Great Dyke strip the root bark from *I. serpentinicola* plants, boil them in water and use them to treat painful conditions and fever. The efficacy or otherwise of this plant species has not been scientifically evaluated. This study evaluates the phytochemical profile of this plant and assesses its cytotoxicity and antimicrobial properties.

Materials and Methods

Collection of plant material

I. serpentinicola roots were freshly collected from Mutorashanga Pass along the Great Dyke of Zimbabwe in January 2014. Plant identification was done *in situ*. Voucher specimens are deposited at the University of Zimbabwe teaching herbarium. Freshly collected roots were washed and oven dried at 48°C for 72 hours.

Crude extraction

Mechanical methods including milling and grinding using mortar and pestle were used to pulverize bark to a powder. Some 108.7 g of the powder was dissolved in 1,087 mL of water using a 1:10 extract: solvent ratio. 102.5 g of the powder was dissolved in 1,025 mL of methanol. Cold maceration with constant agitation was used to extract bioactive compounds over 48 hours. Residue was removed through use of a Buchner flask under vacuum. The aqueous solvent was evaporated from the filtrate at 100°C to concentrate the extract and the methanol solvent was evaporated under reflux at 67°C. Extract was stored at 4°C in a fridge in airtight containers.

Phytochemical screening

Phytochemical evaluation for major phytochemicals was done using standard qualitative methods (Sowofora, 1993; Tiwari et al., 2011). Tests for presence of reducing sugars, alkaloids, anthraquinones, tanins, terpenoids, saponins, oils and fats, flavonoids and cardiac glycosides were carried out on both extracts. The methods used are briefly described below.

Test for reducing sugars (Fehling's test)

1 mL of extract in 10 mL of water was mixed with 5 mL of boiling Fehling's solution (A and B). A brick-red, orange or yellow precipitate showed presence of reducing sugars.

Test for anthraquinones (Borntragor's test)

0.5 mL extract was boiled with 10 mL sulphuric acid and filtered while hot. The filtrate was shaken with 5 mL of chloroform. The chloroform layer was pipetted into a test tube and 1 mL of dilute ammonia added. A dark or violet colour confirmed presence of anthra-quinones.

Test for terpenoids (Salkowski's test)

To 0.5 mL extract was added to 2 ml of chloroform. 3 mL of conc. sulfuric acid was carefully added to the sides of the test tube to form a layer. Reddish-brown colour at the interface shows terpenoid presence.

Test for terpenoids (Lieberman-Buchard test)

A few drops of acetic-anhydride were added to 1 mL of sample and boiled. A few drops of conc. sulphuric acid were added to the sides of the test tube. Brown ring formation at the junction and an upper green layer indicate positive result for terpenoids.

Test for flavonoids

Four methods were used: a) 5 mL of dilute ammonia was added to 1 mL of the extract. 1 mL of conc. sulfuric acid was added and coloration that disappears on standing showed a positive result; b) 1% dilute ammonia was added to the extract. A yellow colouration indicates a positive result; c) 2 mL of extract was heated with 10 mL ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 mL of the filtrate shaken with 1 mL dilute ammonia. A yellow coloration indicates presence of flavonoids; d) 5 mL of sodium hydroxide were added to 5 mL of the extract. Formation of a deep yellow color that lessens when a few drops of dilute sulphuric acid are added indicates presence of flavonoids.

Test for saponins (Foam test)

5 mL distilled water were added to 0.5 mL of the extract in a test tube and shaken vigorously. The solution was observed for the formation of a persistent froth. Emulsion formation on mixture of the froth with olive oil indicates saponin presence.

Test for tannins (Ferric chloride test)

0.5 mL of the extract was diluted with 10 mL water and boiled in a test tube. After boiling it was filtered and a few drops of 0.1% ferric chloride added. A yellow to red precipitate indicated presence of tannins.

Test for alkaloids (Mayer's and Draggendorf's tests)

0.5 mL of extract was diluted with 10 mL of acidified alcohol and boiled and filtered. 5 mL of the filtrate was taken and 2 mL of dilute ammonia added. 5 mL of chloroform was also added while gently shaking the extract. 10 mL acetic acid was used to extract the chloroform layer and this was divided into two portions. To one portion, Draggendorf's reagent which shows a red-brown precipitate if in the presence of alkaloids was added. To the other, Mayer's reagent which forms a cream precipitate in the presence of alkaloids was added.

Test for alkaloids (Wagner's test)

To 5 mL of the extract was added Wagner's Reagent. A reddish-brown precipitate indicates presence of alkaloids.

Test for cardiac glycosides (Keller-Killiani) test

0.5 mL of extract was diluted in 5 mL of water. 2 mL of glacial acetic acid was added with a drop of ferric chloride solution. 1 mL of concentrated sulfuric acid was used to underplay this. A brown ring at interface shows presence of cardenolides.

Tests for fats and oils

Stain test

A small quantity of the extract was pressed between two filter papers. A stain indicates a positive result for oils.

Saponification test

To 2 mL of the extract was added a few drops on 0.5N alcoholic potassium hydroxide. A drop of phenolphthalein was also added and the sample heated for 1-2 hours on a water bath. A positive result for fats and oils is shown by the formation of soap or partial neutralization of the alkali.

Test for phenols (Ellagic acid test)

To 5 mL of the extract was added to 2 mL of 5% glacial acetic acid and 2 mL of 5% sodium nitrite.

Antimicrobial activity evaluation

The test organisms included both Gram negative (*Pseudomonas aeruginosa, Salmonella enterica, Escherichia coli*) and Gram positive (*Cornybacterium diptheriae (Diphteroids), Staphylococcus aureus*). These were obtained from stock cultures maintained at the University of Zimbabwe's Medical School.

Kirby Bauer disk diffusion method

Mueller-Hinton broth and Muller-Hinton agar were prepared as per manufacturer's instructions. The bacterial specimens were cultured from slants in the Muller-Hinton broth for 24 hours at 37°C. The cultures were washed twice using sterile 0.9% saline solution after incubation. The cultures were centrifuged using a Heraeus labofuge 200 centrifuge at 3,000 rpm for 10 min forming a bacterial pellet. Supernatant was decanted and pellet resuspended in sterile 0.9% saline solution to a turbidity of 0.5 McFarland's standard.

At this turbidity, a bacterial suspension has approximately, 1 x 10^8 cfu/mL. McFarland standard was prepared by mixing, 0.5 mL of 0.048M $BaCl_2.H_20$ and 99.5 mL of 0.18M H_2SO4 (1% acid) (Bailey and Puyvelde 1986). 100 µL of each bacterial suspension was uniformly spread onto the sterile Muller-Hinton agar plates in triplicates. Paper discs approximately 6 mm in diameter were made from Whatman filter paper No. 1. These discs were soaked in different serial dilutions with distilled water (10^{-1}-10^{-5}) of extract with a range from 110.3-0.01103 mg/mL for the aqueous extract and 965-0.0956 mg/mL for the methanolic extract. Control discs were soaked with serial dilutions (10^{-1}-10^{-5}) of tetracycline (mg/mL) and, ampicillin (mg/mL) as positive controls. Using aseptic technique, the discs were transferred to the appropriate bacteria pre-inoculated plates.

Discs soaked in sterile water served as the negative control. The plates were incubated for 24 hours at 37°C. The antimicrobial activity was evaluated by measurement of the zones of inhibition and interpreted according to the parameters by Wilker et al., (2007), i.e. zones of inhibition ≤ 19 mm = susceptible, ≤11 mm = resistant and 15-18 mm = intermediate range.

Minimum Inhibitory Concentration (MIC) determination

MIC of the extracts was determined by the disc diffusion assay (Gaudreau et al., 2007). For each extract, 5 concentrations of extract i.e. from 110.3-0.01103 mg/mL for the aqueous and 965-0.0956 mg/mL for the methanolic were used. Filter discs soaked in the different concentrations for each extract were then placed on Muller-Hinton agar plates pre-inoculated with 100 µL of bacterial suspension. The plates were incubated for 24 hours at 37°C. The MIC was determined to be the lowest concentration at which zones of inhibition were observed.

Brine shrimp lethality test

The brine shrimp lethality bioassay by Meyer et al. (1982) was used to test cytotoxicity of the extracts on brine shrimp (*Artemia salina*). 2 g of *A. salina* eggs were hatched in 2% artificial seawater over 24 hours. The nauplii hatched under a bright light with aeration. One litre of sea water was prepared by mixing 12 g NaCl and sterile distilled water. The experiment was done in triplicates using serial dilutions (10^{-1}–10^{-5}) of the extracts as follows: 110.3-0.01103 mg/mL for the aqueous and 965-0.0956 mg/mL for the methanolic extracts. Aliquots of 50 µL were transferred to sterile vials containing 5 mL of aerated artificial seawater. The amount of shrimp added to each vial was counted. 1% DMSO added to the seawater acted as negative control and potassium dichromate (mg/mL) as a positive control. Number of live *A. salina* after 24 hours was counted using a handheld magnifying lens and using a light source.

Data analysis

The data generated by the study was ana-lysed using Microsoft Excel 2010. The same programme was used to draw the graphs and generate equations. Probit analysis was done using linear regression to calculate LC_{50}, for the cytotoxicity determination.

Results and Discussion

Pharmacological evaluations depend to a large extend on effective phytochemical extraction procedures. Our results show that comparatively methanol is a better solvent than water. This is evidenced by methanol's ability to extract additional phytochemicals like flavor-

Table I

Phytochemical screening tests results of *I. serpen-tinicola* root extracts

Test	Solvent	
	Methanol	Water
Reducing sugars	+	+
Anthraquionones	++	+
Terpenoids (Lieberman-Buchard)	++	+
Terpenoids (Salkowski)	++	++
Flavonoids	++	-
Saponins	++	++
Alkaloids (Wagner's)	++	++
Alkaloids (Mayer's)	-	-
Alkaloids (Draggendorf's)	++	++
Phenols	+	+
Oils and fats	++	+
Cardiac glycosides	++	+
Tannins	++	++
Saponification	++	++

++ strong presence; + slight presence; - absent

noids and also its ability to extract higher concentrations of cardiac glycosides and anthraquinones as compared to the water extract (Table I). The Mayer's reagent produced a negative test for alkaloids in contradiction to the Wagner and Dragendorff tests. This may possibly be due to the old Mayer's reagent used in this experiment which may have lost its activity with time. Additionally, there was also a higher extract yield of 45.6% in the methanol solvent than in water which had a yield of 19.9%.

The Figure 1 and 2 below show probit analyses for the brine shrimp mortality data. There was an increase in mortality with increasing extract concentrations. The respective regression lines gave LC_{50} values of 0.158 mg/mL for the aqueous extract and of 0.079 mg/mL for the methanolic extract. The LC_{50} values for both extracts were less than <1000 µg/mL that Meyer et al. (1982) noted to be the threshold of toxicity for plant extracts. This makes the extract's toxicity very high as little amount of the extract is required to bring about mortality of 50% of *A. salina* for both the aqueous and methanol extract.

Cytotoxicity is attributed to phytochemical compounds like saponins and alkaloids (Musa, 2012; Olaleye, 2007), which have been observed in this study. Cardiac glycosides also are a source of toxicity that has been observed in come medicinal plants (Bronstein et al., 2006). *I. serpentinicola,* therefore, is a potential source of cytotoxic agents which could be developed for medical use.

The plant extracts showed some activity against both Gram positive and negative bacteria, indicating a possible broad spectrum of activity. Although the activity was much lower as compared to the standard antibiotics, our results however confirm some activity in concurrence with those obtained by other researchers working on other *Indigofera* species (Leite et al., 2006; Dahot, 1999; Esimore et al., 1999).

Figure 3 presents a generalized diagram of the relative sizes of the zones of inhibition of all the test bacteria against the *I. serpentinicola* extracts and the standard antibiotics.

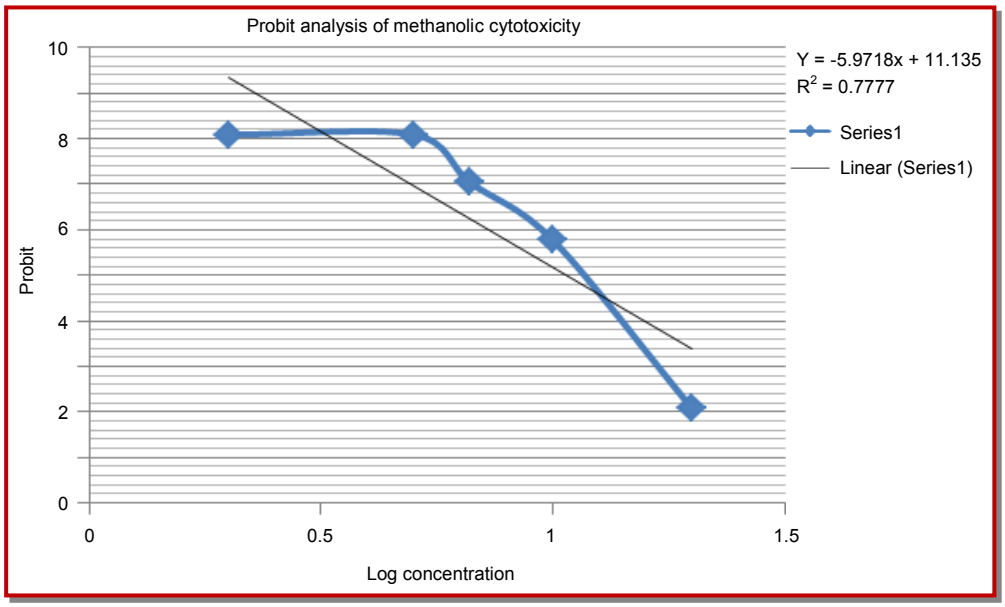

Figure 1: Brine shrimp mortality against methanol extract concentration

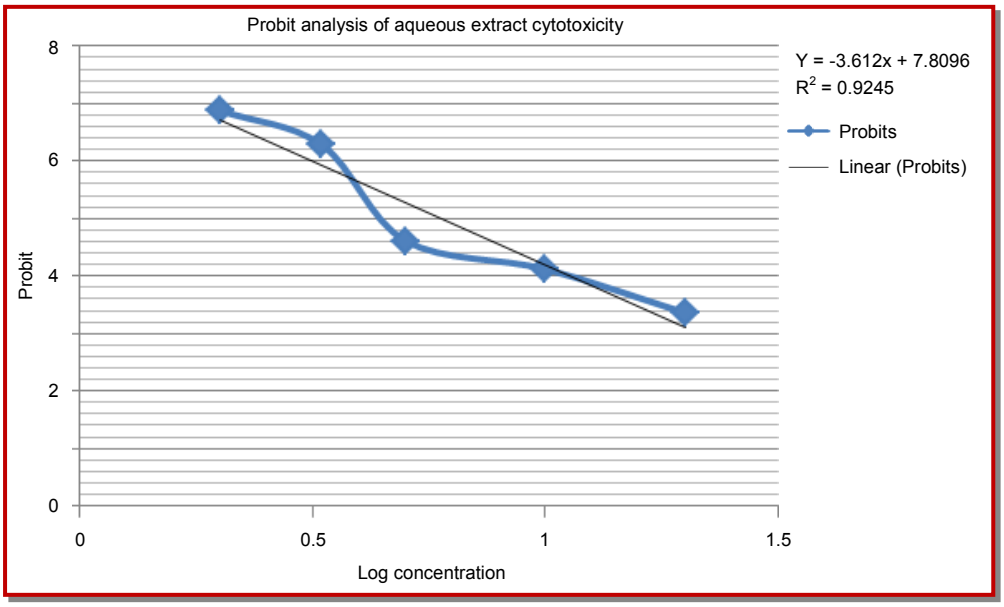

Figure 2: Brine shrimp mortality against water extract concentration

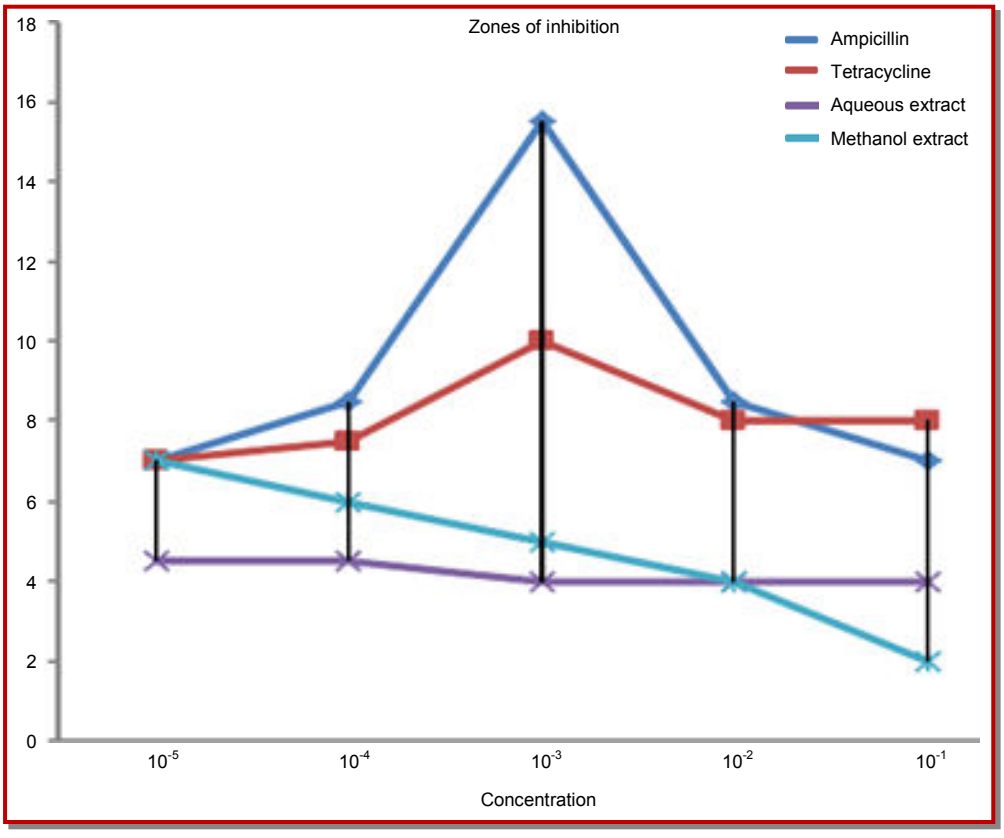

Figure 3: Zones of inhibition of the test bacteria in *I. serpentinicola* extracts

Table II

The bacterial MICs for the aqueous extracts

Test organism	Concentration (mg/mL)				
	110.3	11.03	1.103	0.1103	0.01103
S. aureus	No growth	No growth	Growth	Growth	Growth
S. enteric	No growth	No growth	Growth	Growth	Growth
Diptheriae	No growth	No growth	No growth	Growth	Growth
P. aeruginosa	No growth	No growth	Growth	Growth	Growth

Table III

The bacterial MICs for the methanolic extracts

Test organism	Concentration (mg/mL)				
	965	96.5	9.65	0.965	0.0965
S. aureus	No growth	No growth	No growth	No growth	Growth
S. enterica	No growth	No growth	No growth	No growth	Growth
Diptheriae	No growth	No growth	Growth	Growth	Growth
P. aeruginosa	No growth	No growth	No growth	Growth	Growth

The results show that Gram positive bacteria i.e. *S. aureas* and *C. diphtheriae* are more sensitive to the extract, and this is supported by previous studies (Ceylan and Fung, 2004). The sensitivity may be due to the structural constituents of their cell walls which readily allow the movement of antibiotic into the cells.

Activity of the extract against *P. aeruginosa* as well as *S. enterica* is significant as these pathogens are able to mutate and become resistant to most antimicrobial agents. Gram negative bacteria have been shown to be resistant to most anti-bacterial agents including plant based extracts (Kambizi and Afolayan, 2008). This is due to their possession of an external impenetrable phospholipid membrane containing lipopolysaccharides (Rabe and Staden, 1997).

Antimicrobial activity is often attributed to phytochemicals such as terpenoids, flavonoids, tannins, phenolic compounds or presence of free hydroxyl groups (Rojas et al., 1992). Flavonoid based antimicrobial activity is thought to be a result of their capacity to disrupt enzymatic action in cell division, platelet aggregation and immunological responses and complex formation in the bacterial cell wall as well as extracellular and soluble proteins (Yadav and Agarwala, 2011). Flavonoids are also used by plants in their own defense against microbial agents. Terpenoid activity is said to be from their ability to disrupt membranes while tannins act by interfering with protein synthesis through binding to proline rich areas (Cowan, 1999).

The activity of the plant extract against *S. aureus* and *S. enterica*, gives authentication for its use in abdominal conditions as the bacteria are gastrointestinal pathogens. This means that the *I. serpentinicola* has potential as an antimicrobial agent and could be the solution to many of the antibiotic resistant bacteria such as *P. aeruginosa*. However, due to the low activity, the active antimicrobial agent would have to be identified through further research and then concentrated in a final product to increase its efficiency.

The bacterial MICs for the aqueous and methanol extracts are shown on Table II and III below. Plant extracts are generally considered of pharmacological interest if minimum inhibitory concentration values are less than 1 mg/mL (da Silva et al., 2012). Using this benchmark, the MICs for *S. aureus* and *S. enterica* show values less than 1 mg/mL for the methanol extracts showing their potential pharmacological use. *S. aureus* is Gram positive and *S. enterica* Gram negative showing the broad spectrum activity of the methanol extract.

In conclusion, the use of *I. serpentinicola* as a medicinal plant is justified as pharmacological activity in the form of antimicrobial activity has been demonstrated.

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