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PHYTOCHEMICAL COMPOUNDS AND ANTIMICROBIAL ACTIVITY OF EXTRACTS OF *INDIGOFERA SPICATA* (CREEPING INDIGO) AGAINST *CANDIDA ALBICANS, STREPTOCOCCUS MUTANS* AND *E. COLI*

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ABSTRACT

Indigofera spicata has been used by Kamba community of Kenya as an herbal medicine over a long period to treat coughs and toothaches. There is scanty information on the phytochemical compounds and the antimicrobial activity of *Indigofera spicata* plant extracts on *Candida albicans, Streptococcus mutans and E. coli,* which are known to cause many opportunistic infections to humans. This study aimed at investigating the phytochemical compounds and antimicrobial activity of *Indigofera spicata* leaves, roots and bark extracts against *Candida albicans, Streptococcus mutans and E. coli*. Plants were collected and plant parts dried separately and crushed to obtain a fine powder which was used to extract the crude extracts using methanol solvent. Plant samples were subjected to qualitative phytochemical tests for the identification of chemical constituents present using standard qualitative methods. Phytochemical constituents were analyzed by thin layer chromatography (TLC) in appropriate solvents (n-hexane and methanol, 3:1). The plates were observed under UV lamp at wavelength of 540nm, and Rf values were calculated for each spot. 5mm diameter paper discs were each dipped in a known concentration of the extracts of 0% (distilled water), 25%, 50% and 75% methanol extracts for 2 minutes and placed in either PDA or nutrient agar inoculated with the test organisms. Three discs were placed per petri dish and were incubated at 28 degrees and 48 hours for fungi and 37 degrees for 18-24 hours for bacteria. The experiment was arranged in a completely randomized design in the incubator. The growth diameter of inhibition was measured with a ruler. Data obtained on zone of inhibition was subjected to analysis of variance (ANOVA) by use of SAS statistical package. The methanolic extracts of the plants revealed the presence of tannins, sterols, alkaloids, saponins and terpenoids. However cardiac glycosides, phenols and flavonoids were absent in all the plant parts. Terpenoids were present in the stems and roots. Elution of the column with the n-hexane and methanol led to isolation of uncharacterized active compounds, three in leaves and stems, and two in roots. There were significant differences ($p<0.05$) between the extract concentrations, plant parts and the test microorganisms used. The leaves had the highest inhibitory effects at 75% methanol extract, i.e (5.7+0.2 mm, 6.8+0.2 mm and 8.3+0.7 mm growth diameter of *Candida albicans, Streptococcus mutans and Escherichia coli respectively*) compared to roots (5.7+0.2 mm, 6.0+0.3 mm and 7.0+0.3 mm growth diameter of *Candida albicans, Streptococcus mutans and Escherichia coli respectively*) and stem (5.8+0.2 mm, 5.3+0.2 mm and 7.7+0.2 mm growth diameter of *Candida albicans, Streptococcus mutans and Escherichia coli respectively*). The study suggests the potential of the plant as a source of new antimicrobial agents.

Keywords: antimicrobial activity, Indigofera spicata, microorganisms, opportunistic infections, phytochemical analysis

INTRODUCTION

The success of modern medical science largely depends on drugs originally obtained from natural resources. Plants have provided mankind with a broad and structurally diverse array of pharmacologically active compounds. These chemicals include; flavonoids, alkaloids, carotenoids, tannins, antioxidants and phenolic compounds. These bioactive compounds are used as starting points for antibiotics synthesis in order to treat infectious diseases (**Rahman *et al*., 2018**). From the dawn of civilization, people have developed a great interest in plant-based drugs and pharmaceutical products (**Shahzadi *et al*., 2010**). According to **Musyimi *et al*. (2007)** and **Keter *et al. (2012)***, about 70-80% of the people in the world depend on traditional herbal medicine for primary healthcare needs. Humans use secondary metabolites from different plants as medicines for treatment of different diseases.Only a small percentage of plant species has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even smaller (**Zain *et al*., 2014**). Thus, this explains the need for more research to be done on this area. According to **Banso *et al*. (2006)** investigations into antimicrobial activities of local medicinal plants will expose the plants as potential sources of therapeutic agents. Due to increased demand for herbal medicines, it is important to evaluate the phytochemical compounds found in plants used by herbalists. A major challenge in global health care is the need for novel, effective and affordable medicines to treat microbial infections, especially in developing countries of the world, where up to one-halfof deathsare due to infectious diseases (**Awouafack *et al*., 2013; Srivastava *et al*., 2013**).Currently, the emergence of resistant pathogens to many of the commonly used antibiotics has provided an impetus for further attempts to search for new antimicrobial agents to combat infections and overcome the problems of resistance to currently available antimicrobial agents (**Mahomoodally *et al*., 2008**).

*Indigofera spicata Forssk*is native to Africa, Asia, Madagascar and America. The preferred common name is the creeping indigo. *Indigofera* is the largest genus of the tribe indigofereae, in the Fabaceae (leguminosae) family containing over 700 species found in the tropics and sub-tropics (**Datiles*et al*., 2014**). *Indigofera* species have great promise as forages for ruminants. Their high protein levels and ability to tolerate drought, floods and salinity make them agronomically desirable, while their deep-rooted growth form ability to respond to small rainfall events and resistance to herbivory make them potentially valuable cover crops and forage species for semi-arid and arid areas (**Hassen *et al*., 2006**). According to **Birru *et al*. (2017)** methanolic crude extract of the root of *Indigofera spicata Forssk* has moderate anti-bacterial activity against plasmodium species. *Indigofera spicata Forssk* has been over a long period of time been used for the treatment of toothache, cough and insect bites especially among the Kamba community of Kenya. The leaves are collected and extracted; the extract is administered orally to the patients. Its roots are also known for the treatment of malaria according to Kamba traditions. Root of *Indigofera spicata Forssk* has shown promising anti-diarrheal activity which validates its traditional use (**Birru *et al*., 2016**).

This herbal creeping plant is known in different languages across the world according to **Datilles *et al*. (2014)**; USA: *kolu or indigo*, Brazil: *amendoim-bravo*, German: *Kriechender Indigo strauch*, Indonesia: *basingan*, Thailand: *khram-khrua*, Madagascar: *aika; agitra*, Kenya: *musuusuu* (Kamba Community). The root infusion or sap is used for cough (**Wanzala *et al*., 2016**. There is scanty information about the antimicrobial activity of the plant (**Birru *et al*., 2016**). Similarly, the non-availability and high cost of manufactured synthetic drugs in the market has made it difficult for the local communities to access conventional drugs as a result of economic stress. Therefore, there is need for search of more effective antimicrobial agents among the plant parts with the aim of discovering potentially useful active ingredients that can serve as source and template for the synthesis of new antimicrobial drugs (**Pretorius *et al*., 2003**). Thus, a study by **Birru *et al*. (2017)** revealed that the root extract of *Indigofera spicata* possesses anti-malarial activity.

Thin layer chromatography is a technique used in separation of plant extracts according to their polarities. It is very important to employ this technique when considering the phytochemical analysis of any plant extract. Thin layer chromatography (TLC) gives a fast review of the number of components in a mixture and has been used to support the identity of compounds in a mixture when the retention factor (R_f) of the compounds are compared with the R_f of known compounds (**Svendsen and Verpoorte, 1984**). Thin layer chromatography (TLC) is a quick, sensitive, and inexpensive technique used to determine the number of components in a mixture, verify the identity and purity of a compound, monitor the

progress of a reaction, determine the solvent composition for preparative separations, and analyze the fractions obtained from column chromatography (**Cai, 2014**). Column chromatography helps in separating pure components in a plant extract whereby pure fractions are eluted through a stationary phase (adsorbent) using appropriate solvent or a mixture of solvents. Various solvents of differing polarities must be used to extract different phenolic compounds from plants with a high degree of accuracy (**Wong and Kitts, 2006**). Moreover, researchers have demonstrated that highly polar solvents, such as methanol, have a high effectiveness in extracting antioxidants from plants. Antibiotic effectiveness in almost all the available antibiotics is being threatened by the rising resistance of microorganisms. The major cause of this resistance is the over-use and misuse of antibiotics which is exerting undue selective pressure on microorganisms (**Paphitou, 2013**). This has made infections more difficult to treat which is contributing to the high morbidity and mortality of previously treatable infections (**Byarugaba, 2004**). Infections due to antibiotic resistant organisms are more likely to prolong hospitalization and increase the risk of death (**Graf *et al.*, 2000**). In addition, as the prevalence of multidrug resistant organisms increase, these additional costs will become a greater threat to the local, regional and national medical care systems, many of which are already struggling to survive (**DiazGranados *et al*., 2008**). The pharmaceutical industry finds it increasingly difficult to keep pace with the antimicrobial resistance and is no longer reliable in bringing novel and more effective drugs to the market (**Graf *et al.*, 2000**).There are limited detailed and thorough examinations of *Indigofera Spicata* for their potential role as antimicrobials and phytochemical entities as therapeutic agents. This study aimed at investigating the phytochemical compounds and antimicrobial activity of *Indigofera spicata* leaves, roots and bark extracts against *Candida albicans, Streptococcus mutans and E. coli*.

MATERIALS AND METHODS

Indigofera spicata plant parts were collected from Maseno-Kenya. Plant specimens were then cleaned off the soil or dust by shaking and packaged inside carrier bags then transported to the herbarium for identification in the Department of Botany, Maseno University. The leaves, roots and stem bark were air-dried separately under shade for two weeks and pulverized using an electric grinder to obtain a fine powdered-like texture. This was done to enhance the penetration of the extracting solvents (methanol) into the plant cells, thus facilitating the release of the active principles. The pulverized plant samples were then stored in amber bottles and kept in a cool and dried environment under room temperature.

Extraction, separation and purification

An approximate mass of 50g of dried root, leaves and bark powders were each extracted with equal volumes of the solvent following extraction procedure used by **Kaigongi *et al.* (2014)**.Thin layer chromatography was carried on the three methanol extracts (roots, bark and leaves) of *Indigofera spicata* according to **Musa *et al.* (2017)** to quickly determine the number of compounds in each extract. The n-hexane : methanol, 3:1 solvent was the eluent of choice. Separation took place based on the polarities of the compounds present in the extracts in the mobile and stationary phases. The ratios of distances travelled by the compounds (spots) to solvent front were determined. The spots were visualized by use of a 254 nm UV light. The Rf values were then calculated as shown below.

$$\mathbf{R_f} = \frac{\textit{Distance traveled by the spot(compound)}}{\textit{distance travelled by solvent}}$$

The retention factor **(R_f)** value essentially describes the distance travelled by the individual component.

Qualitative determination phytochemicals

The plant extracts were subjected to various phytochemical tests for the identification of chemical constituents present in the plant material. The phytochemical qualitative tests for major constituents were done using modified procedures described by **Shukla *et al.* (2013)**.

Test for flavonoids

In a boiling tube, a sample of the plant powder was heated with 10mL ethyl acetate over a steam bath for three minutes. Filtration was done and 4mL of the filtrate was shaken with 1 mL of dilute ammonium solution. A yellow coloration observed was an indication of positive test for flavonoids.

Test for alkaloids

A sample of 2g of plant powder was mixed with 40mL of 0.1M hydrochloric acid in a boiling tube and heated in a water bath for 10 minutes. The mixture was then cooled and filtered. Few drops of Mayor's reagent were added to the filtrate. A light turbidity of heavy precipitate indicated presence of alkaloids.

Test for phenols

The extract powder was dissolved in water and a few drops of dilute ferric chloride solution were added. The formation of a red, blue, green, or purple coloration indicated the presence of phenols.

Test for cardiac glycerides

A solution of glacial acetic acid (4.0 mL) with 1 drop of 2.0% $FeCl_3$ mixture was mixed with the 10 mL aqueous plant extract and 1 mL of concentrated H_2SO_4.

Test for tannins

The powder was weighed to 0.5g and dissolved in 1mL of distilled water. Filtration was carried out after addition of 2 mL of $FeCl_3$. Presence of a blue or black precipitate indicated the presence of tannins.

Test for saponins

The powder was weighed to 0.5gm and dissolved in 1 mL of methanol and filtered. Distilled water was added and shaken for a few minutes. Persistence frothing indicated presence of saponins.

Test for steroids

The powder was weighed to 0.5g and dissolved in 10 mL of chloroform and equal volume of concentrated sulphuric acid was added by side of the test tube. If the upper layer turned red and the sulphuric acid layer showed a yellow with green fluorescence, it indicated the presence of steroids.

Test for terpenoids

The plant powder was weighed to 0.5g and then mixed with 10 mL of chloroform and concentrated sulphuric acid was carefully added by side of the test tube. A reddish-brown coloration at the interface indicated the presence of terpenoids.

Antimicrobial bioassays

Bioassays were prepared according to **Bonjar (2004)**. Plant extracts (roots, bark and leaves) were diluted in methanol respectively to make a stock solution. Different concentrations of the various plant part extracts were prepared by diluting the stock extracts to water in the following ratios 0%, 25%, 50% and 75%. Each labeled medium plate was uniformly inoculated with a test organism by using Pour Plate Method to fill the plate surface in a form that lawn growth could be observed.

Sources and maintenance of microorganisms

Gram-positive organisms *Streptococcus mutans,* Gram-negative organisms *Escherichia coli* and fungal yeast *Candida albicans* were obtained and were characterized in the Department of Botany, Maseno University.

Antibacterial assay

The antibacterial assay was performed by Disc Method. The nutrient agar was inoculated with 100 mL of the inoculums and poured in a petri dish. The discs were prepared from whatman number 1 filter paper by punching them. They were then dipped in the various test treatments and placed in the respective Petri dishes as per the labeling. Three discs were placed per petri dish for the purpose of replicability. The dishes were incubated at 37 degrees for 18-24 hours. Zone of inhibition obtained was a measure of antibacterial activity of the subjected extracts and was measured in mm. Distilled water was used as a control **(Mythili *et al.*, 2012; Yin *et al.*, 2013)**.

Antifungal assay

The antifungal assay was performed by Disc Method. The potato dextrose agar was prepared by weighing 28g of PDA and then dissolved in 1000mL of distilled water. After sterilization it was poured into the petri dishes. Distilled water was used as a control. Discs were then made and dipped in the different treatments and placed in the respective petri dishes as per labeling. The plates were maintained at a temperature of 28±2 degrees. After 48 hours, the plates were observed and the diameters of the fungal growth were measured. The zones of inhibitions were measured (**Mythili *et al.*, 2012**).

Disk method

Circular discs, 5mm diameter each was cut from a laboratory grade filter paper punch and each dipped in a known concentration of the extracts for about 2 minutes (**Musyimi *et al.*, 2007**). The diameter of inhibition zones were measured with a ruler and compared with the control disc.The petri dishes were arranged in a completely randomized design.

Data analysis

Data obtained on zone of inhibition was subjected to analysis of variance (ANOVA) by use of SAS statistical package and the means were separated and compared using the least significant difference at P≤0.05.

RESULTS

Extraction, separation and purification

TLC analysis of all the fractions using n-hexane: methanol solvent system (3: 1) revealed the presence of pure spots as shown in (Table 1). The leaves and the stem recorded three spots. The roots showed two distinct spots hence recording the least spots out of the three plant parts.

Table 1 The R_f values of active compounds isolated from the methanol plant extracts of Indigofera spicata using thin layer chromatography (TLC)

	Plant parts					
	Roots		**Stems**		**Leaves**	
No. of spots	**Rf value**	**Visualization**	**Rf value**	**Visualization**	**Rf value**	**Visualization**
1	0.7	UV lamp	0.3	UV lamp	0.5	UV lamp
2	0.9	UV lamp	0.6	UV lamp	0.8	UV lamp
3		UV lamp	0.9	UV lamp	0.9	UV lamp

Qualitative phytochemical tests

The methanolic extracts of the plants revealed the presence of tannins, sterols, saponins and terpenoids. However cardiac glycosides and flavonoids were absent in all the plant parts (Table 2). Saponins were present in all the three plant parts, Tannins were absent in stem but present in roots and leaves. Alkaloids were as well abundant in the three plant parts while phenols were absent in all the parts. Sterols were present in stem and leaves but were absent in roots. Terpenoids were present in both the stem and roots but absent in leaves.

Table 2 Phytochemical screening of the secondary plant metabolites present in the plant parts extracts of Indigofera spicata

Phytocompounds	***Plant parts***		
	Stems	**Leaves**	**Roots**
Saponins	+	+	+
Tannins	-	+	+
Cardiac Glycosides	-	-	-
Flavonoids	-	-	-
Sterols	+	+	-
Terpenoids	+	-	+
Alkaloids	+	+	+
Phenols	-	-	-

Key: (+) Present , (-) Absent

Antimicrobial bioassays

The results presented in Tables 3a & 3b, show that there were significant differences (p≤0.05) between the extract concentrations and plant parts used on growth inhibition of the test microbes. Growth inhibition increased with increasing plant part concentration. The leaves extracts had the highest inhibitory effects compared to roots and stem (Table 3b). This is the first report on the antimicrobial activity of the roots, leaves and the stem of *I. spicata*. The antibacterial activity observed in this study could be attributed to the presence of saponins, terpenoids and sterols found in the plant. There were significance differences in growth inhibition among the three test microorganisms with increasing plant parts extracts concentrations (Tables 3a & 3b). However, there was no significance difference in growth inhibition between the root and stem extracts on the test microbes (Table 3b). The highest growth inhibitory effect was found on *Escherichia coli* with increasing leaves, roots and stem extracts concentration. The lowest growth inhibition occurred in *Candida albicans*.

Table 3a Effects of *Indigofera spicata methanol* extracts concentrations on growth of *Streptococcus mutans*, *Escherichia coli* and *Candida albicans*

Leaf extract concentration	Test microbes		
	Candida albicans	*Streptococcus mutans*	*Escherichia coli*
0% (Control)	5.1+ 0.2	5.5+0.3	5.7+0.3
25%	5.2+0. 3	5.5+0.3	6.2+0.4
50%	5.5+0.2	6.5+0.3	7.5+0.3
75%	5.7+0.2	6.8+0.2	8.3+0.7
Root extract concentration			
0% (Control)	5.2+0.2	5.7+0.2	5.3+0.3
25%	5.2+0.2	5.8+0.2	6.2+0.2
50%	5.3+0.2	6.0+0.3	7.0+0.5
75%	5.7+0.2	6.0+0.3	7.0+0.3
Stem extract concentration			
0% (Control)	5.0+0.0	5.2+0.2	5.2+0.2
25%	5.0+0.0	5.3+0.2	6.7+0.3
50%	5.7+0.3	5.3+0.2	7.2+0.4
75%	5.8+0.2	5.3+0.2	7.7+0.2

Table 3b Analysis of mean data of antimicrobial activity of Indigofera spicata methanol extracts, comparison of the plant parts used, the microbes and the extract concentrations

Microbe	**Growth diameter inhibition (mm)**
Candida albicans	5.4c
Streptococcus mutans	5.8b
E. coli	6.7a
LSD	**0.2**
Extract concentration	
0%	5.3b
25%	6.0a
50%	6.2a
75%	6.2a
LSD	**0.3**
Plant parts	
Roots	5.9b
Stem	5.8b
Leaves	6.1a
LSD	**0.2**

Means with the same letter are not significantly different at p<0.05. Data values are means of the three replicates.

DISCUSSION

The increase of antimicrobial resistance to many available antimicrobial agents has led to the need for the invention of new drugs to accelerate prompt prevention and treatment of microbial infections. The leaves and the stem recorded the highest and equal number of spots each showing three active compounds(Table 1). The roots showed two distinct spots hence recording the least spots out of the three plant parts. These results were in agreement with data obtained in a similar study on the same plant (**Birru *et al.*, 2017**). Interestingly, the leaves which showed highest but equal number of spots to stem, also showed greater inhibition activity against the studied microbes. Methanol is able to extract twice as much polar compounds than water (**Lapornik *et al.*, 2005**).The solvent which is the eluent moves through the plate and goes up by capillary action of the plate carrying the compounds present in the extract which separates and appears as spots on the plate. Solvents used for the extraction of biomolecules from plants are chosen based on the polarity of the solute of interest. The compounds that are closer to the origin shows less movement and are polar than the compounds that move further from the origin (**Musa *et al.*, 2017**).The process of separating active plant materials or secondary metabolites such as alkaloids, flavonoids, terpenes, saponins, steroids, and glycosides from inert or inactive materials in medicinal plants requires extraction using an appropriate solvent and standard extraction procedures.

Phytochemical screening helps to reveal the chemical nature of the constituents of the plant extracts and the one that predominates over the others (**Enwuru *et al.*, 2008**). It may also be used to search for bioactive lead agents that could be used in the partial synthesis of some useful drugs.Phytochemical screening of the plant extracts (Table 2) showed that alkaloids, tannins, sterols, terpenoids and saponins were present in methanolic extract of *I. spicata*. According to **Musyimi *et al.* (2007**), the most important of these bioactive compounds are, Alkaloids, flavonoids, tannins and phenolic compounds. These findings are in agreement to those of **Birru *et al.* (2017)** who reported the presence of alkaloids, tannins, saponins, glycosides, flavonoids, steroids and terpenoids in the plant extract of*I. spicata* as well as the absence of phenolic compounds. In this current study there were no cardiac glycosides and flavonoids in the plant extracts of *I. spicata*; however, the two compounds were present in the roots of the plant extract according to **Birru *et al.* (2017)**. The differences could be attributed to the method of extraction and climate of the regionwhere the plants were collected.Biological and pharmacological activities of phytochemical compounds take into account factors such as species, ecological factors and environmental conditions (**Emitaro *et al.*, 2020**; **Musyimi *et al.*, 2021**). Saponins are characterized by their surface-active properties and they dissolve in water to form foamy solutions and because of surface activity, some drugs containing saponins have a very high long history of usage.

The extract displayed strong antimicrobial potential against the microorganisms tested. Antibacterial activity is due to different chemical agents in the extract, including essential oils (especially thymol), flavonoids and triterpenoids and other nature phenolic compounds or free hydroxyl groups (**Leite *et al.*, 2006**). These are classified as active antimicrobial compounds.Biological activity is attributed to the presence of various secondary metabolites in plants (**Mazid *et al.*, 2011**). The antimicrobial activity of the methanol extract had a broad spectrum of activity since both the Gram positive and the Gram Negative bacterial and the Fungi were sensitive to the extract. Many alkaloids have pharmacological effects and could be associated with inhibition of nucleic acid, protein and membrane phospholipids biosynthesis (**Musyimi *et al.*, 2007**).

The zone of inhibition between *E. coli* (Gram negative*)* and *S. mutans* (Gram positive) was significantly different indicating that there was higher activity on the Gram-negative bacterium than to the Gram-positive one. Differential sensitivity of microbes to the extracts may be explained by the cell wall composition of the gram positive and gram-negative bacteria. This determines and allows different molecules or ions either into or out of the cell and thus the outer membrane serves as a barrier to the passage of many molecules and hence less sensitive to many extracts (**Kaigongi *et al.*, 2014**). The quantity of the phytochemical constituents in a given plant extract determines the extent of extracts' bioactivity. In addition, presence of more than one class of secondary metabolites in a given plant extract will also determine the nature and extent of extract's biological activity (**Wang *et al.*, 2010**). Leaves extracts were more active than both the root and the stem. This could be due to higher concentration of active compounds in the leaves or possibly the synergistic and antagonistic manner of the compounds present. There were no significant differences on the concentrations however the control showed significantly the lowest inhibition. This study is in agreement with **Birru *et al.* (2017)** that root extract of *Indigofera spicata* has antimicrobial activity.

CONCLUSION

This study aimed at identifying phytochemical compounds and evaluating the antimicrobial properties of the compounds from the leaf extracts of *Indigofera spicata*. From this study it can be concluded that the bark, root and leaf extracts of *Indigofera spicata* exhibit growth inhibition against *S. mutans* and *E. coli*. The extracts also showed growth inhibitory activity against the fungus *C. albicans*. This study confirms the presence of tannins, sterols, saponins and terpenoids in *Indigofera spicata* methanol extracts, and suggest the potential of the plant as a source of new antimicrobial agents that can be effective for treatment of infections arising from the three pathogens. This study recommends the use of extracts from *Indigofera spicata* especially the leaves to develop drugs which can act against *C. albicans*, *S. mutans* and *E. coli.* Further purification, identification and characterization of the active compounds of the plant should be prioritized in the future studies.

Conflict of interests: Declare none

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