Original Article

Antibacterial activity of methanol root extract of *Indigofera lupatana* Baker F.

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Abstract. *Indigofera lupatana* Baker F. (locally known as *Mugiti*) has been used by Mbeere community of Kenya to treat cough, diarrhea, pleurisy and gonorrhea. These infectious diseases are caused by pathogenic micro-organisms such as, *Klebsiella pneumoniae*, *Salmonella typhi, Escherichia coli*, *and Neisseria gonorrhea,* among others. Infectious diseases are a cause of morbidity and mortality in humans and animals. Their effects are further aggravated by drug resistance, making it difficult to contain these infections. This calls for search of new drugs that will mitigate these problems. Indigenous plants are promising as a cheap alternative source of new therapeutic agents. Powdered sample of *I. lupatana* Baker F. roots were extracted using methanol solvent. The resultant extract was subjected to anti-microbial assay. The extract showed the highest activity against *Bacillus subtilis* (28.0 mm), *Bacillus cereus* (22.0 mm), *Escherichia coli* (21.7mm), *Staphylococcus aureus* (16.7 mm), *Klebsiella pneumonia* (15.3 mm) and *Proteus mirabilis* (12.3 mm), *Pseudomonas aeruginosa* (11.7 mm), *Salmonella typhimurium* (11.3 mm). The phytochemical studies of extract fractions showed presence of phenolics, flavonoids, tannins, saponins, terpenoids, cardiac glycosides, steroids and phlobatannins. These compounds are responsible for the bioactivity of the sample fractions. The activity was greater among the Gram positive bacteria than Gram negative bacteria. The MIC ranged from between 25 to 400mg/ml.

Key words: *Indigofera lupatana* Baker F., Antimicrobial activity, phytochemical, MIC

1. Introduction

Medicinal plants contribute significantly to rural livelihoods. Apart from the traditional healers practicing herbal medicine, many people are involved in collecting and trading medicinal plants. The result is an increased demand worldwide leading to enhanced new drugs. The World Health Organization (WHO) estimates that 80% of the world's population depends on medicinal plants for their primary health care (Gurib-Fakim and Schmelzer, 2007; Mothana *et al*., 2008).

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Infectious diseases are the world's main cause of human and animal mortality. The situation is further aggravated by the rapid development of multi-drug resistance to available anti-microbial agents(Doughart and Okafor, 2007), their limited anti-microbial spectrum, their side effects (Huie, 2002), and emergence and re-emergence of infections. Therefore studies aimed at finding and characterization of the substances that exhibit activity against infectious micro-organisms, yet showing no cross-resistance with existing antibiotics, are urgently required (Olila *et al.,* 2001). In recent years, pharmaceutical companies have focused on developing drugs from natural products. Also, discovery of new drugs has been made a continuous process to counter the limitations of conventional antibiotics (Doughart and Okafor, 2007).

Indigofera lupatana Baker F. locally called *mugiti* is a woody shrub found in *Acacia-Combretum* ecological zones of Mbeere district in Kenya. Its roots are widely used for their

perceived medicinal value in treating coughs and diarrhea (Riley and Brokensha, 1988), gonorrhea and pleurisy (Kokwaro, 1993; Ngoci *et al.,* 2011). There is apparently no documented scientific report on anti-microbial properties of this plant. This has often constituted a major constraint to consideration of the use of herbal remedies in conjunction with or as an affordable alternative to conventional medical treatment (Okeke *et al.,* 2001).

The main objective of this study was to determine the anti-microbial activity of the extracts of *Indigofera lupatana* Baker F.

2. Materials and methods

2. 1. Collection and Identification of plant samples

The plant sample for the study was collected from Mbeere district, in Eastern province, Kenya. The plant was taxonomically authenticated at the Department of Biological Sciences of Egerton University. A voucher sample was assigned a reference number (NSN1) and banked in the same department herbarium

2. 2. Plant root preparation and extraction

The plant roots were separated, washed, cut into small pieces, air-dried in the dark to avoid decomposition of light sensitive bio active compounds (Houghton and Raman, 1998), at room temperature to a constant weight and ground into a powder by a mill (Thomas-Wiley laboratory mill, model 4). The powder was extracted by use of organic solvent methanol (Houghton and Raman, 1998).

Ground material (150 g) was soaked in the solvent, for 24 hours at room temperature with intermittent shaking followed by decanting and filtration by gravity to separate the debris. Fresh solvent was replaced and agitated for 10 minutes, decanted and filtered. The two volumes were combined together and concentrated in rotary vacuum evaporator (BÜCHI ROTAVAPOR R-205 V805, Flawil, Switzerland) and allowed to air dry (Houghton and Raman, 1998; Wojcikowski *et al.,* 2008).

2. 3. Collection of Test Micro-organisms

A total of eight standard pathogenic bacterial strains were used which were maintained on agar slant at 4 °C in the microbiology laboratory of Biochemistry department of Egerton University. They were: three Gram-positive bacterial species (*Bacillus subtilis* BGA spores suspension which were ready-to-use (Merck, Darmstadt, Federal Republic of Germany), *Bacillus cereus* ATCC 11778 spores which were ready-to-use preparation (Difco Laboratories, Detroit, Michigan, USA) and S*taphylococcus aureus* ATCC 25923 (KEMRI) and five Gram-negative bacteria (*Escherichia coli* ATCC 25922 (KEMRI), *Pseudomonas aeruginosa* ATCC 27853 (KEMRI), *Salmonella typhimurium* ATCC 13311 (KEMRI), *Klebsiella pneumoniae* and *Proteus mirabilis* (clinical isolates from KEMRI).

2. 4. Anti-microbial tests

The tests guide on the choice of appropriate agents for therapy, provide a range of suitable alternatives and accumulated data from which information on the most suitable agents for empirical use can be derived. They are also used to evaluate *in vitro* activity of the anti-microbial agents. The results are either reported qualitatively (as Sensitive, Intermediate, or Resistant) in disc diffusion method or quantitatively (in terms of MIC and MBC) (Collins *et al*., 1995).

2. 5. Culture media

Nutrient agar was used for sub-culturing of the test micro-organisms, at 37 °C for 24 hrs and the Mueller Hinton agar was used for sensitivity assay (Nguemeving *et al.,* 2006).

2. 6. Standards

Chloramphenicol was used as a standard drug for positive control (STD_b) against bacteria. Its choice was based on its properties as a broad spectrum drug, a very stable drug under a variety of conditions of temperature and humidity, and its low toxicity threshold when ingested (Drew *et al*., 1972). The aqueous 1% Dimethylsulfoxide (DMSO) was used as solvent for the extracted samples because it is amphipathic, able to diffuse well in the agar and at this concentration it is non toxic (Moshi *et al*., 2006; Mbaveng *et al.,* 2008). Therefore, aqueous 1% DMSO was used as negative control (STD_a).

2. 7. Anti-microbial susceptibility tests

Media preparation

The media was reconstituted using distilled water according to the manufacturer's instructions, sterilized by autoclaving at 121 ^oC and pressure of 15 psi for 15 minutes. It was then dispensed aseptically into Petri dishes (9 cm diameter), a volume of between 18-25 ml molten agar to achieve a depth of between 3-4mm, and left to solidify and then stored in the refrigerator at 4 ^oC. Before use, the inoculation plates were air dried with the lids ajar until there were no moisture droplets on the petri dish surfaces (Collins *et al.,* 1995).

Table 1. Anti-bacterial activity result for the methanol root extract

Micro organism	Inhibition zones diameter in mm							MIC(mg/ml)	
	Extract concentration (µg x 10^2)					STD_a	STD_b		
	160	80	40	20	10		30µg	E	STD_b
Gram negative bacteria									
E. coli	21.7±0.8	15.5±1.0	13±0.8	10±0.4	0	0	48.3±1.7	100	25
K. pneumoniae	15.3±0.7	9.0±1.2	7.0±0.5	0	0	0	37.7±1.5	200	22.5
P. aeruginosa	11.7±1.3	9.0±0.5	7.0±0	6.0±0	0	0	24.3±2.3	100	NT
P. mirabilis	12.3±0.8	9.0±1	0	0	0	0	34.3±2.3	400	NT
S. typhimurium	11.3±1.2	6.0±0.0	0	0	0	0	29.0±2.6	400	NT
Gram positive bacteria									
S. aureus	16.7±1.2	8.5±0.5	0	0	0	0	37.3±1.5	400	31.3
B. cereus	22.0±0	20.6±0.5	20.0±0	16.5±0.5	15.7±0.7	0	22.3±1.3	25	NT
B. subtilis	28.0±1.2	23.0±0.6	21.7±0.7	19.5±0.5	16.0±1.0	0	32.7±1.5	25	26.3

STD_a –Represents negative control; STD_b – Represents positive control; E – Represents extract fraction and NT – Represent not tested.Values of inhibition zones are in mm (mean±SEM, n=3)

Preparation of discs

Whatmann filter paper (No.1) discs of 6 mm diameter were made by punching the paper, and the blank discs were sterilized in the hot air oven at 160 ^oC for one hour. They were then impregnated with 10 µl of the varying concentration of the methanol extract solution. The methanol extract stock solution (1.6g/ml)) was serially diluted at two folds. The impregnated discs were evaporated at 50 ^oC till when dry (Ayo *et al.*, 2007). The STD_b (chloramphenicol at 30µg/discs) were used as positive controls. Discs loaded with 10µl of aqueous 1% DMSO were used as negative controls (STD_a) (Mbwambo *et al.,* 2007; Mbaveng *et al.,* 2008).

Disc diffusion test

The anti-microbial activity was assayed by disc diffusion method according to Ayo *et al*., (2007), CLSI (2007) and Mbaveng *et al.,* (2008). The bacterial strains were activated by growing them in Nutrient agar at 37 ^oC for 18 to 24 hours. A fresh inoculum was developed by suspending activated colonies in physiological saline solution (0.85% NaCl). An inoculum of bacterial cell suspension of about 1.5×10^6 CFU/ml was determined and standardized using a McFarland turbidity standard No. 0.5. The suspension was authenticated by adjusting the optical density to 0.1 at 600 nm.

This suspension was used to aseptically inoculate by swapping the surface of MHA plates. Excess liquid was air-dried under a sterile hood. The impregnated discs were then planted at equidistant points on top of the inoculated agar medium by sterile forceps. A disc prepared with only the corresponding volume of aqueous 1% DMSO was used as a negative control, while chloramphenicol was used as positive control. The inoculated plates were incubated at 4 ^oC for 2 hours to allow the pre-diffusion of extracts into the media. The plates were then incubated at 37 °C for 24 hrs, after which they were inspected for zones of inhibition. Anti-microbial activity was evaluated by measuring the diameter of the inhibition zones. The lowest concentration of the extract that yielded the lowest zone of inhibition was recorded as the MIC of the extract (Mothana *et al*., 2008).

3. Results

The extract showed the highest activity against *Bacillus subtilis* (28.0 mm), *Bacillus cereus* (22.0 mm), *Escherichia coli* (21.7mm), *Staphylococcus aureus* (16.7 mm), *Klebsiella pneumonia* (15.3

mm) and *Proteus mirabilis* (12.3 mm), *Pseudomonas aeruginosa* (11.7 mm), *Salmonella typhimurium* (11.3 mm) and the lowest MIC of 25 mg/ml in both B. subtilis and B. cereus (Table 1). The activity was broad spectrum and could be due phytochemicals tested in this fraction. The extract tested positive to all phytochemicals tested except alkaloids (Table 2).

Table 2. Phytochemical tests results

Phytochemicals	Methanol extract
Alkaloids	-ve
Flavonoids	+ve
Tannins	+ve
Saponins	+ve
Cardiac glycosides	+ve
Phlobatannins	+ve
Phytosteroids	+ve
Terpenoids	+ve

(+ve)-Represent presence of the tested phytochemicals in the sample extract
(−ve)-Represent absence of the tested phytochemicals in the sample extract

4. Discussion

The plant extract had broad spectrum activity in that it inhibited growth of both Gram positive and Gram negative bacteria. The inhibition zones increased on increasing the concentration of the extract in the discs showing a concentration dependent activity and also varied with the kind of bacteria tested. Although the concentrations of the extract fractions were in the range of 100 times more than the standard antibiotic (chloramphenicol), they showed marked anti-bacterial activity as evidenced by their zones of inhibition. This could be due to the fact that the active components in the extract comprise only a fraction of the extract used. Therefore, the concentration of the active components in the extract could be much lower than the standard antibiotic used. It is important to note that, if the active components were isolated and purified, they would probably show higher antibacterial activity than those observed in this study.

The methanol extract yielded highest overall inhibition of 28.0 ± 1.2 mm in *B. subtilis* and the lowest MIC of 25 mg/ml in both *B. subtilis* and *B. cereus* (Table 1). The activity was broad spectrum and could be due phytochemicals tested in this fraction. A variety of phytochemicals such as tannins, saponins, terpenoids, cardiac glycosides, phytosteroids, phlobatannins, and flavonoids were detected in the plant methanol extracts (Table 2).

These phytochemicals are responsible for the antibacterial activity. Flavonoids have been shown to act by complexing microbial proteins and disrupting microbial membranes (Cowan, 1999; Navarro *et al.,* 2003; Al-Bayati and Al-Mola, 2008; Samy and Gopalakrishnakone, 2008; Kaur and Arora, 2009; Ngoci *et al.,* 2011). Saponins have been demonstrated to act by inhibiting bacterial colonization, lowering surface tension of extracellular medium or by lysing bacterial membranes (Al-Bayati and Al-Mola, 2008; Ngoci *et al.,* 2011). Tannins act by complexing bacterial proteins, interfering with bacterial adhesion, inactivating enzymes and disrupting bacterial cell membrane (Cowan, 1999; Okuda, 2005; Trombetta *et al.,* 2005; Victor *et al.,* 2005; Biradar *et al.,* 2007; Samy and Gopalakrishnakone, 2008; Kaur and Arora, 2009; Ngoci *et al.,* 2011). The activity could also be due to phytosteroids and terpenoids that act by disrupting bacterial membrane (Cowan, 1999; Soares *et al.,* 2005; Ogunwenmo *et al.,* 2007; Roberts, 2007; Samy and Gopalakrishnakone, 2008; Ngoci *et al.,* 2011). Cardiac glycosides and phlobatannins that were also detected could be responsible for antibacterial activity (Kokwaro, 1993).

Although traditional healers make use of water as herbal solvent, studies have shown that methanol solvent is much better and powerful (Wojcikowski *et al.,* 2007). This could be due to the polarity of the solvent that conferred the ability to extract a variety of compounds (Parekh and Chanda, 2006) and could be the justification for the reasons why methanol extract demonstrated high inhibitions. Polarity of the solvent also influences the qualitative and quantitative composition of the active compounds in herbal extracts (Houghton and Raman, 1998; Doughart and Okafor, 2007; Wojcikowski *et al.,* 2007; Tomczykowa *et al.,* 2008).

Gram positive strains were more susceptible to the extract than Gram negative strains. This is in agreement with previous reports that plant extracts are more active against Gram positive bacteria than Gram negative bacteria (Parekh and Chanda, 2006; Mohamed *et al.,* 2010). The higher sensitivity of Gram-positive bacteria could be attributed to their outer peptidoglycan layer which is not an effective permeability barrier as compared to the outer phospholipid membranes of Gram-negative bacteria (Trombetta *et al.,* 2005; Tomczykowa *et al.,* 2008; Kaur and Arora, 2009).

The present work showed the potential of tested extract against the causative agents of nosocomial infections and morbidity among immuno compromised and severely ill patients such as *P. aeruginosa, S. aureus* (Bastos *et al*., 2009; Kaur and Arora, 2009). Infections caused by *P. aeruginosa* and *B. cereus* are difficult to combat (Aliero. and Afolayan, 2005) and therefore their susceptibility to the extract is a pointer to the extract potential as a drug against these bacteria.

The plant extracts also showed recommendable activity toward pathogen responsible for the gastrointestinal disorders that leads to diarrhea, coleocystitis, and urinary tract infections e.g. *E. coli*, *S. typhimurium* (Moshi *et al.,* 2006; Matasyoh *et al*., 2007) and this supports the traditional use of this plant for the treatment of diarrhea (Riley and Brokensha, 1988; Ngoci *et al.,* 2011).

5. Conclusion

Anti-microbial testing showed that *Indigofera lupatana* Baker F. extract had broad spectrum bioactivity as it inhibited both Gram-positive and Gram-negative bacteria. This supports the traditional usage of this plant for therapeutic purposes. Since the activity was shown to be dose dependent, better inhibition against other bacteria could be attained by increasing the extract concentration further.

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