

Full Length Research Paper

Evaluation of antibacterial effects and phytochemical screening of the aqueous and methanolic extracts of *Hibiscus diversifolius*

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***Hibiscus diversifolius* which is widely distributed in Kenya was investigated for its antibacterial effect against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus*. The leaves stem and root of the plant was extracted using aqueous and methanol as solvents. Phytochemical screening was also carried out to determine the phytochemical constituents present in the various parts of the plants used. The results show that both aqueous and methanolic extracts of the different plant parts had antibacterial activity against the various microbes tested. Phytochemical screening revealed the presence of alkaloids, flavonoids, sterols, saponins, terpenoids and cardiac glycosides while tannins and steroids were lacking in all the extracts.**

Key words: Antibacterial, phytochemicals, aqueous, methanol and extracts.

INTRODUCTION

For a long period, plants have been a valuable source of natural products for maintaining human health and impressive number of modern drugs have been isolated from them; many on their use in traditional medicine (Nascimento et al., 2000; Nair et al., 2005). Currently, it is estimated that over 50% of all modern clinical drugs are of natural products origin (Cordell, 2000; Newman et al., 2003). These drugs are employed in the treatment of both infectious and non-infectious diseases. Infectious

diseases remain the leading cause of death and account for one quarter of all deaths in the world (WHO, 1999). To worsen matters, infections due to antibiotic resistant micro-organisms have been on the rise (Pfaller et al., 1998). There is therefore urgent need to come up with new novel antimicrobial agents to combat and curb the spread of these resistant microbes. Higher plants are still poorly explored as sources of new drugs (Hostettman and Terreaux, 2000) and would therefore be a good starting

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point in the search for efficacious novel antimicrobial agents. *Hibiscus diversifolius* is an annual shrub belonging to the family Malvaceae with a wide distribution in Kenya and other parts of the world. Medicinal uses of plants from this family have been reported in traditional folklore medicine and the most frequently cited are antibacterial, antihelminthic and antimalarial. They have reportedly been used in the treatment of cancer, abscesses, bilious conditions, bruises, cough and pneumonia (Olaleye, 2007; Ngari et al., 2010; Agbor et al., 2005). A few members of the genus *Hibiscus* have received scientific attention. Methanolic extracts of *H. sabdariffa* were demonstrated to have antibacterial activity against a number of selected pathogens (Olaleye et al., 2007). *H. cannabinus* have been investigated for their haematinic property in anaemic rats in addition to its phytochemical constituents (Agbor et al., 2005). *H. diversifolius* has never been scientifically investigated. This study was therefore undertaken to determine the antibacterial effect of this plant and also sought to establish the phytochemicals present in this plant as some of these could be used to explain the observed antibacterial effects if any.

MATERIAL AND METHODS

Plant materials and their collection

Plant materials were collected from Oyugis located in Homa-bay county of Kenya during the month of June, 2011. Leaves, stem and root of *H. diversifolius* were collected. The plant was identified in the herbarium, Department of Botany, Jomo Kenyatta University of Agriculture and Technology (JKUAT), where voucher specimens were deposited. The plant materials were dried under shade at temperature below 30°C and pulverized in a hammer mill fitted with a sieve of 0.5 mm pore.

Preparation of methanolic extracts

The ground plant material was extracted twice with methanol as the solvent of extraction. One hundred grams of plant powder was extracted by mixing with 300 ml of methanol. The slurry of solvent and plant powder was stirred and left to stand for 48 h, after which the supernatant was filtered through Whatman® GF/C glass microfibre filter paper and the filtrate concentrated under vacuum at 40°C in Buchii rotary evaporator. The extracts were then dried in a freeze drier and kept desiccated at 4°C until use.

Preparation of aqueous extracts

Plant powders' weighing 300 g was boiled for 20 min in 800 ml of distilled water. After cooling to room temperature, the supernatant was decanted, centrifuged at 5400x gravity for 10 min after which the supernatant was filtered through Whatman® GF/C glass microfibre filter paper, frozen at -15°C and then dried in a freeze drier. The extract was kept desiccated at 4°C.

Antimicrobial screening of *H. diversifolius* extracts

Antibacterial activities of the plant extracts of *H. diversifolius* were

tested by disc diffusion method. Four bacterial strains *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus* were used in this study. The organisms were obtained from a culture collection maintained in the department of Botany, JKUAT. The bacteria were tested for purity by culturing on nutrient agar and maintained on nutrient agar slants.

Preparation of inocula

Stock cultures were maintained on slopes of nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Mueller-Hinton broth (MHB) and reactivated by culturing overnight at 37°C. Cultures were diluted with fresh MHB and compared with McFarland standard to achieve values corresponding to 2×10^6 colony forming units.

Antibacterial activity of the extracts

Antibacterial activity of the plant extracts was tested by disc diffusion method as described by Mbwambo et al. (2007). Four strains of bacteria were used, Gram negative *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and Gram positive *S. aureus* (ATCC 25923), and *B. subtilis* (ATCC 6633). Filter paper discs (Whatman No.1) 6 mm diameter were impregnated with crude extracts. Discs dipped into methanol and distilled water served as negative control. All the bacteria were incubated at 30°C for 24 h by inoculation into nutrient broth. Sterilized Petri- dishes were inoculated with 0.01 ml of one of the above culture media (10^5 - 10^6 per ml). Mueller- Hinton agar sterilized in a flask and cooled to 45- 50°C was distributed by pipette (15 ml) into each inoculated Petri dish and swirled to distribute the medium homogenously. Discs injected with extracts at different concentrations were applied on the solid agar medium by pressing slightly. Standard antibiotic discs of streptomycin (25 ug), tetracycline (100 ug) and gentamycin (10 ug) were also included and tested for their antibacterial activity against test microbes. The treated Petri dishes were placed at 4°C for 1-2 h and then incubated at 35°C for 18-24 h. The discs were tested in triplicate. At the end of the period, the inhibition zones formed on the media were measured with a transparent ruler in millimeters.

Phytochemical screening

Test for the presence of compounds present in plant extracts

The methods described by Nanyemi et al. (2005) and Banso and Adeyemo (2006) were used to test for the presence of alkaloids, flavonoids, sterols and steroids, saponins and tannins.

Determination of alkaloids

Extract of each plant sample was separately stirred with 1% hydrochloric acid (HCL) on a steam bath. The solution obtained was filtered and 1 ml of the filtrate was treated with two drops of Mayer's reagent. The two solutions were mixed and made up to 100 ml with distilled water. Turbidity of the extract filtrate on addition of Mayer's reagent was regarded as evidence for the presence of alkaloids in the extract.

Determination of flavonoids

To 1 ml of each plant extract in a test tube was added a small piece (2mm strip) of magnesium ribbon followed by drop wise addition of

Table 1. Antibacterial effects of aqueous extracts of leaf, stem and root of *H. diversifolius* against test microbes in mm after 3 repeats.

Extract	Microbe	Concentrations (mg/ml)				
		0.01	0.1	1	10	100
Leaf	<i>E. coli</i>	9.0±0.3	8.9±0.3	9.0±0.3	8.1±0.3	9.0±0.3
	<i>P. aeruginosa</i>	11.0±0.2	11.0±0.1	10.0±0.2	9.0±0.3	8.9±0.3
	<i>B. subtilis</i>	10.0±0.1	10.0±0.2	10.1±0.2	9.0±0.1	8.0±0.1
	<i>S. aureus</i>	10.1±0.1	10.4±0.7	10.0±0.2	9.0±0.1	9.8±0.5
Stem	<i>E. coli</i>	8.0±0.2	8.1±0.2	10.0±0.2	8.1±0.2	7.1±0.1
	<i>P. aeruginosa</i>	9.0±0.3	10.0±0.1	10.0±0.1	10.0±0.2	9.1±0.2
	<i>B. subtilis</i>	9.0±0.2	10.0±0.2	10.0±0.1	9.0±0.2	8.0±0.2
	<i>S. aureus</i>	10.0±0.2	10.0±0.2	9.9±0.2	9.0±0.2	9.1±0.2
Root	<i>E. coli</i>	9.0±0.2	9.0±0.3	8.1±0.3	10.1±0.2	10.1±0.2
	<i>P. aeruginosa</i>	9.0±0.2	10.1±0.2	9.0±0.3	10.1±0.2	9.0±0.2
	<i>B. subtilis</i>	8.1±0.3	9.1±0.3	10.1±0.2	9.0±0.2	8.1±0.2
	<i>S. aureus</i>	10.0±0.3	9.0±0.2	9.1±0.2	10.1±0.1	8.9±0.2

concentrated hydrochloric acid. Development of pink or magenta red colors indicated the presence of flavonoids.

Determination of sterols and steroids

One milliliter of extract was put into a test tube in which 0.5 ml sulfuric acid, acetic anhydride and chloroform in similar amount was added. A red coloration indicated presence of sterols while a green color indicated presence of steroids.

Determination of saponins

milliliter of each extract under test was put into a test tube and 50 ml of tap water added. The mixture was then shaken vigorously. Foaming which persisted on warming was taken as an evidence for the presence of saponins but this was subjected to further confirmatory test. This involved dissolving 1 ml of the extract in carbon tetrachloride to which 4 drops of concentrated sulphuric acid was added to the mixture. A blue, green, or red color accompanied by a pink ring confirmed presence of saponins.

Determination of tannins

Ethanol extract of each sample was separately stirred with 10 ml of distilled water and then filtered. To the filtrate was added two drops of 5% iron III chloride (FeCl₃) reagent. Blue- Black or blue green coloration was taken as an indication of the presence of tannins.

Determination of cardiac glycosides

5 ml of extracts were treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was under laid with 1 ml concentrated sulphuric acid. A brown ring at the interface indicates deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring while in the acetic acid layer a greenish ring may form.

Determination of terpenoids

5 ml of each extract was mixed with 2 ml of chloroform, and 3 ml concentrated sulphuric acid. Formation of a reddish brown coloration at the interface was considered a positive test for the presence of terpenoids.

RESULTS

The results of the antibacterial effect of the different concentrations of the aqueous extracts of leaf, stem and roots of *H. diversifolius* are given in Table 1. These extracts of the different plant parts exhibited antibacterial activities against the selected test microbes with zones of inhibition ranging from 8 to 11 mm. For all the aqueous extracts, the antibacterial effect did not differ much between the various concentrations used as the zones of inhibitions formed were almost of the same size regardless of the concentrations used. There were no differences in the antibacterial effects of the aqueous extracts on both the Gram positive and Gram negative as determined from the zones of inhibitions formed.

The results of the different concentrations of methanolic extracts of leaf, stem and root of *H. diversifolius* are given in Table 2. The methanolic extracts of the different plant parts demonstrated antibacterial effect against the different microbes used in this study with zones of inhibition ranging from 6.9 to 12 mm. The antibacterial effect of the different plant parts did not differ much from each other as can be deduced from the resulting zones of inhibitions formed. Varying concentrations of the different extracts did not produce big differences in their antibacterial effect. Again the antibacterial effect of the methanolic extracts against Gram positive and Gram negative organisms tested were almost similar. From the

Table 2. Antibacterial effects of methanolic extracts of leaf, stem and root of *H. diversifolius* against test microbes in mm after three repeats.

Extract	Microbe	Concentrations (mg/ml)				
		0.01	0.1	1	10	100
Leaf	<i>E. coli</i>	10.1±0.2	10.2±0.2	10.0±0.3	9.1±0.2	7.0±0.2
	<i>P. aeruginosa</i>	10.0±0.2	12.0±0.1	10.0±0.2	9.0±0.2	8.1±0.2
	<i>B. subtilis</i>	8.1±0.2	10.1±0.2	8.0±0.1	10.3±0.8	8.0±0.2
	<i>S. aureus</i>	10.1±0.2	8.9±0.2	12.0±0.2	9.0±0.3	7.0±0.2
Stem	<i>E. coli</i>	10.0±0.2	9.9±0.2	9.9±0.4	8.1±0.2	8.9±0.2
	<i>P. aeruginosa</i>	10.0±0.2	6.9±0.2	8.1±0.3	8.0±0.3	7.1±0.2
	<i>B. subtilis</i>	10.0±0.3	9.0±0.2	8.9±0.2	10.3±0.2	9.9±0.2
	<i>S. aureus</i>	9.1±0.2	7.0±0.2	8.1±0.2	9.9±0.2	7.9±0.2
Root	<i>E. coli</i>	10.0±0.3	10.1±0.2	10.0±0.2	9.1±0.3	7.1±0.2
	<i>P. aeruginosa</i>	10.1±0.2	12.1±0.3	10.0±0.3	9.1±0.3	8.1±0.2
	<i>B. subtilis</i>	8.0±0.2	10.4±0.8	7.9±0.2	10.0±0.2	8.1±0.2
	<i>S. aureus</i>	10.1±0.2	9.4±0.3	12.0±0.2	9.0±0.3	7.0±0.2

Table 3. Phytochemical components of leaf, stem and root of *Hibiscus diversifolius* extracted with aqueous and methanolic solvents.

Extract	Phytochemical							
	Alkaloid	Flavonoid	Sterol	Steroid	Saponin	Tannin	Terpenoid	Cardiac glycoside
Methanolic extract								
Leaf	+	+	+	-	+	-	+	+
Stem	+	+	+	-	+	-	+	+
Root	+	+	+	-	+	-	+	+
Aqueous extract								
Leaf	+	+	+	-	+	-	+	+
Stem	+	+	+	-	+	-	+	+
Root	-	-	-	-	-	-	-	+

two tables, the aqueous and methanolic extracts seem to have similar antibacterial activities as the zones of inhibitions produced were almost of similar sizes.

The result of phytochemical screening of the aqueous and methanolic extracts of leaf, stem and root of *H. diversifolius* are provided in Table 3. All the methanolic extracts of the 3 plant parts were found to contain all the phytochemical compounds tested for except steroids and tannins. For aqueous extracts of the leaf, stem and root of *H. diversifolius*, phytochemical screening revealed that the leaf and stem had similar compounds present in them. All the phytochemicals tested for except steroids and tannins were present. This composition was similar to that of the methanolic extracts of leaf, stem and roots. A notable exception was seen with the aqueous root extract which lacked all the phytochemicals except for cardiac glycosides but showed similar antibacterial effects as the other extracts.

DISCUSSION

The present study has demonstrated that all the aqueous

and methanolic extracts of *H. diversifolius* had antibacterial activity against all the microbes tested and that the different plant parts had almost similar antibacterial effect against both the Gram positive and Gram negative organisms used in this particular investigation. It has been published that various plant extracts have been demonstrated to possess antibacterial activity against microbial pathogens (Mahesh and Satish, 2008; Balakrishnan et al., 2006; Mehrgan et al., 2008; Mandal et al., 2000). The antimicrobial activity observed could be due to the varied phytochemicals present. Indeed, several metabolites from herb-species such as alkaloids, tannins, saponins and steroids have previously been associated with antimicrobial activity (Leven et al., 1979). It is necessary to identify the phytochemical components of local medicinal plants because the presence or absence of certain phytochemicals could be used to explain some of the biological activity of certain plant extracts observed. The antibacterial activity of the methanolic extracts of the leaf, stem and root could be attributed to the presence of alkaloids, flavonoids, steroids, saponins, terpenoids and cardiac glycosides.

Phytochemical screening of the aqueous extracts of the leaf and stem of *H. diversifolius* showed that all the phytochemical constituents except steroids and tannins were present and therefore the antibacterial effects could also be associated with these compounds. Phytochemical compounds present in these two aqueous extracts were similar to those present in all the methanolic extracts. It is therefore not surprising that all these extracts had almost similar antibacterial effect as could be discerned from the sizes of the zones of inhibitions formed. The aqueous root extract of *H. diversifolius* lacked all these phytochemicals present in all other extracts except for the presence of cardiac glycosides only. Yet surprisingly, it demonstrated almost similar antibacterial effect comparable to other extracts. Its antibacterial activity can only be attributed to cardiac glycosides as it was the only compound whose presence was revealed by phytochemical screening. Aboaba et al. (2006) has reported that many plants contain toxic glycosides which can get hydrolysed to release phenolics which are toxic to microbial pathogens. It is also possible that there are other compounds besides the ones tested for could be contributing to the antibacterial activity of this particular extract. Indeed, Astal et al. (2005) reported that the water extracts of *Salvadora persica* roots and stems contained potential antimicrobial anionic components such as chloride, sulfate, thiocyanate and nitrate. It is possible that the same compounds may have been present in this particular extract and contributed to the antibacterial effect seen.

Indole quinolone alkaloids, glycoalkaloids, berberine type alkaloids, indole quinolizidine alkaloids have been reported to be active against a range of Gram positive and Gram negative bacteria (Iwu et al., 1999). Flavonoids have been found to show *in vitro* antimicrobial activity against a wide range of bacteria. Their activity has been attributed to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall (Cowan, 1999). There is no information on the antibacterial effects of plant steroids and sterols. Saponins have been reported to possess antifungal activity (Iwu, 2000). Terpenoids are terpenes to which additional elements such as oxygen have been added (Cowan, 1999). Terpenes and terpenoids have been found to possess antimicrobial activity (Mendoza et al., 1997; Amara et al., 1998). The mechanism of action of terpenes on microbes is not yet fully understood, but it is speculated to involve membrane disruption by the lipophilic compounds (Mendoza et al., 1997). Cantrell et al. (2001) investigated a series of terpenoids for their antimicrobial effects and found out that the more lipophilic compounds were significantly more antibacterial than their more polar analogues. Tannins are a large group of polyphenolic compounds that are subdivided into two groups; hydrolysable and condensed tannins. A wide range of anti-infective actions have been assigned to them (Haslam, 1989). It has been postulated that the anti microbial mode

of action for tannin may thus be related to their ability to inactivate microbial adhesins, enzymes and cell envelope transport proteins (Cowan, 1999).

There is evidence that tannins may directly inactivate micro-organisms due to their ability to bind proteins and metals, and also to inhibit the growth of micro-organisms through substrate and metal ion deprivation (Brownlee et al., 1989). The extent to which these phytochemicals present in these extracts of *H. diversifolius* contribute to its antibacterial effect cannot be discerned. These could be exerting their effect through additive or synergistic action of several compounds acting at a single or multiple target sites associated with physiological process.

Conclusion

The aqueous and methanolic extracts of the different plant parts of *H. diversifolius* have demonstrated similar antibacterial effects. Except for the aqueous root extract, all other extracts had similar phytochemical constituents. The result suggests that some of the extracts of this plant should be investigated further as they could serve as source of drugs useful in the chemotherapy of some microbial infections.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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