ijCEPr
International Journal of Chemical, Environmental and Pharmaceutical Research
Vol. 3, No.2, 142-148
May-August, 2012

Inhibitory Effect of Varying Concentrations of Leaves' Extracts of *Centella Asiatica* (Gotu Kola) on Some Microorganisms of Medical Importance

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Article History:
Received: 15 April 2012
Accepted: 9 August 2012

ABSTRACT
The inhibitory concentration of *Centella asiatica* (Gotu kola) on some microorganisms of clinical importance was investigated using standard microbiological methods. The phytochemical screening revealed the presence of Alkaloids, Saponins, Tannins, Flavonnoids, Anthraquinones, Cardiac glycosides and Phlobatannins. The antimicrobial tests were carried out with ethanolic and aqueous extracts using agar disc diffusion method. The bacterial species used were *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Proteus* species, *Shigella* species, *Salmonella typhi* and *Vibrio cholerae*. The results obtained for ethanolic extracts revealed that *Staphylococcus aureus* exhibited the largest zones of inhibition 23.0mm (35mg/ml) while *Shigella* and *Escherichia coli* had the smallest of 15.0mm (15mg/ml). For the aqueous extracts *Proteus* species had the largest zone of inhibition of 18.0mm (35mg/ml) while *Vibrio cholerae* had the least zones of inhibition of 12.1mm (15mg/ml). The higher inhibitory effects of these ethanolic extracts may be due to some bioactive substances present in the extracts. The minimum inhibitory concentrations ranged from 100mg/ml to 500mg/ml for both aqueous and ethanolic extracts. The results obtained qualify *Centella asiatica* to be a medicinal plant that is recommended for use in the treatment of some diseases and infections.
Key words: Inhibitory effect, leaves extracts, bioactive components, *Centella asiatica* (Gotu kola), microorganisms.

INTRODUCTION

It would be of interest to know that most plants that we have around us contain one or more chemical substances which are of therapeutic value. That is why in developing countries especially in rural areas, people often turn to traditional medicine dealers when ill or infected with one particular disease or another.

Herbs had been used by all cultures throughout history to cure or prevent a disease in both animals and man [10]. It has become an integral part of the development of modern civilization. In Nigeria, a variety of herbal preparations are used by traditional medicine practitioners for treatment of different kinds of diseases including microbial infections [13].

The search for new antimicrobial agents led to the screening of bioactive compounds of plants. Medicinal use of plants range from the plant roots, bark, stem, flowers, leaves, seeds and extracts from the whole plants [12]. Some plants whose antimicrobial properties have been demonstrated include some Euphoriaceae plants which the family provides food [13,19].They varied in medicinal properties and are used in ethnobotany such as in the treatment of ailments like respiratory infection, venereal diseases, toothache, rheumatism, cough, ulcer and wounds [1,2,7,12,16]. Others include *Allium sativum*, which is active against *E. coli*, *Salmonella typhi, Staphylococcus aureus* and some fungi.

There is some traditional usage of some plants such as *Centella asiatica* (Gotu kola) in Nigeria. *Centella asiatica* belongs to the kingdom Plantae ,Family of Mackinlayaceae and Genus Centella [5].It is a small herbaceous annual plant of subfamily Mackinlayoideae of the Apiaceae and is a native to India ,Sri Lanka, Northern Australia, Indonesia, Iran, Malaysia, Papua New Guinea,and other parts of Asia[5,15,24]. It has small fan-shaped green leaves with white or light purple to pink flowers. It bears small oval-shaped fruits. The stems are slender, creeping stolons, green to reddish green in colour, interconnecting one plant to another. It has a long –stalked, green reinform leaves with rounded apices which have smooth texture with palmate netted veins. It is mainly found in as a weed growing in waste lands, agricultural lands and river basins. It grows abundantly during the rainy season. It contains no caffeine and is not a stimulant [5,11,15]. The plant has been exploited for several hundred years by traditional medicine dealers for the treatment of wounds, mental fatigue, skin conditions such as leprosy and psoriasis. It has been also used for treatment of syphilis, hepatitis, stomach ulcers, epilepsy, diarrhea, fever and asthma [4,24].

Centella asiatica has been widely used in traditional medicine in Africa, India, China, Japan, Indonesia, Sri Lanka and in South pacific for treatment of microbial infections [3]. *Centella asiatica* is often called one of the miracles elixirs of life because legend has it that an ancient Chinese herbalist lived for more than 200 years as a result of

using this herb [21]. Researchers reported that an American and Europeans herbalist used *Centella asiatica* for disorders that cause connective tissue swelling such as Scleroderma, Ankylising Spondylitis (arthritis of the spine) rheumatoid arthritis, depression and to improve memory [4]. Another reported that *Centella asiatica* has been used traditionally in lowering of high blood pressure, treating of various insufficiency (pooling of blood in the veins usually in the leg), boosting of memory and intelligence, easing anxiety and would healing [21]

Despite all these alleged used, there is need to ascertain some inhibitory effects of various concentrations of this plant extract on some medically important microorganisms especially infectious bacteria. This verification of its inhibitory effects on some of these bacteria of medical importance will help to establish a scientific rationale and basis, thereby validating its use. Hence the reason that proffers this research.

MATERIALS AND METHODS

Plant Collection

Centella asiatica plant part (leaves) was collected from Ikot Abasi Local Government Area of Akwa Ibom State. The leaves were identified by a taxonomist in the Department of Pharmacognosy and Traditional Medicine, University of Uyo, Uyo. The leaves were sun-dried for two weeks. The dried leaves were then grounded into fine particles with mortar and pestle for homogenicity.

Extraction (Ethanolic Extract)

400 grams of pulverized *Centella asiatica* was weighed out in 300ml of 50% ethanol for 72 hours at room temperature and then filtered using Whatman No. 1 filter paper. The filtrate was concentrated in water bath and evaporated to dryness at 40^0C [2,22]. The dried sample was stored in the refrigerator at 4^0C for further analysis.

Table-1: Phytochemical Screening of both Aqueous and Ethanolic Leaf Extracts of *Centella Asiatica*

S.No.	Bioactive Components	Observation	Inference Aqueous Extract	Ethanolic Extract
1	Alkaloid	Creamy precipitate	++	++
2	Saponin	(a) Persistent frothing from frothing test	+	++
		(b) Brown precipitate from sodium bicarbonate test	+	++
3	Tannin	Green precipitate	++++	+++
4	Phlobatannins	Red precipitate from HCl Test	++	+++
5	Anthraquinones	Violet colouration observed from Borntrager's test	+	++
6	Flavonnoids	Orange colouration	+	+
7.	Cardiac glycoside	Reddish brown at interphase with Liberman's test	+	++
		Violent ring observed with Keller-Killani test	+	++

KEY:
+ = bioactive component in small amount
++ = bioactive component moderate amount.
+++ = bioactive component high amount
++ ++ = bioactive component much higher amoun

Aqueous Extract

400 grams of pulverized *Centella asiatica* weighted out into 300ml of distilled water. The beaker was shaken every 30 minutes for 6 hours before leaving it undisturbed on a work bench for 48 hours. The mixture was then filtered using Whatman No. 1 paper. The filtrate was kept in the refrigerator at 4^0C until used [2,22].

Phytochemical Screening

To test for alkaloids, 0.5g of the extracts were stirred with 5ml of 1% hydrochloric acid (HCl) on a steam bath and then filtered. 1ml of the filtrate was treated with few drops of Dragnendroffs reagent and observed [22].

In Saponin test, two methods were employed, namely frothing test and sodium bicarbonate test. (a) Frothing test, about 0.5g of the plant's extracts were shaken vigorously with distilled water in a test-tube and

allowed for 15 minutes then observed [22]. (b)) Sodium bicarbonate test, about 0.5g of the plant's extracts was added with 5% sodium bicarbonate and Fehling's solutions A and B were added and boiled. Then observation was made [23].

Also to test for the presence of tannin, about 0.5g of the extracts were dissolved with 100ml of distilled water and filtered. The filtrated 5% Ferric Chloride reagent was added and observed. Hydrochloric acid test was used for detection of *Phlobatannins*. 0.5g of the extracts were dissolved in water and filtered, the filtrate was boiled with 1% hydrochloric acid and observed [22]. Borntrager's test was used in detecting the presence of *anthraquinones* whereby 0.5g of the extracts were shaken with 10ml benzene and filtered. To the filtrates, 10% ammonia solution was added to each tube and the mixture were shaken and observed [23]. To test the presence of Flavonnoids was carried according to method described by [23]. Whereby few pieces of magnesium metal were added to 5ml of each plant extract solution. The solution was obtained by dissolving the extract in concentrated hydrochloric acid. The presence of cardiac glycosides was confirmed using Liberman's test, and Keller-Killani test [9,22,23].

Table-2: Zones of Inhibition (mm) Showing Inhibitory Effect of Varying Concentration of *Centella asiatica* Aqueous Leaves Extracts on the Test Organisms

Test Organisms	Concentration (mg/ml) and Zones of Inhibition (in mm)				
	100mg/ml	200mg/ml	300mg/ml	400mg/ml	500mg/ml
Staphylococcus aureus	14.2	15.1	15.9	16.4	17.0
Streptococcus pyogenes	12.0	12.5	14.9	15.4	16.4
Escherichia coli	12.9	13.1	13.9	14.6	15.7
Proteus mirabitis	14.8	15.2	15.5	16.6	18.0
Shigella spp	12.1	12.1	12.5	14.6	15.7
Salmonella spp	13.1	15.9	14.4	15.1	16.00
Vibrio cholerae	12.1	15.1	15.3	16.2	17.0

Collection of Bacterial Test Isolates

Bacterial isolates used for this analysis were obtained from Microbiology Laboratory, University of Uyo, University of Uyo Health Centre as well as University of Uyo Teaching Hospitals, all in Akwa Ibom State, Nigeria. The organisms used were *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Proteus mirabilis, Shigella* species*, Proteus mirabilis, Salmonella* species *and Vibrio cholerae.*

Evaluation and Antimicrobial properties of the Plant's Leaf Extracts

The ability of the plant's leaf extract to inhibit growth of the bacterial isolates of medical importance was determined using the disc diffusion method [17]. Sterile filter paper discs of 6mm in diameter were soaked in equal volumes of varying concentrations of the extracts and left for 2 hours undisturbed. The 0.2ml of 18 hours peptone water culture of each of the test bacterial isolates was spread on the sterile Mueller Hinton Agar plates. The discs about 6mm in diameter containing varying concentrations of these extracts were picked with sterile forceps and placed at different areas on the surface of each plate were incubated at 37^0C for 24 hours. Control experiments comprising of plates were also set up. Antimicrobial activity of extracts or the inhibitory effect of the extracts on the test organisms was determined by measuring the zones of inhibition in Milliliter (mm) diameter of the respective disc.

Measurement and Interpretation of Zones of Inhibition

The diameters of the zone of inhibition of the growth were measured by the use of scale ruler in milliliter (mm) clear zones of inhibition indicated the susceptibility of the organism to the extracts while absence of such zones showed resistance or no inhibitory effect of extracts on the test organism.

Table-3: Zones of Inhibition (mm) Showing Inhibitory Effect of Varying Concentration of Ethanolic Leaves Extracts of *Centella asiatica* on the Test Organisms

Test Organisms	Concentration (mg/ml) and Zones of Inhibition (in mm)				
	100mg/ml	200mg/ml	300mg/ml	400mg/ml	500mg/ml
Staphylococcus aureus	16.2	18.8	20.0	23.0	23.0
Streptococcus pyogenes	14.3	16.7	17.7	19.0	20.0
Escherichia coli	15.0	18.0	18.8	19.3	20.0
Proteus mirabilis	16.0	17.9	18.2	20.1	20.5

Shigella spp	13.0	14.0	16.9	17.4	18.6
Salmonella spp	15.1	18.7	19.0	21.0	22.0
Vibrio cholerae	13.3	14.7	17.7	19.0	20.0

Susceptibility Test of the Organisms of Selected Commercial Antibiotics

A control experiment was set up to test the susceptibility of these test organisms on the commercial antibiotics. Each test isolates were inoculated and spread on sterile Mueller-Hinton Agar plates and multi-discs of antimicrobial drugs were placed on each plate using a sterile forceps and incubated also at 37^0C for 24 hours [8].

Table-4: Zones of Inhibitions of Gram-Positive Isolates to Commercial Antibiotics Used

Antibiotics Used	Disc Concentration	Test Organisms/Zones of Inhibitions (mm)	
		Staphylococcus aureus	*Streptococcus pyogenes*
RD	20µg	11.3	13.1
FLX	20µg	14.0	12.5
E	30µg	13.0	16.0
CH	20µg	14.5	11.5
APX	20µg	11.0	14.2
CPX	10µg	16.0	15.1
S	30µg	15.0	11.4
LC	20µg	16.5	13.4
NB	10µg	12.5	16.5
CN	10µg	16.1	16.0

Zones of Inhibition > 12mm = Sensitive
Zones of inhibition < 12mm = Resistance

Key:

RD	=	Rifampicin	S	=	Streptomycin
FLX	=	Floxapen	LC	=	Lincocin
E	=	Erythromycin	NB	=	Norfloxacin
CH	=	Chloramphenicol	CN	=	Gentamycin
APX	=	Ampiclox	<12mm	=	Resistant
CPX	=	Ciprofloxacin	>12mm	=	Sensitive

Table-5: Zones of Inhibitions of Gram-Negative Isolates to Commercial Antibiotics Used

Antibiotics Used	Disc Concentration	Test Organisms/Zones of Inhibitions (mm)				
		E.c	*P. m*	*S. pp*	*Sal. spp*	*V. c*
OFD	10µg	13.0	14.2	15.1	12.1	13.5
PEF	10µg	11.1	14.0	12.0	12.5	12.5
CPX	10µg	12.5	13.1	16.1	16.1	16.1
AU	30µg	15.2	16.2	15.2	15.1	15.6
CN	10µg	16.1	17.1	14.3	18.1	14.1
SXT	20µg	13.5	18.1	15.3	16.1	15.2
PN	30µg	14.3	15.8	16.3	15.2	12.0
CEP	10µg	16.8	17.1	16.3	16.2	13.1
NA	30µg	15.4	12.2	15.1	12.4	14.2
S	30µg	14.5	13.3	14.1	12.0	15.3

Key:

OFD	=	Tarivid	CEP	=	Ceprorex
PEF	=	Peflacine	NA	=	Nalidixic acid
CPX	=	Ciprofloxacin	S	=	Streptomycin
AU	=	Augmentin	E. c	=	*Escherichia coli*
CN	=	Gentamycin	*P. m*	=	*Proteus mirabilis*
SXT	=	Septrin	*S.* pp	=	*Shigella* species
PN	=	Ampicillin	*Sal.* Spp	=	*Salmonella* species
			V. c	=	Vibrio cholerae

Table-6: Minimum Inhibitory Concentrations of Aqueous Leaves Extracts of *Centella asiatica* on the Test Organisms

Test Organisms	Test Organisms/Zones of Inhibitions (mm)				
	500	250	125	62.5	31.25
S. a	-	-	-	+	+
St. p	-	-	+	+	+
E. c	-	-	+	+	+
P. m	-	-	-	-	+
S. pp	-	+	+	+	+
Sal. spp	-	-	-	+	+
V. c	-	-	-	-	+

Keys:			Minimum Inhibitory Concentration
S. a	=	*Staphylococcus aureus*	125mg/ml
St. p	=	*Streptococcus pyogenes*	250mg/ml
E. c	=	*Escherichia coli*	250mg/ml
P. m	=	*Proteus mirabilis*	62.5mg/ml
S. pp	=	*Shigella* species	500mg/ml
Sal. spp.	=	*Salmonella* species	125mg/ml
V. c	=	*Vibrio cholerae*	62.5mg/ml
+	=	Growth	
-	=	No growth	

Table-7: Minimum Inhibitory Concentrations of Ethanolic Leaves Extracts of *Centella asiatica* on the Test Organization

Test Organisms	Test Organisms/Zones of Inhibitions (mm)				
	500	250	125	62.5	31.25
S. a	-	-	-	-	+
St. p	-	-	-	+	+
E. c	-	-	+	+	+
P. m	-	-	-	+	+
S. pp	-	+	+	+	+
Sal. spp	-	-	-	-	+
V. c	-	-	-	+	+

Keys:			Minimum Inhibitory Concentration
S. a	=	*Staphylococcus aureus*	62.5mg/ml
St. p	=	*Streptococcus pyogenes*	125mg/ml
E. c	=	*Escherichia coli*	250mg/ml
P. m	=	*Proteus mirabilis*	125mg/ml
S. pp	=	*Shigella* species	500mg/ml
Sal. Spp .	=	*Salmonella* species	62.5mg/ml
V. c	-	*Vibrio cholera*	125mg/ml
+	=	Growth	
-	=	No growth	

Determination of Minimum Inhibitory Concentration of the Extracts of Each Isolates

The minimum inhibitory concentration (MIC) of the leaf extracts on each isolate was determined using tube dilution method as described by [8]. Different concentrations of the extract ranging 31.5mg/ml to 500mg/ml were prepared by making a serial dilution with the 500mg/ml to the power of five for each of the extracts. The different concentration was seeded on sterile Petri-dishes and then a molten nutrient agar was poured on these seeded Petri-dishes and the swirled, allow to cool test. Organism was inoculated on these Petri-dishes with different concentration of the extracts and controls for all the cultures were set up without the extracts. The plates were incubated at 37^0C for 24 hours. The concentration without any visible growth was reported as the minimum inhibitory concentration of the plant's leaf extracts for that particular organism.

RESULTS AND DISCUSSION

The phytochemical screening of both Aqueous and Ethanol extract of *Centella asiatica* leaves carried out showed the presence of some bioactive components in varying amounts. The components are alkaloids, Saponins, tannin,

Phlobatannins, Flavonnoids, anthraquinones and cardiac glycosides. This is shown on Table 1. The result of phytochemical analysis of *Centella asiatica* leaves showed that the leaves contain some bioactive components namely Saponins, tannins, Phlobatannins, anthraquinones, Flavonnoids, alkaloid and cardiac glycoside. These bioactive components when found on a plant, have some inhibitory effects on some microorganisms, some of which were reported in literature as antimicrobial constituents. For instance, Flavonnoids are known to be antimicrobial in nature [20]. Tannins were also identified to have antimicrobial activities [6].

The aqueous and ethanolic extracts obtained from *Centella asiatica* leaves of various concentrations were used for antimicrobial sensitivity assay to determine the inhibitory effects of these extracts on the test organisms. The results revealed that both in aqueous and ethanolic extracts, the inhibitory effects determined by the zone of inhibition (in mm) increases with increase in concentration of extracts. However the highest zones of inhibitions were greatly recorded with ethanolic extracts than that of aqueous extract. A zone of inhibition of 14.2mm was observed with *Staphylococcus aureus* at the aqueous concentration of 100mg/ml but as the concentration of the extracts increased to 200mg/ml, the inhibition effect of the extracts also increased with zones of inhibition of 15.1mm. Zones of inhibitions by other test organisms also increased with increased concentrations. Similarly in ethanolic extracts, inhibitory effect was also recorded for *Staphylococcus aureus* and *Salmonella* species with clear zones of inhibition of 16.2mm and 16.1mm respectively, with an extracts concentration of 100mg/ml but 18.8mm and 18.7mm when extracts concentration was 200mg/ml. However the highest zone of inhibitions was observed with *Staphylococcus aureus* with 23.0mm at the ethanolic leaves extracts concentration of 400mg/ml and 500mg/ml respectively. These are illustrated on Tables 2 and 3. Although the aqueous leaves extracts produced some inhibitory effects on the clinical isolates, the ethanolic leaves extracts were observed to produce high inhibitory effects. This confirmed the report of [18] who reported ethanol to be the best solvent for extraction over aqueous when working with plants of medicinal importance.

It was discovered that in the commercial antibiotics which are commoly used as a routine drugs, *S.* aureus was sensitive to some antibiotics such as Floxapen, Erythromycin, Ciprofloxacin, Streptomycin and Gentamycin but resistant to Rifampicin and Ampiclox. Also sensitivity patterns for other organisms were also observed. However, the results showed that the highest zones of inhibitions when using the commercial antibiotic susceptibility discs to be 16.5mm from *Staphylococcus aureus* on Lincocin (LND) and 16.5mm was observed with *Streptococcus pyogenes* on Norfloxacin (NB) while 18.1mm were recorded for *Salmonella* species on Gentamycin (CN) and *Proteus* species on Septrin (SXT) respectively (Table 4 and 5). Thus, since higher zones of inhibitions from the leaves extracts of *Centella asiatica* on these organisms were observed on these isolates, the plants leaves could be used to cure infections from the tested isolates in the place of commonly used antibiotics which the isolates have started to develop resistance.

Minimum inhibitory concentrations (MIC) of both the aqueous and ethanolic leaves extracts of Centella asiatica were determined for each test organism. In aqueous extracts *Streptococcus pyogenes* had the MIC of 250mg/ml while MIC for *Staphylococcus* aureus was 125mg/ml and 62.5mg/ml was observed *Proteus mirabilis.* In ethanolic extracts, the lowest MIC of 62.5mg/ml was observed with *Staphylococcus* aureus and *Salmonella* species respectively, while the highest MIC was observed with *Shigella* species with 500mg/ml (Table 6 and 7).

The results of antimicrobial activities of the extracts revealed that *Centella asiatica* extracts have high antimicrobial activity with some zones of inhibition of $\geq$15mm from ethanolic leaves extracts against some clinical isolates while some susceptibility patterns were also observed with aqueous extracts. Thus, both aqueous and ethanol could be used as solvents to extract bioactive components from this plant. However, the strong inhibitory effects of these extracts over commercial antibiotics and zone of inhibition greater than 12mm in diameter for the isolates are noteworthy as some organisms used were noted to be resistant to commercial antibiotics, for instance *S. aureus* was resistant to Ampiclox but highly sensitive to the extracts. The MIC of both aqueous and ethanolic extract of *Centella asiatica* leaves showed the lowest concentration of 62.5mg/ml for some isolates, thus, small quantity of the plants leaves could be used to treat infections from these organisms.

CONCLUSION

The findings in the study showed the inhibitory effects of both the aqueous and ethanolic leaves extracts of *Centella asiatica* on some clinical organisms commonly involved in wound infection, urinary tract infection, gastrointestinal tract infections, sore throat, respiratory tract infection and inflammations, thereby justifying its use for therapeutic purposes. Therefore there is need to conserve this plant species for exploitation by pharmaceutical industries for the production of new drugs.

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