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Phytochemical screening and antimicrobial activity of *Theobroma cacao on Staphylococcus aureus, Escherichia coli, Samonella spp and Shigella spp*

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Abstract

Cocoa leaf and root were studied for medicinal prospective and the focus was to screen for phytochemicals present, Minimum Inhibitory Concentration, Minimum Bactericidal Concentration and antimicrobial activity of the cocoa root and leaf against pathogenic bacteria *Staphylococcus aureus, Escherichia coli, Salmonella spp, Shigella spp*. Extraction of the samples was done by filtration and concentration in water bath with methanol and aqueous as the solvents, followed by phytochemical analysis, antimicrobial activity, Minimum Inhibitory Concentration and Minimum Bactericidal Concentration. The phytochemical screening identified presence of glycosides, steroids, quinones, phenols, proteins, flavoids, tannins and alkaloids. The antimicrobial assay by agar well diffusion method of cocoa leaf and root extracts elicited ranges of zone of inhibition measured in millimetre (mm) against the selected pathogenic bacteria; *Staphylococcus aureus, Escherichia coli, Salmonella spp, Shigella spp* at various concentration (500Mg/ml, 250Mg/ml, 200Mg/ml, 125Mg/ml and 100Mg/ml). The aqueous leaf extract at 500Mg/ml to 100Mg/ml concentration elicited zone of inhibition ranging from 26.33±1.53 to 12.00±1.00 against *S. aureus*. Against *E. coli* the zone of inhibition ranges from 22.67±1.16 to 9.00±1.00. Similarly at 500Mg/ml to 100Mg/ml concentration the aqueous leaf elicited zone of inhibition ranging from 19.67±1.53 to 8.33±0.58 against Salmonella spp and 17.00±1.73 to 8.33±1.16 against Shigella spp. The methanolic leaf extract also elicited zone of inhibition at various concentration (500Mg/ml to 100Mg/ml) ranging from 21.33±1.53 to 11.67±1.53 against *S. aureus*, 26.67±2.52 to 14.33±0.58 against E. coli, 20.00±1.00 to 12.67±3.06 against Salmonella sppand 13.00±2.00 to 5.67±0.55 against shigella spp at concentration of 500Mg/ml to 100Mg/ml respectively. Lastly the root bark extract elicited zone of inhibition ranging from 22.66±2.09 to 16.00±0.00 against *S. aureus*, 16.33±2.31 to 10.33±1.16 against E. coli, 17.00±2.00 to 8.67±1.16 against Salmonella spp and 20.00±1.00 to 8.33±0.58 against Shigella spp at concentration of 500Mg/ml to 100Mg/ml. The MIC was at 50Mg/ml concentration for all test organisms using the three different extracts while organisms were resistant at all concentration for MBC using all extracts. It is recommended that *T.cacao leaf* and root are used for antibacterial agent with further purification and proper processing, it is also recommended for toxicology test to know the level of human cell tolerance.

Keywords: Antimicrobial activity; *Theobroma cacao;* Phytochemistry; Microorganisms; Aqueous extract; Methanolic extract

1. Introduction

Medicinal plants are considered a rich resources of ingredients which can be used in drug development pharmacopoeia, non-pharmacopoeia or synthetic drugs. Some plants are considered important source of nutrition and as a result of that they are recommended for their therapeutic values (Mahtab, 2016). Medicinal plants are the hope of health continuity worldwide, with so many cases of serious diseases which need medical attention (WHO, 2010). Medicinal plants contain substances that can be used for therapeutic purposes. There some medicinal plants whose therapeutic properties and

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constituents have been established scientifically, while others are regarded as medicinal but not yet subjected to thorough scientific study.

Cocoa has become an important ethno medicinal plant since it has a unique chemical composition of more than 500 different compounds (Crown and Hurst, 2009). The vast contribution of cocoa for human health includes: anti-inflammatory, regulation of blood pressure, insulin resistance, oxidative stress (Valeria Ludovici *et al.,* 2017), anti atherogenic, anti-microbial (Wollgast, 2000), immunomodulatory, vasodilatory, analgesic and antibacterial (Santos *et al.,* 2014) activities.

Almonds or cocoa beans constitute raw material for the production of chocolate and its derivatives, which are used in different forms worldwide. Processing of cocoa on the farms begins with opening of the fruit (pods) in the field under the trees, where the seeds are separated from the husks. The seeds or cocoa beans harvested are fermented and then dried to reduce moisture content and water activity. The drying process interferes with biochemical reactions initiated during fermentation, leading to a reduction in the bitterness, acidity of cocoa beans (Ziegleder, 2009). The cultivation of cocoa is of economic importance for several countries such as Ghana, Ivory Coast, Nigeria, Indonesia, Malaysia, and Brazil (Azizah *et al*., 2007)

As science is advancing recent discoveries are being made on plants that have bio-active compounds (Fernanda *et al.,* 2019 and Moghadamtousi *et al.,* 2015) capable of inhabiting the development of disease caused by microorganisms. Medicinal plants can as well serve as source of wealth for a country since they serve as raw materials for the manufacture of conventional drugs (Sadha and Roy, 2016)

In this part of the country, most plants fruit and vegetables are consumed as food which contain several medicinal potency and prevent the body from microbial infections which could result to disease manifestation that may require the use of synthetic treatment that might have side effects (Rasool, 2013).

Aim

The aim of this research is to test for the phytochemical and antimicrobial activity of *T.cacao* leaves, and root extracts on pathogenic bacteria (*Salmonella spp, Staphylococcus aureus, Shigella spp and Escherichia coli).*

2. Methodology

2.1 Sample Collection

The cocoa leaves and root were explanted from cocoa plantation in Ukwo, Owukpa, Ogbadibo local government area of Benue state. The samples were collected in sterile polythene bags and transported to the microbiology laboratory of University of Agriculture Makurdi through Benue link transport. They were further washed under tap water for few minutes followed by distilled water for 5-10 minutes. Then, dried under room temperature for 2weeks.

2.2 Sample Preparation

The collected, dried sample of cocoa leaves and cocoa root were pounded using mortar and pestle was sieved to make a coarse powder.

2.2.1 Preparation of crude Extraction

120g of each of the plant material (root and leaf) was weighed and transferred into a container and soaked in 500ml each of methanol and aqueous solvent differently for 3days (72hrs) and filtered with sieve to remove debris. The filtrate was evaporated in a water bath at 49.7°C to get the crude extract. They were then stored until required and was used for phytochemical and antimicrobial analysis. The method of (Idris *et al.,* 2009) was used.

2.3 Phytochemical Screening of the Plant Extracts

The crude extract of each solvent (methanol and aqueous) were partitioned and screened for glycosides, protein, quinines, terpanoids, alkaloids, flavoids, saponins, steroids, tannin and phenolic acids using standard method as described by Ngbele *et al.,* (2008). The color intensity of the precipitate which was formed was used as analytical test control.

2.3.1 Test for Glycosides

A 5ml of diluted H_2SO_4 was added to 1ml of the plant extract in a 100ml flask. It was boiled for 15 minutes, it was cooled and neutralized with 10% NaOH. 1ml of Fehling solution A and B was added to the neutralized solution and a brick red precipitate of reducing sugar indicated the presence of glycoside.

2.3.2 Test for Steroids

1g of the plant extract was dissolved in a few drops of acetic acid. It was gently warmed and cooled under the tap water and a drop of concentrated H_2SO_4 acid was added along the sides of the test tube. The appearance of a green colour indicated the presence of steroids.

2.3.3 Test for Saponins

About 3ml of the extract was mixed with equal amount of distilled water and was shaken vigorously, a copious lather formation indicated the presence of saponins.

2.3.4 Test for Quinones

About 2ml of the extract was mixed with concentrated sulphuric acid. The appearance of blue-green or red colour formation indicated the presence of quinones.

2.3.5 Test for Phenol

A few drops of Ferric Chloride solution was added to 2ml of the extract in a test tube, the presence of a green color indicated the presence of phenol.

2.3.6 Test for Terpernoids

About 2ml of chloroform was added to 1ml of the extract and 5ml of concentrated H_2SO_4 was added. A reddish brown precipitate produced immediately indicated the presence of terpernoids.

2.3.7 Test for Proteins

The plant extract was treated with 2 to 3 drops of concentrated Nitric acid. Yellow colour precipitation in the test tube indicated the presence of protein.

2.3.8 Test for Flavonoids

About 2ml of the extract was mixed with 1g of magnesium turnings, the mixture was boiled for 5minutes. The appearance of orange to red color indicated the presence of flavoids.

2.3.9 Test for Tannins (Wohler's test)

A few drops of lead acetate solution was added to 1ml of the extract, the appearance of a white precipitate indicated the presence of tannin.

2.3.10 Test for Alkaloids (Meyer's test)

About 1ml of the extract was added to few drops of dilute hydrochloric acid and Mayer's reagent were added to the solution, the formation of a white precipitate indicated the presence of alkaloid.

2.4 Test Organisms

Cold stored agar slant cultures of gram positive and gram negative organisms were used in this study. Isolates were obtained from the microbiology laboratory, Department of Microbiology, University of Agriculture Makurdi. Viability test of organism: *Staphylococcus aureus*, *Escherichia coli, Shigella dysenteriea, Salmonella sp*. was carried by resuscitating the organisms in buffered peptone broth and thereafter sub-cultured into nutrient agar medium and incubated at 37°C for 24hrs. The probable identity of the clinically sourced isolates were further subjected to biochemical tests for confirmation which include; coagulase, indole, citrate and oxidase tests as described by Collins *et al.* (2004) and Sharma (2009). The result of the biochemical reactions elicited by the test isolates were compared with standard identification keys as described by Collins *et al.* (2004).

2.5 Preparation of Concentration of the Plant Extracts.

One gram (1g) each of aqueous and methanolic extracts was added to 2ml of distilled water and methanol respectively to give a concentration of 500mg/ml. other concentrations of 250mg/ml, 200mg/ml, 125mg/ml and 100mg/ml were prepared by double broth dilution method as described by udochukwu *et al.,* (2015).

2.6 Antibacterial Susceptibility Testing of the Extract with the Test Organisms.

Susceptibility testing was carried out using agar well diffusion method, according to the recommendation of the National Committee for Clinical Laboratory Standards (2000).

In this method, the inoculum were prepared by inoculating the normal saline broth and incubated at 37°C for 24hrs. The cultures were diluted to 0.5 McFarland turbidity standard after the incubation. About 0.5ml each of the cultured organisms was pipetted onto the petri dish after which prepared Muller Hinton agar was pour plated and allowed to solidify. After the culture plates have gelled wells were bored on the surface of the agar plates using 4mm cork borer. About 0.2 ml of the different concentrations of each extract were transferred into the well using Pastuer pipette. The wells were sufficiently spaced to prevent the resulting zone of inhibition from overlapping. The plates were incubated for 37°C for 24hrs. The experiment was performed in triplicates and the resulting zone of inhibitions measure the diameter of the well using a ruler.

2.6.1 Minimum Inhibitory Concentration (MIC)

The mic of the potent extracts was determined according to the macro broth dilution technique described by (Baron and Feingold, 1990). Standardized suspension of the test organisms was inoculated into a series of test tubes of normal saline broth containing two fold dilution of the extracts and inoculated at 37°C for 24hrs. The MIC were recorded as the least concentration that inhibited the growth of the test organisms.

2.6.2 Minimum Bactericidal Concentration (MBC)

The MBC of the respective extracts was determined by procedure described by Asewata *et al*., (2013). Aliquot were taken from the MIC tubes with no visible growth and sub-cultured on freshly prepared nutrient agar plates and later incubated at 37°C for 24hours. The MBC was recorded as the concentration of the extract that did not show any growth on new set of agar plates.

2.7 Statistical Analysis

Data were analyzed for mean and standard deviation. Difference in parameter was tested for statistical difference at $P < 0.05$ using ANOVA. All the analysis were done using statistical package service solution (SPSS) version 21.

3. Results

3.1 Zone Of Inhibition

Table 1 shows Zone of inhibition of the aqueous leaf extract of *Theobroma cacao*on four different test organisms; *Staphylococcus aureus*, *E. coli, Salmonella spp, and Shigella spp*. *Staphylococcus aureus* had the highest zone of inhibition when the highest concentration of 500mg/ml was used as compared to the control. All test organisms were susceptible to the leaf extract of *Theobroma cacao* at the different concentration ranging from 500mg/ml to 100mg/ml.

Zone of inhibition of the methanolic leaf extract which was used against four different test organisms are shown in table 2. *E. coli* had the highest zone of inhibition of 26.67±2.53 when the concentration of 500mg/ml was used as compared to the control. All test organisms was susceptible to the leaf extract except *Shigella spp* which was resistant to the methanolic leaf extract at the concentration of 125mg/ml and 100mg/ml.

The zone of inhibition elicited by methanolic root extract of *Theobroma cacao* using as *S. aureus, E. coli, Salmonella spp, Shigella spp* as test organisms is represented in table 3. The highest zone of inhibition was recorded against *S. aureus* at the concentration of 500mg/ml as compared to the control. The test organisms were all susceptible to the root extract of *Theobroma cacao* at different concentration ranging from 500mg/ml to 100mg/ml.

Table 4describes the Minimum Inhibitory Concentration of the aqueous leaf extracts in which at 50% concentration they was no turbidity, at 25%, 12.5% and 6.25% they was moderate and high turbidity.

Table 1 Zone of Inhibition of Aqueous Leaf Extract of *Theobroma cacao* on selected organisms

Organism	500 Mg/ml	250 Mg/ml	200 Mg/ml	125 Mg/ml	100 Mg/ml	(Ciprofloxacin 10µg)
S. aureus	26.33±1.53	22.33±1.16	19.33±1.16	15.33±2.52	12.00±1.00	17.75±1.35
E. coli	22.67±1.16	13.67±1.53	11.67±2.08	10.33±0.58	9.00±1.00	18.56±1.52
Salmonella spp	19.67±1.53	14.67±2.52	15.33±1.16	12.67±2.08	8.33±0.58	16.00±1.57
Shigella spp	17.00±1.73	16.33±0.58	14.00±1.00	11.67±0.58	8.33±1.16	15.00±1.00

df= 4;P=0.02

Table 2 Zone of Inhibition of The Methanolic Leaf Extract of *Theobroma cacao* on selected test organisms

Organism	500Mg/ml	250Mg/ml	200Mg/ml	125Mg/ml	100Mg/ml	(Ciprofloxacin 10µg)
S. aureus	21.33±1.53	18.33±1.53	15.67±1.16	14.33±1.16	11.67±1.53	17.75±1.35
E. coli	26.67±2.52	21.67±0.58	18.33±1.16	16.33±0.58	14.33±0.58	18.56±1.52
Salmonella spp	20.00±1.00	17.00±1.73	16.33±2.31	14.00±3.00	12.67±3.06	16.00±1.57
Sbigella spp	13.00±2.00	11.67±1.16	9.67±1.60	7.32±2.03	5.67±0.55	15.00±1.00

df = 4; P=0.01

Table 3 Zone of Inhibition of The Methanolic Bark Extract of *Theobroma cacao* on selected test organisms

Organism	500 Mg/ml	250 Mg/ml	200 Mg/ml	125 Mg/ml	100 Mg/ml	(Ciprofloxacin 10µg)
S. aureus	22.66±2.09	19.67±1.53	15.67±5.77	18.00±1.73	16.00±0.00	17.75±1.35
E. coli	16.33±2.31	15.33±0.58	13.33±1.16	11.33±1.16	10.33±1.16	18.56±1.52
Salmonella spp	17.00±2.00	13.00±2.65	11.00±1.00	9.33±0.58	8.67±1.16	16.00±1.57
Shigella spp	20.00±1.00	18.33±0.58	14.67±1.53	11.33±1.16	8.33±0.58	15.00±1.0

df= 4; P=0.02

Table 4 Minimum Inhibitory Concentration of Aqueous Leaf Extract on Test Organisms

Organism	Extracts	50%	25%	12.5%	6.25%
S. aureus	Methanol	-	+	+	++
E. coli	Methanol	-	+	+	++
Salmonella spp	Methanol	-	+	++	++
Shigella spp	Methanol	-	+	++	++

Key (-) no turbidity (+) moderate turbidity (++) high turbidity

The Minimum Inhibitory Concentration of the methanolic leaf extract is presented in table 5. At 50% concentration they was no turbidity, at 25%, 12.5% and 6.25% they was moderate and high turbidity.

Table 6 displays the Minimum Inhibitory Concentration of the methanolic root extract in which at 50% concentration they was no turbidity, at 25%, 12.5% and 6.25% they was moderate and high turbidity.

The Minimum Bactericidal Concentration of the aqueous leaf extract of the selected test organisms were resistant to the plant extracts at all concentration as shown in table 7.

The display on table 8 explains the Minimum Bactericidal Concentration of the methanolic leaf extract on the selected test organisms. The organisms were resistant at all concentration.

In table 9 Minimum Bactericidal Concentration of the methanolic root extract is presented at all concentration. The selected test organisms were resistant to the extracts.

Table 10 shows the result for phytochemical screening of *Theobroma cacao* leaf and bark. Glycosides, steroids, saponins, quinones, phenols, proteins, flavoid, tannins, alkaloids were all present except terpenoids.

Table 5 Minimum Inhibitory Concentration of Methanolic Leaf Extract on Test Organisms

Organism	Extracts	50%	25%	12.5%	6.25%
S. aureus	Aqueous	-	+	+	++
E. coli	Aqueous	-	+	+	+
Salmonella spp	Aqueous	-	+	++	++
Shigella spp	Aqueous	-	+	+	+ +

Key (-) no turbidity (+) moderate turbidity (++) high turbidity

Table 6 Minimum Inhibitory Concentration of Methanolic Root Extract on Test Organisms

Pathogen	50%	25%	12.5%	6.25%
S. aureus	-	+	+	++
E. coli	-	+	+	++
Salmonella spp	-	+	+	++
Shigella spp	-	+	+	++

Key (-) No turbidity, (+) Moderate turbidity (++) High turbidity.

Table 7 Minimum Bacterial Concentration of Aqueous Leaf Extract on Test Organisms

Organism	50%	25%	12.5%	6.25%
S. aureus	R	R	R	R
E. coli	R	R	R	R
Salmonella spp	R	R	R	R
Shigella spp	R	R	R	R

Key: (R) Resistant

Table 8 Minimum Bacterial Concentration of MethanolicLeaf Extract on Test Organisms

Organism	50%	25%	12.5%	6.25%
S. aureus	R	R	R	R
E. coli	R	R	R	R
Salmonella spp	R	R	R	R
Shigella spp	R	R	R	R

Key: (R) Resistant

Table 9 Minimum Bacterial Concentration of Methanolic Root Extract on Test Organisms

Organism	50%	25%	12.5%	6.25%
S. aureus	R	R	R	R
E. coli	R	R	R	R
Salmonella spp	R	R	R	R
Shigella spp	R	R	R	R

Key: (R) Resistant

Table 10 Result for Phytochemical Screening of *Theobroma Cacao* Leaf and Bark

Phytochemical constituents	AQ LE	METH LE	METH RTE
Glycosides	+	+	+
Steroids	+	+	-
Saponins	-	+	-
Quinones	+	+	+
Phenols	+	+	+
Terpenoids	-	-	-
Proteins	+	+	+
Flavoids	+	+	+
Tannin	+	+	+
Alkaloids	+	+	+

Key; (+) present, (-) absent; AQ LE= Aqueous leaf extract, METH LE = Methanolic leaf extract and METH RTE = Methanolic Root extract.

4. Discussion

The phytochemical screening of *Theobroma cacao* leaf and root in aqueous and methanolic solvent revealed the presence of bioactive compounds such as glycosides, steroids, tannins, flavonoids, saponins, proteins, alkaloids, quinones and absence of terpenoids. This may be due to environmental or physiological conditions experienced by the plant and also may be due to species difference. The distribution of saponins, tannins, flavonoids and steroids could be the reason for its biological activity. This agrees with the research of Jiyoung *et al* (2014) who reported the presence of phytochemicals in cocoa to be responsible for its health promoting effects. The cocoa plant extracts were tested for antimicrobial activity against the test organisms (*Staphylococcus aureus, E. coli, Salmonella spp, Shigella spp*) on Muellar Hinton agar in which the methanolic and aqueous extract of leaf and root elicited different ranges of zone of inhibition at all concentration with highest inhibition zone at 500mg/ml for all extracts, this agrees with the report of Jayanthi Abraham *et al* (2015) that the plant has biological activities potentials. According to plant database (2008) phytochemical components have antibacterial properties which were confirmed in this study. The presence of tannins in the plant extracts agrees with the report of Evans (1998) that tannins are important in herbal medicine. The plant extracts also showed Minimum Inhibitory Concentration (MIC), the selected test organisms were susceptible at 50% concentration and Minimum Bactericidal Concentration (MBC) was also tested in which the organisms were resistant all concentrations.

5. Conclusion

The result of phytochemical screening in this study reveals the presence of secondary metabolites to include tannins, alkaloids, flavonoids, saponins in *Theobroma cacao* leaves and root. Extracts elicited zone of inhibition indicating therapeutic potentials as a result of the bio-components present whose antibacterial potentials are highly comparable with the antibiotic ciprofloxacin against the test organisms (*Staphylococcus aureus, E. coli, Salmonella spp, Shigella spp*). The minimum inhibitory concentration was at 50% concentration and organisms were resistant to extracts at all concentration in Minimum Bactericidal Concentration testing.

Therefore, there is a great chance of these cocoa plant parts to be used as antibacterial agent with potential applications in pharmaceutical industry for controlling infections caused by the organisms used in this study.

Recommendations

- *Theobroma cacao* leaf and root are recommended as antibacterial agent for treatment of diseases caused by the test organisms with further purification and proper processing.
- It is recommended that these extracts be subjected to toxicology test to know the extent at which human cells can tolerate it.
- It is recommended that other parts of this plant are studied for antimicrobial efficacy.

It is recommended that cocoa is cultivated more for abundant availability to pharmaceutical industries.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors have no conflicts of interest relevant to this article.

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