INHIBITORY EFFECT OF ANTIMICROBIAL ACTIVITY OF HIBISCUS CALYX EXTRACT ON BACTERIAL ISOLATES OBTAINED FROM FOOD SAMPLES

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Abstract: Antimicrobial activity of hibiscus calyces extract was carried out on isolates of bacteria obtained from "kunu" and bread samples determine the inhibitory effect of the hibiscus calyces extracts on the bacteria isolates from the food samples. The hibiscus calyces were extracted with water and ethanol at concentrations of 0.1, 0.15 and 0.20 g/ml, respectively. The following species of bacteria were identified from the food samples of bread and kunu; *Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Micrococcus* species The antimicrobial activity of *H. sabdariffa* calyces extract at three different concentrations (0.1, 0.15 and 0.20 g/ml) were tested against the bacteria isolated from the food samples using agar well diffusion method. The aqueous extracts at concentrations of 0.1, 0.15 and 0.20 g/ml did not show any antimicrobial growth effect on all the test microorganisms. At 0.10 g/ml, the ethanolic extracts showed similar results to that of the aqueous extract. At 0.15 g/ml, the antimicrobial effectiveness varied slightly among test organisms. The ethanolic extract at 0.20 g/ml concentration showed the highest potency on all the test microorganisms. Ethanolic extracts of the calyces had a notably higher antibacterial activity on all the test organisms than the aqueous extracts which showed no microbial inhibitory effects at the selected concentrations. The results obtained from this study therefore showed that *Hibiscus sabdariffa* calyces ethanolic extract at 0.20 g/ml concentration can be used as a non-synthetic food preservatives.

Keywords: Antimicrobial activity, *Hibiscus sabdariffa*, calyces extracts, inhibitory effect, preservatives

Introduction

Hibiscus sabdariffa L. belongs to the large family of Malvaceae (Osman *et al.*, 2011) and is commonly known as "sule" or "zobo"' (in Hausa) in Nigeria (Dokossi, 1998). The Genus Hibiscus has more than 300 known species which are used as ornamental plants. It grows up to 5 – 7 feet in height, with lobed leaves sometimes used for greens. The narrow leaves and stems are reddish-green in colour. It is a medicinal plant commonly found in the tropics and subtropics. *Hibiscus sabdariffa* L. calyces are widely used in the preparation of beverages. The calyces contain compounds that exhibit antimicrobial activity, yet little research has been conducted on their possible use in food systems as antimicrobials.

The *Hibiscus sabdariffa* plant is consumed as food and also has medicinal value (Stephens, 2012). The young shoots and leaves are used as vegetables and potherbs. The red calyces of *H. sabdariffa* are used in the preparation of a flavourful, tart cold or hot beverage and jams. These calyces have been shown to contain numerous bioactive compounds. The majority of compounds found within the calyces that exhibit antimicrobial activity are polyphenolic compounds. One group of polyphenolic compounds present in Hibiscus calyx extract is the flavonoids, which includes the plant pigments called anthocyanins. Other compounds found in the calyces include phenolic acids such as gallic and protocatechuic acids (Ramirez-Rodriguez *et al.*, 2011).

The calyces are also used as colouring matter (Dokosi, 1998). *Hibiscus sabdariffa* is medicinally used as a laxative, an anticarcinogenic, an antihypertensive, and a cholesterol lowering medicine. It also exhibits great antioxidant activity, lowers hepatoxicity and reduces fevers. In some parts of Africa, it is used as a remedy for abscesses, bilious conditions, cough, sores, wounds, dysuria, and scurvy (Morton, 1987). In folk medicine, an infusion from the calyces is used as a diuretic and to treat gastrointestinal disorders, liver diseases, fever, and hypertension (Monroy-Ortiz and Castillo-Espana, 2007).

There is little literature about the antimicrobial property of *H. sabdariffa* plant though some scientists have attributed it to the presence of the secondary metabolites (alkaloids, flavonoids, phenolics, and biterpenoids) in the extract (VanEtten *et al.*, 1994; Badreldin *et al.*, 2005; Olaleye, 2007). Fullerton *et al.* (2011) determined the antimicrobial activity of sorrel (*Hibiscus sabdariffa*) on *Escherichia coli* O157:H7 isolated from food, veterinary, and clinical samples. Ethanolic extracts of *Hibiscus sabdariffa* calyces have been studied and shown to have antimicrobial agents effective in inhibiting *E. coli* O157:H7. The *Hibiscus sabdariffa* plant therefore holds a great promise as an antimicrobial agent. According to Sharaf *et al*. (1966), the colouring matter of the calyces is said to be lethal to *Mycobacterium tuberculosis*. Other works done by Sharaf *et al*. (1966), similarly showed that the aqueous extracts of *H. sabdariffa* prevented the growth of *Pasteurella, Pseudomonas, Proteus and Streptococcus* bacteria. Oboh and Elusiyan (2004) also studied the nutrient composition and antimicrobial activity of sorrel drinks against *P. aeruginosa, Lactobacillus sp., Bacillus sp.*, and *Corynebacterium* and found out that the aqueous extracts of *H. sabdariffa* inhibited the growth of *P. aeruginosa, Lactobacillus sp., Bacillus sp.*, and *Corynebacterium sp.* Currently, the main preservative agents for the inhibition of food spoilage microorganisms are synthetic chemicals. However, the misuse and overuse of preservatives has become the key factor for the emergence of health issues. Researchers are now turning their attention to herbal products. The therapeutic use of plants especially as antimicrobials has been reported by many scientists (González-Lamothe *et al.*, 2009). Reports of antimicrobial activity of *H. sabdariffa* show various levels of microbial growth inhibition against Gram positive and Gram negative bacteria (González-Lamothe *et al.*, 2009). Therefore, there is need for investigation into new technologies to develop more health-friendly preservatives against food spoilage microorganisms.

Materials and Methods

Sample collection

The samples of *Hibiscus sabdariffa* calyces were purchased from Kaduna market, Kaduna State, Nigeria.

Preparation of plant extract

Extraction of the calyces was done using two different solvents namely; ethanol and water. The calyces were sun dried for two consecutive days. The dried calyces were then pounded using a sterile wooden mortar and pestle and sieved using a 100 mesh (150 microns) sieve. The powder was divided into three parts: 10, 15 and 20 grams and stored in sterile glass containers.

Alcoholic and aqueous extraction of Hibiscus sabdariffa calyces

Procedure similar to the one used by Benjamin *et al.* (2016) with slight modification was used. The modification was that ethanol and sterile distilled water were the only solvents used. Each portion of the powdered calyces of 10, 15 and 20 grams, respectively was dissolved in 100 mls each of the solvents used (distilled water and ethanol). The different solutions were shaken vigorously at intervals for about an hour and subsequently left undisturbed for 18 h, respectively. At the end of the 18 h, both aqueous and ethanolic extracts were taken and filtered through a sterile muslin cloth.

Isolation and enumeration of microorganisms

Procedures as described by Obasi *et al.* (2014) were used to enumerate microorganisms from bread and kunu samples. The sterile culture plates of MacConkey agar, Mueller Hinton agar, Nutrient agar, Salmonella Shigella agar, and Triple sugar iron agar were used for enumeration of the bacteria cells.

Enumeration of total viable count

Each of the various samples of bread and kunu were analysed. One gram (1g) of the bread sample dissolved in 9 mls of sterile distilled water in a glass test tube. Each sample was serially diluted in sterile peptone water and 0.1 ml amounts of appropriate dilutions were poured on various agar media using pour plating. All samples were plated in duplicates and incubated at 37ºC for 24 – 48 h. All enumerations were expressed as colony forming units per millilitre (Cfu/ml) of plated samples.

Identification of microorganisms

Isolate representatives of growth on each medium used for enumeration were picked and purified by sub-culturing several times on the same medium and the purified isolates were stored at 5-10ºC on slants of the same medium of isolation.

The purified bacterial isolates were Gram stained and examined for Gram reaction and morphology.

Biochemical tests

Various biochemical tests were carried out to test for the physiological characterisation and confirmation of the isolated organisms as described by Baker *et al*. (2009).

Catalase test: A small amount of growth was taken from the cultured agar using a sterilized wire loop and placed on a glass slide creating a smear. A few drops of 3% hydrogen peroxide (H_2O_2) was added onto the smear. A positive result is the rapid evolution of oxygen (O_2) as evidenced by bubbling while a negative result gives off no bubbles or only a few scattered bubbles.

Coagulase test (Slide method): A drop of physiological saline was placed on each end of a slide. With a flamed wire loop, a portion of the isolated colony was emulsified in each of the drops to make two thick suspensions. A drop of human plasma was added to one of the suspensions and mixed gently. Clumping of the organisms was looked for within 10 seconds. No plasma was added to the second suspension to differentiate any granular appearance of the organism from true coagulase clumping. Agglutination or clumping of bacterial cells within 5-10 seconds is taken as a positive result.

Citrate test: A fresh (16- to 18-h) pure culture was used as an inoculum source. The inoculum was taken using a flamed wire loop and dipped into the prepared citrate broth already in a test tube. The inoculated medium was then incubated at 37ºC for about 18 h. The blue colour change indicates positive result, while green is negative. The citrate test is shown in Plate 1.

Indole test: A tube of tryptone broth was inoculated with a small amount of a pure culture and incubated at 37°C for 24 h. To test for indole production, 5 drops of Kovác's reagent were added directly to the tube.

A positive indole test is indicated by the formation of a pink to red colour ("cherry red ring") in the reagent layer on top of the medium within seconds of adding the reagent. If a culture is indole negative, the reagent layer will remain yellow or be slightly cloudy.

Oxidase test: With a sterile swab, a small amount of organism from the cultured Petri plates was obtained and placed on a piece of Whatman filter paper. One drop of reagent was placed onto the smear on the filter paper. Positive reactions turn the bacteria violet to purple immediately or within 10 to 30 seconds.

Triple sugar iron test (TSI): Using a flamed inoculation loop, the top of a well isolated colony was touched and inoculated into TSI agar by stabbing through the centre of the medium to the bottom of the test tube. The mouth of the test tube was then covered with cotton wool. The inoculated medium was then incubated at 37ºC for about 18 h. The test is shown in Plate 2.

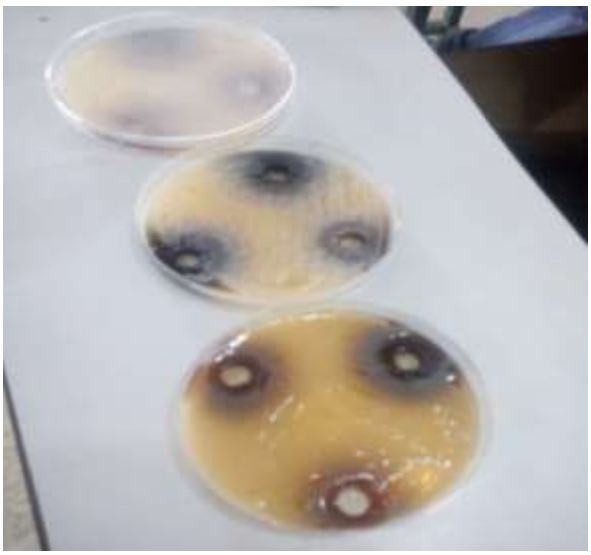

Plate 1: Picture showing sensitivity test results using aqueous extracts of hibiscus calyces

Plate 2: Picture showing sensitivity test results using ethanolic extract of hibiscus calyx extract

Sensitivity test

The method as described by Abbas *et al.* (2016) was used in the determination of the antimicrobial effectiveness of the plant extracts on the isolates obtained. The bacterial isolates used were obtained from food samples of bread and kunu. These isolates include: *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, staphylococcus aureus* and *Micrococcus spp.* Petri dishes containing Mueller Hinton Agar were seeded with the different microbial isolates. Wells were made using a flamed cock borer measuring 5 mm in diameter in the agar. Three evenly spaced wells were made in each Petri dish. The wells were labelled according to the various concentrations of the calyx extracts. Slightly over 1 ml of the various extracts were dispensed into the appropriate wells and incubated at 37ºC for 18 h.

Results and Discussion

Tables 1 and 2 showed the results from the sensitivity tests using aqueous and ethanolic extracts, respectively. The results obtained from the three selected concentrations (0.10, 0.15 and 0.20 g/ml) showed that ethanolic extracts of 0.20 g/ml concentration had significant inhibitory effects on each isolate contrary to the negative result obtained from the aqueous extracts at the three selected concentrations. The growth inhibition at low concentrations of (0.10 g/ml) of the extracts against all the test microorganisms were not significant because the plant extracts at that concentration had no bacteria inhibitory effect. The ethanolic extracts of the *Hibiscus sabdariffa* calyces showed inhibitory effects on both Gram positive and Gram negative bacteria (especially at concentrations of 0.20 g/ml). This shows the broad spectrum nature of the ethanolic extract at 0.20 g/ml concentration against the bacteria cells. The results of this study is in agreement with the report of Olaleye (2007) where *Hibiscus sabdariffa* extracts had a wide range of antimicrobial activity against bacteria such as *Staphylococcus aureus, Bacillus stearothermophilus, Micrococcus luteus, Serratia marcescens, Clostridium sporogenes, Escherichia coli, Klebsiella pneumonia, Bacillus cereus,* and *pseudomonas sp*.

Table 1.0 Antimicrobial activity of aqueous extracts (Sensitivity test 1)

Isolate code	Concentration of extract		
	0.1 g/ml	0.15 g/ml	0.20 g/ml
BMC_1	-	-	-
BMC_2	-	-	-
BMC_3	-	-	-
$BSSA_1$	-	-	-
$BSSA_2$	-	-	-
KSSA	-	-	-
KMC_1	-	-	-
KMC_2	-	-	-

+ => Showed inhibitory effect, - => No inhibitory effect. BMC_1, BMC_2& BMC_3 => Bacteria isolates from bread sample cultured on MacConkey Agar. $BSSA_1$ & $BSSA_2$ => Bacterial cells from bread sample cultured on Salmonella shigella agar. KSSA => Bacterial isolates from kunu sample cultured on Salmonella shigella Agar KMC_1 & KMC_2 => Bacterial isolates from kunu sample cultured on McConkey Agar

Table 2: Antimicrobial activity of ethanolic extracts (Sensitivity Test 2)

Isolate code	Concentration of extract		
	0.1 g/ml	0.15 g/ml	0.20 g/ml
BMC_1	-	-	+
BMC_2	-	±	+
BMC_3	-	+	+
$BSSA_1$	-	±	+
$BSSA_2$	-	-	+
KSSA	-	±	+
KMC_1	-	+	+
KMC_2	-	±	+

=> No inhibitory effect, ± => Minimal growth, + => Showed inhibitory effect. BMC_1, BMC_2& BMC_3 => Bacteria isolates from bread sample cultured on McConkey Agar. $BSSA_1$ & $BSSA_2$ => Bacterial cells from bread sample cultured on Salmonella shigella agar. KSSA => Bacterial isolates from kunu sample cultured on Salmonella shigella Agar. KMC_1 & KMC_2 => Bacterial isolates from kunu sample cultured on McConkey Agar

Also, the slightly higher potency of the ethanolic extract over the aqueous extract in the Agar Diffusion assay agrees with the reports of Frimpong (2008) and Khalaphallah and Wagdi (2014) where ethanol extracts also showed a higher growth inhibition compared with the water extracts. At 0.20g/ml concentration, the ethanolic extracts were notably effective against *Escherichia coli, Pseudomonas aeruginosa, Micrococcus spp. B. subtilis* and *Staphylococcus aureus*. These findings were also similar to studies done by Oboh and Elusiyan (2004) who found *H.sabdariffa* plant extracts to be effective against *Pseudomonas aeruginosa;* Frimpong (2008) also detected the plant extract to be effective against *S. aureus, E. coli* and *Pseudomonas aeruginosa*. Recently, Khalaphallah and Wagdi (2014) have shown that the extract of the plant is effective against *Pseudomonas aeruginosa, E. coli* and *Bacillus subtilis*. This may be attributed to the fact that ethanol was able to extract constituents from *Hibiscus sabdariffa* calyces with more potent anti-microbial activity than water. This detected difference may be due to insolubility of the active compounds in water. The mechanism of action of the extract may be by inhibition of electron transport, protein translocation, phosphorylation steps, and other enzyme-dependent reactions, followed by an increase in plasma membrane permeability and finally ion leakage from the bacterial cells (Walsh *et al.*, 2003) and may be related to the permeability of the bacteria cell surface to the extracts (Cowan, 1999).

Conclusion

It can be concluded from this study that, ethanolic extracts from *H. sabdariffa* calyces have shown antimicrobial activity against all of the test organisms, have great potential for use as non-synthetic preservatives in food systems.

Conflict of Interest

Authors declare that there is no conflict of interest in this study.

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