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In vitro evaluation of antilipid peroxidation and antimicrobial potentials of methanol extract of *Hibiscus sabdariffa* (Malvaceae): An index of potential use in male infertility treatment

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Abstract
Hibiscus sabdariffa (Malvaceae) is widely known in Nigeria for production of beverage drink (Zobo drink) as well as male infertility treatment among others. This research is principally focused on evaluating the effect of the plant calyx on some pathogenic organisms as well as on polyunsaturated fatty acid peroxidation which are parts of major causes of male infertility in Nigeria. Phytochemical studies of the plant (calyx) showed the presence of saponins, steroids, phenolic compounds, reducing sugars, flavonoids and glycosides. A remarkable bactericidal property against all test organisms was noted with highest activity observed against *Proteus mirabilis* (15 mm) while Ampiclox (standard antibiotics) showed no activity, least activity was observed against *Enterobacter aerogenes* (7 mm). A weak anti-lipid peroxidation activity was observed in the plant in concentration independent manner relatives to Vitamin E (alpha-Tocopherol) at $P<0.05$. An excellent scavenging activity against DPPH radical relative to Vitamin C (standard) at $P<0.05$ was also observed in *H. sabdariffa*. Also, a similar activity observed in the lipid peroxidation studies was also noted in reducing power activity of the plant. However, the entire findings of this research scientifically justify the male infertility ethnobotanical claim of the plant in Nigeria.

Keywords: Male infertility, lipid peroxidation, radicals, *Hibiscus sabdariffa*

1. Introduction
Infertility is a principal problem affecting the people socially, medically and psychologically. Major contributory factor to infertility has been attributed to male factor. Statistically, 15% of all couples in the United States are infertile and 25% of these cases are attributed to male infertility [1, 2].

Male infertility refers to inability of male to impregnate a fertile female. Outside the common causes of this aberration, such as low sperm count (Azoospermia), trauma, cystic fibrosis, obstructive lesions, hormonal imbalance, alcohol and illegal drug uses. Oxidative stress and microbial infections have been identified as major initiators of male infertility [4]. During oxidative stress, ROS attacks polyunsaturated fatty acids on the spermatozoa plasma membrane resulting into a cascade of chemical reactions leading to malondialdehyde [5]. Moreover, this in turn has decreased or detrimental effect on the motility of the sperm in a mechanism not understood [6]. DNA damage on sperm cell (minimal DNA damage can easily be self or oocyte repaired, but extensive damage result to apoptosis of the sperm cell leading to severe male infertility) [7].

Moreover, microbial infection is another cause of male infertility partly because of the anatomical association of urinary tract (containing some pathogenic organisms mainly *E.coli* and *Staphylococcus aureus* during urinary tract infection) with reproductive tract [8]. Microbial infections have been reported to induce male infertility via reduction of sperm viability [8, 9]. However, search for phytoactive compounds from medicinal plants with antimicrobial and scavenging properties are greatly imperative due to high cost, contraindications, unavailability and toxicity associated with some commonly used conventional drugs used in management of male infertility cause by microbial infection and oxidative damage of spermatozoa. In view of the above, ethno-pharmacological evidence has suggested a number of medicinal plants to be useful in male infertility treatment, part of which is *Hibiscus sabdariffa* [10].

H. sabdariffa belongs to the Malvaceae family, the calyx are prolific in some commercial blends of drinks due to its pleasant taste, culinary, decorative and medicinal uses [11]. The leaves are alternate, 7.5-12.5cm long, green with reddish veins and short or long petioles. The flowers are 8-10cm long and 7-10cm in diameter. Hypotensive, anticancer, antimicrobial, antidiabetes properties have been reported in the plant [12, 13, 14].

The aim of this research is to experimentally verify the ethnomedical claim of the plant in the treatment of male infertility apart from its main beverage drink attribute (Zobo drink in Nigeria), via investigations on its prevention of lipid peroxidation and antimicrobial properties using *in vitro* models.

2. Materials and Methods

2.1 Collection and Extraction of Plant Material

Dried *H. sabdariffa* calyx was brought in Auchi market, Edo State Nigeria, the plant was authenticated by Mr. Emmanuel Amodu at Herbarium of Paxherbal Clinic and Research Laboratories, Edo State was voucher specimen was deposited. The plant was further dried using laboratory oven at 40^{o}C. The completely dried plant material was ground to coarse powder and extracted with method by Soxhletextraction and extract was concentrated to dryness in vacuum.

2.1.2 Preliminary phytochemical screening

Phytochemical screening was performed using standard methods described by [15, 16].

2.2 Antimicrobial Screening

2.2.1 Organism Source

The organism used were clinical isolates which include, *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterobacter aeruginosa, Proteus mirabilis.* They were obtained from the Department of Medical Microbiology University of Benin Teaching Hospital (UBTH), Benin City, Nigeria. All the organisms were checked for purity at Paxherbal Clinic and Research Laboratories by a Microbiologist and maintained at 40 °C in slants of nutrients agar for bacteria.

2.2.2 Preparation of the inocula

A loopful of the test organism was taken from their respective agar slants and sub cultured into test -tubes containing Mueller-Hinton broth for bacteria. The test tubes were incubated for 18hr at 37 °C for bacteria, and the obtained microorganisms in the broth were standardized using normal saline to obtain a population density of 10^{8}cfu/ml.

2.2.3 Assay for Antimicrobial Activity

The methanol extract *of H. sabdariffa* was assayed for its antimicrobial activity using the disc diffusion techniques by Kirby - Bauer as described by Isuand Onyeagba (1998) and Ibekwe. Whatman Filter paper (Whatman No.l Filter paper) were cut into size of 6mm diameter with office perforator and sterilized at 105°C for 1hour. The sterile discs were impregnated with 2 mg/ml of the plant sample and dried in the oven at 60°C for about 15-30mins. Mueller-Hinton agar plates were seeded with standardized broth culture of test organisms containing 10^{8} cfu/ml equivalents to 0.5 McFarland standards (NCCLS) and the prepared disc containing 2 mg of the sample were placed on the plates. They were incubated at 37°C for 24hrs and observed for clear zones of inhibition against the organisms. The zones diameters were measured witha transparent ruler and the result recorded in millimeter (mm). The assay was done in triplicate. Sterilized disc soaked in sterile distilled water were used as negative control and another soaked in 2 mg/ml of Ampicillin-Cloxacillin (Ampliclox) as positive control.

2.2.4 Minimum Inhibitory Concentration-Broth Dilution Method

The minimum inhibition concentration of the crude extract was carried out using macro broth dilution technique as described by Boron and Fingold, 1990. The nutrient broths were prepared according to the manufacturer's instruction. The standardized inocula (0.1 ml) of the microbes were incubated at 37 °C for 1-7 days for bacterial and observed for turbidity. The lowest concentration which showed no turbidity in the test tube was recorded as the MIC.

2.2.5 Minimum Bactericidal Concentration -Macro Broth Dilution Method

Fresh Mueller Hinton agar medium was prepared, sterilized at 121°C for 15mins and was poured into sterile petri-dishes and left to cool and solidify. The contents of the MIC tubes (that is the tubes that showed no growth) were then sub-cultured on to the media incubated at 37 °C for 24hours and 30 °C for 1-3 days and observed for colony growth. The MBC/MFC was the plates with lowest concentration of extract and without colony growth.

2.3 Antioxidant Studies

2.3.1 Lipid peroxidation assay

Lipid peroxidation inhibition assay was carried out using slightly modified method described by Ohkawa (2011). Liver homogenate was prepared from commercially available goat liver. The liver was washed several times with ice-cold saline solution. A 10% of liver homogenate was prepared using ice-cold KCl (0.15 M) in a blender. Lipid peroxidation was initiated in 1ml of tissue homogenate incubated with varieties concentration of extracts (20-100mg/ml), by the addition of 0.1 ml of ferric sulphate (25 µM), 0.1ml of ascorbate (100 µM) and 0.l ml of KH_2PO_4 (10mM) and the volume was made up to 3ml with distilled water and incubated at 37 °C for 1 hour. Then 1 ml of 5% trichloroacetic acid (TCA) and 1ml of 0.67% TBA was added to this reaction mixture and the tubes were boiled for 30 min in boiling water bath. This was centrifuged at 3500 rpm for 10 mins. The extent of inhibition of lipid peroxidation was evaluated, through estimation of thiobarbarturic acid reactive substance (TBAS) levels.

2.3.2 Total Antioxidant studies

This was determined using the DPPH method (Ohinishi *et al.,* 1994; Oke and Hamburger, 2002). Briefly, 0.1 mM DPPH solution was added to different concentrations of the extract with gentle shaking. Triplicate measurements of the optical density change were done 60 minutes later after incubating in the dark at 517nm.

2.3.3 Ferric reducing power

Ferric reducing power was determined by mixing various concentrations of plant extract and standard ascorbic acid solution (viz. 10, 20, 40, 60, 80 and 100 µg/ml) in 1 ml of methanol with phosphate buffer (2.5 ml, 0.2 M at pH 6.6) and potassium ferricyanide [K_3 $Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of 10% tricholoro acetic acid (TCA) was added to the

mixture, which was then centrifuged at 3000g (rpm) for 10 min at room temperature. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride ($FeCl_3$) (0.5 ml, 0.1%), and the absorbance of the reaction mixture was measured at 700 nm as indicative of increased reducing power. All the tests were performed in triplicate and a graph was plotted for the average of three observations [14, 15].

3. Results and Discussion

Plants over the years have provided a source of novel drugs, as phytomedicines have been shown to exhibit significant contribution towards human health (Didry *et al.,* 1998).

Table 1: Phytochemical screening of *H.sabdariffa*

Class of Phytochemical	*H. sabdariffa*
Glycosides	++
Cardiac glycosides	-
Saponins	++
Flavonoids	+
Phenolic compounds	++
Hydrolysable Tannins	-
Condensed tannins	++
Phlobatannins	-
Alkaloid	-
Terpenoids	-
Starch	-
Reducing sugars	++
Steroids	++

Phytochemical evaluation of *H. sabdariffa* showed the presence of glycosides, saponins, phenolic compounds (condensed tannins), reducing sugar and steroid in moderate amount. Also the presence of flavonoid was also observed, as shown in Table 1.

Table 2: Antimicrobial screening (zone-of-inhibition, mm) of *H. sabdariffa*

Microorganism	Negative control (distilled water)	Positive control (Ampiclox)	*Hibiscus sabdariffa*
Bacillus subtilis	0	19	8
Escherichia coli	0	0	10
Enterobacter aeruginosa	0	0	7
Klebsiella pneumonia	0	0	10
Pseudomonas aeruginosa	0	17	10
Staphylococcus aureus	0	19	12
Proteus mirabilis	0	0	15

Table 3: MIC and MBC (mg/ml) Evaluation of *Hibiscus sabdariffa*

Microorganism	MIC	MBC
Bacillus subtilis	40	60
Escherichia coli	40	60
Enterobacter aeruginosa	40	60
Klebsiella pneumonia	-	-
Pseudomonas aeruginosa	40	60
Staphylococcus aureus	20	40
Proteus mirabilis	20	40

Antimicrobial investigation of *Hibiscus Sabdariffa* against some pathogenic organisms such as *E.coli* (Gram negative) and *S.aureus* (Gram positive) causing primary male infertility partly due to their ability to colonize the urinary tract which in turn have detrimental influence on the male reproductive system (Momoh *et al.,* 2011). Table 2 showed that *H. Sabdariffa* exhibited a good sensitivity against all test organisms, with the highest activity observed against *Proteus mirabilis* (15mm) while Ampiclox (positive control) showed no activity, least activity was observed against *Enterobacter aeruginosa* (7mm). Minimum inhibitory concentration and minimum bactericidal concentration are shown in Table 3 above and their result further corroborate with the antimicrobial activity shown in Table 2. The antimicrobial activity shown in this research confirms the report by Kurijan*et al.,* 2010.

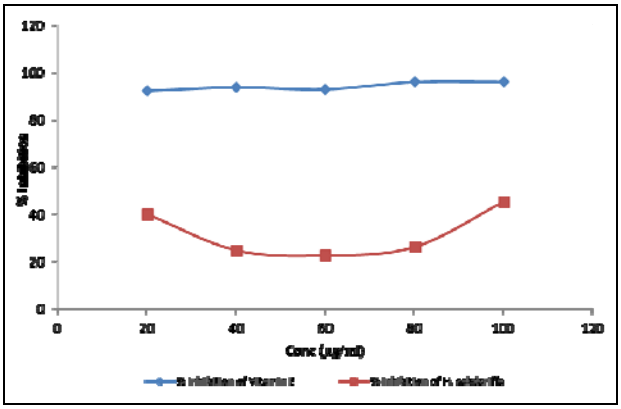

Fig 1: Lipid peroxidation studies of *H. sabdariffa*

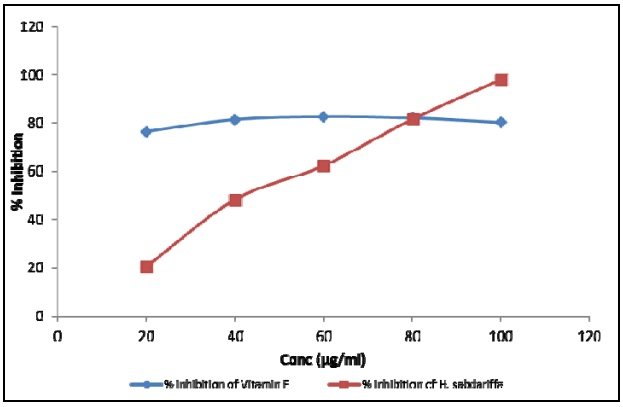

Fig 2: Total antioxidant studies of *H. sabdariffa*

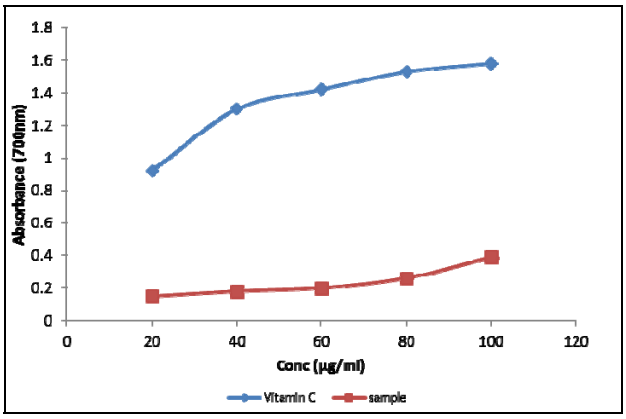

Fig 3: Reducing power studies of *H. sabdariffa*

Moreover, the plasma membrane of sperm cells are rich in poly unsaturated fatty acids (PUFA), these fatty acids (PUFA) are susceptible to ROS attack during oxidative stress causing a series of lipid peroxidation reactions which result in

destruction of sperm viability, motility, DNA and sperm functions as shown in Figure 1. A weak antilipid peroxidation activity was noted in *H. sabdariffa* in a concentration-independent manner relative to vitamin E which was the positive control at $P<0.05$. Lowest and highest activities were observed at 60 µg/ml and 100 µg/ml respectively as shown in Figure 1.

Evaluation of radical scavenging potential of *H. sabdariffa* is shown in Figure 2. Hence ability of a plant to contain compounds that can scavenge radicals or prevent occurrence of oxidative stress will aid in the prevention of both male fertility and other diseases arising from oxidative stress.

A very good radical scavenging activity against DPPH radical was observed in *Hibiscus sabdariffa* relative to vitamin C (standard) at $P<0.05$. Also, lowest and highest activity was noted at 20 µg/ml and 100 µg/ml respectively in a concentration as shown in Figure 2.

A concentration-dependent activity was also observed inFigure 3, in a highly statistically different at $P<0.05$ from activity noted in vitamin C. However, this indicated that *H. sabdariffa* exhibited a very weak ability to reduce metals which might be highly reactive and detrimental to male reproductive health via their ability to attack PUFA in a series of complex biochemical reactions.

4. Conclusion

From the laboratory findings in this research, the ethnobotanical claim of *H. sabdariffa* in the management of male infertility, apart from beverage purposes, was investigated and experimentally validated because of the ability of the bactericidal property of the plant against some pathogenic organisms that can cause infection which in turn can alter sperm viability, morphology, motility and integrity. Moreover, the plant also showed a promising ability to reduce peroxidation of PUFA in the membrane of sperm cell during ROS attack as well as scavenge radicals which can either directly or indirectly induce oxidative stress that may serve as a precursor to male infertility (ROS induced) and other diseases in human.

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