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Phytochemical Testing and *In vitro* Antibacterial Activity *of Bixa orellana* (Annatto) Seed Extract

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MA and ASA wrote the study protocol and designed the study. Author ASA together with authors DB, RP and GD supervised MA's collection of the data. Authors ASA and MA performed the statistical analysis and wrote the first draft of the manuscript. Authors MA, ASA and JSC managed the analyses of the study. Authors MA and ASA managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: The present study was designed to investigate the antibacterial activity of the seeds of *Bixa orellana* (Annatto, family *Bixaceae*) extracts.
Methods: Powdered seed material was extracted using either organic solvents or acid-base protocols and the crystals obtained were washed with deionized water, oven-dried for about 12 hours at 45ºC and stored inair-tight containers. The antimicrobial activity of the ethanolic, dimethyl sulphoxide and aqueous extract of *B. orellana* seed was tested *In*

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vitro against *Escherichia coli*, *Staphylococcus aureus, Salmonella typhimurium* and *Bacillus subtilis* using agar well diffusion method.
Results: The antibacterial activity of the seed extract was observed at low concentration (250µg/ml) in ethanolic and dimethyl sulphoxide solution while, in potassium hydroxide, antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* was observed at 750µg/ml. The minimum inhibitory concentration (MIC) values range from75 to 750µg/ml while the minimum bactericidal concentration (MBC) values occurred between 75 and 1500µg/ml respectively against *Staph. aureus* and *B. subtilis* respectively.
Conclusion: Results of this study indicated that the seed extracts of *Bixa orellana* possess significant inhibitory and bactericidal activities against select resistant strains of gram-positive bacteria.

Keywords: In-vitro; antibacterial; *Bixaorellana*; extracts.

1. INTRODUCTION

Plants with wide variety of chemical constituents offer some promising sources of new antimicrobial agents with general as well as specific activities [1]. Reports abound in the literature on the presence of antimicrobial compounds in various plants [2-4]. Recent screening of bioactive compounds from plants has led to the discovery of new medicinal substances possessingcyto-protective protective and therapeutic effects in various ailments including cancer [5,6]. Novel phytochemical substances of plant origin have been shown toinhibit the growth or destroy bacterial cells, which were resistant to existing synthetic agents. The differences in chemical structure of these phytochemicals compared with the existing synthetic agents indicate possible differences in mechanism of action (Fabricant and Farnsworth, 2001) [7]. Attempts have been made to categorize phytochemical constituents of medicinal plantsaccording totheir pharmacological activities [8].

Annatto plant (*Bixa orellana,* family *Bixaceae*) is a profusely fruiting shrub, reaching 6-20 in height [9]. The prickly heart shaped pods contain small reddish-orange seeds; each pod contains approximately 50 seeds. It is found mainly in the forest ecosystem of the Brazilian humid tropics but it is now cultivated in the tropics throughout the world [10]. It is extensively used as colorant in food and cosmetic industry as condiment or as remedy in traditional medicine. In folklore medicine, the pulp surrounding the seed has been used to treat burns, bleeding dysentery, gonorrhea, constipation and fever [11]. Historically, the South American Indians have used the plant extract to treat wounds, diarrhea and asthma [5]. The objective of the present study was to evaluate the antimicrobial activity of annatto coloring fraction against select resistant strains of bacteria, using agar diffusion method.

2. MATERIALS AND METHODS

2.1 Materials

Fresh plant materials were collected in batches from a single source (farmer) in the parish of Manchester during the peak season (August to January). Plant material was identified at the Rural Agricultural Development Authority (RADA) and by a botanist at the Botany Department of the University of the West Indies Mona Campus. The plant material was air-dried for about 2 weeks; the seeds were freed from the pod and dried further in hot-air oven at 50ºC for 12 hours.

All apparatuses and materials used in the preparation and inoculation of culture media including spatula, conical flasks, measuring cylinders, aluminum foil, autoclave, weighing balance, Bunsen burner and tripod stand were handled following aseptic technique and all operations were performed under a laminar flow hood (Enviro Corporation Albuquerque, New Mexico).

2.2 Preparation of Plant Extract

The plant extracts were prepared as previously reported [12]. Briefly, three by 250g of annatto seed were soaked in 900ml of 1M KOH and stirred for 30 minutes at 70ºC on a magnetic stirrer in a Labconco protector laboratory hood (Labconco Corporation, USA). The mixture was filtered, the residue washed repeatedly with fresh 900ml of 1M KOH with stirring for 30 minutes and filtered. The combined filtrate was acidified with 3M HCl to precipitate the crystals overnight. The crystal was washed repeatedly with distilled water and oven dried at 45ºC for about 72 hours. The lumps were pulverized in a mortar with pestle and the resulting powder was stored in airtight container.

2.3 Phytochemical Screening

Phytochemical screening was performed following literature mathods [1].

2.3.1 Saponins

Approximately 0.5g of seed was boiled in 5mL of distilled water for 2.5min and filtered. 2.5mL of the filtrate was added to 10mL of distilled water in a test tube. The test tube was corked, shaken vigorously and examined for any frothing that persisted for about 30 seconds. The froth, if formed, was mixed with few drops of paraffin oil and shaken vigorously, then observed for any formation of an emulsion.

2.3.2 Tannins

Approximately 0.5g of seed was boiled in 20mL of distilled water for 5 minutes and filtered. A few drops of 5% ferric chloride were added and observed and the mixture for brownish green or a blue-black coloration.

2.3.3 Terpenoid (salkowski) test

Five (5)mL of the aqueous alkaline crude extracts of annatto seed was mixed with 2mL of chloroform; 3mL of concentrated sulphuric acid was carefully added to avoid mixing and form an interface. The mixture was examined for the presence of a reddish brown coloration at the interface, which is indicative of terpenoid content.

2.3.4 Steroid

Two (2)mL of acetic anhydride was added to 5mL of ethanolic extract, followed by 2mL of concentrated sulphuric acid. Formation of a blue or green color indicates the presence of steroids.

2.3.5 Glycosides

Approximately 0.5g of seed was boiled in 5mL of distilled water for 5 minutes. The sample was filtered and 5mL of the filtrate was treated with 2mL glacial acetic acid containing one drop of ferric chloride solution. This was under-laid with 1mL of concentrated sulphuric acid. Brown ring coloration at the interface indicates the presence of deoxysugar, characteristic of cardenolides.

2.3.6 Carbohydrate

Approximately 0.5g of the aqueous alkaline crude extracts of annatto seed was heated with 1mL of concentrated sulphuric acid. Occurrence of blackening and effervescence indicates the presence of carbohydrate.

2.3.7 Flavonoids

Approximately 0.5g of seed was boiled in 5mL of distilled water, this was then filtered and 5mL of the filtrate was treated with 5mL of dilute ammonia solution followed by drop-wise addition of concentrated sulphuric acid. A yellow coloration indicates the presence of flavonoid.

2.3.8 Resins

About 5mL of boiled ethanol was added to 0.5g of the seed and allowed to stand for 5 minutes. The mixture was filtered and the filtrate was treated with 1% aqueous hydrochloric acid. Formation of resinous precipitate indicates the presence of resins.

2.3.9 Alkaloids

About 1% dilute hydrochloric acid was added to 5mg of aqueous alkaline extract of annatto seeds while heating in a steam bath. The mixture was filtered and 1mL of the filtrate was treated with few drops of Draggendorf's reagent and the other 1mL portion was treated with Wagner's reagent. The formation of precipitates (red for Draggendorf's reagent and reddish brown for Wagner's reagent)indicated the presence of alkaloids.

2.4 Antibacterial Susceptibility Test (AST)

2.4.1 Microorganisms

Pure cultures of bacteria isolated from clinical specimen were obtained from the Molecular Biology Laboratory at University of West Indies. The organisms are *Escherichia coli* ATCC 25932, *Salmonella typhimurium* ATCC 13311, *Staphylococcus aureus,* ATCC 29213 and *Bacillus subtilis* ATCC 6633. The organisms were maintained on Nutrient agar slants at 4°C and sub-cultured for use in testing. Ampicillin 10µg/mL and streptomycin 1mg/mL were used as positive control for the sensitivity test against bacterial strains. Sterile distilled water, potassium hydroxide, ethanol and dimethylsulfoxide were used as negative control.

2.4.2 Sterilization of materials

All glassware were washed, dried and wrapped in aluminum foil and sterilized in hot air oven (Lab-Line L-C 101 F, Mumbai) at 160°C for 90 minutes. All media, distilled water, and

McCartney bottles were sterilized in the autoclave at 121ºC for 15 minutes. The laboratory bench was swabbed with 70% alcohol before and after each round of experimentation.

2.4.3 Preparation of culture media

Nutrient agar, Mueller Hinton agar, and Nutrient broth were prepared according to manufacturers' specifications. Nutrient agar powder (14g) was dissolved in a sterile conical flask containing 500ml of sterile distilled water. The suspension was heated to boiling and autoclaved at 121ºC for 15 minutes. After autoclaving, the media was allowed to cool to a temperature of about 45ºC and poured into Petri dishes.

2.4.4 Preparation and standardization of bacterial inoculum

Preparation and standardization of each inoculum was done using the method described by Anibijuwon et al. [13]. The test organism growing as a pure culture on solid media was transferred to sterile nutrient broth and incubated for 18hours at 37°C. The optical density of the culture was determined using Cecil UV-VIS 9000 series (Cecil Instruments Ltd. Cambridge) at 600nm. Each standardized inoculum was used for antibacterial test.

2.4.5 Agar-well diffusion assay

The medium was inoculated with 0.5mL microorganism suspended in Nutrient Broth. The agar was allowed to solidify and seven wells of 6mL each were punched in the solidified agar plate. The aqueous alkaline extract of annatto seed was dissolved in either of three different solvents, namely 0.1 M KOH, 50:50 ETOH: H_2O and 0.2mL DMSO in 1mL of distilled H_2O. Forty (40)μL each of aqueous alkaline crude extracts of annatto seed at concentrations of 250, 500, 750, 1000, 1500 and 3,000μg/mL was poured into the respective well and numbered 1 to 6; the blanks (0.1M KOH, 50:50 ETOH:$H_2O$and 0.2mL DMSO in 1mL of distilled H_2O) were poured into well # 7 of each test batch. Forty (40)μL of the standard antibacterial agents (Ampicillin 10μg/mL and streptomycin 1mg/mL was poured into a different agar plate). The test was carried out in triplicate, the plaques were incubated at 37ºC for 24 hours and the zones of inhibition were measured in mm. The antibacterial activity was calculated using Eq. 1:

$$\% RIZD = \frac{\{(IZD_{sample})/(Conc_{Sample}) - IZD_{Negativecontr.}\}}{\{(IZD_{antibacterialstd})/Conc_{antibacterialStd}\}} X\ 100\% \quad (1)$$

Where RIZD is the percentage of relative inhibition zone diameter, IZD_{sample} is the inhibition zone diameter (mm) of annatto, $IZD_{negative\ contr.}$ is the inhibition zone diameter (mm) of the solvent and $IZD_{antibiotic\ standard}$ is the inhibition zone diameter (mm) of ampicilin or streptomycin. The RIZD of sample and standard are weighted on their respective concentrations.

2.4.6 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the aqueous alkaline crude extracts of annatto seed was determined by broth dilution method [14]. The reconstituted annatto extract was diluted to give 11.74, 23.4, 46.9, 93.75, 187.5, 375 and 750mg/mL of extract in 0.1M potassium hydroxide and 50:50 ethanol: water respectively, and 9.38, 18.75, 37.5,75, 150 and 300μg/mL in DMSO final concentrations and added to the sets of test tubes containing nutrient broth. Using the sterile micropipette, 40μl of the standard microbial broth

cultures were introduced into each test tube. Another set containing only the medium and each of the test bacteria was set up separately as a control. The test tubes were incubated at 37°C for 24hr. in an incubator (Sheldon Manufacturing Inc., U.S.A).

2.4.7 Minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) of the aqueous alkaline crude extracts of annatto seed was determined following [14]. Serially diluted subculture test tubes, which did not yield any visible turbidity (growth) in the minimum inhibitory concentration (MIC), were streaked on freshly prepared nutrient agar plates, incubated at 37°C for 24h and the MBC determined.

3. RESULTS AND DISCUSSION

Phytochemical screening of aqueous alkaline extract of annatto seed tested positive for saponins, tannins, terpenoids, steroids, glycosides, carbohydrates, and flavonoids but test negative for alkaloids and resins (Table 1). The presence of wide range of phytochemical constituents provides some evidence in support of the various ethnobotanical claims for its use [15].

The active compounds identified in this study exhibited antibacterial activities against *B. subtilis* and *Staph aureus* (Fig. 1) but had no activity against *E. coli* and *S. typhimurium*. Several plants, which are rich in alkaloids, flavonoids, tannins and glycoside, have been shown to possess antimicrobial activity against a number of microorganisms.

Banso [2] reported some antibacterial activities of ethanolic extract of *Acacia nilotica* against S. *viridians*, *B. subtilis*, *S. aureus*, *E. coli* and *S. sonnei. Acacia nilotica* contains terpenoids, tannins, alkaloids, saponins and glycosides. The activity of annatto extract could be attributed to the presence, mainly, of flavonoids and tannins. Flavonoids are widely distributed class of naturally occurring pigments present in vascular plants. They exhibit broad spectrum of biological activities including cardio-protection, antioxidant, anticancer, anti-inflammatory and antimicrobial [16]. They also trigger, biologically, the production of natural enzymes that fight diseases [17]. Flavonoids have been the principal physiologically active constituent in folk medicine and have been used historically by physicians to manage various conditions [18]. Tannins are naturally occurring plant polyphenols that are obtained from various parts of plants, which combine with protein and other polymers to form stable complexes. They are generally recognized as safe (GRAS) food additives [19]. Tannins can be classified into hydrolysable and non-hydrolysable (condensed) tannin; tannic acid is a substantial hydrolysable class [20]. Tannins extracted from different plant species have specific physical and chemical properties. On the antimicrobial activity, tannins have shown activities against pathogenic bacterial strains. The ability of plant polyphenols to complex with polymers and minerals has been suggested as the principal reason for their inhibitory effect on bacteria. The complexation induced by tannins may account for tannin toxicity and the inhibition of microbial enzymes [21].

This inhibitory mechanism involves direct interaction with membranes, cell walls and/or extracellular proteins [22,23]. The inhibitory effects of the aqueous alkali extract of annatto seed in different solvent (DMSO, ethanol and potassium hydroxide) against different test organism are shown in (Fig. 1). The antibacterial activities of the extract were compared with two standard antibiotics (ampicillin and streptomycin). The antibacterial activity was observed to be dose-dependent in the range of 250 - 3000µg/mL against some of the tested

strains. Extract prepared in dimethylsulfoxide (DMSO) and ethanol showed activity against Gram–positive bacteria cells of *Staph. aureus* and *B. subtilis* at concentrations between 250 and 3000µg/mL, while the extract in potassium hydroxide solution were active only in the concentration range of 750 to 3000µg/mL. Gram-negative cells including *E. coli* and *S. typhimurium* did not show sensitivity to the extracts (Fig. 1). This observation is consistent with previous report by Irobi et al. [24], which indicated that the leaf extracts of annatto possess significant antibacterial activity against standard strains of Gram-positive bacteria including *B. subtilis, Strept. faecalis and Staph. aureus*. In addition, flavonoids have been reported to show significant activity against Gram-positive bacteria but not with Gram-negative bacteria [17]. The difference in cell wall structure between Gram-positive and Gram-negative bacteria may be responsible for the differences in susceptibility to phytochemicals. In gram-negative, presence of lipopolysaccharide layer outside the peptidoglycan layer makes the cell wall more complex and renders it almost impermeable to many environmental substances and antibiotics. However, Gram-positive bacteria have peptidoglycan and no lipopolysaccharide in their cell structure. Peptidoglycan provides less resistance to permeability by various agents, and thus shows greater sensitivity [17,25].

Table 1. Phytochemical screening of aqueous alkaline crude extract of annatto seed

S/N	Phytochemical test	Reagents used (test performed)	Observation	Interference
1	Saponin	Foam Test	Produce foam that lasts for more than 10 minutes	Positive
2	Tannins	Ferric chloride test	Brownish green coloration was observed	Positive
3	Terpenoid (salkowski test)	Steroidal ring test	A reddish brown coloration of the interface was formed (steroidal ring)	Positive
4	Steroid	Acetic anhydride and Conc. sulphuric acid.	Blue-green coloration	Positive
5	Glycosides	Glacial acetic acid ferric chloride solution Conc. sulphuric acid.	Formation of faint brown ring	Positive
6	Carbohydrate	Conc. sulphuric acid.	Extract turned black and effervescence occurred	Positive
7	Flavonoids	Shinoda's test	An orange color was observed	Positive
		Lead ethanoate test	A pale yellowish- brown color was observed (buff –colored)	Positive
8	Resins	Aqueous HCl	No resinous precipitate formed	Negative
9	Alkaloids	Dragendorff's and Wagner's reagents	No precipitate	Negative

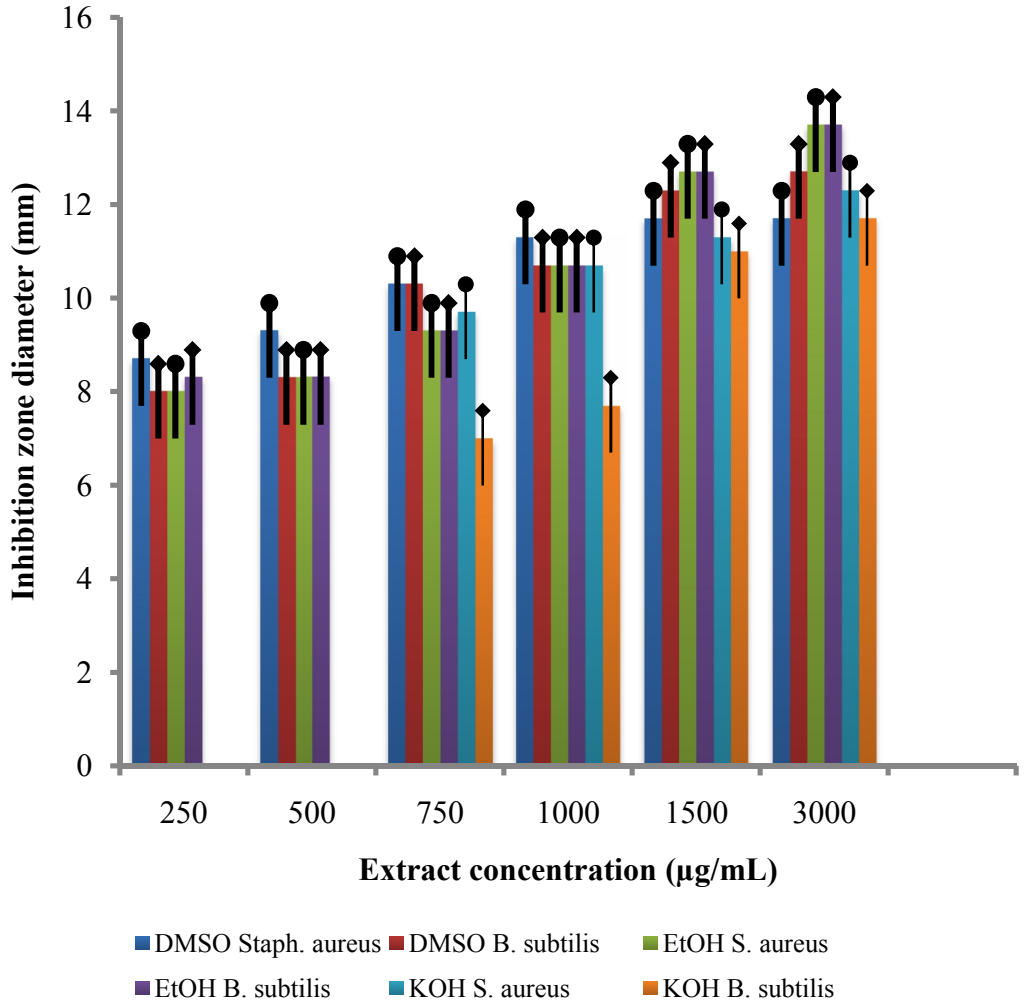

Fig. 1. Zones of inhibition against *B. subtilis* and *Staph. aureus* versus concentrations of annatto extracts prepared in different media

***KOH extract showed no activity against B. subtilis and S. aureus at 250 and 500µg/mL*

Similarly, as shown in (Fig. 1), the aqueous alkali solution of annatto seed extract in potassium hydroxide has the lowest inhibitory effect followed by the DMSO and ethanol respectively. This observation may be due partly to the existence of active constituents of the extract in unionized form in both ethanol and DMSO while they exists mainly in ionized form in potassium hydroxide. The bacteria cell membrane is lipophillic in nature and known to be preferentially permeable to only unionized molecules. The degree to which a molecule is ionized in solution depends on the nature of the molecule and the pH of the solution in which it is present as expressed by the Henderson–Hasselbalch equation:

$$pH = pK_a + \log\left(\frac{protonacceptor}{protondonor}\right) \quad (2)$$

Hence, for an acid (XH), the relationship between the ionized and unionized form is given by:

$$pH = pK_a + \log\frac{X^-}{XH} \quad (3)$$

Where X^- isthe salt of bixin (bixinate) and XH is the bixin.

It appears that, at 250 and 500µg/ml, bioactive constituents of annatto seed extract in potassium hydroxide solution were extensively ionized and will not be able to penetrate through the bacteria cell wall and membrane structure; this may be responsible for the absence of antibacterial activity. At higher concentrations, some proportion of the annatto extract would exist in unionized form, which may be responsible for the smaller zone of inhibition observed in comparison with the solutions of extracts in DMSO and ethanol.

The test organisms were selected from pathogenic bacteria strains that have developed resistance to existing antibiotics due to indiscriminate use of antimicrobial drugs in treating infectious diseases [26,27]. The world wide emergence of *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus* and many other β-lactamase producers has become a major therapeutic problem. Multi-drug resistant strains are widely distributed in hospitals and are increasingly being isolated from community acquired infections [26,27]. All these have resulted in severe consequences including increased treatment failure and health care cost. Results of this study indicate that annatto extract may provide alternative or complimentary approaches to managing some resistant strains of *Staph. aureus* and *B. subtilis* to existing antibacterial agents. Zone of inhibition produced by the crude extract in agar diffusion against susceptible bacteria rangedfrom 8 to 13.7mm, while the ampicillin and streptomycin used as positive controls produced 3 to 12 mm inhibitory zone diameters (Fig. 1). Solvents (blanks) used to prepare the extract and fractions were also evaluated separately as negative controls to determine inherent antibacterial activities of the solvents. Negligiblezones of inhibition were recorded for the solvents.MIC and MBC produced by the standard antibiotics (ampicillin and streptomycin, Table 2) were used as baseline in calculating the relative potencies of annatto extracts in different solvents (i.e. DMSO, ethanol and potassium hydroxide).

The potency of annatto extract in DMSO at 250 to 500µg/mL against *Staph. aureus* and *B. subtilis* was about 9 and 3 times respectively that of ampicillin. Similarly, the ethanolic solution at 250 to 500µg/mL showed potency of about 8 and 3 times that of ampicillin against *Staph. aureus* and *B. subtilis* respectively. Extract in aqueous potassium hydroxide solution was active at concentrations above 500µg/mL. Furthermore, the potencies against *Staph. aureus* and *B. subtilis* were respectively 10 and 11 times (in DMSO solution), 12 and 16 times (in ethanolic solution) and, 18 and 25 times (aq. potassium hydroxide solution) the potency of streptomycin respectively (Figs. 2 and 3).

Table 2. Antibacterial activities of the positive standards

Antibiotic	Zone of Inhibition (mm)	
	***B. subtilis* (±SD)**	***S. aureus* (±SD)**
Ampicillin(10µg/mL)	12±0.0	4.0±0.5
Streptomycin(1000µg/mL)	3.5±0.0	3.0±0.0

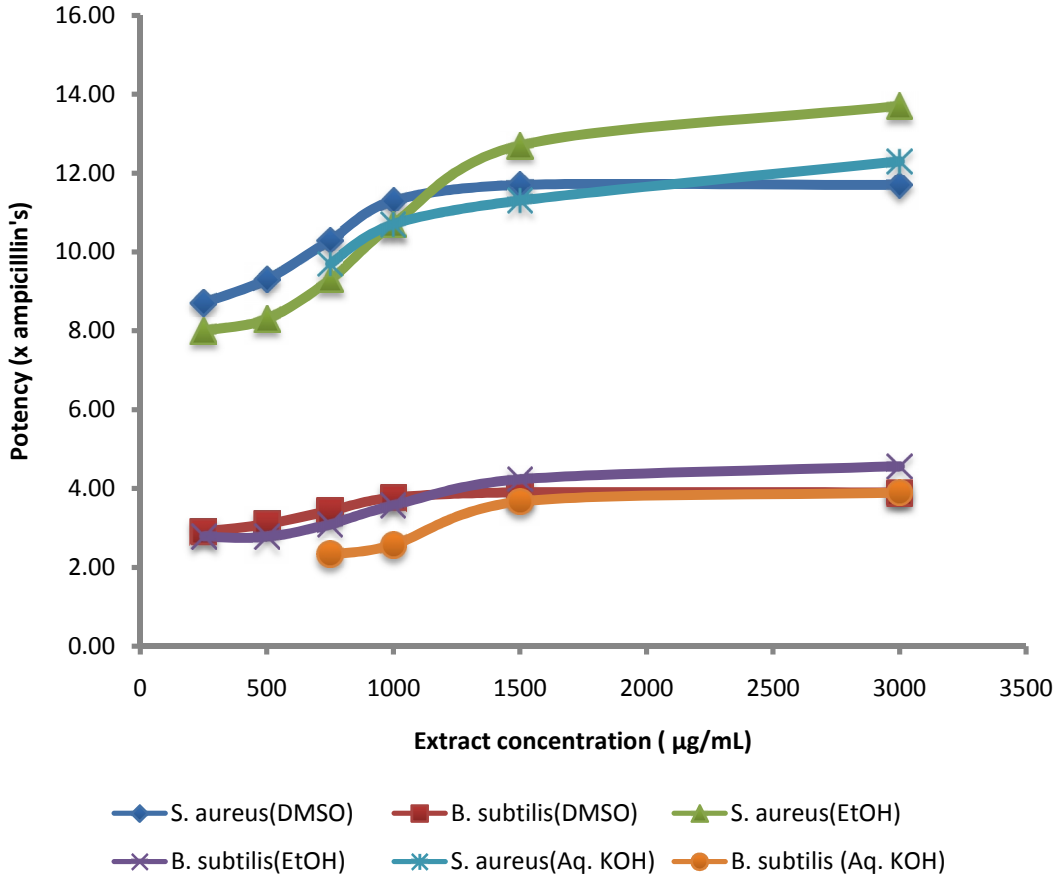

Fig. 2. *In vitro* potency of annatto extracts against bacteria species relative to the standard antibacterial agent ampicllin

**Relative potencies were calculated using Eq. 1. Data represent extract's potency compared with that of drug standard (ampicillin) at equivalent concentrations*

Minimum inhibitory concentration (MIC) values of crude extract of *Bixa orellana* against susceptible bacteria cells ranged from 75 to750µg/ml (Fig. 4). The crude extract in DMSO showed the lowest MIC of 75µg/ml while the extract in the potassium hydroxide have the highest MIC of 750µg/ml. The permeability enhancing effect of DMSO may be responsible for the higher activity of its annatto extract. Therefore, in can be concluded that the crude extracts of *Bixa orellana* in ethanol and DMSO show bactericidal effect on the test organisms at low concentrations (75–100µg/ml), while extract in aqueous potassium hydroxide solution was only bactericidal to *Staph aureus* at concentrations above 500µg/ml.

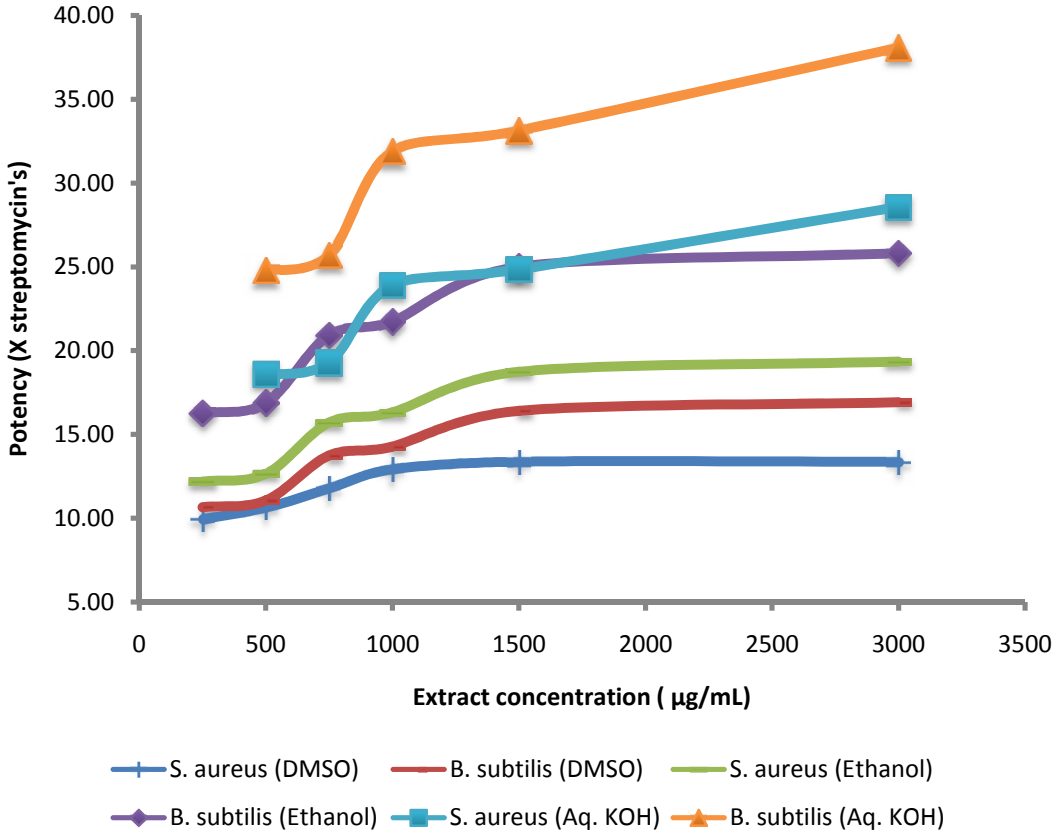

Fig. 3. *In vitro* potency of annatto extracts against bacteria species relative to the standard antibacterial agent streptomycin

**Relative potencies were calculated using Eq. 1. Data represent extract's potency compared with that of drug standard (streptomycin) at equivalent concentrations*

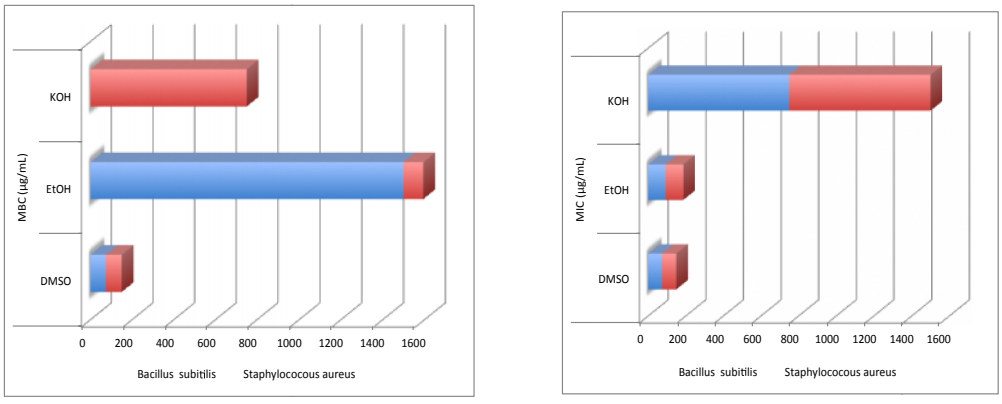

Fig. 4. Minimum inhibitory and bactericidal concentrations of annatto extracts in various solvents against *B. subtilis* and *Staph. aureus*

4. CONCLUSION

The *Bixa orellana* seed extracts showed bacteriostatic and bactericidal activities against Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*. The extracts contain the bioactive constituent saponins, tannins, terpenoids, steroid, glycoside, carbohydrate and flavonoids. Results suggest *Bixa orellana* seed as potential source of potent antibacterial agents. Against *B. subtilis*, MIC ranged from 75µg/mL to 750µg/mL while MBC ranged from 75µg/mL to 1500µg/mL, depending on solvent of extraction. Similarly, MIC against *Staph. aureus* ranged between 75 µg/mL and 750 µg/mL while MBC ranged from 75µg/mL to 750µg/mL. Further work is needed to isolate the secondary metabolites for which the extracts have tested positive and evaluate their *In vivo* potencies against bacterial strains.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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