Plasmid Profiles of Extremophiles Associated with Indigenous Black Soaps and Functional Groups Present

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ABSTRACT

In the present study, plain indigenous black soap market sample and black soap fortified with brown and white eggshell powder effectively at 20 g/250 mL of molten soap were analyzed microbiologically using standard method after which the bacterial isolates were profiled for presence of plasmid. Structural elucidation of the black soaps was achieved by Gas Chromatography Mass Spectrometry (GCMS) analysis of the methylated fraction. Fungal isolates from plain black soap were Chrysosporium spp. and Aspergillus granulosus while the bacterial isolate: Bacillus vedderi contained plasmid with an estimated molecular weight of 17578 bp. Ramulispora javanicus and Aspergillus flavus while Bacillus vedderi and Bacillus faraginis were fungal isolates were also recorded from black soap fortified with brown and white eggshell powder, respectively. The GCMS analysis revealed fatty acid as the predominant functional group. Plasmid presence in these bacteria may confer extremophily status on them and thus aid their survival in this environment. Antimicrobial activities of the indigenous black in folkloric applications may be predicated on the presence of its fatty acid compounds.

Key words: Extremophiles, plasmid, saponification, black soap, egg shell, fatty acid

INTRODUCTION

Microbial skin infections in the tropics can be very severe and florid often with protean manifestations. This together with constant evolution of resistant microbial strains has worsened the scenario with unprecedented, not pleasant effects on the economy of these developing states in terms of huge budgetary allocation to health care with little or no inspiring results, confining it into a cactus fence of boring harmonic motion. Far from the distant past, the West African natives had always preferred the indigenous black soap mainly for cosmetic, traditional and even spiritual cleansing purposes (Bella, 2011). It is known as ‘Sabulun salo’ in Hausa, ‘Anago’, ‘Alata samina’ in Ghana and eko-zhiko’ in Nupe (Aliyu et al., 2012). Among the Yoruba people of south western Nigeria, it is known to as ọsédúdú or ‘abuje’ (Bella, 2011). The indigenous black soap is suitable for bathing purposes because of its soft nature coupled with its unique natural scent (Ugbogu et al., 2006). It has been discovered to be very pleasant to the skin and at the same time kills microorganisms that cause skin infection thereby ensuring a smooth, healthy, disease free skin (Ikpoh et al., 2012). The Indigenous black soap is also believed to be rich in active plant phytoconstituents (Oladunmoye et al., 2009).
The eggshell is a waste product that can be collected from hatcheries in large quantity. Apart from its negative impacts on the environment when disposed, it can also serve a very useful purpose. The eggshell powder has been used as a home remedy for acne and other skin associated infection as a result of which it is believed to contain bioactive components. Nakano et al. (2003) has also submitted that eggshell may contain biologically active compounds. Eggshell was presented as being composed of 93.70% calcium carbonate, 4.20% organic matter, 1.30% magnesium carbonate and 0.8% calcium phosphate (Winton, 2003). Formula variation of empiric antimicrobial compounds such as the black soap is therefore very vital to winning the war against microbial resistance. There is need for novel based investigations, directed at demystifying the plausible active constituents of the indigenous black soap.

MATERIALS AND METHODS

Collection of samples and test organisms: Plain black soap sample was bought from the local market. Black soap was prepared with ashes from dried cocoa pod husks by saponification (hot method) and then fortified with brown and white eggshell powder effectively at 20 g/250 mL of molten soap before allowing it to cure in a mould for two weeks.

Preparation of culture media: Microbiological media were prepared according to the appropriate manufacturer’s specifications.

Microbiological analysis of the black soap samples: Dilution plate methods were used separately and appropriately for plain black soap. Precisely 1 g of the soap samples were suspended into 9 mL of sterile distilled water in properly labeled test tubes to make a stock solution. Dilutions were made to $10^{-5}$ by removing 1 mL from the first test tube ($10^{-1}$) aseptically and adding to successive tubes. With a sterile 1 mL pipette, 1 mL aliquot from the higher dilutions ($10^{-4}$ and $10^{-5}$) obtained for each sample were aseptically transferred to already labeled nutrient and potato dextrose agar plates and spread with the aid of sterile glass spreader. The nutrient agar plates were incubated upside down at 37°C for 24 h while Potato dextrose agar plates at 25°C at 72 h.

Characterization and identification of microbial isolates: The pure culture of the bacterial isolates were examined and subjected to microscopic examination, staining techniques (Gram and Spore) and the following biochemical tests; motility, catalase, oxidase, starch hydrolysis, carbon sources utilization, in vitro growth at pH 10 according to the methods described by Olutiola et al. (2000). Bacterial isolates was identified with reference to the Bergey’s Manual of Systematic Bacteriology and the Advanced Bacterial Identification Software (ABIS) online Encyclopedia. Pure fungal isolates were identified based on macroscopic and microscopic characteristics with robust reference to Barnet and Hunter’s illustrated genera of imperfect fungi (Barnett and Hunter, 1998).

Plasmid analysis of bacterial isolates

Plasmid extraction: Plasmid extraction was accomplished based on the methods of Molina-Aja et al. (2002) with little modification. A single bacterial colony was picked up and grown in 5.0 mL of Muller Hilton broth overnight in an Eppendorf tube and centrifuged at 10,000 rpm for
2 min. The cell pellets obtained were re-suspended in 150 μL EDTA-Tris buffer and vortexed to mix. This was followed by the addition of 175 μL of 2% Sodium Dodecyl Sulphate (SDS) and 175 μL of 0.4 N NaOH. The tube was mixed vigorously, 250 μL of cold 5 M potassium acetate was added vigorously, the tube was centrifuged at 12000 rpm for 5 min and the supernatant was transferred to a sterile 1.5 mL Eppendorf tube and equal volume of cold isopropanol was added. After inverting gently, the mixture was immediately centrifuged at 12000 rpm for 10 min and the DNA pellet was washed with 650 μL of cold (40°C) 70% ethanol by centrifuging at 12000 rpm for 15 min. The supernatant was discarded and the pellet was dried for 30 min and re-suspended in 40 μL of sterile deionized water.

**Agarose gel electrophoresis:** Agarose gel electrophoresis was achieved by weighing 0.8 g of agarose powder and 100 mL of 1X Tris Borate Buffer (TBE buffer) was added, the buffer was dissolved by boiling in a microwave oven and allowed to cool to about 60°C and then 10 μL of ethidium bromide was added and mixed by swirling. The agarose was then poured into electrophoresis tank with the comb in place to obtain a gel thickness of about 4-5 mm and was allowed to solidify for about 20 min and the comb was removed, the tray was then placed in the electrophoresis tank. This was followed by the addition of 1X TBE buffer, this was then poured into the tank ensuring that the buffer covered the surface of the gel. The sample 15 μL was mixed with 2 μL of the loading dye and was carefully loaded into the wells created by the combs (marker was loaded in line 1). Electrodes were connected to the power pack in such a way that the negative terminal is at the end where the sample was loaded; electrophoresis was run at 60-100 V until loading dye has migrated about three-quarter of gel. Electrodes were disconnected and gel was removed from the tank and visualized in UV-trans-illuminator (FOTO UV 1-1430).

**Preparation of indigenous black soap for GCMS analysis:** Indigenous black soaps were prepared for GCMS analysis according to the methods described by AOAC (2007). About 1.0 g of soap was dissolved in 0.2 mL of toluene. To the solution, 1.5 mL of methanol and 0.3 mL of the 8.0% hydrogen chloride (HCl) solution were added in this order. The tube was vortexed and then incubated at 45°C overnight (14 h or longer) for mild methanolysis/methylation. After cooling to room temperature, 3 μL of sample was injected into the column of GCMS (QP2010 Plus Shimadzu, Japan).

**RESULTS AND DISCUSSION**

Plasmids are extra-chromosomal, double stranded DNA found in some bacteria as additional genetic endowment, often saddled with molecular blue print for survival (Onifade and Oladoja, 2015). The presence of plasmid DNA in the bacteria isolates especially *B. vedderi* (Fig. 1) has further corroborated the previous findings of (Agbagwa et al., 2012) as mentioned earlier. Before now, no attention has been placed both on the microbiological analysis of the black soap and plasmid profiling of its isolates. This will indeed be a vista for mapping potential extremophiles with useful metabolites. Plasmids frequently carry genes for antibiotic resistance, toxigenicity and can as well confer extremophily status on microorganisms. Plasmids are useful markers in Recombinant DNA technology and as such this makes plasmids indispensable tool in Molecular Biology (Gohar et al., 2015). The results from this investigative study can be used as an
Fig. 1: Electrophoretic patterns for plasmid profile of bacterial isolates from indigenous black soap, M: Molecular weight marker, 1: *Bacillus vedderi*, 2: *Bacillus faraginis*, bp: Basepair, 1 bp: 3.4 Angstrom (Å) while 1000 bp: 1 k base pairs

Fig. 2: Chromatogram of methylated fraction of indigenous black soap market sample

epidemiological tool for the typing of isolates from black soap samples. The more plasmids exist in an organism, the more specific is the plasmid profile as a marker for a single isolate. Most *Bacillus* species, regardless of their source, harbour at least one indigenous plasmid (Molina-Aja *et al.*, 2002; Reyaz *et al.*, 2013; Lobova *et al.*, 2015). These bacterial isolates have further corroborated the submission of Gao *et al.* (2011) that *Bacillus* spp., have evolved several genetic mediated mechanisms to survive in extreme environment.

Results from the GCMS analysis of the methylated fraction of the black soap samples (Fig. 2-4) have revealed peak ranges between 9 and 10 with their respective retention times. On a general
Fig. 3: Chromatogram of methylated fraction of indigenous black soap fortified with brown eggshell powder

Fig. 4: Chromatogram of methylated fraction of indigenous black soap fortified with white eggshell powder

note, fatty acids were the mostly identified (Table 1-3). Previous submission by Parsons et al. (2012) that fatty acids have bactericidal effect on vital pathogenic microorganisms including drug resistant S. aureus by membrane disruption mechanisms may as well be applicable to other gram positive bacterial pathogen as a result of structural similarities in their cell wall. Oils like sheabutter for instance is highly valued and used locally to treat skin eruptions. Facts from the current GCMS analyses of the black soap have once again further confirmed the position of Aliyu et al. (2012) which attributed the antimicrobial action of the traditional medicated soap specifically against gram positive organisms like S. aureus to the ability of the soaps long chain fatty acid content to distort the peptidoglycan present in its cell wall. Desbois and Smith (2010) also posited that fatty acids are known to create pores that may penetrate the cell membrane which induces portions of the lipid bilayer to be released and disruption of membrane permeability takes place. The previous submission of Lograda et al. (2012) that fatty acids have been demonstrated to be bactericidal to important pathogenic microorganisms including antibiotic resistant S. aureus has further lent credence to this serendipity.
### Table 1: Retention time of separated compounds from plain indigenous black soap

| Peak/No. | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Retention time (min) | 11.099 | 13.545 | 15.931 | 18.027 | 19.499 | 21.113 | 21.434 | 23.774 | 26.208 |
| Compounds | n-Decanoic acid | Tertadecanoic acid | Undecanoic acid | Pentadecanoic acid | n-Hexadecanoic acid | 9-Octadecenoic acid | Octadecanoic acid | 1-Decen,2,4dimethyl 1,2-Benzenedicarboxylic acid |
| Structure | ![Structure](image1) | ![Structure](image2) | ![Structure](image3) | ![Structure](image4) | ![Structure](image5) | ![Structure](image6) | ![Structure](image7) | ![Structure](image8) |

### Table 2: Retention time of separated compounds from indigenous black soap fortified with white eggshell powder

| Peak/No. | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Retention time (min) | 8.63 | 11.200 | 13.834 | 14.145 | 16.069 | 19.546 | 21.116 | 22.213 | 26.183 |
| Compounds | Octanoic acid | n-Decanoic acid | Tetradecanoic acid | Tributyl phosphate | Octadecanoic acid | n-Hexadecanoic acid | 16-Octadecanoic acid | Oleic acid | 1-pentanol |
| Structure | ![Structure](image9) | ![Structure](image10) | ![Structure](image11) | ![Structure](image12) | ![Structure](image13) | ![Structure](image14) | ![Structure](image15) | ![Structure](image16) | ![Structure](image17) |
| Peak/No. | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Retention time (min) | 11.089 | 13.591 | 14.760 | 15.593 | 16.930 | 18.014 | 19.159 | 22.179 | 24.434 | 26.210 |
| Compounds | n-Decanoic acid | Pentadecanoic acid | 2-tridecen-1-ol | 1-Tetradecene | n-Hexadecanoic acid | Hexadecanoic acid | 1-Octadecene | 1-Hecaconanol | 1-Eicanol | 1,2-Benzeneacrylic acid |
| Structure | ![n-Decanoic acid](image1.png) | ![Pentadecanoic acid](image2.png) | ![2-tridecen-1-ol](image3.png) | ![1-Tetradecene](image4.png) | ![n-Hexadecanoic acid](image5.png) | ![Hexadecanoic acid](image6.png) | ![1-Octadecene](image7.png) | ![1-Hecaconanol](image8.png) | ![1-Eicanol](image9.png) | ![1,2-Benzeneacrylic acid](image10.png) |
CONCLUSION
This study has provided information on the plasmid profiles of extremophilic bacteria associated with indigenous black soap and functional groups present. Presence of these plasmids could be the molecular basis for their existence in such environment. Antimicrobial activities of the indigenous black soap could also be as a result of the presence of these oils in their relatively partially unsaponified state.

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