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Full Length Research Paper

The use of extracts from four local Nigerian plants for the staining of selected bacteria and moulds

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Dyestuffs from four Nigerian plants were extracted and their staining ability on some species of bacteria and moulds were determined. The dyestuffs were extracted using water and organic solvents. Acetic acid treatment of the extracts increased the ability of the extract to stain both bacteria and moulds. Treatment with ammonium hydroxide reduced the staining of the plant extracts except the zobo (*Hibuscus sabderiffa*) extract. The best staining result was observed with uhe (*Pterocarpus osun*) extracts. Annato (*Bixa orellana*) and zobo extracts had moderate effect; whereas Lalle *(Lawsonia inermis*) showed little or no staining potential. The stains however had greater affinity for moulds than bacteria. The results obtained indicate that extracts with good staining ability have the potential for use in the morphological identification of moulds and in diagnostic bacteriology.

Key words: Plant extracts, staining, moulds, bacteria.

INTRODUCTION

Dyes are chemical substances of chemical or synthetic origin, soluble in a medium used to impart a desired colour to a non food material like paper, leather, wood, textile and even cosmetics in a process known as dying (Green, 1995). Dyes are also referred to as stains and can be used to add colour to tissues and microbes to make them optically distinct (Ragaswami and Bagyaraj, 1993).

Dyestuffs are dyes obtained from a natural source, that is, from plants or animals. Pigments are the specific chemical compounds responsible for the visible colour in the plant parts (Green, 1995). In histopathology, the most commonly used dye is haematoxylin obtained from a South African tree known as logwood (*Haematoxylium campechianum.* L).Studies conducted by Avwioro et al. (2005) have shown that the red dyestuff obtained from *Pterocarpus osun* species was used in staining tissue section for histopathological diagnosis of diseases. Microbial stains are used to impart colour in order to make the cells and tissues more distinct (Onyeike and Osuji, 2002).

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Although, microorganisms can be seen with the aid of a light microscope, they need to be fixed and or stained to increase visibility, accentuate morphological features and sometimes preserve them for further study (Prescott et al., 1999). Ragaswami and Bagyaraj (1993) reported that bacterial cells are attracted to the opposite charge ions of the stain. Prescott et al. (1999) are of the opinion that the pH of the dye may alter staining effectiveness since the nature and degree of the charge on cell component changes with pH.

Most dyes in current use are chemically synthesized. Besides been expensive, they are also hazardous to human health (Bhuyan and Saikia, 2004). Some dye components are carcinogenic or at least strongly allergenic resulting in their withdrawal as their hazard becomes recognized (Bhuyan and Saikia, 2004; Blender, 1982). In this report, the staining potentials of four Nigerian plant extracts were determined using selected bacteria and moulds. The effectiveness of the extracts was also compared to conventional staining reagents.

MATERIALS AND METHODS

Sources of raw materials

Plants and plant parts used are as follows: (i) seed of *Bixa orellena*

(annatto) (ii) heartwood of *Pt. osun* (uhe) (iii) flowers (calyces) of *Hibiscus sabderiffa* (zobo) and (iv) young leaves of *Lawsonia inermis* (henna). Zobo calyces and uhe wood were sourced in a local market while annatto seeds and henna leaves were obtained from an ornamental garden all in Owerri, Imo State, Nigeria.

Description of raw materials

B. orellena L, belonging to the family Bixaceae is a small tree, native to central and south American, but now grown in the tropics for ornamental purposes. The seed gives a red colour and is used as food colouring. It is locally called annatto by the Latin American. The principal pigment is bixin (Green, 1995).

Pt. osun belonging to family Papilonaceae is commonly called *uhe* or *ufie* by the Igbo speaking part of Nigeria. It is used to add colour to locally made palm kernel oil known as '*Mmanuaki*'. It is a deciduous tree of Indian origin (Green, 1995). In Europe, redwood is used to add colour and flavour to sea foods, sauces meat products, snacks, food and alcoholic drinks (Thadens and Verstrygne, 1989). The principal pigments are santalin A and B which are insoluble in water but soluble in organic acid and alkali (Green, 1995).

H. sabdariffa L belonging to the family Malvaceae is a native of Malaysia but is also cultivated in India, Nigeria and tropical America for its juice. Its edible calyces is acidic (Morton, 1987). Locally it is known as *zobo* in Nigeria and drinks made from it have a deep purple colour which stains the teeth and tongue temporarily. The nutritional and medicinal properties had been reported by Morton (1987).The major pigment has been identified as daphniphylline. Small amount of delphinidin-3- monoglucoside are also present (Morton, 1987). *L. inermis* L. belonging to the family Lythraceae is a shrub commonly used as a fragrant hedge. It is found in Senegal, Liberia, Nigeria and other African countries and grown for its dyes. Commonly known as *lalle*, the aqueous extracts from its leaves are used for hair and skin dyeing and other cosmetic purposes, and has acquired significance in Islamic culture (Chourasis, 1989). The major pigment in henna is law some (2 hydroxy-1, 4-naphaquine).This fixes strongly to protein hence its fast dyeing properties (Green, 1995).

Preparation of raw materials

Wood

The heartwood of *Pt. osun* was sun dried and chopped into pieces and further dried in a Uniscope SM9053 digital laboratory oven for 4 days at 60°C. The chips were broken into smaller pieces using laboratory mortar and pestles and further reduced to powder by milling (Avwioro et al., 2005). The powder was sterilized in a hot air oven at 120°C for 1 h.

The young fresh leaves of *L. inermis* were grind to paste with a laboratory mortar and pestle (Drapper, 1976) and sterilized in a hot air oven at 120°C for 1 h. The dried calyces of *H. sabdariffa* were grind with a laboratory mortar and pestle to a rough powder (Drapper, 1976). The powder was heat sterilized in a laboratory oven at 120°C for 1 h. The thorny pods containing the seeds of *Bixa orellena* were broken with the hand. The seeds were separated from the other particles and sterilized in hot air oven at 120°C for 1 h.

Extraction of pigment

Two extraction methods were employed. The plant materials were boiled in distilled water followed by soaking the plant materials in organic solvents.

Procedure of extraction

Two grams of *uhe* and ten grams of the other plants were soaked in 20ml and 100 ml respectively of the solvents (80% ethanol and absolute ethanol, methanol and chloroform) and aqueous solutions (hot and cold) in 250 ml Uniscope conical flasks. The mouths of the flasks were stoppered with cotton wool wrapped with aluminum foil and allowed to stand in the dark for 48 h at ambient temperature. The quantities of the plant materials were boiled in 20 and 100 ml distilled water respectively for 5 min and allowed to cool before purification.

Purification of extracts (dyestuff)

Filtration:

The content of each flask was filtered (Avwioro et al., 2005). The residue from the chloroform extracts were put back into the conical flask and 20 ml of distilled water added into it and shaken gently until a strong colour became visible and the contents filtered again.

Evaporation of solvents

The organic solvent in the extracts were evaporated by passing warm air over the mouth of the flask. The extracts were further dried in the hot air oven at 60ºC for 1h.

Reconstitution of dyestuffs

The dried extracts (except annatto) were all reconstituted by adding 20 ml of distilled water. The reconstituted extracts were transferred into a screw capped bijou bottle, corked tightly, labeled and preserved in the refrigerator prior to use.

Preparation of staining reagents (dyestuff) from plant extracts

Two milliliters each of the reconstituted dyestuffs, except annatto were transferred into a test tube and 0.2 ml glacial acetic acid was added and the whole contents shaken to mix then corked and labeled appropriately. The same treatment was repeated using ammonium hydroxide solution (Avwioro et al., 2005). Three milliliters each of 0.1N glacial acetic and ammonium hydroxide were separately added into the test tubes containing equal quantities of dried annatto extract and mixed thoroughly to dissolve. Boiled water extracts were not treated.

Microorganisms

Pure cultures of *Escherichia coli* and *Staphylococcus aureus* were collected from the diagnostic unit of the Federal Medical Centre (FMC), Owerri, Imo State, Nigeria. The pure cultures were screened to confirm their identity and viability using standard methods (Cheesbrough, 2000).

Aspergillus and *Rhizopus* species were isolated from moist bread cultivated on Sabouraud Dextrose Agar (SDA) and incubated at ambient temperature for 5 days. The features of the isolates (e.g, spore arrangement and mycelia formation) were cross matched with those present in Barnett and Hunter (1987).

Staining of tested microorganisms

Staining of moulds

A drop of prepared staining solution was placed on a clean grease

Table 1. Extractability of dyestuffs from some selected plant materials.

Source of dyestuff	Extracting solvent	Colour of extract	Degree of extraction
B. orellena (annatto)	Boiled water	Amber	-
	Abs ethanol	Red	+++
	80% ethanol	Red	+++
	Methanol	Orange red	++
	Acetone	Orange red	++
	Acet + water (1:1)	Orange red	++
	Chloroform	Red	+ ++
	Chlo + water (1:1)	Red	+++
Pterocarpus osun (uhe)	Boiled water	Pale amber	-
	Abs ethanol	Red	+++
	80% ethanol	Red	+++
	Methanol	Orange	++
	Acetone	Red	+ ++
	Ace + water (1:1)	Red	+++
	Chloroform	Pale amber	-
	Chlo + water (1:1)	Red	+++
Hibiscus sabdarrifa (zobo)	Boiled water	Red mauve	+++
	abs ethanol	Mauve	+++
	80% ethanol	Mauve	+++
	Methanol	Mauve	++
	Acetone	Amber	-
	Ace + water (1:1)	Mauve	+++
	Chloroform	Amber	-
	Chlo + water (1:1)	Red mauve	+++
Lawsonia inermis (lalle)	Bolied water	Brown	++
	Abs ethanol	Brown	+++
	80% ethanol	Brown	+++
	Methanol	Brown	+++
	Acetone	Pale brown	+
	Ace + water (1:1)	Brown	++
	Chloroform	Pale brown	+
	Chlo + water (1:1)	Brown	++

+++, very deep colour; ++, moderate colour; +, light colour; -, colourless.

free microscope slide. Red hot flamed mounting needle was used to collect a tiny portion of the moulds grown on SDA and placed on a pool of the staining reagents on the slide. With another mounting needle, the mycelia were teased apart and a cover slip gently placed over the specimen in the pool to avoid bubbles and excess fluids blot dried (Beishir, 1987; Harrigan and McCance, 1990). The slide was allowed to stand for 3 min and the morphology of the organism viewed at 40× magnification. Control slides were prepared and stained with lactophenol cotton blue. Different combinations of the extracts were also used to stain the moulds.

Staining of bacteria

A smear of the tested organisms (24 h) was made on clean grease free slide and heat fixed (Beishir, 1987; Baker et al., 2001; Cheesbrough, 2000). Gram staining reagents (crystal violet, lugols iodine and acetone) were used except for the counter stain (safranin). In one set of the test, ammonium hydroxide treated dyestuff was used as counter stain. Control slides were also prepared and stained by Grams method (Prescott et al., 1999). The procedure was repeated with the hot water plant extracts.

RESULTS

Extraction of dyestuffs

Absolute ethanol and chloroform (mixed with water in the ratio of 1:1) was the best solvent in the extraction of plant materials. Boiled water was good in the extraction of *zobo* and *lalle* as *uhe* and *annatto* dissolved poorly in it (Table 1). However, all dyestuffs were soluble in 80%

Table 2. Staining abilities of untreated dyestuff extracts on selected moulds.

Plant dyestuff	Type of extract	*Rhizopus* sp	*Aspergillus* sp
B. orellena	Boiled water	-	-
	Abs ethanol	-	+
	80% ethanol	+	+
	Methanol	-	-
	Acetone	-	-
	Ace + water (1:1)	-	-
	Chloroform	+	-
	Chlo + water (1:1)	-	-
P. osun	Boiled water	-	-
	Abs ethanol	+	+
	80% ethanol	+	+
	Methanol	+	+
	Acetone	-	+
	Ace + water (1:1)	+	+
	Chloroform	-	-
	Chlo + water (1:1)	-	+
H. sabdarriffa	Boiled water	+	+
	Abs ethanol	+	+
	80% ethanol	+	+
	Methanol	+	+
	Acetone	-	+
	Ace + water (1:1)	+	+
	Chloroform	-	-
	Chlo + water (1:1)	-	+
L. inermis	Boiled water	-	-
	Abs ethanol	-	-
	80% ethanol	-	-
	Methanol	-	-
	Acetone	-	-
	Ace + water (1:1)	-	-
	Chloroform	-	-
	Chlo + water (1:1)	-	-

+, organisms stained; -, organisms not stained.

ethanol and produced colours as strong as absolute ethanol. The effectiveness and or level of extraction were determined by the concentration of the colour as summarized in Table 1.

Acid and base treatment

Treatment with glacial acetic acid did not change the colour of any extract, however, the colour of *zobo* extract changed on addition of ammonium hydroxide. All ethanol extract changed from mauve to black, while acetone and chloroform extracts turned blue-black on treatment with ammonium hydroxide.

Staining abilities of plant extracts

Untreated plant extracts

Untreated organic solvent extracts of plant materials stained moulds differently as shown in Table 2. Boiled water extracts of *zobo* stained fungi mycelia and spores more strongly than other boiled water extracts (Figure 1). The acetone and methanol extracts of *uhe* proved most effective in staining moulds (Figures 2 and 3). However,

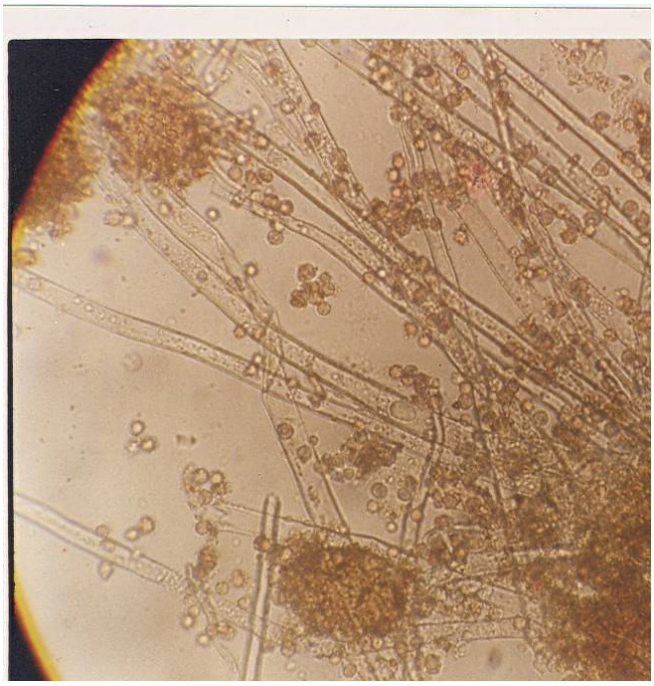

Figure 1. *A. oryzae* stained with hot water extract of zobo.

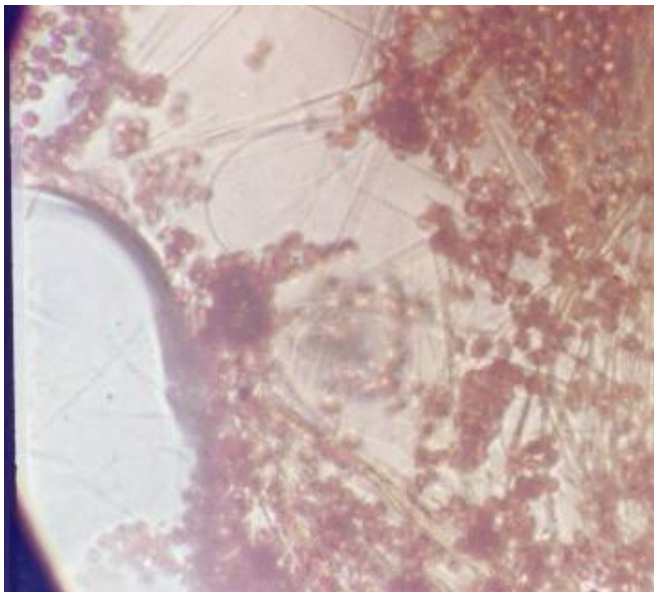

Figure 2. *A. oryzae* stained with acetone extract of uhe treated with glacial acetic acid.

none of the untreated extracts were able to stain bacteria.

Glacial acetic acid treated extracts

The staining proficiency of acetic acid treated extract is shown in Table 3. Improvement on the staining ability of

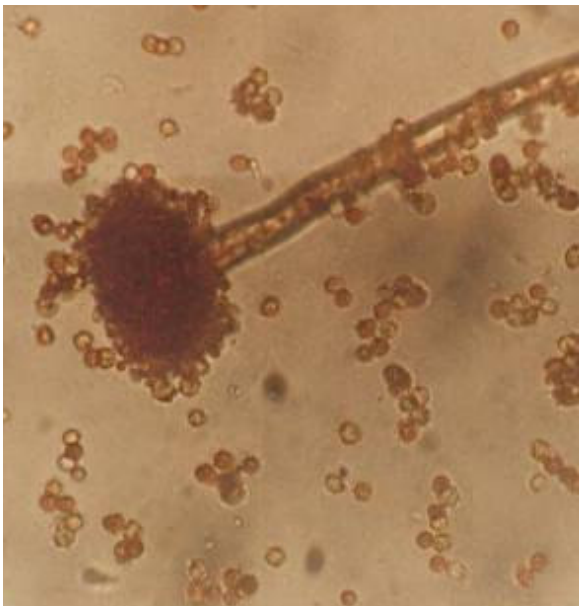

Figure 3. *A. oryzae* stained with methanol extract of uhe.

acid treated plant extract is demonstrated in Figures 4 and 5, and compares very well with conventional stains (lactophenol cotton blue) shown in Figure 6. Cells of *E. coli* were visibly stained when ethanol extracts of *uhe* treated with glacial acetic was used as the counter stain (Figure 7), although *S. aureus* stained poorly.

Ammonium hydroxide treat extracts

Addition of ammonium hydroxide only improved the staining capability of hot extract of *zobo* and *annatto* (Table 3). Ammonium hydroxide treatment greatly reduced the staining ability of *uhe* and *lalle* as shown in Table 3. A synergistic effect of *zobo* and *uhe* treated with acetic acid and ammonium hydroxide is shown in Table 4. Extracts treated with acetic acid proved effective on all the test organisms. Moulds stained better than bacteria in all the extracts treated with acetic acid. Ammonium hydroxide treated extracts stained poorly (Table 4).

DISCUSSION

Dyestuff prepared from *zobo* is soluble both in water and organic solvents. Others were soluble only in organic solvents (Table 1). *L. inermis* was moderately soluble in boiled water. Acetone extracts appeared darker after one week. The chemistry behind this is not yet understood.

Ethanol was adjudged the best as all dyestuff was soluble in it (Table 1).*Uhe* and *annatto* dissolve poorly in boiled water, probably because of their oil based nature as they form oil film in the final formulation. *Uhe* congealed and formed clumps when refrigerated at 4°C

Table 3. Staining of moulds and bacteria with treated plant extracts.

Plant extracts	Glacial acetic acid treatment				Ammonium hydroxide treatment			
	A	B	C	D	A	B	C	D
P. osun								
80% ethanol	++	++	+	+	-	-	-	-
Abs ethanol	+++	+++	+	+	-	-	-	-
Chloroform	-	-	-	-	-	-	-	-
Chlo + water(1:1)	++	+	+	++	-	+	-	-
Methanol	++	++	++	-	-	-	-	-
Acetone	+	+	-	-	-	-	-	-
Ace + water(1:1)	+	++	++	+	+	+	-	-
H. sabdariffa								
80 ethanol	+	-	+	-	+	-	-	-
Abs ethanol	+	-	+	-	++	-	-	-
Chloroform	-	-	-	-	-	-	-	-
Chlo + water(1:1)	+	-	-	-	-	-	-	-
Methanol	+	-	-	+	-	-	-	-
Acetone	-	-	-	-	-	-	-	-
Ace + water(1:1)	+	-	-	+	-	-	-	-
Hot water	++	+	+	-	-	-	-	-
B. orellena								
80% ethanol	+	-	-	-	++	+	+	-
Abs ethanol	++	++	+	-	-	+	-	-
Chloroform	-	-	-	-	-	-	-	-
Chlo + water(1:1)	+	+	+	-	+	+	-	-
Methnol	+	+	-	-	-	-	-	-
Acetone	-	-	-	-	-	-	-	-
Ace + water(!:1)	-	-	-	-	-	-	-	-
Hot water	-	-	-	-	-	-	-	-
L. inermis								
80% ethanol	-	-	-	-	-	-	-	-
Abs ethanol	-	-	-	-	-	-	-	-
Chloroform	-	-	-	-	-	-	-	-
Chlo + water(1:1)	-	-	-	-	-	-	-	-
Methanol	-	-	-	-	-	-	-	-
Acetone	-	-	-	-	-	-	-	-
Ace + water(1:1)	-	-	-	-	-	-	-	-
Hot water	-	-	-	-	-	-	-	-

A, *Aspergillus oryzae*; B, *Rhizopus stolonifer*; C, *Escherichia coli*; D, *Staphylococcus aureus*.

and dissolved at 30°C. Dyestuffs prepared from water developed fungal growth after 3 weeks even at refrigeration temperature.

Alcohol extracts of *uhe, zobo* and *annatto* showed varied staining reactions on moulds and bacteria, whereas *lalle* did not stain any of the tested microorganisms (Table 2). The poor staining potential of *lalle* could be attributed to chemical modification which prevents penetration of the dyes into the cell walls of the organisms. The dyestuffs showed stronger staining affinity on moulds than bacteria (Table 3). Organic compounds treated with acetic acid and ammonium hydroxide were found to facilitate the extraction of active components in the plants. This was demonstrated by the high quality micrographs. Baker et al. (2001) reported that tissue elements such as nucleus have great affinity

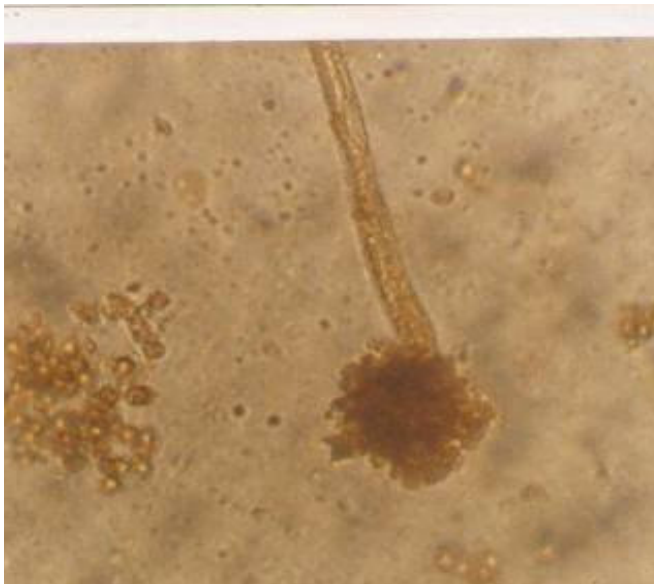

Figure 4. *A. oryzae* stained with chloroform extract of bixa treated with glacial acetic acid treated with glacial acetic acid.

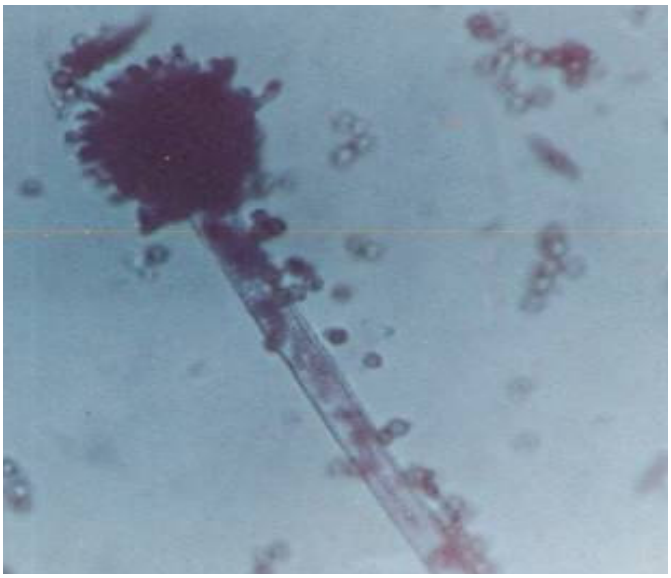

Figure 6. *A. oryzae* stained with lactophenol cotton blue treated with glacial acetic acid.

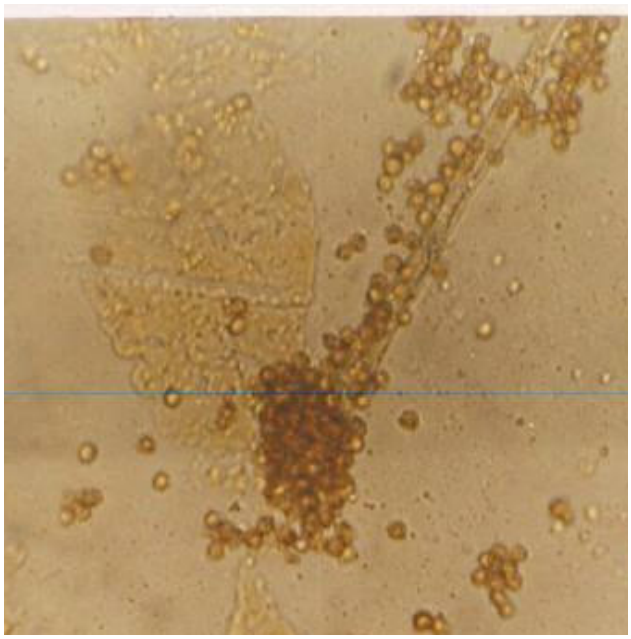

Figure 5. *R. stolonifer* stained with methanol extract of bix.

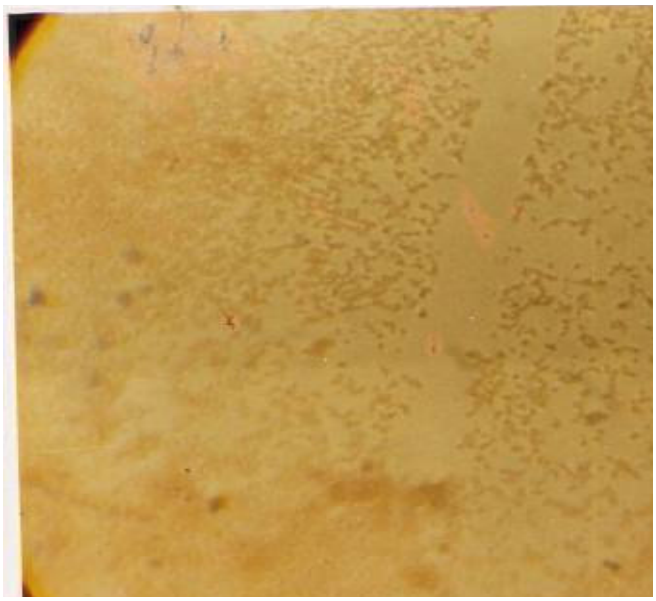

Figure 7. *E. coli* stained with ethanol extract of uhe treated.

for a basic stain while cytoplasm which is basic in character has affinity for an acidic stain. This statement placed *uhe* as an acidic dye because of its greater affinity for the cytoplasm of moulds and gram negative bacteria. At high pH (with addition of ammonium hydroxide), the staining potential of *uhe* was reduced. This supported the report of Hoffman and Banknocht (1999), that pH of staining solution often affect staining reactions. *Zobo* extracts from water and ethanol stained moderately. Ethanol extract of *zobo* enhanced with ammonium hydroxide stained *Aspergillus* sp visibly, but could not stain bacteria. Water extract of *annatto* did not stain bacteria and moulds; however the organic extracts stained moderately when treated with acetic acid. Treatment with ammonium produced bubbles, probably due to effervescence nature of the base.

Extracts of *uhe* formed clumps at the edge of the cover slip when dried, although the stained organisms

Table 4. Synergistic effect of the extracts of two treated dyestuffs on selected moulds and bacteria.

Treated plant extracts	Glacial acetic acid				Ammonium hydroxide			
	A	B	C	D	A	B	C	D
uhe + zobo (abs ethanol)	+++	+++	++	-	+	-	-	-
uhe + zobo (methanol)	+++	+++	-	-	+	-	+	-
uhe + zobo (acetone + water)	++	++	++	+	-	-	-	-
uhe + zobo (chlo + water)	++	+	+	+	-	-	-	-

A, *Aspergillus oryzae*; B, *Rhizopus stolonifer*; C, *Escherichia coli*; D, *Staphylococcus aureus* -; not stained; +, weak staining; ++, moderates.

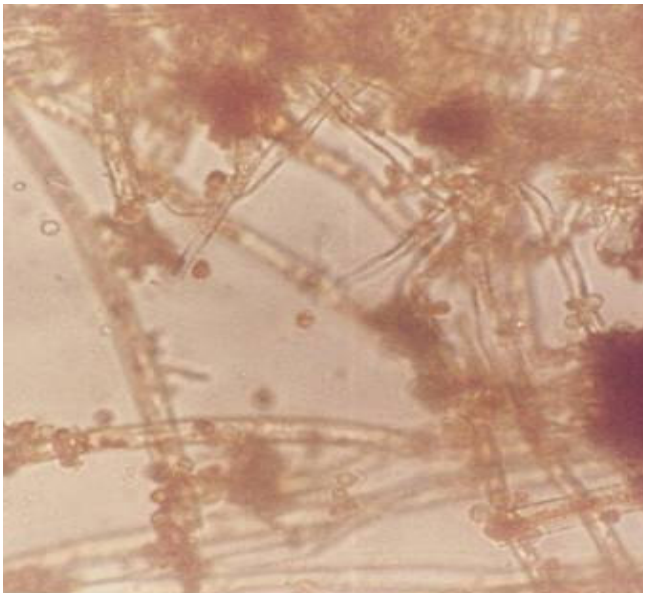

Figure 8. *A. oryzae* stained with ethanol extract of zobo with glacial acetic acid treated with ammonium hydroxide.

remained intact. *Uhe* stained bacteria better than *zobo* and *annatto*, even though it could not differentiate gram positive organisms from gram negative organism. The combined effects of *zobo* and *uhe* extracted with organic solvent and treated with acid and base improved staining potentials for moulds. This is most evident especially for acid treated dyes (Figures 1 - 8). Some of the plant extracts stained moulds very well and can be used as a substitute to the conventional stains.

Conclusion

The staining potentials of four Nigerian plant extracts on selected bacteria and moulds showed that some of the plant extracts could stain moulds as shown in the micrographs. The plants are *B. orellena* (annatto), *Pt. osun* (uhe), *H. sabderrrifa* (zobo) and *L. innermis* (lalle). The active components were extracted with acetone, ethanol, methanol, chloroform and aqueous solution (hot and cold). The extracts were treated with glacial acetic acid and ammonium hydroxide to enhance their staining proficiencies. Lactophenol cotton and Grams reagents were used as the control stains for moulds and bacteria respectively. The result showed that moulds stained better than bacteria.

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