

Staining of Ova of Intestinal Parasites with Extracts of *Hibiscus Sabdariffa* and *Azadirachta Indica*

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ABSTRACT: Ethanol extracts of two indigenous Nigerian plants *Hibiscus sabdariffa* and *Azadirachta indica* extracted using the soxhlet extraction method, were used to stain malaria parasites. The extracts were made with different percentages of ethanol (96%, 75%, 50% and 30%) and diluted to various concentrations. There was no previous record known on the use of *H. sabdariffa* & *A. indica* in staining intestinal protozoa according to the author's knowledge. By double dilution method, 0.13g/ml, 0.16g/ml, 0.11g/ml and 0.01g/ml to 0.08g/ml were made for *H. sabdariffa*, and 0.18g/ml, 0.09g/ml, 0.07g/ml, 0.05g/ml, 0.04g/ml, 0.03g/ml 0.02g/ml and 0.01g/ml for *A. indica*. Eggs of worms such as the ova of *Ascaris lumbricoides*, ova of Hookworm and *Trichuris trichiura* were deeply stained alike by high concentration of both dyes. Cyst of *E. histolytica* and *E. coli* stained grey in *H. sabdariffa* but were highly refractive with *A. indica*. In lower concentrations the outer covering of *A. lumbricoides* and *T. trichiura* were stained with the colour of the respective dyes while the granular segments retained its yellow-brown colour.

KEYWORDS: Staining, Ova, Intestinal parasites, Extracts, *H. sabdariffa*, *A. Indica*

INTRODUCTION

Dyes are frequently used in biology and for viewing with different microscopes. They may be used to define and examine bulk tissues such as muscle fiber, connective tissue, cell population of blood cells or organelles within individual cells. Staining procedures are required for detailed study and to prepare permanent preparations. Biological stains are prepared from dyes which have been manufactured to rigid specification for this purpose, or have been subject to rigid quality assurance procedures to ensure that they are suitable for this specialized purpose (Baker *et al*, 1998).

Historical development of dyes has previously been documented. (Baker *et al*, 1998). In 1981, Banerjee and Mukherjee found these dyes as Santalin and Santarubin. During the 19th century, fabrics were dyed with dyes obtained from madder roots until mid 1880's when it was replaced by a synthetic substitute. Woad was used in middle ages by Europeans to create a blue fabric dye. This woad is a shrub which grew abundantly in parts of Europe. The pigment that impacted the colour to the fabrics was found in leaves which was dried and ground to powder and a paste was made out of it by mixing with water. Indigo, a shrub commonly known by the ancient Egyptians and Indians later replaced woad. Its coloring pigment is also found on the leaves which were extracted as with woad but in addition was fermented, purified and pressed to cakes. It therefore prevailed as blue dye being substantive i.e. needing no mordant and extremely fast to washing and light In 1905, Adolf Von Baeyer won a Nobel Prize for the discovery of the molecular structure of Indigo. Woad was being raised extensively in Germany in 1290 AD and was among the three major blue dyes: Woad, Madder, and Weld. Brazil wood in 1321 AD was first mentioned as a dye from East Indies and India. Furthermore, France, Holland and Germany began

The cultivation of dye plants as an industry in 1507. Drebber, a Dutch Chemist in 1630, produced a new brilliant red dye from Cochineal and tin. While searching for the cure for malaria, William Henry Perkin in 1856 discovered "Mauve" a basic dye. The development of these industries continued up to the 20th century

Previous studies using some plant extracts have shown that they can be explored in biology and medicine to reveal or identify cellular components or parasites infecting blood cells and tissues. Some of these works include the use of Nigerian plant extract *Rhizophora racemosa* for differential staining of blood smears from toad, chicken, and albino rats (Odinohirin, 1982). Dyes from *Baphia nitida*, *Rhizophora racemosa* and *Rothmania hispida* were used to stain red blood cells and intra-cellular malaria parasites at various concentrations of the dyes and results from these proved that these dyes are viable to be used as stains for diagnostic purposes in Medicine and Biology (Okolie, 2006). The dyes were extracted using the dry extraction method at various concentrations of ethanol and the best concentration of the extract was obtained using lower concentrations of ethanol extract. Other plant extracts which were also explored include *Diocela reflexa*, *Aframomum danieli*, *Basella alba*, *Cola acuminata* and *Cola nitida*.

The fact that there is a high dependence on synthetic dyes manufactured by developed countries for use by Nigerian hospitals and Laboratories has necessitated the study on natural dyes. The synthetic dyes are expensive and no industry in Nigeria is known to produce them (Okolie, 2006). Therefore the need for exploratory studies using some local extracts from indigenous plants *Hibiscus sabdariffa* and *Azadirachta indica* for the staining of intestinal and malaria parasites has become imperative. It is hoped that at the end of this work, extracts from these plants will be adopted in Nigeria for diagnostic purposes

MATERIALS AND METHODS

The Study Area

The research was carried out in the Medical Laboratory of Imo State University Owerri, between the months of January and March, 2008. Already dried leaves of *Hibiscus sabdariffa* were purchased from the local market (Ekeonunwa market) in Owerri, Imo State, where it is known as Zobo leaf, while *Azadirachta indica* was obtained from a tree along the Imo state University junction. by works layout

Processing Of the Plants

The dried leaves of *Hibiscus sabdariffa* and *Azadirachta indica* were ground to a powder using the electric and manual grinding machine. A smooth consistency was ensured to attain good surface areas for pigment extraction. The dry powders of the respective plants were weighed separately using an electric weighing scale (Mettler PN 163) and they weighed 25.5g and 14.76g respectively. These were then soaked in ethanol in percentages of 96%, 75%, 50%, and 30% for 24 hours undisturbed. The extraction was preceded using the Soxhlet extractor (extraction with heating mantle, an electrochemical standard model, India). The extract was then transferred to a rotatory evaporator (Model-labarato 4000, England) for concentration to take place. There was further drying of the extract in a drying oven (Accumax model, India) at 60°C. The temperature enabled the preservation of the active ingredients of the extract after it was obtained in a powdered form. Dilutions of the resulting solution using the double dilution method described by Ojiegbe (2005) were done to attain lower staining concentrations. Dilutions were only made up to the third tube because with increased dilution, the colour intensity of the solution decreased.

Staining Procedure

Wet preparations of the stool samples were made using lugol's iodine and saline. The same technique was applied using the plant extracts.

Concentration of the stool sample using zinc sulphate solution was done and the concentrate was examined using the extract. A little portion of the pooled stool sample was emulsified into a drop of lugol's iodine or plant extract on a clean grease-free slide, This was covered using a cover slip making sure there was no air bubble. It was then examined under the microscope using the x10 and x40 objectives respectively.

Concentration Technique Using Zinc Sulphate Solution

Principle of the technique is as follows: A zinc sulphate solution is used which has a specific gravity (relative density) of 1.180-1.200. Faeces are emulsified in the solution and the suspension is left undisturbed for the eggs and cysts to float to the surface. They are collected on a cover glass. (Cheesbrough, 1999).

The procedure involves the following: Into a test tube about one quarter of the zinc sulphate solution was filled. About 1 gram of faeces was emulsified into the solution. The tube was then filled with the zinc sulphate solution and mixed well. The faecal suspension was strained to remove large

faecal particles. The suspension was returned to the tube and allowed to stand in a vertical position in a rack. A further solution was added to the tube with the aid of a Pasteur's pipette. This was to ensure the tube is filled to the brim. A clean grease-free cover glass was placed on top of the tube avoiding air bubbles. This was left for 30-45 minutes for the cysts and eggs to float. The cover glass was carefully lifted from the tube by a straight pull upwards. It was placed face down on a slide and examined microscopically using the x10 and x40 objective. Few drops of the extract were applied through the side of the cover glass before examination.

RESULTS

Hibiscus sabdariffa dye extract retained its pinkish colour at the various dilutions with ethanol. Staining of stool and blood smears were done using the following concentrations of 96% ethanol extract; stock (0.13g/ml), 0.07g/ml, 0.03g/ml, 0.02g/ml and 0.1g/ml. Stool samples of eggs of *A. lumbricoides* appeared dark pink at concentrations of 0.13g/ml and 0.07g/ml using x10 and x40 objectives. The outer covering and nucleus were evenly stained dark pink. At concentrations of 0.03g/ml, 0.02g/ml and 0.01g/ml the outer covering was stained pink while the nucleus retained its yellow-brown colour in both x10 and x40 objectives. With 75% ethanol extract and dilutions of 0.16g/ml, 0.08g/ml, 0.04g/ml and 0.02g/ml, the ova of *Ascaris lumbricoides* absorbed the stain and was seen as pink outer covering with yellow-brown granular mass, the cyst of *E.coli* and *E.histolytica* were seen as pinkish spherical bodies with grayish nucleus clearly differentiated in both cysts. Malaria parasites were seen as red chromatin dots with brown nucleus in both thick and thin films. (Tables 1 -2).

The 50% ethanol extract and at dilutions of 0.05g/ml, 0.02g/ml and 0.01g/ml with the stock having a concentration of 0.11g/ml, the ova of *Ascaris lumbricoides*, ova of hookworm, and cysts of *E.coli* and *E.histolytica* were clearly defined producing the same characteristics as that of 75% ethanol extract. The same result was obtained for the 30% ethanol extracts at dilutions of 0.07g/ml, 0.03g/ml and 0.02g/ml while the stock had a concentration of 0.13g/ml. In all, best staining results were obtained at lower concentrations of 0.03g/ml, 0.02g/ml and 0.01g/ml in all percentage extraction of the dye.

Dyes from *Azadirachta indica* had shades of green in all the percentage extraction of the dye except the 30% extract which gave a brownish colouration. At 96% ethanol extract, dilutions were made at concentrations of 0.09g/ml, 0.05g/ml, 0.02g/ml and 0.01g/ml. Stool examinations using these various dilutions were able to clearly differentiate the ova of hookworm and *A.lumbricoides*. The ovum of Hookworm was clearly identified with a pale green cytoplasm and a grayish nucleus. The same colour characteristics were produced by the ova of *A.lumbricoides*. It was clearly differentiated as green cytoplasm and grayish nucleus. Cyst of *E.coli* and *E.histolytica* were difficult to identify using *A.indica*.

This could be explained by the fact that *E.coli* and *E.histolytica* were refractile to *A.indica* extract.

At concentrations of 0.03g/ml, 0.02g/ml and 0.01g/ml (stock 0.07g/ml), the same result was obtained with wet preparations of stool using the extract. Concentrations used for the 50% and 30% extract are 0.05g/ml, 0.02g/ml and 0.01g/ml respectively (stock: 0/09g/ml). These also produced staining characteristics as that of 96% ethanol extract. Its various dilutions have no bearing on the results. Controls using saline and iodine for the wet preparation of stool was similar in comparison with those gotten using the extracts. Photomicrographs of slide preparations of stool samples stained with the extracts are shown in figures 1 and 2.

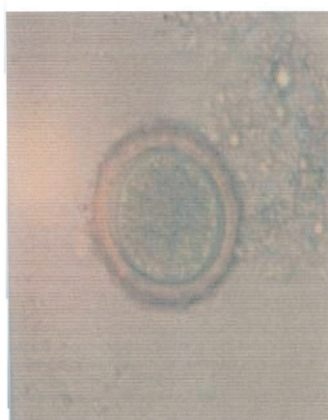


FIGURE 1 OVA OF ASCARIS LUMBRICOIDES IN WET PREPARATION OF STOOL USING LOW CONCENTRATION OF HIBISCUS SABDARIFFA

TABLE 1: Result of wet preparation using *H. sabdariffa* at various concentrations.

CONCENTRATION OF EXTRACT (g/ml)	OF INTESTINAL PARASITE	WET PREPARATION OF STOOL
03	<i>E. coli</i> and <i>E. histolytica</i>	Pink cytoplasm with grey nucleus
02	<i>E. coli</i> and <i>E. histolytica</i>	Pink cytoplasm with grey nucleus
03	Ova of <i>A. lumbricoides</i>	Pink cytoplasm with yellow-brown nucleus
03	<i>T. trichuria</i>	Pink cytoplasm with yellow-brown nucleus
03	Ova of Hook worm	Pink cytoplasm with yellow-brown nucleus
02	Ova of <i>A. lumbricoides</i>	Pink cytoplasm with yellow-brown nucleus
02	<i>T. trichuria</i>	Pink cytoplasm with yellow-brown nucleus
02	Ova of Hook worm	Pink cytoplasm with yellow-brown nucleus
13	<i>A. lumbricoides</i>	Cytoplasm and nucleus evenly stained pink
13	<i>T. trichuria</i>	Cytoplasm and nucleus evenly stained pink
13	Ova of Hook worm	Cytoplasm and nucleus evenly stained pink
16	<i>A. lumbricoides</i>	Cytoplasm and nucleus evenly stained pink
16	<i>T. trichuria</i>	Cytoplasm and nucleus evenly stained pink
16	Ova of Hook worm	Cytoplasm and nucleus evenly stained pink

DISCUSSION

The use of alcohol as extractant suggested by Okolie (2006) produced reasonable results. The dye stuffs were very soluble in the ethanol used in the extraction. He had reported some degree of success in the use of pigments from red mangrove and *cola nitida* extracted from 50%

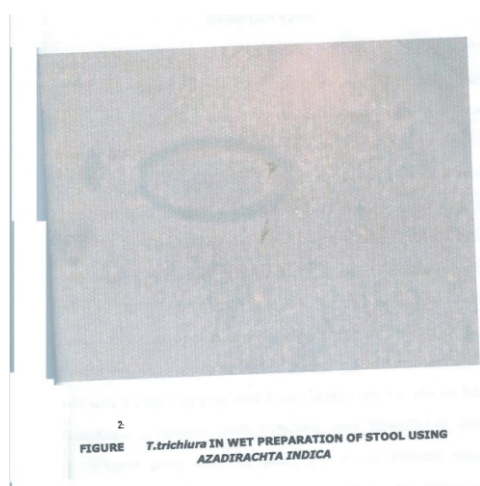


FIGURE 2 *T.trichuria* IN WET PREPARATION OF STOOL USING AZADIRACHTA INDICA

TABLE 2: Result of wet preparation made with *A. indica* at various concentrations

CONCENTRATION OF EXTRACT (g/ml)	OF INTESTINAL PARASITE	WET PREPARATION OF STOOL
03	<i>E. coli</i> and <i>E. histolytica</i>	Protozoa refractile to the dye
02	<i>E. coli</i> and <i>E. histolytica</i>	Protozoa refractile to the dye
03	Ova of <i>A. lumbricoides</i>	Cytoplasm and nucleus dye grey
03, 0.18, 0.09	<i>T. trichuria</i>	Cytoplasm and nucleus dye grey
03, 0.18, 0.09	Ova of Hook worm	Cytoplasm and nucleus dye grey

alcohol for staining nematode parasite. Also as recommended by Okolie (2006), the use of ethanol as extractant prevented fungal growth during storage and its volatile nature increased the permeability of the dye stuff and produced greater staining affinities to the parasites. It is important to note that the colour of the dyes for *Hibiscus sabdariffa* remained pink at its various concentration dilutions with lower concentration taking the lower shades of pink. This also applied with the *Azadirachta indica* dyes. Only the 30% extract had shades of brown giving lighter brown colour at the lower dilutions of 0.02g/ml and 0.01g/ml.

Part of this work involved the use of various percentages of ethanol to make dye extracts of the plants involved but in the course of study it was observed that the percentage of ethanol used for the extraction had no effect on the staining abilities of the dyes. Each dilution in the various concentrations produced the same result with difference made only in the colour intensity of the dyes. Higher concentrations stained darkly on faecal wet preparations, thus making visibility/identification of intestinal protozoa very strenuous. Therefore, there was need to open the iris of the microscope a little bit in order to allow light to penetrate the wet preparation. Intestinal parasites like the ova of *Ascaris lumbricoides*, ova of hookworm, *Trichuris trichuria*, and larvae of *Strongyloides stercoralis* were less evenly stained by these (higher concentrations of the extracts).

Therefore since lower concentrations of the extracts, (0.03g/ml, 0.02g/ml and 0.01g/ml) gave better staining results, it is recommended that for diagnostic purposes that these dyes should be produced commercially in these concentrations.

Wet preparation of faecal materials gave clear differentiation of some of the intestinal parasites which were clearly identified. The embryo and ova of the eggs of worms were clearly stained at lower concentrations. The *Hibiscus sabdariffa* undiluted stock dye stuff gave an uneven staining of *Trichuris trichura* and *A. lumbricoides*. Wet preparation of *Trichuris trichuria* under saline and iodine reveals it as yellow-brown and barrel shaped with a colourless protruding mucoid plug at each end. It also contains a central granular mass which is the unsegmented ovum. The walls and ova absorbed the dye extracts and were stained alike being pinkish in colour.

Lower concentration stained outer covering pink while the granular mass retained its yellow-brown colour. This could be explained by the fact that the dye was able to penetrate the albuminous coat of the ova by the increased permeability of the ethanol used in its extraction. This further supports the view that alcohol not only gives a good dye extraction but also a good staining result. *Strongyloides stercoralis* larva was also evenly stained by the diluted extract. The walls and the rhabditiform large bulbed oesophagus were dyed pink; at higher concentrations but in lower concentrations only the rhabditiform large bulbed oesophagus dyed pink enabling clear differentiation and identification. The difference between the staining abilities of the high and lower concentrations could be explained by the fact that decreases in the concentration of the dye increases its permeability. There was no alteration in the structural morphology of the dyed materials. It is therefore apparent that *Hibiscus sabdariffa* can be used in place of synthetic dyes for diagnostic purposes.

Furthermore, *Azadirachta indica* dye extracts produced the same colour characteristics when used in staining wet preparation. The results compared favourably with the control (the faecal samples stained with iodine). Intestinal parasites were clearly identified and its structural morphology was not altered by the stain. As with *Hibiscus sabdariffa* higher concentration of 0.18g/ml, 0.07g/ml, and 0.09g/ml made visibility difficult such that the ova of worms were intensely stained and difficult to identify. Such applied to the ova of *Ascaris lumbricoides*, ova of hookworm and *Trichuris trichura*. Okolie (2006) had observed in his work using other plant extracts including *Azadirachta indica* that the extracts at high concentrations tended to clump the faecal materials and trapped the organisms in the clump, thus rendering visibility virtually impossible. Intestinal parasites were best stained at 0.03g/ml and 0.02g/ml. At such concentrations, the walls of *Ascaris lumbricoides* absorbed the dye and were stained grey to pale green while the granular mass retained its yellow-brown colour. This was in contrast to the conventional saline and iodine wet preparations which revealed *A. lumbricoides* as a yellow-brown oval shell with a central granular mass. The ovum of *Trichuris trichuria* was evenly stained but not as intense as with lower concentrations of the dye. It was rather stained light enough to be identified.

In conclusion, *H. sabdariffa* and *A. indica* (at concentrations 0.03g/ml and 0.02g/ml) produced satisfactory results in comparison to conventional methods used in identifying

intestinal parasites on a wet preparation of faecal sample. It is therefore recommended that they be produced in commercial quantities, at the specified concentrations and be used for diagnostic purposes in biology and medicine. It is recommended that more research be carried out on production of dried concentrates of these dyes in order to improve on their quality. Medical laboratory science Council of Nigeria (MLSCN) should be involved in securing approval from the biological stain commission for the production of the dyes in commercial quantity.

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