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EVALUATING THE STAINING POTENTIAL OF DYE EXTRACTED FROM *BAPHIA NITIDA* (CAMWOOD) ON MALARIA PARASITE.

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ABSTRACT
This study examines the staining potential of aqueous and ethanolic extracts from *Baphia nitida* (indigenous herbaceous plant species) as a staining agent for malaria parasite. A total of 100 subjects were recruited for this study. 25 were apparently healthy subjects (controls) that had their blood films stained with the plant extract and the remaining 75 were subjects already diagnosed of malaria whose blood films were stained with the plant extract and Giemsa/Leishman stain (controls) respectively. The result obtained showed that dye extracted from *Baphia nitida* was unable to stain the malaria parasite. However, it was able to detect some of the blood cells such as red blood cells and white blood cells. It was noted that distilled aqueous hot extract with mordant gave the best staining result with 32% of the blood films stained with the above extract having an excellent staining quality, 16% being very good and 52% with a good staining quality. The distilled cold water extract (poor=60% and good=40%) stained much better than the ethanolic extract (poor=44%, good=52% and very good=4%). Generally, dye extract from *Baphia nitida* gave a less contrasted appearance in comparison to the conventional stains. The relationship between the method of dye extraction used and the quality of stain obtained was extremely significant ($p<0.05$). This implies that the quality of stain obtained following staining is largely dependent on the method of dye extraction use.

KEYWORDS: *Biphia nitida, Malaria Parasite, Giemsa stain, Leishman stain.*

INTRODUCTION

1.1 BACKGROUND OF STUDY

Dark blue trophozoites (ring form), dark red chromatin, red schuffners' dot are all results derived from staining blood film with conventional laboratory stains such as Leishman or Giemsa stains using blood obtained from a malaria infected patient with certain colored chemical compounds. These chemical compounds are called dyes or rather, more scientifically 'stains'. A dye is an aromatic organic compound and is fundamentally based on the structure of benzene. To us, benzene appears to be a colorless fluid. However, it absorbs electromagnetic radiation just as dyes do, but it does so at about 200nm so that we don't see it (as wavelengths just outside the visible range are considered colorless). Dyes are generally applied in an aqueous solution and may require a mordant to improve the fastness of the dye on the fiber. They impact more or less permanent color to other materials. They are also described as colorants mixed in oil-like mineral spirits or in water as a carrier. The molecular size of the dye particle is so small that they allow light to pass through virtually unhindered. The use of dyes dates from centuries back. Its origin is speculated to have arisen accidentally as a result of staining from berries or other plant parts. The deliberate use of these plants to produce colors probably followed soon after.[1]

Malaria has remained a major public health problem in Nigeria with high morbidity and mortality. Five species can infect and be spread by humans.[2] These include *Plasmodium falciparum, P.ovale, P.vivax, P.malariae* and *P.knowlesi.* In Nigeria, 98% of the malaria cases are as a result of Plasmodium falciparum which is the most virulent and has the greatest propensity for developing resistance.[3] Due to its high prevalence, diagnosis of malaria is one of the most common conventional tests carried out in majority of the Nigerian laboratories. Diagnosis is by usually microscopy (examination of stained blood films using a microscope). Microscopy is the most commonly used method to detect the malarial parasite—about 165 million films were examined for malaria in 2010.[4] It continues to be the gold standard for diagnosis. Nigeria like some other developing countries depend so much on products manufactured by developed countries and thus is branded a consumer country. The stains used in most Nigerian laboratories are purchased

from other countries hence the need for a new dye product that is simple easy to use and more importantly available locally.

Isolating herbal dye from extracts of leaves, flower, bark, root, and other parts of some ornamental plant species mostly involve powdering, mixing with other materials, boiling in water, and dissolving in inorganic or organic solvent.[5] Until the late nineteenth century, the natural dyes extracted from plants, animals and minerals were used as primary staining material in the textile, paint, and other industries. However, with the pace of development of synthetic dyes as it is cheaper, brighter, more color fast and easy to use, the use of natural dyes have decreased.[6] As the public becomes aware of ecological and environmental problems related with the synthetic dyes the use of natural dyes has once again gained interest.[7] Natural dyes can stand as a much-needed alternative to the complex world of chemical dyes.[8] Although methods of preparation of herbal dyes are more complex than the commercial dyes, but their staining quality and stability are far better than the synthetic dyes. Vibrant colors can be produced from natural dyes by mixing them with each other in different proportion. Furthermore, these natural dyes could consequently provide a new economy source to the country.

The tropical countries of which Nigeria is inclusive have a greater diversity of dye producing plants these include *Baphia nitida* amongst others. *Baphia nitida* is also known as camwood, barwood or African sandlewood. *Baphia nitida* Lodd is belonging to Fabaceae family commonly known as camwood.[9] The heart wood is pale brown when fresh and turning rapidly to dark red or orange upon exposure. The heart wood and roots of this plant yields a red dye (camwood dye). The shavings of the dried heartwood are ground into fine powder to obtain extract. The extract is more soluble in alcohol and alkali but much less soluble in water. It is an irony that with all its apt staining potential, not much is generally known about dye extract from *Baphia nitida* as a biological stain. The ensuing work is aimed at attempting the use of dye extract from the plant *Baphia nitida* as a biological stain for the malaria parasite and white blood cells.

SUBJECTS, MATERIALS AND METHODS

3.0 STUDY AREA

This study was conducted at the Nnamdi Azikiwe University Teaching Hospital (NAUTH). This hospital is a secondary healthcare facility serving many smaller towns and villages both in Anambra and neighbouring States. Nnamdi Azikiwe University College of Health Sciences Okofia, Nnewi Campus (NAU-CHS), Wave Diagnostic Laboratory and life Diagnostic and Research Laboratory all located in Nnewi-North Local government Area, Anambra State. Nnewi is located in the south eastern part of Nigeria and the second largest city in Anambra State. Geographically, Nnewi falls within the tropical rain forest region of Nigeria with a longitude of 6^0 1^1 N and a latitude of $6^0 55^1$E.

3.1 STUDY DESIGN

This research is a cross-sectional study involving 100 subjects categorized into three groups viz;
GROUP I — 75 Malaria parasite infected subjects whose blood film was stained with the plant extract.
GROUP II — 75 Malaria parasite infected subjects as mentioned above whose blood film was stained with Giemsa and Leishman stain (controls).
GROUP III— 25 Normal healthy subjects whose blood film was stained with the plant extract (controls).

3.2 STUDY POPULATION

A total number of one hundred (100) subjects were randomly selected for the study. This includes seventy-five (75) malaria positive subjects that served as both test and controls. In addition, twenty-five (25) apparently healthy subjects that also served as controls.

3.3 SAMPLING SIZE AND TECHNIQUE

A total of one hundred subjects were recruited for this study by simple random sampling technique from the sample population in the study area.

The sample size was calculated using the using the Taro Yamane formula

$$n = \frac{N}{1 + N(e)}$$

Where,
n= sample size
N= population size
e= acceptable sampling error
1= unity (constant)
n= 100

3.4 INCLUSION CRITERIA

- The subjects selected include patients who have already been diagnosed of malaria.
- Controls consist of the above named subjects along with apparently healthy subjects (also controls).

3.5 EXCLUSION CRITERIA

- For the purpose of the research and to ensure authenticity of the result, the test subjects who were negative to the malaria parasite test were excluded
- Control subjects who had malaria were equally excluded.

3.6 COLLECTION OF BLOOD SAMPLE

- About two (2) ml or more of blood from subjects already diagnosed of malaria previously collected into ethylene diamine tetracetic acid containers were obtained from the Haematology unit of the Nnamdi Azikiwe University Teaching Hospital (NAUTH) and Wave Diagnostic Laboratory.

- About two (2ml) or less in some subjects of venous blood was collected asceptically from the subjects (controls) using standard vene puncture technique

3.7 COLLECION OF PLANT MATERIAL

The finely ground powder of the heart wood of *Baphia nitida* was purchased from retail outlets of the Ogbette main market, Enugu State.

3.8 MATERIALS

- Labtech Centrifuge
- Slides
- Microscope
- Cotton wool
- Bunsen burner
- Giemsa and Leishman staining solution

3.9 ETHICAL CONSIDERATION

The ethical approval for this research was applied for from the Faculty of Health Sciences and Technology, College of Health Sciences, Nnamdi Azikiwe University.

3.10 METHODS OF SAMPLE ANALYSIS

DYE EXTRACTION

The finely ground powder of *Baphia nitida* were used to prepare cold water, hot water and ethanol extracts as follows:

1. Distilled hot water extraction

Twenty gram (20g) of the shavings from the plant heart wood was weighed and mixed with 100ml of distilled water then heated (boiled) for some minutes. For this, 25g of potassium alum was added to the 20g of the shavings from the heart wood (serving as a mordant) before dissolving in the 100ml of distilled water then boiled.

Distilled cold water extraction

Twenty gram (20g) of the shavings from the plant heart wood was also weighed then mixed with 100ml of cold distilled water. This is similar to the above method with the exception that heat was applied to the hot distilled water extraction method.

Ethanol extraction method

Equivalent amount (20g) of the plant material will be soaked in 140ml of 80% ethanol (80ml of absolute alcohol and 20ml of water). This was heated for some minutes using a bunsen burner then used for staining.

PURIFICATION OF EXTRACT

Following dye extraction, the filtrate was spun at 3000rpm for 15 minutes (for the cold extraction method) using a centrifuge. The supernatant obtained was dispensed into reagent bottles and labeled accordingly.

BLOOD FILM STAINING

Giemsa Staining

Principle of test

This is a classic blood film stain for peripheral blood smear. The Giemsa solution consists of methylene blue (azure B) a basic dye, which when treated with an alkali forms methylene azure, that stains the acidic component of the malaria parasites and eosin an acidic dye directed against the basic parasite components. The Giemsa stain is usually used to stain thick films.

Procedure

1. The blood film was fixed with methanol for 3 minutes.
2. The slide was flooded with the stain and allow for 15 minutes.
3. The slide was washed and differentiated with the buffer solution.
4. It was drained of excess stain and allowed to air dry.

Leishman staining.

Procedure

1. The blood film was covered with Leishman stain for 3 minutes
2. The stain was diluted with twice the equal volume of the buffer solution then allowed to stain for 15 minutes
3. The slide was drained of excess water then, allowed to air dry

Staining using plant extract

Procedure

1. For the thin and thick film, the slide was flooded with the different staining solutions prepared from the plant extract.
2. This was allowed to stand for 15 minutes
3. Then washed with water
4. The back of the slide was wiped clean and allowed to air dry

MICROSCOPIC EXAMINATION

Procedure

1. Sufficient amount of immersion oil was placed on the well-made blood film.
2. The blood films were placed on the slide holder.
3. With the condenser sufficiently closed, the blood film was examined using ×40 objective lens and ×100 objective lens with the condenser iris fully closed.

STATISTICAL ANALYSIS

Analysis was done using the Statistical Package for Social Sciences (SPSS) version 16 and 20 Comparison of the various parameters gotten from the different groups was done with Pearson's chi-square test. The level of significance was set at <0.05.

In this study, one hundred subjects were employed and divided into three groups with seventy-five test and control subjects whose blood films were stained with the

plant extract and Giemsa/Leishman staining solution respectively as well as twenty-five subjects whose blood films were stained with the plant extract only. The presence of malaria parasite in blood films stained by the plant extract, together with the background color and the quality of blood films stained with the plant extract were evaluated. The results were analysed using the Statistical Package for Social Sciences (SPPS) version 16 and 20. The Chi-Square test and P-value were determined and $p<0.05$ was considered significant.

RESULT

In the course of the study, it was observed that dye extracted from *Baphia nitida* was unable to detect the malaria parasite present in the blood of test subjects. The quality of staining obtained by using dye extracted from *Baphia nitida* on blood films were grouped into four grades: poor, good, very good and excellent. This was based on their ability to stain the background of the blood film distinctly (Fig. 1 and 2), its capacity to stain and hence distinguish a large proportion of the red blood cells as well as its ability to detect the white blood cells.

Table I: Distribution of the staining quality of blood films stained using the distilled cold water extraction method.

Staining quality	Frequency	Percentage (%)
Poor	15	60.0
Good	10	40.0
Total	25	100

From the table above, most of the blood films stained poorly (60%) while the remaining were considerably good (40%).

Table II: Distribution of the staining quality of blood films stained using the distilled hot water extraction method with mordant.

Staining quality	Frequency	Percentage (%)
Good	13	52.0
Very good	4	16.0
Excellent	8	32.0
Total	25	100

From the table above, a large number of the blood films had a good staining quality (52%), followed by an excellent (32%) and very good (16%) staining quality.

Table III: Distribution of the staining quality of blood films stained using the ethanol extraction method.

Staining quality	Frequency	Percentage (%)
Poor	11	44.0
Good	13	52.0
Very good	1	4.0
Total	25	100

This shows that while no malaria parasite (*Plasmodium spp*) was detected by the plant extract, the blood films had an orange background with staining qualities ranges from poor, good to very good.

Table IV: Distribution of malaria among subjects.

	Frequency	Percentage (%)
1. Positive	75	75
2. Negative	25	25
Total	100	100

The above table indicates that out of a total of one hundred (100) subjects that participated in the study, seventy-five (75) have already been diagnosed of malaria (malaria positive) while twenty-five (25) were healthy (malaria negative).

Table V: Frequency distribution of method used for staining blood films with dye extracted from *baphia nitida*.

Method	Frequency	Percentage (%)
1. Distilled cold water	32	32.0
Extraction		
2. Distilled Hot water	37	37.0
Extraction		
3. Ethanol extraction	31	31.0
Total	100	100

This table shows that a total of one hundred blood films were stained with plant extract out of which thirty-two were stained using the distilled cold water extraction method, thirty-seven with the distilled hot water extraction method to which a mordant was added and thirty- one were stained with the ethanol extraction method.

Table V: Frequency distribution of the staining quality obtained from staining blood films with dye extracted from *baphia nitida.*

Staining quality	Frequency	Percentage (%)
Poor	28	28
Good	59	59
Very good	5	5
Excellent	8	8
Total	100	100

This indicates that a large number of blood films stained with the plant extract had a good staining quality (59%), this was closely followed up by 28% that stained poorly, 8% that had an excellent staining quality and 5% whose staining quality were very good.

Table VI: The relationship between methods of dye extraction used and quality of stain obtained

Staining quality	Distilled cold water extraction Method	Distilled hot water extraction with mordant Method	Ethanol extraction method	p-value
Poor	14	0	14	
Good	18	25	16	
Very good	0	4	1	0.000
Excellent	0	8	0	
Total	32	37	31	

Pearson chi-square testing revealed a significant relationship between the method of extraction used and the resultant staining quality ($p<0.05$).

Table VII: Outline of result obtained from staining blood films with extract from *baphia nitida* and giemsa/leishman staining solution (malaria parasite detection).

Result	*Baphia nitida*	Giemsa/lieshman
Negative	100	25
Positive	0	75

The table above shows that dye extracted from *Baphia nitida* could not detect the malaria parasite present in the blood of test subjects in contrast to the conventional Giemsa/Leishman staining solution that was able to detect the parasite.

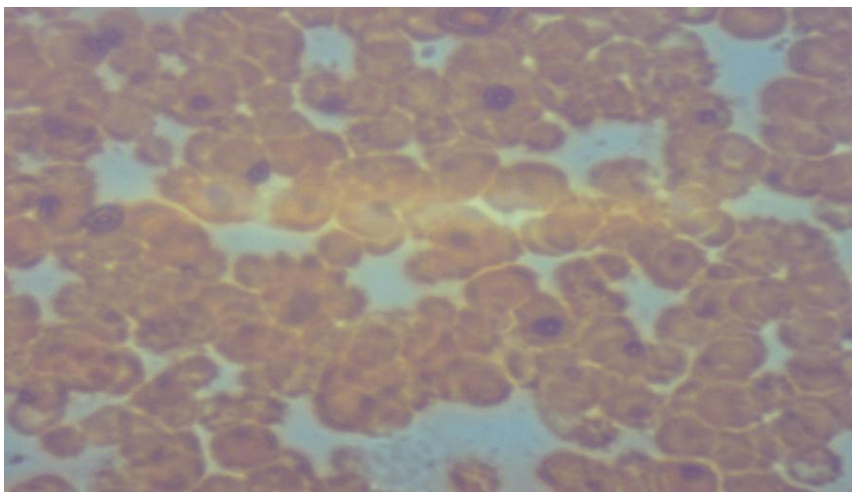

Figure I: Red and white blood cells stained with dye extracted from *Baphia nitida*.

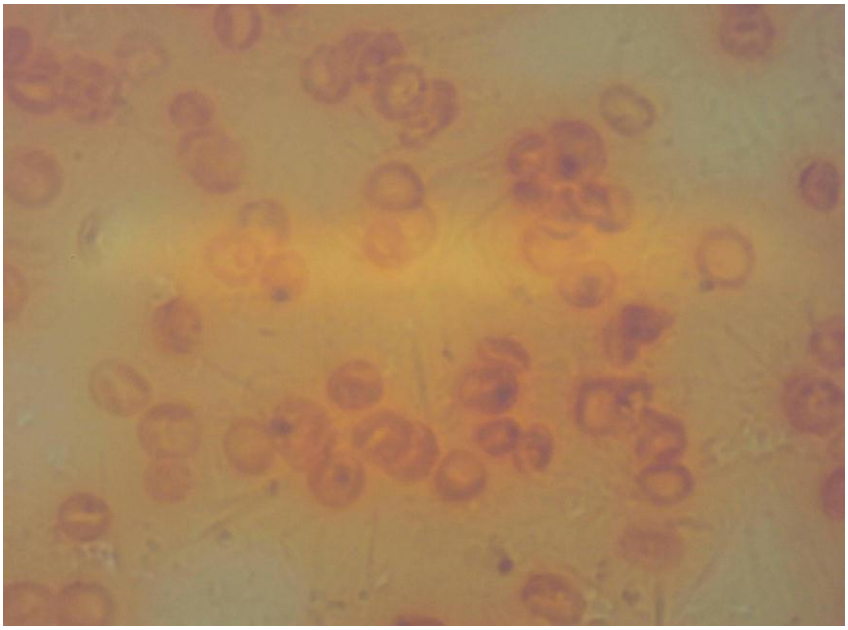

Figure II: Red blood cells stained with dye extracted from *Baphia nitida*.

DISCUSSION, CONCLUSION AND RECOMMENDATION

DISCUSSION

Several research papers have discussed the therapeutic benefits and aesthetic uses of dye extracted from *Baphia nitida.* On the contrary, few researchers have tried to apply extracts of the plant in diagnostic medical laboratory procedures. Apart from the work Endeshaw *et al*[10] as well as other works carried out anonymously, not much is known on the previous studies carried out to determine staining of parasites with the extract of the above mentioned plant. Affinity is the result of attractive forces between the dye molecule and molecules within the tissue. Dyes have a greater affinity for tissue molecules than solvent molecules. The affinity of dyes for tissue elements is affected by a number of factors such as structure, shape, and charge distribution of the dye molecule and the solvent characteristics.[11]

In this study, the extract from *Baphia nitida* imparted an orange colour to the background of the blood film as well as to the red blood cells with the staining intensity varying with the method of dye extraction used. The white cells also stained orange. However, the dye wasn't able to differentiate between the various types of white blood cells (i.e. neutrophils, eosinophils, lymphocytes etc.) while giving the granules of the white blood cells a purple colour. This could be as a result of the fact that they are enriched with many pigment components like the principle dyeing substances: isoflavonoid dimers santalins A and B and santarubins A, B and C. They as well, contain several other compounds such as baphic acid, baphin, deoxysantarubin, homopterocarpin, maackiain, pterocarpin and santal which all contribute to the coloring properties.[11]

Distilled water (cold and hot with mordant) as well as alcohol extracts of *Baphia nitida* were unable to stain the malaria parasite. In contrast, in the blood films of the controls, the malaria parasite appeared as delicate ring forms with a characteristic purple colour. Their inability to detect the malaria parasite could be as a result of lack of addition of certain constituents of the normal Romanowsky stain such as glycerol, acetic acid etc. which has been known to enhance stain uptake. According to staining theory, acidic structures are stained by basic dyes while basic structures are stained by acidic dyes.[11] The stains treated with acid and bases were reported to improve staining potentials for moulds[12] and this can be applicable to other microbes. In addition, inadequate pH buffering during the staining process could have also affected the dyes' staining action as the ability to stain specific tissue structures is determined by the pH values of stain.[13]

The dye was observed to be more soluble in cold distilled water, less soluble in ethanol and much less in hot distilled water with mordant (potassium alum) added it. This contradicts the information obtained from a similar work which indicated that the dye stuffs in *Baphia nitida* were very soluble in alcohol, moderately soluble in boiling water (without addition of a mordant) and slightly soluble in cold water.

The study recorded a highly significant relationship between the method of dye extraction used and the staining quality obtained ($p<0.05$). The distilled hot water extract with mordant provided the best staining quality out of all the extracts used. The distilled cold water extract stained the red blood cells quite effectively in contrast with the ethanol extract which stained the blood cells least. This is in contradiction with Worall[14] who in his work listed ethanol as an ideal extract as it can extract maximum dyes soluble in organic solvents, being volatile in nature highly permeable to the tissues/tegumental coat of parasites and an important

anti-microbial agent. In the study carried out by kumar, he noted that the staining quality observed while using aqueous and alcoholic extract of sugar beet (*Beta vulgaris*), China rose (*Hibiscus rosa-sinensis*) and red rose (*Rosa hybrida*) does not differ significantly as per extraction methods adopted for the extraction of the colorant from the plant materials.[15] The outstanding staining quality observed while staining using the distilled hot water extract with mordant can be attributed to the addition of a mordant (potassium alum). Mordants are hydroxides or salts of divalent or trivalent metals, which form salt bridges between the tissue and dye. Without a mordant, a staining reaction cannot take place in certain dyes where there is no direct union between dye molecules and tissue.[11] Mordants effect their action by attachment of the mordant metal to the tissue by covalent and coordinate bond formation[16] causing the dye to produce a much better staining quality. There is a strong influence on color strength of many different natural dyes by the nature, type and concentration of different mordants.

5.1 CONCLUSION

The local plant *Baphia nitida* was selected and its aqueous and ethanolic extracts were used as an eco-friendly and cost effective biological stain to determine its ability to serve as a suitable alternative to the conventional stains Giemsa/Leishman stain. This study concludes that dye extract from *Baphia nitida* (cold or hot aqueous and ethanol extracts) possess the potential to be used as a tool for diagnosis in the laboratory provided that further studies be carried out and other constituents such as acetic acid, glycerol etc. be added to it to enhance the staining quality of the dye with the distilled hot water extraction method with mordant being a much appropriate substitute.

5.2 RECOMMENDATION

Baphia nitida is a cheap natural efficient staining dye. This plant species is readily available and affordable at low market price. The use of extracts from *Baphia nitida* as staining agent will reduce the problems associated with over-dependence on toxic, expensive and scarcely available exotic stains and could mark the end of an era of dependence on foreign biological stains. Further researches should be conducted on the nature of the tissues-molecule reactions of active chemical substances in the dye extracts. There is also a need to investigate the potential use extracts in the staining of other parasites. Other works should also be carried out on the shelf-life of the stain in order to boost its production to commercial scale. However, larger studies with more details in pH buffering and more chemical constituents to be added to improve its staining quality are highly recommended. It is envisaged that enhanced studies aimed at determining the optimal concentration of the plant extracts and commercial production of the same could mark the beginning of self-reliance for Nigeria in areas of biological stain production.

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