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Research article

APPLICATION OF METHANOLIC EXTRACTS FROM *HISBISCUS SABDARIFFA* LINN AS A BIOLOGICAL STAINING AGENT FOR SOME FUNGAL SPECIES.

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ABSTRACT: The efficacy of methanolic extracts from Hibiscus sabdariffa, an indigenous herbaceous plant species for use as staining agent on three fungal species is reported in this paper. *Apergillus niger*, Rhizopus stolonifer and Penicillium notatum were stained using methanolic extracts from H. sabdariffa with the pH value of 2.7 which was mordanted with potassium alum and acidified with 65% acetic acid. These were compared microscopically with preparations using Lactophenol-in-cotton blue with a pH value of 3.6. H. sabdariffa preparations gave a more contrasted appearance as against preparations involving Lactophenol-in-cotton blue stain. The absorbance was determined at different wavelengths ranging from 400-640nm in which the absorbance of the extracts showed higher values than that of Lactophenol-in-cotton blue stain. Although, absorbance values were strongly correlated (r=0.776) the correlation was not significant at (df=5, p=0.01, r=0.875). The analysis of H. sabdariffa tissues used for the extracts indicated that moisture content was rather low (30.76%). The results of this study suggest that methanolic extracts from H. sabdariffa could be used as a mycological stain.
Key words: Methanolic extracts, Hibiscus sabdariffa, *Apergillus niger*, Rhizopus stolonifer and Penicillium notatum.

INTRODUCTION

Staining techniques originated from the second half of the last century and stains have been used to enhance accurate descriptions of the microscopic structure of tissues, which is necessary for histopathologic diagnosis. It is an auxiliary technique used in microscopy to enhance contrast in the microscopic image [12]. Plant and insect parts have found place in histological staining due to their colouring and dying effects. For instance, plants and insect parts used in histological staining as natural dyes are *Haematoxylon campechiaumn,* from which haematoxylin is obtained and *Dactylopius cacti,* from which carmine stain is obtained [7]. Although most of the dyes in current use in histopathology laboratories are of synthetic origin, natural dyes still hold promise as a potential source of cheaper dyes and consequently providing employment opportunities in developing countries. It is interesting to note that over 2000 dyes are synthesized from various parts of plants, of which only about 150 have been commercially exploited [8]. Among such plants is the *H. sabdariffa* Linn (Family**:** Malvaceae), which is the true roselle plant, a very important dye-yielding annual/perennial plant species. Apart from the use of the extract in biological staining, the extracts can also be used as a therapeutic, a laxative, an antihypertensive, and a cholesterol lowering medicine [3]. Also, it exhibits great antioxidant activity, lowers hepatoxicity, reduces fever, diuretic and antiscorbutic in action (11). In Nigeria, it has two main uses; i.e. as vegetable and for preparation of beverage called zobo drink. No report was known on the use of the plant extract in biological staining and particularly on the three species of fungi (*A.niger, R. stolonifer* and *P. notatum)*. Fungal stains remain an important tool in the histology laboratory as diagnostic methods for identifying infectious microorganisms. A fungus is a member of a large group of eukaryotic organisms that includes microorganisms such as yeasts and molds, as well as the more familiar mushrooms [9]. Fungi are important as agents of biodegradation and responsible for the majority of plant diseases and several diseases of animals (including humans). They are also used in industrial fermentation processes and in commercial production of many biochemical products, beneficial in agriculture, horticulture and forestry [13].

Although, many chemical dyes (stains) are applied not only to plants, animals and fungal tissues, but also bacterial cells, some of the stains are expensive, scarcely-available and hazardous to humans and the global environment. Therefore, the potential of methanolic extracts from *H. sabdariffa* as a staining agent for fungal species was investigated in this study.

MATERIALS AND METHODS

Methanolic Extract of *Hibiscus sabdariffa*

Methanolic extracts of *Hibiscus sabdariffa* were obtained from 320g of powdered calyces using the soxhlet extraction method (AOAC, 2000) in the Chemistry Department of Bingham University. The calyces were chopped into smaller pieces, dried at a temperature of 37°C for four weeks, and then ground to fine powder, using a blender and stored in an air-tight glass container prior to the extraction process [10]. The powdered calyces were soaked for 24 hours to allow for effective percolation of the extracting solvent prior to the extraction process. The soaked powder was then extracted in methanol (absolute) for 7 hours using Soxhlet Extractor. The extracts were then concentrated for 5 hours by transferring it to a rotary evaporator set at 3000 rev/min at 65°C (AOAC, 2000).

Quantitative proximate analysis of *Hibiscus sabdariffa*

Quantitative proximate analysis was carried out in order to determine the moisture content of the plant samples using standard methods (AOAC, 2000). The moisture content of 10 X 320g samples of the calyx of *H.sabdariffa* were dried in an oven set at 103˚C to a constant weight.

$$\%\text{Moisture content} = \frac{\text{Dry weight of sample}}{\text{Wet weight of the sample}} \times 100$$

Treatment of Cultures of *Aspergillus niger, Penicillium notatum* and *Rhizopus stolonifer*

Three fungal species, *A. niger, R. stolonifer* and *P. notatum* were cultured using standard techniques in the laboratory of the Biological Sciences Department, Bingham University using potato dextrose agar. The agar was prepared by weighing 3.9g of dehydrated potato dextrose agar which was mixed with 100 cm^3 of water and autoclaved at 121 degrees Celsius for 15minutes at 15 PSI. The agar was removed and cooled to 45 degrees Celsius and poured into sterile Petri-dishes and allowed to set [1]. The agar was inoculated by wiping a swab containing the culture across the surface of the agar. The inoculated Petri-dishes were incubated upside down at 25^0C for 5 days before growth were observed. This procedure was used for the 3 species of fungi *(A. niger, R. stolonifer* and *P. notatum)* [1].

Determination of pH and Spectrophotometry:

The pH of methanolic extracts and Lactophenol-in-cotton blue were determined using pH meter (model Cyber Scan 500), standardized with buffer solutions of 4.0 and 7.0 pH according to [4]. The absorbance of the methanolic extracts and Lactophenol-in-cotton blue stain were determined using the uv spectrophotometer. Methanol was used as blank for the dye from *H. sabdariffa,* while distilled water was used as blank for the Lactophenol-in-cotton blue. Each of the dye samples was run on UV/ visible spectrophotometer, which automatically subtracted the effect of the blank and plotted the graph of Absorbance (*A*) against wavelength [2]. The absorbance (*A*) values for both the methanolic extracts and the Lactophenol-in-cotton blue at the wavelengths of 400-640nm were correlated to observe the relationship:

$$r = \frac{n.\sum xy - \sum x.\sum y}{\sqrt{[n.\sum x^2 - (\sum x)^2][n.\sum xy^2 - (\sum x)^2]}}$$

Where:

r = correlation coefficient.
n = no of samples.
x = independent variable (Absorbance values of *H. sabdariffa* at wavelengths of 400-640nm).
y = dependent variable (Absorbance values of Lactophenol-in-cotton blue at wavelengths of 400-640nm).

Microscopy

A drop of the methanolic extracts mordanted with potassium alum and acidified with glacial acetic acid was placed on a clean glass slide. Using an inoculating needle, pieces of *A. niger, P. notatum* and *R. stolonifer* were picked and placed on the glass slide, teased, and a cover slip was gently added and the preparation was examined under the microscope. This same procedure was used in examining preparations in Lactophenol-in-cotton blue stain. Photomicrographs of the three species of the fungi were taken [2].

RESULTS AND DISCUSSION

The percentage yield of the methanolic extracts.

In Table1 below, the result of the yield of methanolic extracts from 320g weighed samples of *H. sabdariffa* calyces indicated a yield of 65.5%. This was calculated from the weight of samples after the extraction was made.

Table 1: The percentage yield of the extract from *H. sabdariffa*

Plant species	Weight of sample used (grams)	Weight of samples after extraction (grams)	Percentage yield (%)
H. sabdariffa	320	110.4	65.5

Absorbance rate of the methanolic extracts and Lactophenol in cotton blue stain.

The moisture content of *H. sabdariffa* extracts and the pH values of the extracts and Lactophenol in cotton blue are shown in table 2 below. Both are acid with 2.7 for *H. sabdariffa* and 3.6 for Lactophenol-in-cotton blue respectively. The moisture content of *H. sabdariffa* 30.76 per 320g weight.

Table 2: pH value and moisture content of *H. sabdariffa* extract and Lactophenol-in-cotton blue stain

Extracts/Stain	pH Value	Moisture content (320g)
H. sabdariffa	2.7	30.76 ± 0.15*
Lactophenol-in-cotton blue	3.6	-

*Moisture content value is by means of triplicate determination ±standard deviation (SD).

In Table 3 & Fig 1 below, the result showed that, the highest value of absorbance rate (0.945*A*) was observed for methanolic extracts and 0.329*A* for lactophenol cotton blue at the wavelength of 400nm. The lowest absorbance values of 0.107*A* and 0.121*A* were observed at the wave length of 640nm in Lactophenol-in-cotton blue and the extracts respectively. Also, as illustrated in Figure 1, at the wave length of 640nm the absorbance spectra for each of the stains tends towards equality (wavelength: 640nm, absorbance: 0.121A and 0.107A for for *H.sabdariffa* and Lactophenol-in-cotton blue respectively).

Table 3: Absorbance rate of *H. sabdarifffa* extracts and Lactophenol-in-cotton blue.

Wavelength (nm)	ABSORBANCE(*A*)	
	H.sabdariffa	**Lactophenol-in-cotton blue stain**
400	0.945	0.329
440	0.902	0.204
480	0.190	0.146
520	0.163	0.143
560	0.143	0.132
600	0.133	0.110
640	0.121	0.107

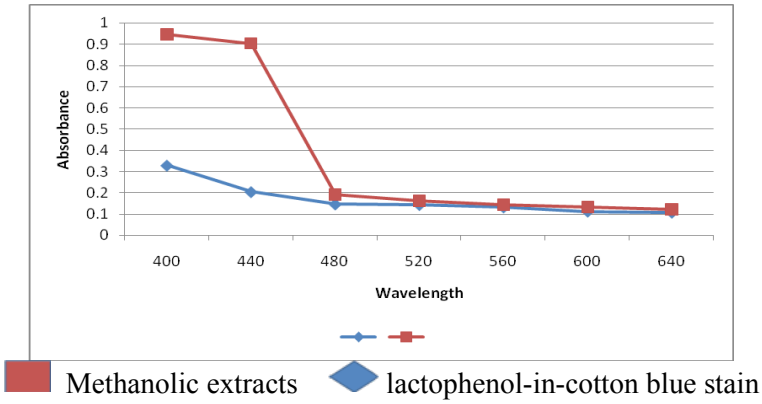

Figure 1: Absorbance rate of *H.sabdarifffa* extract and Lactophenol-in-cotton blue.

Photomicrographs of the stained *A. niger, P. notatum* and *R. stolonifer* using the methanolic extracts and Lactophenol-in-cotton blue.

In Fig. 2 below, the result shows the comparative photomicrographs of stained *A. niger, P. notatum* and *R. stolonifer* using the methanolic extracts and lactophenol in cotton blue. The 3 species of fungi stained with *H.sabdariffa* dye extract show better staining than the lactophenol-in-cotton blue. *R.stolonifer* stained with the extracts showed clearer view of the organelles better than the sample stained with Lactophenol-in-cotton blue. *A. niger* and *P. notatum* stained with the methanolic extract shows clearly the vesicle, sporangiosphores and sporangia better than the Lactophenol-in-cotton blue which show poor contrasts of the organelles.

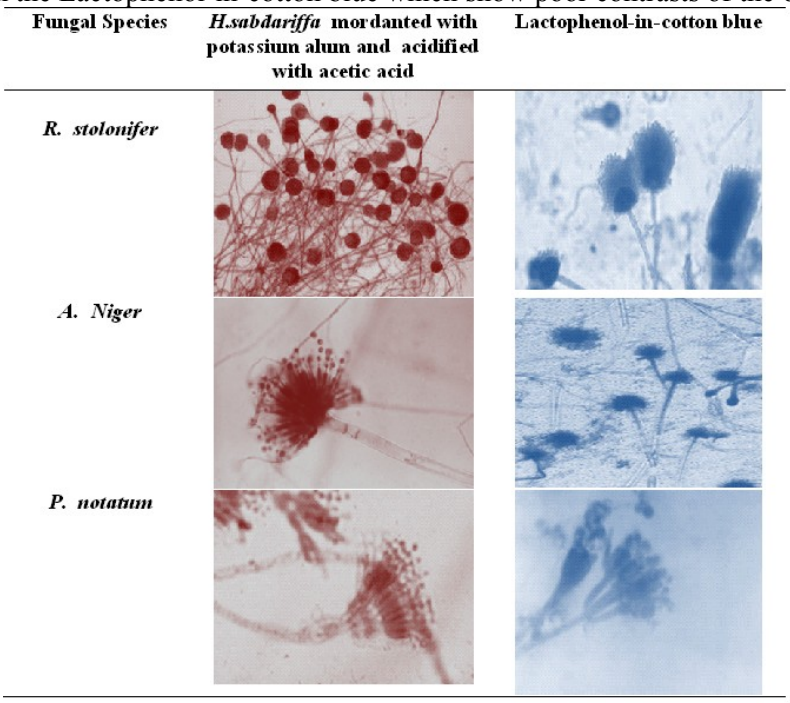

Fig 2: Comparative photomicrographs of the stained *A. niger, P. notatum* and *R. stolonifer* using methanolic extracts of *H.sabdariffa* and Lactophenol-in-cotton blue

The combined properties of good yield of *H. Sabdariffa* extracts and intense colouration of the cells and tissues of the three fungi used in this study indicate that its use as a mycological staining agent could be acceptable. The pH values of the methanolic extracts and Lactophenol-in-cotton blue are both acidic with 2.7 for the methanolic extracts and 3.6 for Lactophenol-in-cotton blue (Table 2 above). As demonstrated by (6), the ability of a dye to stain specific tissue structures is determined by the pH of the stain. Acidic structures are stained by basic dyes while basic structures are stained by acidic dyes. This shows why the methanolic extracts and Lactophenol-in-cotton blue was able to stain appropriately the 3 species of fungi.

The absorbance spectra of the methanolic extracts and Lactophenol-in-cotton blue stain revealed a range of wavelengths of absorption from 400nm to 640 nm. The result obtained showed that the highest value of absorbance rate of 0.945*A* was observed for methanolic extracts and 0.329*A* for Lactophenol- in-cotton blue at the wavelength of 400nm. The lowest absorbance values of 0.107*A* and 0.121*A* were observed at the wave length of 640nm for Lactophenol-in-cotton blue and the methanolic extracts respectively (Fig.1). The absorbance values were correlated (r=0.776), but the correlation was not significant at (*df*=5, *p*=0.01 and r=0.875).

This falls within the visible region of the electromagnetic spectrum and shows the presence of colour imparting chromophores in the dye extracts (2). Lactophenol-in-cotton blue stain shows low colour impartation due to its low absorbance compared with dye extract from *H.sabdariffa* which shows higher absorbance. This was also observed in *B.orellana*, *C. domestic* and *L. cyanesce* by (2).

The photomicrographs of stained *A. niger, P. notatum* and *R. stolonifer* using the methanolic extracts from *H.sabdariffa* showed more contrast than those in Lactophenol-in-cotton blue (Fig 2). Tissue of *R. stolonifer* stained with the methanolic extracts showed clearer components than those stained with Lactophenol-in-cotton blue. *A. niger* and *P. notatum* stained with the methanolic extracts showed clearly the sporangiophores and sporangia better than preparations in Lactophenol-in-cotton blue.

CONCLUSION

The result of this study show strong evidence that methanolic extracts from *H.sabdariffa* could be used as a staining agent for *A. niger, P. notatum* and *R. stolonifer*. Methanolic extracts from *H. sabdariffa* could be a good replacement for Lactophenol in cotton blue stain because, this plant species is available at affordable low market price. The use of methanolic extracts from *H.sabdariffa* as staining agent reduces the problems associated with over-dependence on toxic, expensive and scarcely available exotic stains. Further research could 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 methanolic extracts in the staining of other microorganisms. Work should also be carried out on the shelf-life of the stain in order to boost its production to commercial scale.

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