



## SEPARATION OF ANTIOXIDANT COMPOUNDS FROM THE STEM BARK EXTRACTS OF *HAEMATOSTAPHIS BARTERI* HOOK F. (ANACARDIACEAE) USING CHROMATOGRAPHIC TECHNIQUES

<sup>\*1</sup>Ezekiel, J. S., <sup>2</sup>Adamu H. M., <sup>2</sup>Chindo I. Y., <sup>2</sup>Garba I. H., <sup>3</sup>Tadzia K.

<sup>1</sup>Umar Suleiman College of Education, Gashua, Yobe State

<sup>2</sup>Abubakar Tafawa Balewa University, Bauchi

<sup>3</sup>Army University Biu, Boro State

\*Corresponding authors' email: [nehemiaeze@gmail.com](mailto:nehemiaeze@gmail.com)

### ABSTRACT

In the search for plants with antioxidant properties, *Haematostaphis barteri* Hook f. (Anacardiaceae) was found to be a plant often used in Nigeria and other nations for the management of cancer, anemia, haemorrhoid and many other ailments. The ethyl acetate and acetone extracts of the stem bark in previous research by the same author were assayed for phenolic compounds and the phenolic compounds were found to possess antioxidant properties when TLC chromatograms were stained with iodine vapour and several spots were revealed. The ethyl acetate and acetone fractions were further chromatographed on TLC with several solvent systems and the mixtures from toluene, ethyl acetate and formic acid (TEF) in the ratio of 4/2.5/3.5 and 3/5/2 and n-butanol, acetic acid and water (BAW) in the ratio of 12/4/6 gave better resolutions. When chromatograms were obtained and developed with a solution of p-anisaldehyde in concentrated sulphuric acid, different spots were visualized with different colours signifying the presence of different bioactive metabolites. The Presence of antioxidant compounds was determined by bioautography TLC chromatograms were stained with a solution of 0.2% DPPH (2, 2-Diphenyl-1-picrylhydrazyl) solution in methanol and dried with heat gun and the appearance of yellow spots on purple background signifies the presence of antioxidants. A selected spot with an R<sub>f</sub> value of 0.31 was monitored and isolated from column chromatography. The purified bioactive metabolite was crystallized, weighed and saved for further analysis.

**Keywords:** cancer, ethyl acetate extract, chromatography, bioautography

### INTRODUCTION

*Haematostaphis barteri* Hook f. (Anacardiaceae) is a tropical plant found most often in savannah forests around mountains and creeks and, the stem bark has been used by traditional healers in northern Nigeria for the management of ailments such as cancer (Kubmarawa *et al.*, 2007), stomach ache, and vomiting (Rabo and Sanusi, 2001), anemia and hemorrhoid and tumor (personal interview with Kalamu, a noted community leader). Different ethnic groups in Ghana use the leaves and bark infusion of *H. barteri* in the treatment of malaria, hepatitis and sleeping sickness (Boampong *et al.*, 2015). Similarly, Wurochekke *et al.* (2014) reported the use of stem bark extract of the same plant in the management of trypanosomiasis.

The water extract of the stem bark of *Haematostaphis barteri* Hook f. has been reported for its use as traditional medicine and, the ethyl acetate fraction of the extract was found to contain some phytochemicals which showed antioxidant properties (Ezekiel *et al.* 2015). In the current study, chromatography was employed to separate secondary metabolites that were isolated from *H. barteri* using ethyl acetate solvent and which earlier showed the presence of the substantial amount of phenolic compounds from phytochemical assay and antioxidant properties as reported in some relevant literature and Ezekiel *et al.*, (2015).

Among the several types of chromatography methods, Thin Layer Chromatography (TLC) and Column Chromatography (CC) were utilized in the separation and purification of the antioxidant compounds from the stem bark extracts of *H. barteri*. The choice of these methods was determined by their simplicity, high capacity and low cost of the adsorbents such as silica gel (Zhang *et al.*, 2018). Silica gel is the most widely used adsorbent in phytochemical analysis. TLC investigation revealed the phytochemicals present in the Ethyl acetate extract of the stem bark of *H. barteri* by noting the number of

various spots on the TLC plates. Purification and separation of antioxidant compounds from the ethyl acetate fractions of *H. barteri* was facilitated by bioassay-guided analytical procedures; the bioautographic thin layer chromatography and finally by column separation.

### MATERIALS AND METHODS

#### Purification of Plant Extracts

Ethyl acetate (EA) extract of *H. barteri* that tested positive for antioxidant activity as reported from literature (Ezekiel *et al.*, 2015) was obtained and subjected to purification processes by first performing solvent/solvent fractionation in a separation flask. The fraction from the solvent/solvent fractionation with ethyl acetate gave EA/AC and that from acetone gave AC/EA (acetone/ ethyl acetate fraction) which among other solvents and solvent fractions previously tested positive for antioxidant activity using bioautography with 0.2% DPPH solution. The extract solutions were prepared for chromatographic separation.

#### Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) analysis was conducted on pre-coated TLC plates (silica gel 60 F<sup>254</sup>). Ethyl Acetate fractions only were chromatographed because they showed substantial antioxidant properties which crystallize well. Different solvent systems were tried for the optimization of TLC elution to separate the different constituents present in the ethyl acetate fractions. Some of the TLC chromatograms that showed good resolutions were chromatographed with a solvent system made from the mixture of toluene, ethyl acetate and formic acid (TEF) in the ratio of 4/2.5/3.5 and 3/5/2 and n-Butanol, acetic acid and water (BAW) in the ratio 12/4/6. These eluents were previously termed phenolic eluents because it was previously found to be the choicest

eluent for phenolic compounds (Wojciak-Kosior and Oniszczyk, 2008; Schellenberg and Kabrodt, 2015).

After elution with various eluents (TEF: 4/2.5/3.5 and 3/5/2 and BAW: 12/4/6), the developed TLC chromatograms were observed for spots using iodine vapour and P-anisaldehyde in concentrated sulphuric acid as staining agents. After the chromatograms were evaporated in a hot air oven, and when they were saturated in iodine vapor they all appeared as yellow spots. But when a solution of p-anisaldehyde in concentrated sulphuric acid was used as a staining agent by spraying, the chromatograms subsequently dried using heat gun, and different coloured spots varying from violet, red and blue showed up signifying the presence of various phytochemical components (Reach Devices, USA, 2015).

#### Bioautography Determination of Antioxidant Compounds

The TLC process above was replicated to obtain more chromatograms. In bioautography, the chromatograms were stained with 0.2% DPPH solution in methanol. When the chromatograms were dried using heat gun a characteristic yellowish spots on a purple coloured background indicated the presence of antioxidant compounds (Wang *et al.*, 2012).

#### Column Chromatography (CC)

The ethyl acetate fractions after TLC analysis to determine the types and number of various phytochemicals in each fraction were subjected to column chromatography for purification and separation. A column was loaded with silica gel (Loba chemie 60-120 mesh) using toluene as packing solvent. After column separation by gradient elution using TEF (4/2.5/3.5 and 3/5/2 and BAW (12/4/6) as mobile phase, the various column fractions thus obtained were collected in several 100 ml beakers.

Several fractions were collected from each of the column chromatographic separation and were concentrated in a hot air oven at 60°C after which further analysis by TLC was performed to select a metabolite with an R<sub>f</sub> value of 0.31, that is the particular group of metabolite with similar R<sub>f</sub> values were pooled, crystallized and saved for further analysis.

#### RESULTS AND DISCUSSION

The result of the research was presented in tables and Figures as seen below. Ethyl acetate extract fractions of *Haematostaphis barteri* stem bark were chromatographed on pre-coated TLC plates (Loba chemie 60 F<sup>254</sup>). The chromatograms gave various spots (Figures 1 and 2) with various R<sub>f</sub> values as presented on Table 2 below.

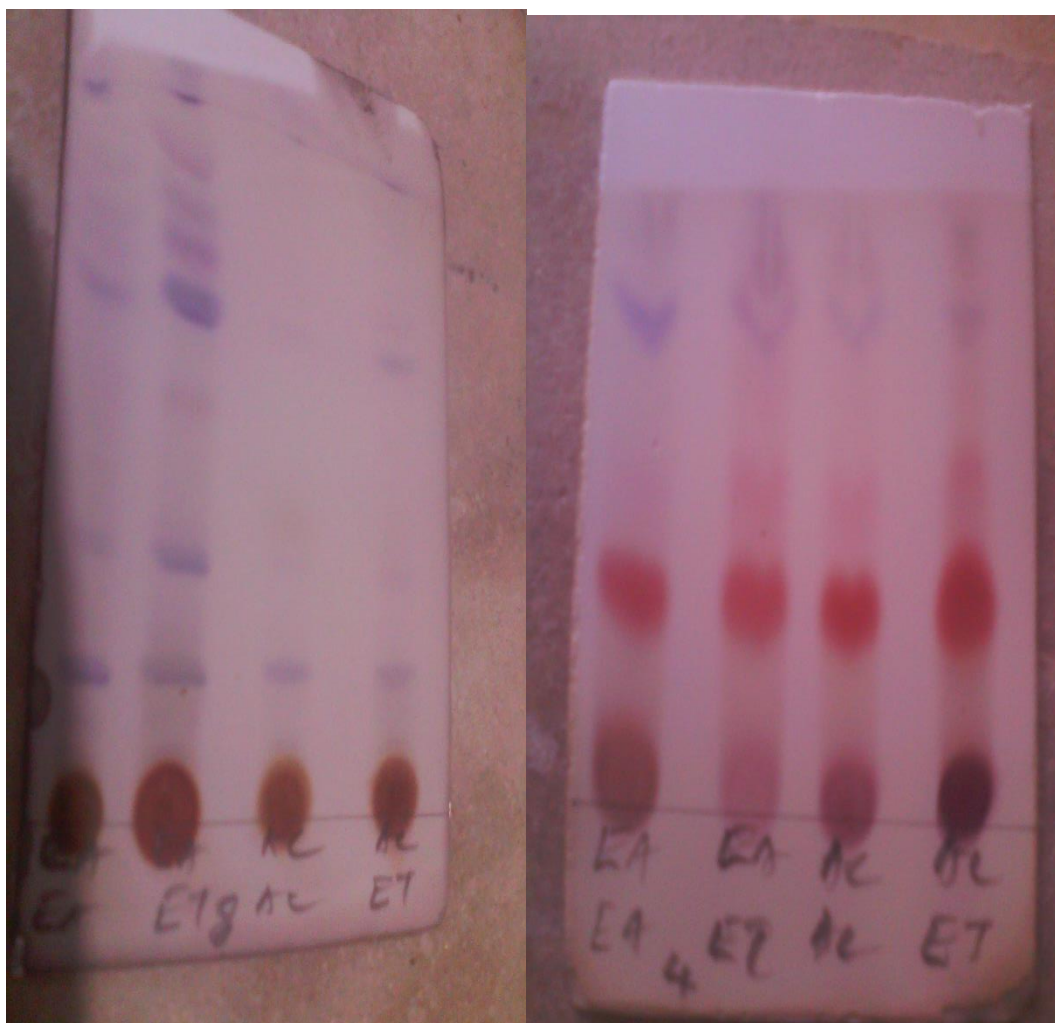


Figure 1 & 2: TLC Chromatograms of Ethyl acetate and Acetone Fractions

The abbreviations EA/EA; EA/AC; AC/AC; AC/ET on the chromatograms stand for fractions obtained after solvent/solvent fractionation. EA/EA signifies ethyl acetate

fraction obtained from fractionation of ethyl acetate extract with ethyl acetate; EA/AC signifies ethyl acetate fraction obtained from fractionation of ethyl acetate extract with

acetone; EA/ET signifies ethyl acetate fraction obtained from fractionation of ethyl acetate extract with ethanol; AC/AC signifies acetone fraction obtained from fractionation of

acetone extract with acetone and AC/ET signifies fraction obtained from fractionation of acetone extract with ethanol.

**Table 2: Rf values of EA and AC solvent/solvent fractions from *H. barteri* extracts**

Constituent number	Rf value of spot			
	EA/EA	EA/EtOH	AC/AC	AC/EtOH
1	0.17	0.17	0.17	0.17
2	0.31	0.31	0.31	0.31
3	0.64	0.64	-	0.64
4	0.71	0.71	-	-
5	0.74	0.74	-	-
6	0.78	0.78	-	-
7	0.95	0.95	0.95	0.95

The result of the TLC served as a clue for column chromatography. The solvent systems that gave good resolution in TLC were employed in column separation. After column separation, various fractions were obtained and re-chromatographed on TLC and all fractions that had similar Rf

values were pooled together (Fig. 3 and 4). The pooled column fractions were re-chromatographed on micro-column to obtain a pure compound (Figure 5) which was crystallized and saved for further analysis.



Figure 3, 4 and 5: TLC plates of column fractions showing pooled fractions and re-eluted to obtain a single spot signifying a clean pure isolate

## DISCUSSION

### Bioactivity-guided Fractionation of Antioxidant Compounds

The ethyl acetate and acetone extracts of the stem bark of *H. barteri* were found to possess antioxidant properties on 2, 2-Diphenylpicryl-1-hydrazyl (DPPH) stable free radical

(Ezekiel *et al.*, 2015). The ethyl acetate and acetone fractions which were previously fractionated on solvent/solvent fractionation were subjected to chromatographic purification to separate the bioactive compound having antioxidant properties



Thin Layer Chromatography (TLC) was used to detect the presence of different secondary metabolites in the ethyl acetate and acetone fractions. A mixture of toluene, ethyl acetate and formic acid (TEF) with varying ratios used as eluents formed a better solvent system for the separation of phenolic compounds (Schellenburg and Kabrodt *et al.*, 2015). When TEF was used as a solvent system, several spots were visualized on the TLC chromatograms which after bioautography with 0.2% DPPH methanol solution, most of the spots showed antioxidant properties.

#### Thin Layer Chromatography (TLC) in Tandem with Bioautography

TLC was carried out on the ethyl acetate fraction using TEF at various ratios from (4/2.5/3.5 and 3/5/2) and BAW in the ratio 12/4/6 on which the chromatograms were visualized with both iodine vapour and P-anisaldehyde solution in sulphuric acid. The developed TLC plates were sprayed with 0.2% DPPH methanol solution and the resultant chromatograms after evaporation showed yellow spots on a purple background signifying components with antioxidant properties.

#### Column Chromatography (CC)

Columns were loaded with silica gel using toluene as loading solvent and subsequently eluted with the three solvent systems determined from TLC and run in a gradient pattern. Several fractions were collected from the columns in 100 cm<sup>3</sup> beakers. When tested on TLC again, fractions having similar R<sub>f</sub> values were pooled together and crystallized. Crystals resulting from pooled column fractions were chromatographed on TLC and visualized with iodine vapour as shown in Figures 3 and 4. The pooled column fraction tagged F1-9 was re-chromatographed on the column again which gave a clean fraction as shown in Figure 5. The R<sub>f</sub> value of the clean isolate corresponds to one of the spots with an R<sub>f</sub> value of 0.31 on the TLC chromatogram of the crude extract (Fig. 1 and 2) which on bioautography gave a positive test for antioxidant.

#### CONCLUSION

In conclusion, the ethyl acetate and acetone fractions obtained from solvent/solvent fractionations when chromatographed on TLC revealed the presence of several bioactive metabolites. The bioactive metabolites on bioautographic chromatography showed the presence of antioxidant components when they were developed with P-anisaldehyde/sulphuric acid by the appearance of different colours on the chromatograms. The solvent systems that gave good chromatograms on TLC were employed in subsequent column chromatography and clean column fractions were obtained with an R<sub>f</sub> value of 0.31 corresponding to the purple coloured spots on TLC chromatogram. The purple-coloured spot with an R<sub>f</sub> value of 0.31 showed antioxidant property on bioautographic assay.

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