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Research Article

Physical Charaterization of Chromatographic Components from the Bioactive Aqueous Fraction of *Vernonia amygdalina* [Esteraeceae] Leaf

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Abstract: The objective of this study was to isolate and characterize the bioactive saponin from *Vernonia amygdalina* leaf using its physical and chromatographic properties. Standard procedure of liquid-liquid extraction was employed to isolate crude saponin and column chromatography to obtain fractionate for bio-evaluation. Antipyretic evaluation of the crude saponin and fractionate was carried out using *Saccharomyces cerevisiae* induced pyrexia. Pure components from fractionate were separated using Reverse-Phase High Performance Liquid Chromatography. The physical properties of these components were evaluated according to standard procedures using organoleptic, ignition and solubility tests. Obtained data indicated three pure colored components with retention times 7.78±0.19, 8.76±0.16 and 13.54±0.87 min. Data obtained showed all the components to be aromatic and polar in character. Finding suggests that the bioactive saponin is triterpenoidal.

Keywords: Antipyretic, organoleptic, reverse-phase, saponin, solubility, triterpenoidal

INTRODUCTION

Phytochemical principles have become the traditional pool for present day allopathic agents. Many synthetic agents in contemporary use for health management bear their root in the complete or modified structural form of phytochemicals, as contained in their parent herbal source. The effectiveness of traditional use of herbs in managing common ailments has been attributed to these phytochemicals. The use of phytomedicines as prepared in various ethno-formulations, date back to many years ago. These herbal formulations and constituent secondary metabolites have been well documented for some of the medicinal plants (*Nunn, 2002; NIIR, 2004).*

Because of the bitter taste, *Vernonia amygdalina* is commonly called "bitter leaf". It can be considered as one of the numerous medicinal vegetables widely distributed in African and the Arabian Peninsula. It grows in a range of ecological zones (Bonsi *et al*., 1995). Some of the local names associated with the herb include "omululuza" (West and Central Uganda); "olusia" (Luo, Kenya); "ewuro" and "olugbo" (Southern and Eastern Nigeria); and awonoo (Southern Ghana) (Asase and Oppong-Mensah, 2009). The leaf is used for both its nutritional and therapeutic benefits. In Southern Ethiopia, dairy farmers feed the boiled leaf to their stock during the dry period (Bonsi *et al*., 1995). Studies have identified a number of bioactive phytochemicals in the leaf extract of this plant (Izevbigie, 2005; Adiukwu *et al*., 2011). Principles like saponins, alkaloids, tannins and glycosides have been attributed to be the reason for the bitter taste of the leaf (*Ijeh and Ejike, 2011)*. Usually, the leaves are processed by soaking and washing in warm water to reduce the bitter taste before human consumption (Ridout *et al*., 1991).

Saponin is widely distributed in plants (Güçlü-Ustündağ and Mazza, 2007). It is a glycoside made up of the A-glycone (lipophilic region) which may be triterpenoidal or steroidal in nature and the glycone (hydrophilic region), characterized by the number of sugar chains. The sugar group is attached to the triterpene or steroid aglycone backbone called sapogenin through a glycosidic bond. Because of the bitter and unpleasant taste, saponin use has been highly limited (Güçlü-Ustündağ and Mazza, 2007). The increasing evidence of saponin benefits in health care has galvanized a renewed focus and interest in this group of compounds (Shi *et al*., 2004). Studies have highlighted the contributions of some saponin containing foodstuffs to health (Liu and Henkel, 2002;

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Muir *et al*., 2002). The saponin constituent of *Vernonia amygdalina* has been shown by studies to be responsible for some of the medicinal benefits (Adiukwu *et al*., 2013a). However, studies to sufficiently elucidate and/or characterize the chemical structure of this principle have been limited. Therefore, the objective of this study was to investigate the physical and chromatographic properties of the saponin principle that has been identified in the earlier study of the bioactive fraction of *Vernonia amygdalina* leaf extract.

MATERIALS AND METHODS

Chemicals, drugs and test agents: Analytical grades of methanol, n-butanol, diethyl ether, chloroform, acetone and xylene from BDH, Uganda as well as, acetic acid (Sigma-Aldrich, Germany) were used in this study. Active dry *Saccharomyces cerevisiae*: brewer's yeast (Griffchem®) was used to induce pyrexia in the antipyretic study. A dose of 300 mg/kg acetylsalicylic acid (ASA) (Pinewood, Caprin®) as reference standard, 5 mL/kg normal saline solution (Albert David, India) as placebo, *V. amygdalina* leaf aqueous extract as test sample 1, crude saponin as test sample 2 and Va-SB (*V. amygdalina* saponin fraction B) as test sample 3, were used in the animal study.

Plant material and extraction: The fresh leaves of *Vernonia amygdalina* identified by a botanist were collected in the morning, between June and July (raining season) in south-western Uganda. Specimen was retained with voucher number 16-20 in the Departmen of Pharmacy, Faculty of Medicine, Mbarara University of Science and Technology. The leaves were shade air-dried and ground into a coarse powder that was sieved to 2000 g fine powder. The moistened powder was allowed to stand for 15 min before maceration for three hours in warm (<80°C) distilled water at a modified ratio of 131 g to 9.0 L with intermittent shaking (Adiukwu *et al*., 2013b). The obtained infusion was filtered while warm. The filtrate was further filtered using buchner filter assemblage (aided by a suction pump) and subsequently evaporated to dryness in an oven (at ≤ 80°C). The total amount of yield (leaf residue) obtained was 18%. This was stored in a desiccator at room temperature (25°C) until required for use.

Preliminary screening: Screening of the aqueous extract for phytochemicals was carried out using standard procedures (Harborne, 1973). Test procedures carried out were foaming assay for saponins, dragendorff's test for Alkaloids, baljet test for sophisticated lactones, liebermann-burchard test for triterpenes and steroids, fehling test for reducing sugars, ninhydrine test for amino acids, shinoda assay for flavonoids, borntragers test for quinines, salkowski test for terpenoids, ferric chloride test for tannins and kedde's assay for cardiotonic glycosides.

Isolation and fractionation: The liquid-liquid extraction technique as described by Obadoni and Ochuko (2001) was adopted for the isolation. A forty milliliter solution was prepared in distilled water using 20 g of the dried aqueous extract of *V. amygdalina* leaf. This was extracted thrice with 20 mL diethyl ether. The diethyl ether layer was discarded and the retained aqueous layer extracted further with 60 mL n-butan-1-ol (four times). The n-butan-1-ol extracts were bulked together and washed four times using 10 mL of 5% NaCl. The washed extract was concentrated at <80°C in an oven and air dried at room temperature to yield 1.81 g (9.1% w/w) of crude saponin residue. The residue was screened for saponin using the foaming test (Harborne, 1973).

Flash column chromatographic fractionation: Procedure as described by Adiukwu *et al*. (2013b) was adopted for the column fractionation. The crude saponin dissolved in methanol was adsorbed onto a TLC grade silica gel (CSI 010, Unilab) at a ratio of two to five and dried in an oven at <80°C to produce a 21 g free flowing powder. The powder was loaded and fractionated on a silica gel (May and Baker Dagenham, England: 0.2-0.5 mm, pore size 40 amgstrom, 30-70 mesh) containing flash column. The column was eluted with a gradient mobile phase solvent system of increasing polarity starting with xylene; combination of chloroform and methanol (one to one); and methanol, in multiples of 100 mL. An air pump (Merck, Germany) was used to facilitate the rate of elution. Each 100 mL effluent collected was profiled using TLC (Thin Layer Chromatography) with a mobile phase system of acetone, chloroform and methanol (at a ratio of one to four to two) (Stil *et al*., 1978). Spots were located using saturated iodine chamber. Effluents with similar profile were combined together, concentrated over a water bath and allowed to evaporate to dryness at room temperature. This resulted in 2 fractions: *Vernonia amygdalina* saponin fraction A (Va-SA) 0.932 g, eluted with chloroform/methanol and *Vernonia amygdalina* saponin fraction B (Va-SB) 1.35 g, eluted with methanol. A preliminary screening of both fractions for saponinusing foaming test was conducted (Harborne, 1973). Va-SB was positive and was preserved in a desiccator for further use.

Animal handling: Twenty-five Wistar rats weighing between 120-200 g of both sexes were placed in standard cages where they were maintained on standard animal pellets (Nuvita Feeds Ltd., Kampala) and water *ad libitum*. The rats for the antipyretic evaluation were selected based on their measured basal anal temperature

(≤ 37°C) using a thermistor probe (with a resolution of 0.0005 and accurate to 0.001°C) and also achieving a minimum of 0.1°C increase in anal temperature in response to 10 mL/kg intra-peritoneal (*i.p*) administered 15% w/v *Saccharomyces cerevisiae*. The animals were randomly divided into five groups: placebo group (1), reference group (2) and test groups (3 to 5). Each group consists of five animals which were fasted over-night.

National Institute of Health (1978) guide for the care and use of laboratory animals as approved by the Institutional Ethical Committee was adopted for the animal protocol in this study.

Preparation and administration of drug: Reference (ASA) and tests samples (crude saponin and Va-SB) were prepared using sterile normal saline solution which also, served as placebo in the antipyretic study. Oral administration was carried out using a cannula-syringe assemblage.

Antipyretic study: Standard procedure as previously described, was adopted for this study (Okokon and Onah, 2004). Wistar rats earlier selected and induced pyrexia as described above, were randomly divided into five groups of five animals each. The animals were allowed to starve overnight in their respective cages. Twenty hours after the administration of *Saccharomyces cerevisae* solution, the anal temperature of each animal in their respective group was measured. Oral administration of normal saline to group one, ASA to group two and crude saponin (200, 400 and 600 mg/kg) to groups three to five, was immediately carried out after the anal temperature measurement. Four hours after the administration of the dose, the anal temperature of each animal was measured again.

Using the same placebo and reference standard as above, similar antipyretic evaluation procedure was conducted for the study of Va-SB. Dose levels of 200, 300 and 400 mg/kgVa-SB were used for test groups three, four and five respectively. Doses employed in this study were based on prior study of the crude saponin content in *V. amygdalina* leaf (Adiukwu *et al*., 2011).

RETENTION TIME DETERMINATION OF Va-SB PURE COMPONENTS USING ANALYTICAL REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC)

Sample preparation: Standard procedure as described by Hostettmann and Marston (1986) was followed in the sample preparation. Sample was freshly prepared by dissolving Va-SB in 80% methanol and sonicated to form a 5×10^{-2} g/mL solution. This was filtered with a membrane filter of 0.45 µm pore size and 0.25 mm diameter (coat number: 08020177). The filtrate was preserved in an ultra-low temperature freezer for subsequent use.

Mobile phase preparation: Following a preliminary TLC evaluation of the Va-SB fraction, a linear gradient mobile phase of methanol and water was adopted for this study. The freshly prepared mobile phases were 100, 70, 60, 40, 20 and 0% methanol solutions. Each of the solutions were sonicated for 6 min and mounted with their respective sample bottle (Adiukwu *et al*., 2013b).

Stationary phase preparation: Using a 2.3×60 mm diameter and 5 µm particle size reverse phase C_{18} column (Agilent® PN 880975-905, Germany) at a pressure between 162 and 171 Kgf with a flow rate of 0.2 mL/minute, the stationary phase was conditioned (base line). The baseline determination (or conditioning procedure) was performed using double beam UV-vis detector HPLC equipment (Shimadzu 1601, Germany).

Chromatogram development: The sample prepared as above was placed in the oven at 25°C using sample vials of 15 µmL. After a prior conditioning of the stationary phase as described above, the chromatogram for the various mobile phases at a run time of 25 min, were developed in accordance with standard method (Hostettmann and Marston, 1986).

System optimization: Using 70% methanol as the mobile phase with the best resolution and the stationary phase as described above, the system was optimized at a UV-vis detection of 365 nm. The retention times of the obtained pure components were determined and documented.

PREPARATORY RP-HPLC COLUMN ELUTION AND RECOVERY OF Va-SB PURE COMPONENTS

Following the chromatographic conditions as optimized above, 120 µmL sample vials and a reverse phase C_{18} column (Agilent® PN 880975-905, Germany) of 9.2×480 mm diameter with 5 µm particles size were employed in the purification and recovery of pure effluents, at the prior documented retention times. The obtained effluents were evaluated for purity using rapid TLC and concentrated using the rotatory evaporator at 5 rpm and 45°C. Concentrates were dried at 2 atmospheric pressure and -50° to -55°C using the freeze dryer (Edwards®, Germany). The obtained pure components were preserved at -16 to -37°C using an ultra-low temperature freezer.

PHYSICAL EVALUATION OF RECOVERED Va-SB PURE COMPONENTS

Test for saponin: Three pure components: Va-SB_1, Va-SB_2 and Va-BS_3 recovered in the preparatory RP-HPLC were tested for saponin using the foaming test technique (Harborne, 1973).

Organoleptic evaluation: The technique as described by Ralph *et al*. (2010) for color, taste and odor was adopted for the organoleptic characterization of the pure components. Following similar procedure, the color of the respective pure components were observed in the neutral (distilled water), dilute acid and alkaline solutions.

Ignition test: Using the spatula, a speck of each pure component was separately exposed to bunsen burner flame in accordance with standard method (Ralph *et al*., 2010) and observed for resulting flame color change.

Solubility test:

Water solubility: The flask method according to the OECD (1992) (Organization for Economic Co-operation and Development), guidelines was adopted for this test. A preliminary estimation of required volume of distilled water was carried out at room temperature using a corked testtube. With 1.0×10^{-3} g of the crushed pure component in the tube, water was added incrementally with intermittent vigorous shaking until sample was observed visually to have dissolved in 6 mL. The evaluation was repeated using 6 mL of the distilled water (as prior estimated) and 5.0×10^{-3} g of the same pure sample in a corked test tube. The mixture was vigorously shaken and placed in a Branson CPX1800Hsonicator bath maintained at 30°C for 24 h. It was allowed to equilibrate for a further 24 h in the same sonicator at room temperature. 5 mL of the solution was filtered into a centrifuge tube and centrifuged for 2 min at 50 rpm. 3 mL of the resulting homogenous solution was decanted into a prior weighed test tube and evaporated to dryness in an oven at 45° to 50°C. The final weight of the test tube was determined after drying using analytical weighing balance. The solubility of the pure component was considered as the weight (in grams) of the solute in 1 ml of the solution. The evaluation was similarly carried out for all the three pure components in triplicates.

Alkaline solubility: Adopting technique as described by Ralph *et al*. (2010) 1 mL of 5% NaOH was added in incremental small portions to the test tube containing small (not weighed) quantity of the pure component. The mixture was shaken vigorously after the addition of each portion of the alkaline solution and visually observed for solubility. Color change, homogeneity and/or evolution of gas or heat were used as solubility indicator. 1 mL blank 5% NaOH solution in a separate test tube was used as control. This was technique was followed in the evaluation of each pure component in duplicate.

Acid solubility: The procedure as described in the alkaline solubility test was adopted for this study. The different components were similarly evaluated using 5% HCl acid (instead of 5%NaOH as above).Color change, homogeneity and/or evolution of gas or heat were used as solubility indicator. 1 mL blank 5% HCl acid in a separate test tube was used as control. Each pure component was evaluated in duplicate.

Data analysis: Data and calculations were based on the numerical expression of mean±SEM (standard error of mean) in the animal study; mean±SD (standard deviation) in the chromatographic and solubility study. Analysis of Variance (ANOVA) was used for values within groups and student T-test, for data between groups. $p\leq0.05$ was taken as level of significance in all cases where applied.

RESULTS

Preliminary screening: Standard test for phytochemical constituents revealed the presence of saponins, alkaloids, sophisticated lactones, triterpenoids, reducing sugars, amino acids, flavonoids, terpenoids, tannins and cardiotonic glycosides but absence of quinine.

Antipyretic study: The antipyretic study showed a dose dependent response with the crude saponin (Table 1) and Va-SB fraction (Table 2). Statistical analysis using ANOVA indicated no significant ($p\leq0.05$) difference in the yeast induced anal temperature within and among groups. T-test analysis indicated significant ($p\leq0.05$) dose induced anal temperature decrease for all the test samples in the different evaluations.

RP-HPLC Evaluations: Data obtained in Table 3 showed that Va-SB_1 has the least retention time: 7.78±0.19 min, among the recovered pure components. This is an indication of higher polarity relative to Va-SB_2 and Va-SB_3.

Physical evaluation of pure components:

Test for Saponin: This test was positive for all the pure components.

Organoleptic evaluation: Observed organoleptic characters showed that the dry samples of Va-SB_1, Va-SB_2 and Va-BS_3 were similarly yellow with sour or un-agreeable taste. No clear odor was associated with these pure components. In the alkaline solution, all the pure components were intensely dark yellow while they retained their associated dry state yellow color in the acidic solution.

Ignition test: Sooty flame was observed for all the components.

Solubility test: Obtained result indicated all the pure components were soluble in aqueous, alkaline and acidic solutions. Though the extent of their solubility in

Table 1: Effect of the crude saponin on the anal temperature in rats 4 hrs after administration

		20 hrs after yeast administration, the anal Temp.°C readings are	
Group	Dose	0 hrs (after Dose admin.)	4 hrs (after Dose admin.)
Placebo	Normal saline 5 mL/kg	39.054±0.12	39.04±0.11
Standard	ASA 300 mg/kg	39.10±0.11	38.95±0.08
Test	Crude saponin 200 mg/kg	38.75±0.25	38. 62±0.24
Test	Crude saponin 400 mg/kg	38.78±0.19	38.49±0.18
Test	Crude saponin 600 mg/kg	38.66±0.11	38.29±0.11
Group	Dose	Dose induced anal temp.°C decrease	
Placebo	Normal saline 5 mL/kg	0.014±0.02	
Standard	ASA 300 mg/kg	*0.15±0.04 ($p = 3.95x10^{-3}$)	
Test	Crude saponin 200 mg/kg	*0.14±0.02 ($p = 2.4x10^{-4}$)	
Test	Crude saponin 400 mg/kg	*0.29±0.01 ($p = 1.2x10^{-4}$)	
Test	Crude saponin 600 mg/kg	*0.39±0.01 ($p = 1.06x10^{-5}$)	

Data are mean±SEM (standard error of mean) value (n = 5). *Significantly ($p \leq 0.05$) different from placebo

Table 2: Effect of the *Vernonia amygdalina* chromatographic fraction, *Va*-SB on the anal temperature in rats 4 hrs after administration

		20 hrs after yeast administration, the anal Temp.°C readings are	
Group	Dose	0 hrs (after Dose admin.)	4 hrs (after Dose admin.)
Placebo	Normal saline 5 mL/kg	38.01±0.29	37.96±0.28
Standard	ASA 300 mg/kg	37.84±0.22	37.64±0.23
Test	Va-SB 200 mg/kg	37.87±0.27	37.81±0.27
Test	Va-SB 300 mg/kg	37.83±0.28	37.69±0.28
Test	Va-SB 400 mg/kg	37.82±0.25	37.67±0.25
Group	Dose	Dose induced anal temp.°C decrease	
Placebo	Normal saline 5 mL/kg	0.05±0.04	
Standard	ASA 300 mg/kg	*0.19±0.01 ($p = 4.7x10^{-4}$)	
Test	Va-SB 200 mg/kg	*0.06±0.01 ($p = 3.1x10^{-3}$)	
Test	Va-SB 300 mg/kg	*0.14±0.01 ($p = 2.2x10^{-3}$)	
Test	Va-SB 400 mg/kg	*0.15±0.01 ($p = 1.45x10^{-3}$)	

Data are mean±SEM (standard error of mean) value (n = 5). *Significantly ($p \leq 0.05$) different from placebo

Table 3: Mean of retention time and area of UV absorption of pure components

S/N	Components	Retention time (minutes)	Area of UV-Absorption (μm^2)	Relative abundance (%)
1	Va-SB_1	7.78±0.19	1.58×10^8±1.62	49.28
2	Va-SB_2	8.76±0.16	1.56×10^8±1.14	48.66
3	Va-SB_3	13.54±0.87	6.59×10^6±1.43	2.06

Data are mean±SD (standard deviation of mean), value (n) = 3

the alkaline and acidic solutions were not estimated, they exhibited varying degree of aqueous (distilled water) solubility: Va-SB_1 at $1.67\pm0.20\times10^{-4}$ g/mL,Va-SB_2 and Va-SB_3 at $1.55\pm0.15\times10^{-4}$ g/mL and $9.82\pm0.19\times10^{-5}$ g/mL respectively (data is mean±SD and value (n) = 3).

DISCUSSION

Qualitative and quantitative evaluations are major steps in determining the extent a medicinal product (allopathic or herbal) meets set standards and specifications for a product. In phyto-medicine, especially when the source of the starting material is herbal, a key component in designing standards for product regulation is the qualitative and quantitative definition of the phyto-principles (Zöllner and Schwarz, 2013). As routine checks of properties associated with such products, profiling of constituent phytochemicals can be measures to identify adulterations (Zöllner and Schwarz, 2013). Obtained preliminary screening of phytochemical constituents of the *Vernonia amygdalina* aqueous extract in this study agrees with previous findings (Izevbigie, 2005; Adiukwu *et al*., 2011). Data showed the presence of saponin and other phyto-principles.

The antipyretic study showed a dose dependent anal temperature decrease which was significant ($p \leq 0.05$) for all the test samples in the different antipyretic evaluations. This is in conformity with earlier reports (Okokon and Onah, 2004; Adiukwu *et al*., 2011). Induction of pyrexia in laboratory animals, as adopted in this study, is considered to be inhibited by the inhibitory effect of the test samples on the prostaglandins E_2 synthesis in the hypothalamus (Adiukwu *et al*., 2013a; Aronoff and Neilson, 2001).

Chromatographic evaluation of the fraction, Va-SB showed the presence of three pure components: Va-SB_1, Va-SB_2 and Va-SB_3. Generally, chromatography is a physical separation technique that depends on the relative affinity of the principles for the two separate phases: solid and liquid or solid and gaseous or liquid

and gaseous phases (Baraem and Nielsen, 2010). The challenge in the use of this technique in phyto-medicine is the unclear profile or nature of phyto-constituents. However, the polarity theory of solvents used in the extraction of herbal materials has found a good measure in providing working data for the physical properties of phyto-principles. Hence, most herbal materials obtained or prepared using aqueous medium are usually evaluated with chromatographic technique where the mobile phase is considered more polar to the stationary phase (Zöllner and Schwarz, 2013). In this study, reverse phase technique was used for the HPLC evaluation. The retention time data obtained for the pure components using the analytical RP-HPLC, indicated Va-SB_3 (13.54±0.87 sec) to be the least polar.

Based on the principle of chromatographic separation, mobility of molecules with higher partition coefficient for the stationary phase are usually retarded and with longer retention time (Baraem and Nielsen, 2010). This may explain the polarity and retention time obtained for Va-SB_3 relative to the other pure components. At 2.06% relative abundance, Va-SB_3 was also shown to be the least in content. This is with due cognizance of the area of ultraviolet (UV) spectra absorption, as shown by the SPD-10A VP UV-vis detector on the chromatogram. Usually, the magnitude of absorption or emission by an electromagnetic active substance is quantitatively proportional to that substance. With respect to the chromatogram, it is considered proportional to the area of absorption (Culler, 1993).

Va-SB_1, Va-SB_2 and Va-SB_3 recovered with the preparative RP-HPLC were positive to foaming test, indicative of saponin. Observed organoleptic characteristics indicated that the pure components were colored both in the dry state and in solution. Sooty flame was also observed for all the components in the flame test. The solubility test showed all the pure components were soluble, however, water solubility profile indicated Va-SB_1 (1.67±0.20×10-4 g/mL) to be the most aqueous soluble.

Compounds that are colored are often considered to be highly conjugated (Gouterman, 1978). Extended conjugation is common with aromatic compounds and it confers chromophoric properties on molecules where it is present. Hence, such molecule alone or in chemical bonding with other chemical structures/molecules, has the intrinsic ability to absorb electromagnetic radiation at the visible region of the spectrum (Workman, 2016a). This absorption of light radiation at longer wavelengths (or lower energy level) due to $\pi \rightarrow \pi^*$ transition may suggest why the pure components are colored. Some groups like the hydroxyl (OH-group as present in some basic compounds) are auxochromic and can extend the conjugation of a chromophore (or a less conjugated molecule) by the sharing of nonbonding electrons: $n \rightarrow \pi^*$. This action usually results in the shift to longer wavelength of maximum absorption (λ_{max}) which could be in the in the visible region. This bathochromic or red shift may explain the increase in colour intensity when the pure components were dissolved in the alkaline solution (Workman, 2016b).

The sooty flame observed in the ignition test is characteristic of aromatic compounds (Ahluwalia and Dhingra, 2000). The solubility data suggests that the pure components are organic acids. Most organic acids are carboxylic or phenols/polyphenols hence can react with 5% NaOH (Ahluwalia and Dhingra, 2000). Solubility in the acidic solution without colour change may suggest the surface active property of the components. However, observed sooty flame and solubility in dilute alkaline solution is common characteristic of aromatic organic acid, typical of triterpenoidal saponin (Hostettmann and Marston, 1995).

CONCLUSION

Data obtained in this study agreed with previous findings on the bioactive property of the crude saponin and the fractionate Va-SB from the aqueous extract of *Verninia amygdaline* leaf. RP-HPLC of Va-SB identified three pure components with distinct retention times. This was further supported by the difference in the water solubility data obtained for these three components. However, data in the organoleptic, ignition and all the solubility tests put together, showed that the compounds are similar in physical characteristics. Such peculiarity in properties is common with compounds in the same group or family. Evidence in this study suggests that this group compounds are saponin with triterpenoidal sapogenin.

Data in this study was not sufficient to show the bioactivity or chemical properties of the pure components. Further studies in these areas, as well as, structural elucidation of Va-SB_1, Va-SB_2 and Va-BS_3, will be valuable in the understanding and rational use of *Vernonia amygdalina* as phyto-medicine.

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AUTHOR(S) DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

Adiukwu, P.C., A. Amon and G. Nambatya, 2011. Pharmacognostic, antiplasmodial and antipyretic evaluation of the aqueous extract of *Vernonia amygdalina* leaf. Int. J. Biol. Chem. Sci., 5(2): 709-716.

Adiukwu, P.C., F.I.B. Kayanja, G.K. Nambatya, S. Rugera, J.O.C. Ezeonwumelu, J.K. Tanayen, B.J. Murokore, O. Twikirize, S. Twinomujuni, D. Byamugisha and L. Imanirampa, 2013a. Antipyretic and antinociceptive properties of the aqueous extract and saponin from an edible vegetable: *Vernonia amygdalina* leaf. Afr. J. Food Agric. Nutr. Dev., 13(2): 7587-7606.

Adiukwu, P.C., F.I.B. Kayanja, G. Nambatya, B. Adzu, S. Twinomujuni, O. Twikirize, A.A. Ganiyu, E. Uwiduhaye, E. Agwu, J.K. Tanayen, P. Nuwagira and P. Buzaare, 2013b. Anti-inflammatory and anti-pyretic activity of the leaf, root and saponin fraction from *Vernonia amygdalina*. Brit. J. Pharmacol. Toxicol., 4(2): 33-40.

Ahluwalia, V.K. and S. Dhingra, 2000. Comprehensive Practical Organic Chemistry (Qualitative Analysis): Preliminary Examination. Universities Press, Hyderabad, pp: 3-14.

Aronoff, M.D. and E.G. Neilson, 2001. Antipyretics: Mechanisms of action and clinical use in fever suppression. Am. J. Med., 111(4): 304-315.

Asase, A. and G. Oppong-Mensah, 2009. Traditional antimalarial phytotherapy remedies in herbal markets in Southern Ghana. J. Ethnopharmacol., 126(3): 492-499.

Baraem, I. and S. Nielsen, 2010. Basic Principles of Chromatography. In: Nielsen Suzanne (Ed.), Food Analysis. Springer, New York, pp: 481-495.

Bonsi, M.L.K., P.O. Osuji, A.K. Tuah and N.N. Umunna, 1995. *Vernonia amygdalina* as a supplement to teff straw (*Eragrostis tef*) fed to ethiopian menz sheep. Agroforest. Syst., 31(3): 229-241.

Culler, I.S.R., 1993. Diffuse Reflectance Infrared Spectroscopy: Sampling Techniques for Qualitative/Quantitative Analysis of Solids. In: Coleman, P.B. (Ed.), Practical Sampling Techniques for Infrared Analysis. CRC Press, Washington, DC., pp: 93-106.

Güçlü-Ustündağ, O. and G. Mazza, 2007. Saponins: Properties, applications and processing. Crit. Rev. Food Sci., 47(3): 231-258.

Gouterman, M., 1978. Optical Spectra and Electronic Structure of Porphyrins and Related Rings. In: Dolphin, D. (Ed.), The Porphyrins Volume III, Part A. Academic Press, New York, pp: 1-165.

Harborne, J.B., 1973. Phytochemical Methods. Chapman and Hall, London, pp: 113.

Hostettmann, M. and A. Marston, 1986. High Pressure Liquid Chromatography. Preparative Chromatography Techniques. Springer-Verlag, New York, pp: 56-61.

Hostettmann, K. and A. Marston, 1995. Chemistry and Pharmacology of Natural Products: Saponins. Cambridge University Press, New York, pp: 140-186.

Ijeh, I.I. and C.E.C.C. Ejike, 2011. Current perspectives on the medicinal potentials of *Vernonia amygdalina* Del. J. Med. Plants Res., 5(7): 1051-1061.

Izevbigie, E.B., 2005. Phytochemotherapy for cancer. U.S. Pat.: 6,849,604; Issued on: February 1.

Liu, J. and T. Henkel, 2002. Traditional Chinese Medicine (TCM): Are polyphenols and saponins the key ingredients triggering biological activities? Curr. Med. Chem., 9(15): 1483-1485.

Muir, A.D., D. Paton, K. Ballantyne and A.A. Aubin, 2002. Process for recovery and purification of saponins and sapogenins from quinoa (*Chenopodium quinoa*). U.S. Patent No. 6,355,249 B2.

National Institute of Health, 1978. Guide for the Care and use of Laboratory Animals (revised). NIH Publication, pp: 83-23.

NIIR (National Institute of Industrial Research), 2004. Compendium of Medicinal Plants. National Institute of Industrial Research, Delhi, pp: 320.

Nunn, J.F., 2002. Ancient Egyptian Medicine. University of Oklahoma Press, Oklahoma, pp: 15.

Obadoni, B.O. and P.O. Ochuko, 2001. Phytochemical studies and comparative efficacy of the crude extracts of some haemostatic plants in edo and delta states of Nigeria. Global J. Pure Appl. Sci., 8(2): 203-208.

OECD, 1992. Guidelines for the testing of chemicals: Water solubility. Official J. Eur. Commun., L 383 A, 2: 54-62.

Okokon, J.E. and M.I. Onah, 2004. Pharmacological studies on root extract of *Vernonia amygdalina*. Nigeria J. Prod. Med., 8: 59-61.

Ralph, L.S., C.K.F. Hermann, T.C. Morrill, D.Y. Curtin and R.C. Fuson, 2010. The Systematic Identification of Organic Compounds. 8th Edn.,Wiley, New York, pp: 25-75.

Ridout, C.L., K.R. Price, M.S. DuPont, M.L. Parker and G.R. Fenwick, 1991. Quinoa saponins: Analysis and preliminary investigations into the effects of reduction by processing. J. Sci. Food Agr., 54(2): 165-176.

Shi, J., K. Arunasalam, D. Yeung, Y. Kakuda, G. Mittal and Y. Jiang, 2004. Saponins from edible legumes: Chemistry, processing and health benefits. J. Med. Food, 7(1): 67-78.

Stil, W.C., M. Kahn and A. Mitra, 1978. Rapid chromatographic technique for preparative separations with moderate resolution. J. Org. Chem., 43(14): 2923-2925.

Workman, J., 2016a. Ultraviolet Spectroscopy. In: The Concise Handbook of Analytical Spectroscopy. (Vol. 1: Theory of Ultraviolet Spectroscopy, Light Interaction with Matter). World Scientific, New Jersey, pp: 25-44.

Workman, J., 2016b. Visible Spectroscopy. In: The Concise Handbook of Analytical Spectroscopy (Vol. 2: Theory of Visible Spectroscopy, Light interaction with matter). World Scientific, New Jersey, pp: 23-40.

Zöllner, T. and M. Schwarz, 2013. Herbal reference standards: Applications, definitions and regulatory requirements. Rev. Bras. Farmacogn., 23(1): 1-21.