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Original Article

PREFORMULATION CHARACTERIZATION OF AQUEOUS EXTRACTS FROM THE LEAVES OF ANTIMALARIAL PLANTS-*ARTEMISIA ANNUA* L., *VERNONIA AMYGDALINA* DEL., AND *MICROGLOSSA PYRIFOLIA* (LAM.) KUNTZE

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ABSTRACT

Objective: The current study aimed at characterising relevant physical and chemical properties of antimalarial aqueous extracts from the leaves of *Artemisia annua* (Aa), *Vernonia amygdalina* (Va), and *Microglossa pyrifolia* (Mp) to build a solid foundation for the development of stable dosage forms.

Methods: The aqueous extracts were profiled for key antimalarial chemical markers, aqueous solubility, partition coefficient, permeability, and powder flow properties using standard procedure with modifications where applicable. The powder compaction behaviours were studied using Kawakita and tablet ability models.

Results: Aa extract had 11.2 % of total flavonoids and 0.27 % of artemisinin as its antimalarial chemical markers. Va and Mp extracts contained 0.07 % and 28.5 % total terpenoids as their respective chemical markers. All the extracts exhibited high solubility and low permeability, qualifying them as class III crude drugs based on the biopharmaceutical classification system (BCS). Mp had excellent flow (angle of repose 18.9, Hausner Ratio 1.2, and Carr's Index 13 %) while Va and Aa had passable flow, thus requiring a glidant. The powder samples underwent plastic deformation, according to the Kawakita plot. Aa also showed the highest level of tabletability, followed by Va, and lastly, Mp (Area under curves of 18.5, 9.2, and 7.8 for Aa, Va and Mp, respectively).

Conclusion: Based on their chemical and physical properties, the Aa, Va, and Mp aqueous extracts can be incorporated into stable, bioavailable, and modern herbal drug delivery systems or dosage forms.

Keywords: Preformulation, Physicochemical parameters, Tabletability, *Artemisia annua*, *Vernonia amygdalina, Microglossa pyrifolia*

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INTRODUCTION

Preformulation is the first stage in formulation development that focuses on characterising or profiling a new drug's physical and chemical properties to guide the selection of suitable formulation pathways and make scientifically sound decisions in the design of robust dosage forms [1]. In the context of synthetic drug formulation development, preformulation studies encompass a wide range of properties. Drug solubility, partition coefficient, and pKa are the key parameters to be determined at the earlier preformulation stage. Once these properties are known, other parameters such as organoleptic properties (odour, colour, and taste), pH, and ionisation can also be assessed to build the drug's chemical profile. Physical and bulk properties, including crystallinity, polymorphism, hygroscopicity, particle size and shape characterisation, powder flow characteristics, compressibility and compactibility, can also be evaluated to inform formulation decisions on the New Chemical Entity (NCE).

The concept of preformulation emerged in the late 1950s and 1960s due to a technological shift in industrial pharmaceutical product development [2]. Improvements in the analytical methods provided an opportunity and sparked the need to generate prior information before venturing into developing stable and bioavailable pharmaceutical dosage forms that can be manufactured consistently on a large scale. The application of preformulation studies has been mainly restricted to the development of synthetic medicines consisting of a single drug molecule as the active principle. Therefore, most of the conventional techniques developed and properties extensively studied are relevant to synthetic drugs, making some scientists doubt their applicability to the formulation of herbal medicines with complex phytochemistry [3]. However, with the emergence of a new class of medicines known as phytopharmaceuticals, there is a need to modify the structure of preformulation to address the formulation needs of these drugs. Phytopharmaceuticals are processed or unprocessed herbal materials or extracts in a dosage form for internal or external use in humans or animals for diagnosis, treatment, and prevention of any disease but do not include administration by parenteral route [4]. It also contains purified active principles from plant sources that can be formulated into standard dosage forms [3]. Unlike preformulation studies in synthetic drugs where extensive guidance and techniques are available, the area of phytopharmaceuticals is void of the same resources. Therefore, the creative adoption of many measurement techniques is needed to study the properties of phytopharmaceuticals and formulate high-quality modern dosage forms comparable to synthetic pharmaceuticals, as current trends in herbal drug delivery systems demand.

Complete preformulation characterisation of an NCE is possible using five vital analytical techniques, including UV spectroscopy, Thin Layer Chromatography (TLC), High-Performance Liquid Chromatography (HPLC), Differential Scanning Calorimetry (DSC), and Dynamic Vapor Sorption (DVS) [5]. However, the innovative adoption of these techniques for profiling essential physicochemical parameters of crude herbal extracts or active phytopharmaceutical ingredients (API) is an area not well exploited in natural product formulation development. The primary focus has been on the phytochemical characterisation of the active compounds in these phytopharmaceutical extracts, leaving the formulation scientist with no scientific data for dosage form design. Possible approaches to study relevant preformulation parameters for crude extracts were previously suggested [4]. Some of these were adopted in physicochemical characterisation of several phytopharmaceutical extracts: aqueous stem bark extract of *Bridelia ferruginea* Benth [6], *Pugun Tano*, *Embilica officinalis* and *Glycine max* Merrill ethanolic

extracts [7, 8], *Ambrosia peruviana* seed ethanolic extract [9], and hydrothanolic extracts of *Zingiber officinale* and *Piper nigrum* [10].

Artemisinia annua, *Vernonia amygdalina*, and *Microglossa pyrifolia* emerged as essential antimalarial plants with potential for further product development in a review of Ugandan antimalarial plants [11]. Most of the studies on these plants focused on their antiplasmodial or antimalarial activities at preclinical and clinical stages, general or preliminary phytochemical screening of the compounds, isolation of the active principles, and others. A comprehensive preformulation studies to guide formulation decisions have never been conducted. Therefore, this work aimed at characterising relevant physical and chemical properties of aqueous extracts from leaves of *Artemisia annua*, *Vernonia amygdalina*, and *Microglossa pyrifolia* to build a solid foundation for a stable and consistently bioavailable dosage form that can be produced massively using high-level technologies.

MATERIALS AND METHODS

Materials

The fresh plant leaves were collected from different regions within Uganda depending on their ethnobotanical use for malaria (*Artemisia annua*, *Microglossa pyrifolia*, and *Vernonia amygdalina* from Fort portal-0.6668° N, 30.2854° E; Bushenyi-0.524° N, 30.2173° E; and Mbarara-0.6152° S, 30.6522° E respectively). Solvents and reagents of both analytical and HPLC grade, including ammonia, methanol (HPLC grade), n-butanol, glacial acetic acid, n-octanol, acetonitrile (HPLC grade) and sulphuric acid used in the study were obtained from Loba Chemie (India). Chemical standards, including artemisinin, vernodalol, and linalool, were obtained from TargetMol® (USA) and quercetin from Sigma-Aldrich Co. (USA).

Plant authentication and processing of aqueous extracts

The herbarium specimens of the plant materials were authenticated at the national herbarium of Makerere University, Department of Botany. Accession numbers 51145, 51146, and 51147 were issued for *M. pyrifolia* (Mp), *V. amygdalina* (Va), and *A. annua* (Aa), respectively. The collected study samples were shade-dried at room temperature for two (2) weeks and milled to a coarse powder. The powder materials were infused in hot distilled water (1:8 powder: distilled water) for 1 h before filtering using a muslin cloth. Later, with Whatman's filter paper (No. 1). The filtrates were concentrated *in vacuo* using a rotary evaporator (IKA, Germany) at 50 ⍰ and finally freeze-dried (FD-1CL, USA). The final extracts (dry or semi-solid) were packaged and labelled in amber glass bottles and stored in a fridge (-4 ⍰) till use.

The percentage yield of the extracts obtained was calculated using the following equation.

$$\text{Percentage yield} = \frac{\text{Weight of dry extract (g)}}{\text{Weight of plant leaf powder(g)}} \times 100 \quad \text{..... Eq. 1.}$$

Phytochemical quantification of key antimalarial chemical markers

Total flavonoids in *Artemisia annua* extract

Total flavonoid content was estimated using the aluminium chloride method based on the previously described procedure [12]. The *A. annua* extract (0.5 ml) was separately mixed with 1.5 ml of solvent, 0.1 ml of 10% aluminium chloride to develop colour, 0.1 ml of 1 M potassium acetate was added to provide an alkaline pH and 2.8 ml of distilled water. The mixture was kept at room temperature for 30 min, and the absorbance of the reaction mixture was measured at 430 nm using a spectrophotometer (Jenway 6705, England). Quercetin standard was prepared following the same procedure in 1-9 µg/ml concentrations, and absorbance was measured to plot a calibration curve. Total flavonoid content was obtained using the standard plot and expressed as µg quercetin equivalent per mg of aqueous extract.

Artemisinin quantification in *Artemisia annua* extract

A standard plot was generated using dilutions of artemisinin (1, 10, 40, 80, and 160 µg/ml). These were prepared from the initial stock solution of artemisinin in methanol (5.6 mg in 5.6 ml of solvent). The stock solution (1 mg/ml) was diluted with the HPLC mobile phase to prepare the different dilutions, which were later filtered and injected into the HPLC system (Shimadzu, Tokyo, Japan).

The aq. extract of *Artemisia annua* was dissolved in distilled water (10 mg/ml) and labelled as solution I. Then, 1 ml of solution I was diluted with diethyl ether (9 ml) to make a final 1 mg/ml concentration. The mixture was then filtered and injected into the HPLC system.

The stationary phase was a thermostat column compartment on a Geminin® column (5 µm NX-C18 110Å; 250 mm x 4.6 mm), maintained at 30 °C. The volume of each sample injected was 10 µl, and the mobile phase system used consisted of acetonitrile: methanol: water in ratios of 50:20:30. Flow rate and the detection wavelength used were 0.8 ml/min and 220 nm, respectively. The quantity of the artemisinin was calculated using the standard plot and expressed as µg per mg of aqueous extract.

Vernodalol quantification in *Vernonia amygdalina* aqueous extract

Total sesquiterpenes lactones was determined using a method described earlier [13].

A standard plot was generated using dilutions of vernodalol standard (5, 20, 40, 80, and 200 µg/ml). These were prepared from the initial stock solution of vernodalol in methanol (1 mg/ml). The stock solution was diluted with the HPLC mobile phase to prepare the different dilutions, which were later filtered and injected into the HPLC system (Shimadzu, Tokyo, Japan).

The aq. extract of Va was dissolved in distilled water (10 mg/ml) and labelled as the solution I. Then, 1 ml of solution I was diluted with methanol (9 ml) to make a final concentration of 1 mg/ml. The mixture was then filtered and injected into the HPLC system.

The stationary phase was a thermostat column compartment on a Geminin® column (5 µm NX-C18 110Å; 250 mm x 4.6 mm), maintained at 35 °C. The volume of each sample injected was 20 µl, and the mobile phase system used consisted of acetonitrile: 1 % acetic acid in ratios of 45:55. Flow rate and the detection wavelength used were 1 ml/min and 254 nm, respectively.

The quantity of the vernodalol was calculated using the standard plot and expressed as µg per mg of aqueous extract.

Total terpenoids in aqueous *Microglossa pyrifolia* extract

Total terpenoids in the extract were determined using a method previously described with modifications [14].

A standard solution of linalool was prepared by dissolving it in 95 % methanol (1 mg/ml). The linalool solution (1 ml) of various quantities (0.2, 0.4, 0.6, 0.8, and 1 mg) was added to test tubes containing chloroform (2 ml) and vortexed for 3 min. Concentrated sulphuric acid (0.2 ml) was added slowly by the sides of the test tube, and the mixture was incubated in the dark at room temperature for 2 h. At the end of the incubation time, a reddish-brown precipitate was formed. The reaction supernatant was carefully decanted without disturbing the precipitate, which was dissolved in 95 % methanol (3 ml) and vortexed. The absorbance of the final solutions was measured at 302 nm using a UV spectrophotometer (Jenway 6705, England) and 95 % methanol as a blank. A graph of absorbance against concentration (mg/ml) was plotted (standard plot).

The aqueous extract of Mp was dissolved in distilled water (50 mg/ml). The solution was diluted with 95 % methanol in the ratio of 1:4 (extract: methanol). Then, 1 ml of the solution obtained was added to test tubes (in triplicates) containing chloroform (2 ml) and taken through the steps described above for the linalool standard for forming the precipitate. The absorbances were measured after that as stated for the linalool standard till measurement of the absorbance. The total terpenoid quantity of the extract as linalool equivalent was calculated using the regression equation of the linalool standard curve and expressed as mg per g of the plant extract.

Organoleptic properties

The organoleptic parameters (colour, odour, and taste) were evaluated using a panel of ten (10) volunteers as described by Abdulai and co-workers [15, 16]. The participants were presented with aqueous solutions of the extract in concentrations equivalent to the expected clinical dose.

For colour determination, the solution (20 ml) was poured on a Petri dish, and participants compared their observations to the colours on a standard chart provided. The colour of the extract was assigned based on their assessment. The most mentioned or suggested colour among the panellists for each sample was accepted.

For odour determination, each panellist was required to rate the smell of the samples based on their perceptions from 1 (unpalatable smell) to 7 (excellent smell). The panellists' scores were later converted to a % odour score.

For taste/bitterness, the panellists were provided with the solution samples (10 ml each). The 'rinse and spit' design was used with a swirling time of 20 s. Each panellist scored the bitterness on an anchored 7-point scale, where 1 is "not bitter at all", and 7 is "strongest bitterness imaginable". The bitterness was also converted to a % bitterness score. A dispersion of quinine (2×10^{-3} gl^{-1}) was prepared 24 h before the sensory assessment and used as a standard.

Solubility

An excess of the extract was dissolved in distilled water maintained at a temperature of 37 ⍰. The resulting saturated solution was filtered (using Whatman filter paper No. 5), and the concentration and quantity of the dissolved extract in the filtrates were determined by UV spectrophotometry (Jenway 6705, England). A calibration plot was prepared using the crude extract as standard at concentrations of 0.02, 0.04, 0.06, 0.08, and 0.1 mg/ml. The maximum wavelength of each extract was determined from a scan (200-800 nm), and the wavelength of the highest peak (absorbance) was employed.

Partition coefficient and permeability determination

This was determined using the shake-flask method as described previously [5]. Equal volumes of the saturated aqueous solution of the extracts were mixed vigorously with n-octanol in a separating funnel for 30 min. The two phases were then allowed to separate for 5 min, and the extract concentration remaining in the aqueous phase was determined. By difference, the concentration of the extract in the n-octanol phase was calculated as follows:

$$C_{n\text{-octanol}} = C_{aq.\ initial} - C_{aq.\ final} \quad \text{Eqn 2}$$

Where $C_{n\text{-octanol}}$–concentration in the n-octanol layer, C_{aq}–concentration in the aqueous layer

The partition coefficient was calculated as:

$$P_{o,w} = \frac{C_o}{C_w} \quad \text{Eqn 3}$$

Where $P_{o,w}$–partition coefficient, C_o–concentration of the extract in n-octanol and C_w–concentration of the extract in the aqueous phase.

The permeability was calculated using the following equation:

$$\text{Permeability} = \frac{\text{Conc in n-octanol}}{\text{Aqueous solubility}} \times 100 \quad \text{Eqn 4}$$

Hygroscopicity

The hygroscopicity was evaluated using a weather resistance and stability chamber maintained at 25±2 ⍰ and 80±2% RH [17]. Samples of each extract (300 mg) were weighed onto open and tarred glass Petri dishes of known weights and then placed in the chamber. After 24 h of storage, the samples were removed, and the final mass of each was determined. The percentage increase in mass (P) of each sample was calculated as:

$$P = \frac{M_3 - M_2}{M_2 - M_1} * 100 \quad \text{Eqn 5}$$

Where M_1–is the mass of the empty petri dish plate, M_2–is the initial mass of the plate and sample, and M_3–is the final mass of the plate and sample. The samples were classified for their hygroscopic nature as per the EP classification system.

pH

A 1 % (w/v) of extract solutions in neutralized distilled water were prepared, filtered and the pH read on a calibrated pH meter (Elektro Genesis, Germany). The procedure was repeated in triplicates.

Powder properties

The extract powders were prepared depending on their nature–the dry solid one (*V. amygdalina*) was merely milled and sieved (mesh 40) while the extracts existing in semi-solid state (*A. annua* and *M. pyrifolia*) were converted into granules using selected fillers as follows. The samples (Aa and Mp) were mixed separately with the fillers (table 1) in quantities sufficient enough to form a suitable wet mass utilizing the residual liquid in the semi-solid extracts without adding a granulation fluid. The wet mass was sieved (mesh 20) and oven-dried at 60 ⍰ for 40 min. The dry granules were milled and sieved (mesh 40) and then assessed for the following physical powder properties using standard methods [18].

Moisture content

This was determined using an automated moisture analyzer (Metler Toledo MJ33, Switzerland). The powder samples (≥0.5 g) were introduced to the equipment after tare. Upon closing the unit, the percentage moisture content was recorded for each sample after complete heating or evaporation. The procedure was done in triplicates for data reproducibility.

Angle of repose

The powder sample (10 g) was carefully poured through a funnel (stem diameter 10 mm, clamped 10 cm above the bench) onto a clean sheet of paper. The height (h) and the radius (r) of a heap of cone formed were measured (in cm). The angle was calculated as,

$$\alpha = \tan^{-1}(h/r) \quad \text{Eqn 6}$$

Bulk and tapped densities

The powder sample (20 g) was carefully poured into a measuring cylinder tilted at an angle of 45° to minimize tapping. The cylinder was placed on its base, and the volume occupied by the powder was noted, V_b. Thereafter, the cylinder containing each powder sample was tapped until a constant volume was obtained, and the final volume, Vt, was noted. The densities were therefore calculated as;

$$\rho_{bulk} = \frac{\text{Weight}}{V_b} \quad \text{Eqn. 7}$$

$$\boldsymbol{\rho_{tap}} = \frac{\text{Weight}}{V_t} \quad \text{Eqn. 8}$$

Compressibility index (CI)

This was calculated according to the equation,

$$CI = \frac{\rho_{tap} - \rho_{bulk}}{\rho_{tap}} * 100 \quad \text{Eqn. 9}$$

Hausner's ratio (HR)

This was also calculated from tapped and bulk density according to the following relationship,

$$HR = \rho_{tap}/\rho_{bulk} \quad \text{Eqn. 10}$$

Compressibility and tablet ability

The extract powder (Va) and granules (Aa and Mp) weighing 700 mg were compressed separately using a tabletting press at three different compression pressures ($P_1 < P_2 < P_3$). A dwell time of 30 s was maintained for each compression. Before compression, the die cavity (13.0 mm diameter) and the punches were lubricated using 0.5% dispersion of magnesium stearate in acetone. The compacts were kept in a desiccator for 24 h (to allow for elastic recovery, hardening and prevention of falsely low yield values) before any test. Dimensions of weight, radius (r) and thickness (h) were measured. The apparent density (ρ_A), of the compacts, was calculated using the formula $\rho_A = \frac{\text{Weight } (g)}{\text{Volume } (\pi r^2 h)}$ ..... Eqn. 11

Table 1: Granulation of Aa and Mp extracts

Ingredient	F1 (%)	F2 (%)
Va extract	43.9	-
Mp extract	-	63.5
PVP	8.5	8.5
Lactose	4.8	4.8
Maize starch (BP)	42.8	23.2
Total (%)	100	100

Aa–*Vernonia amydalina* aqueous extract, Mp–*Microglossa pyrifolia* aqueous extract, PVP–Polyvinylpyrrolidone

The Crushing strength (CS) of the compacts was determined using an automated hardness tester (VTHT-500, India) and their corresponding tensile strength (TS) was calculated as,

$$TS = \frac{2CS}{\pi dh} \quad \text{..... Eq. 12}$$

Where d is the diameter.

As previously described [18, 19], the following models were used to analyze the mode of deformation, compressional, and compaction characteristics of the samples.

A Kawakita plot of P/C Verses P was used to establish the modes of deformation and compressional characteristics of the powder samples, where $C = 1 - \frac{\rho_{bulk}}{\rho_A}$.

A graph of tensile strength against compression pressure was plotted to evaluate the tabletability of the powder sample (Va) and granules (Aa and Mp) [19].

Statistical analysis

The results were summarized as the mean±SD descriptive statistics. The data was analyzed using GraphPad Prism software version 8.0.2. The means of percentage of different groups were compared using one-way analysis of variance (ANOVA) for any significant differences among the groups, and Tukey's multiple comparison test was conducted to compare the means between the groups. Statistical significance was considered at $P \leq 0.05$.

RESULTS AND DISCUSSION

Quantification of antimalarial phytochemical markers

The percentage yields of aqueous extracts were 12.14, 18.5, and 15.5 % for *Microglossa pyrifolia* (Mp), *Vernonia amygdalina* (Va), and *Artemisia annua* (Aa), respectively. The calibration curve of the quercetin standard used for quantifying total flavonoids in Aa extract is shown in fig. 1. The Aa extract contained 11.14±0.26 % of total flavonoids quercetin equivalent and 0.27 % of artemisinin, as shown in table 2. The standard plot of the artemisinin used for calculating its concentration is also shown in fig. 2, and the accompanying HPLC chromatogram of the artemisinin standard and Aa extract are displayed in fig. 3. Flavonoids and artemisinin are the main phytochemical compounds in Aa that are known to be responsible for their antimalarial activity. Apart from boosting immunity through elevations of body defensive cells such as white blood cells and phagocytes [20], flavonoids in Aa have also been reported to increase bioavailability of artemisinin, thus contributing to its antimalarial effect [21].

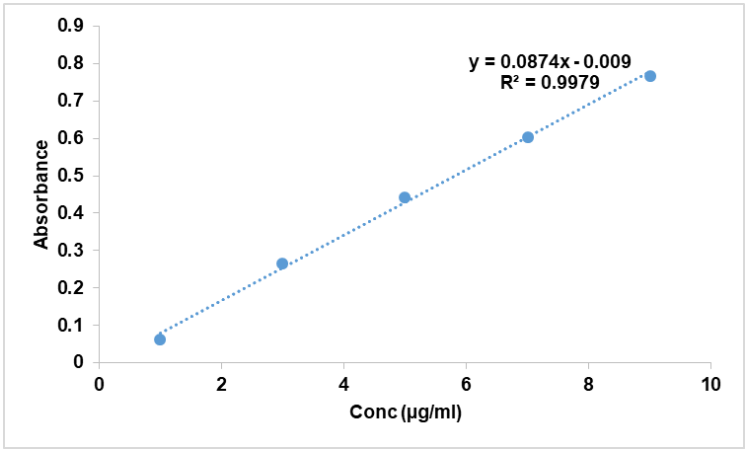

Fig. 1: Calibration curve for quercetin standard

The effect of Aa flavonoids on immunity has supported the use of plant infusions for malaria prevention in many parts of Uganda [20]. Therefore, using total flavonoid levels in the Aa extract as a phytochemical marker is important for monitoring the quality of the extract or its formulations and assuring the efficacy of such standardized products in the prevention or treatment of malaria. The concentration of total flavonoids in this study was higher than that in a previous study conducted on plant powder in Uganda [22]. This difference could be attributed to variations in the methods of quantifications, including the usage of casticin as a standard as opposed to quercetin used in this study. Similar total flavonoid contents (8-9 %) were reported in a cultivar of Aa grown in Brazil [23] and also in Iran (11.6 %) on *Artemisia annua* L. [12]. Therefore, total flavonoid quantification is a good phytochemical marker for Aa extract intended for malaria prophylaxis and treatment. The low level of artemisinin content in the aqueous extract is expected considering the method and solvent of extraction, and the quantity obtained in this work is not far from the previously reported in Aa infusions [20, 21]. The level of the compound in the extract is too low to be responsible for its antimalarial activity, so it works with other phytochemicals, as validated earlier [24]. However, using it as a phytochemical marker for monitoring the quality of products developed using the crude Aa extract as an active ingredient is a noble approach for natural dosage form standardization.

The vernodalol concentration of 0.07 % (table 2) in the Va aqueous extract was determined using the calibration plot presented in fig. 4, and the chromatograms for both the vernodalol compound and extract are shown in fig. 5. The sesquiterpene lactone compound is one of the known antimalarial active ingredients isolated from *Vernonia amygadalina* and its efficacy has already been reported [25]. The presence and quantity of the compound in a product containing Va extract is, therefore, useful as a chemical marker in its standardization.

The amount of vernodalol in this study was lower than the one reported in a previous study that focused on the isolation of the compound from Va and reported 0.7% of compound per ethanolic extract and 0.04 % per leaf powder [25, 26]. The variations in the levels of the compound may be attributed to the differences in the methods of extractions (aqueous vs ethanolic). On the contrary, another quantitative study that determined the level of vernodalol from both leaf and root powders extracted using aqueous and ethanolic (70 %) maceration and soxhlet techniques [27], reported that the compound was detected in the leaf extract but in unquantifiable levels. Geographical locations may be responsible for these differences, but in areas where the plant leaves are used for malaria treatment, studies have confirmed vernodalol as one of the key actives responsible for the therapeutic benefits [11, 24].

Table 2: Percentage levels of key antimalarial phytochemical markers in aqueous extracts

Phytochemical marker	Aa (Mean±SD)	Va (Mean±SD)	Mp (Mean±SD)
Total flavonoids (% quercetin equivalent, mg/mg)	11.24±0.26	-	-
Artemisinin (%, µg/mg)	0.27±0.02	-	-
Vernadolol (%, µg/mg)	-	0.07±0.00	-
Total terpernoids (% Linalool equivalent, mg/mg)	-	-	28.50±4.46

Aa–*Artemisinia annua*, Va–*Vernonia amygdalina*, Mp–*Microglossa pyrifolia*. Data presented as mean±SD, n=3.

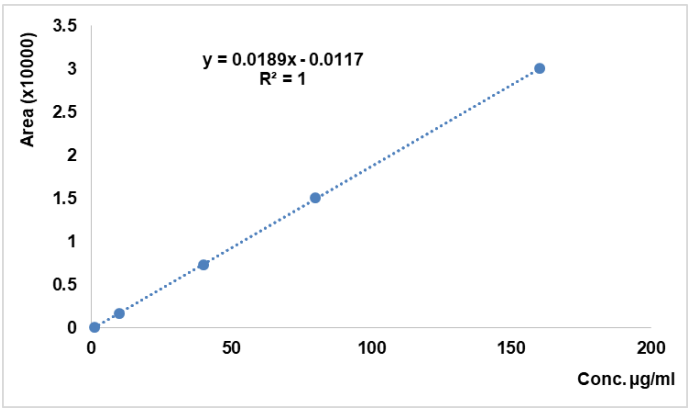

Fig. 2: Standard calibration plot for artemisinin, data plotted as mean, n=3

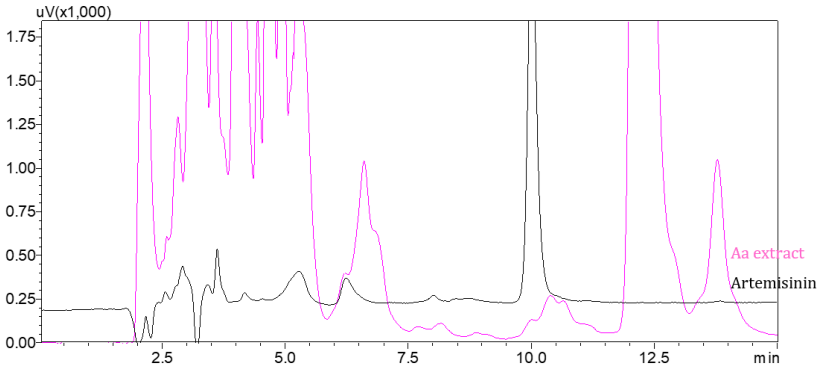

Fig. 3: HPLC chromatograms of artemisinin standard and Aa extract showing artemisinin peak at 10 min

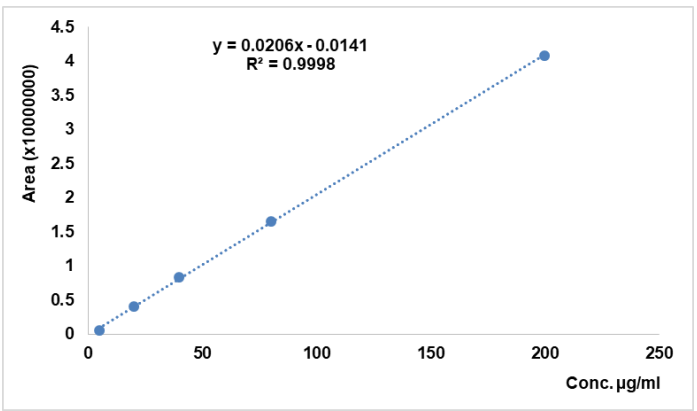

Fig. 4: Standard calibration plot for vernodalol

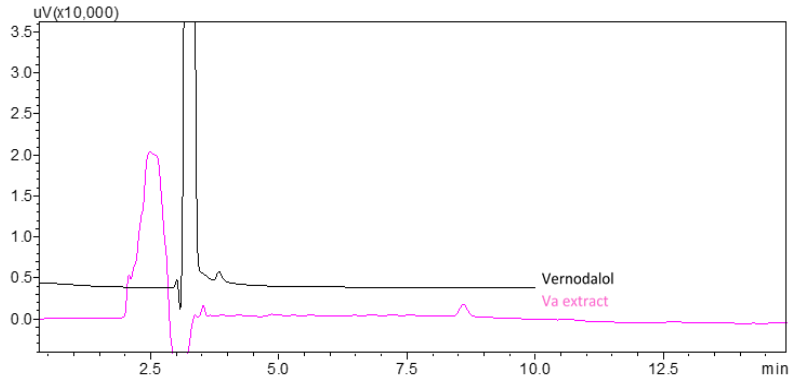

Fig. 5: HPLC Chromatograms for vernodalol standard and Va aqueous extract showing vernodalol peak at 3.3 min

The aqueous extract of Mp showed a high level of total terpenoids (Linalool equivalent), as shown in table 2. These have also been extensively studied as the main antimalarial actives in the plant with some specific terpenoids, including E-phytol, 1,3-hydroxyoctadeca-9Z, 11E, 15-trien-oic-acid and 6E-geranylgeraniol-19-oic-acid which have been isolated and their anti-plasmodial activities studied [28]. Quantification of the total terpenoids as a major antimalarial phytochemical marker is important because the crude aqueous extract had better efficacy in bioassay-guided fractionation compared to the isolated compounds [28], suggesting synergistic interaction among the terpenoids in the plant extract. Therefore, Mp-based antimalarial product formulation can be standardised using the total terpenoids, and no previous reports on the quantification of total terpenoids in the Mp plant exist in the literature to make comparisons.

Organoleptic analysis

The organoleptic analysis of the extracts (fig. 6) indicates that Aa and Va possess high bitterness comparable to that of the standard quinine solution (P = 0.84). Mp extract exhibited significantly lower bitterness than Aa and quinine sulfate (fig. 6A). This is in agreement with previous clinical studies on Va and Aa, where patients (especially children) showed low compliance with the infusion of the plants due to bitterness [29, 30]. The taste of the extracts also necessitates the need for sweeteners in a formulation containing the extracts to increase their palatability or other approaches that mask bitterness. In terms of odour, there was no significant difference among the extracts, though Aa showed the best smell (fig. 6B). However, the odour scores for the extracts are moderate and flavouring agents may be necessary, especially for oral antimalarial formulations, to improve product smell or aroma. Regarding the colour of the extracts, 80 % of the panellists assigned brown to Aa, and 10 % each assigned yellow-brown and greenish brown. Eighty-six percent (86 %) of the panellists gave brown colour for Va, and the remaining 14 % suggested black. Mp was considered brown by 60 % of the panellists, 20 % gave it colour black, and 10 % each assigned it coffee brown and greenish brown. Considering the highest scores for each extract, they are all brown. Unless deemed necessary for adding colourants in a formulation, the natural brown colour of herbal extracts is generally acceptable for their product formulations.

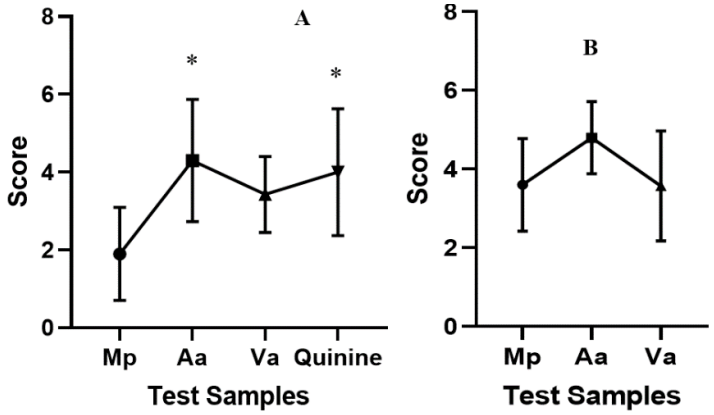

Fig. 6: The bitterness A and odour B scores are represented as mean±SD, n=10. *Statistically significant differences between Mp and the respective samples (Aa and quinine)

Solubility and partition coefficient/permeability

Aqueous solubility is a critical physicochemical parameter of new chemical entities (NCEs) or active pharmaceutical ingredients (APIs) as it influences the possibility of developing a product into an oral dosage form, determines the extent of drug bioavailability, and dictates the choice of solvents and excipients formulation stage [31]. Based on the study findings in table 3, Aa exhibited the highest aqueous solubility, followed by Mp, and Va showed the lowest. Meanwhile, Mp demonstrated the highest partition coefficient (P_{ow}), followed by Va, and lastly, Aa. The differences in the partition coefficients of the various extracts were statistically significant. The calibration plots for aqueous extracts of Mp, Va, and Aa used for estimation of their solubility and partition coefficient are shown in fig. 7. Drug compounds with aqueous solubility over 1 % (10 mg/ml) are considered to be bioavailable, while those between 1–10 mg/ml need techniques such as salt formation, cosolvency, and among others to improve their bioavailability if they are to be delivered by oral route [32]. Based on this, all extracts that demonstrated aqueous solubility's in the range, 49–147 mg/ml have a high chance of being bioavailable if given by oral route.

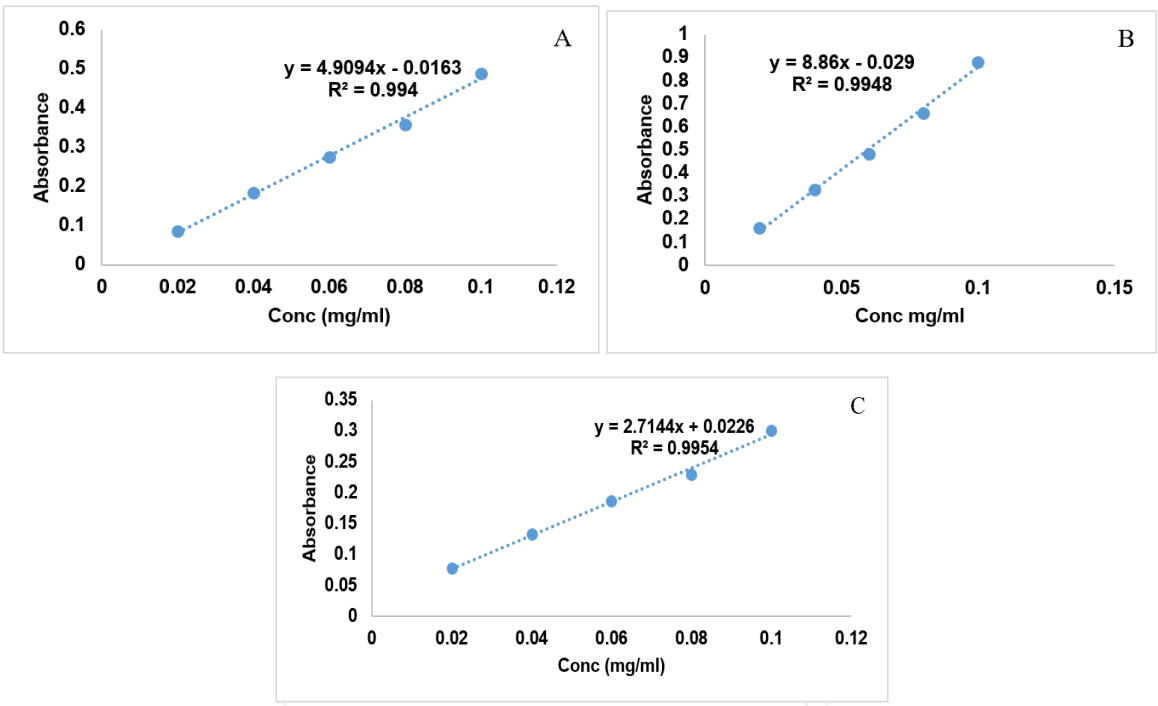

Fig. 7: Calibration plots for aqueous leaf extracts of A *Microglossa pyrifolia* (Mp), B *Vernonia amygdalina* (Va), C *Artemisia annua* (Aa)

As much as the high aqueous solubility of a drug is essential for easy drug dissolution in the body, which precedes drug absorption, the lipophilic nature of the cell membranes makes it challenging for such polar compounds to be absorbed. Therefore, a good NCE should have a balance between aqueous solubility and some lipophilicity for it to be bioavailable. Based on the polarity and lipophilicity classifications, drugs with P_{ow}<1 and LogP<0 are hydrophilic, and those with P_{ow}>1 and Log>0 are lipophilic [5], meaning Va and Aa extracts are hydrophilic while Mp is highly hydrophobic. The differences could be attributed to the nature of phytochemicals controlling the overall polarity of the extracts. For example, the aqueous extract of Mp is previously known to contain high levels of short chain fatty acids and terpenoids such as 6E-gera-nylgeraniol-19-oic-acid despite their non-polar character [28]. Therefore, the lipophilic behavior of Mp extract could be attributed to the presence of such compounds or phytochemical groups.

The LogP constant is also used to predict possible sites of drug absorption. For example, drugs targeting the central nervous system (CNS) should have a LogP of around 2, those for oral and intestinal absorption should have a LogP of 1.35-1.8, and LogP>5 is ideal for a sub-lingual drug delivery system [33]. However, Lipinski's rule of 5 states that a drug intended for oral administration should have a LogP<5. LogP of the extracts are below 5, as shown in table 3, so ideally, they should be suitable for the oral delivery route as used in local communities in the form of infusions.

According to the biopharmaceutical classification system (BCS), drugs are classified into four classes based on their solubility and permeability, with class I having high solubility (highest dose dissolvable in ≤ 250 ml) and high permeability (>90 % bioavailability in humans) [34]. Our extracts had solubility exceeding the specifications of class I considering their doses (200 mg/kg for Va and Aa, and 100 mg/kg for Mp) established in previous studies, but with low approximated permeability of less than 90 %, as shown in table 3. Based on the BCS, all the extracts fall under class III due to their high solubility and low permeability [34-36]. This indicates the need for techniques to improve permeability at the formulation stage. The high aqueous solubility of the extracts and low permeability could be attributed to the nature and complexity of phytochemical compounds possessed by each. However, the BCS definition of high permeability applies to the extent of absorption in humans, not our values obtained from the shake-flask method using n-butanol to mimic cell membranes. Therefore, our adoption of this principle is inconclusive; it just raises possible preliminary concerns that the formulation scientist should consider in developing a robust drug delivery system or dosage forms that ensure adequate bioavailability of the active phytochemicals in the extracts and satisfactory therapeutic benefits.

Hygroscopicity and pH

Hygroscopicity measures the ability of a material to absorb water or moisture when exposed to a humid environment. All the extracts showed high percentages of hygroscopicity ranging from 98 to 118 %, with Mp having values similar to Aa and Va. Meanwhile, Va had the highest hygroscopicity, statistically significant at P ≤ 0.05 compared to Aa (table 3). According to the European Pharmacopoeia (PhrEur) criteria for the classification of pharmaceutical ingredients based on hygroscopicity, those absorbing over 15 % (w/w) of moisture at the specified conditions adopted in our study are categorized as very hygroscopic [37]. Therefore, the three aqueous extracts fall under this class of hygroscopicity and need precaution regarding storage conditions and packaging to prevent chemical degradation, microbial growth, and other forms of instability due to moisture sorption.

The extracts also exhibited weakly acidic (Aa, pH = 6) to alkaline (Va, pH = 7) properties as presented in table 3. The pH of an NCE is usually important in predicting the sites along the GIT where it would be greatly absorbed, especially for those that undergo ionisation. According to this philosophy, weakly acidic drugs are more absorbed in acidic environments and weakly basic in alkaline sites since they will remain in their un-ionized state for easy permeability. This concept may also be applied to plant extracts with multiple phyto-compounds to predict the possible site of absorption of most compounds based on the extract's pH, but using the Henderson-Hasselbach equation to calculate the extent of ionisation may not be possible due to the many compounds it contains with varying and unknown pKa's. However, using a chemical marker in the extract with known pKa could solve this challenge. Therefore, based on the pH of our extracts, Aa would be absorbed more in the acidic regions ranging from the stomach (pH 1-3) to jejunum (pH 4.4–6.6), while Va and Mp in the ileum (pH 6.8–8.0) [38].

Table 3: Physicochemical parameters of the extracts

Parameter	Aa (Mean±SD)	Va (Mean±SD)	Mp (Mean±SD)
Solubility (mg/ml)	147.39±2.67**	49.06±0.43**	122.82±1.29**
Partition coefficient (P_{ow})	0.12±0.01**	0.40±0.00**	4.74±0.18**
LogP	-0.94±0.04**	-0.40±0.00**	0.68±0.02**
Permeability (%)	10.33±1.04	28.53±0.12	82.56±1.10
Hygroscopicity (%)	98.90±9.60*	118.08±6.82*	112.83±4.13
pH	5.94±0.01**	7.08±0.01**	6.82±0.09**
Moisture content (%)	14.44±0.58**	10.65±1.01**	3.62±1.49**
Angle of repose (◦)	18.75±0.59	17.58±0.33	18.98±0.93
Bulk density (g/ml)	0.51±0.02*(Va)	0.62±0.00*(Aa)	0.61±0.02*(Aa)
Tapped density (g/ml)	0.714±0.00	0.81±0.02	0.71±0.01
Carr's index (%)	28.10±3.23*(Va)	22.95±1.96*(Mp,Aa)	13.16±4.18*(Va)
Hausner's ratio	1.39±0.06*(Mp)	1.30±0.03 *(Mp)	1.15±0.06*(Va,Aa)

Aa–*Artemisia annua*, Va–*Vernonia amygdalina*, Mp–*Microglossa pyrifolia,* Data presented as Mean±SD, n=3. *-the means are statistically significant for the parameter, **-the means of all the groups or samples are statistically different from each for the particular parameter, *(Va,Mp,Aa)– the mean of the group is statistically significant to the group in bracket.

Powder properties

The aqueous extracts contained varying moisture contents ranging from 3.6 % for Mp to 14.4 % for Aa (table 3), and these were statistically significant at $P \leq 0.05$. The moisture content of a powder material is essential in ensuring its stability, as high levels of moisture promote the growth of microbes. In herbal extracts, it is usually influenced by the method, extent of drying and the nature of the extracts. Those containing some oily components and existing in a sticky semi-solid state may show higher moisture content values consisting of water and volatile oils. According to World Health Organization (WHO) specifications for herbal powder materials, the maximum allowable level should be<12 % [39]. Comparing this to our results for the extracts, only Aa has higher values, possibly due to its semi-solid nature. Therefore, it may require extensive drying using other methods, such as spray drying, to achieve lower levels of moisture, and the extract also needs to be stored at lower temperatures (below 4℃) to prevent microbial growth.

The angle of repose, which measures powder flowability, was low (<20°) in all the samples, as shown in table 3. A powder with an angle of repose<30° is considered to have excellent flow [32]. All the powder or granule samples were within the specification for excellent flow and are expected to flow freely during tabletting and other technological processes of producing solid dosage forms. Aa had the lowest bulk density (0.5 g/ml), which was significantly different from Mp and Va, while Mp had the highest bulk density (0.6 g/ml), similar to Va (table 3). The tapped density of all the powder samples had similar tapped densities ranging from 0.7 to 0.8 g/ml, comparable to previous studies [40]. These parameters (bulk and tapped densities) guide in understanding the packing characteristics of a powder material–the higher the density, the better the level of packing under free fall and agitation for bulk and tapped, respectively [41]. Consequently, Aa has the lowest packing density propensity at free fall. Still, all three powder samples are packed at the same level on tapping and are expected to occupy similar volumes during capsule filling, which utilises the tapped density property to know the quantity of powder a specific capsule shell can accommodate. The compressibility and Hasner's indices are also additional indirect methods for classifying powders in terms of flow. Aa showed the highest compressibility index (28 %), followed by Va, then Mp (13 %), as shown in table 3. The same trend was observed with Hausner's ratio (HR), Aa>Va>Mp, ranging from 1.3 to 1.2. The lower the indices, the better the flow; therefore, the flow order is Mp>Va>Aa. Further classifications for CI values between 5–15 % indicate excellent flow as exhibited by Mp, 12–16 % as good flow, 18-21 as fair to passable flow, and 23–35 as poor, as shown by Va and Aa [6]. HR below 1.25 is also classified as good flow, and>1.25 indicates poor, but within between 1.25 and 1.5, the flow can be improved by adding a glidant [5]. The HR values of our samples still agreed with the previous flow trend (Mp>Va>Aa), with Va and Aa powders requiring additions of glidants in future solid dosage form development. The poor flow parameters (HR and CI) reported in this study for Aa and Va powders could be attributed to their higher moisture contents since this increases cohesion between particles and decreases flowability [42].

Compressibility and tabletability

The compressibility of the powders was evaluated using Kawakita's model, in which a plot of P/C (degree of powder volume reduction) against P (compression pressure) was obtained, as shown in fig. 8 A. The Kawakita constants *a* and *b*, which relate to the initial densification of the powder before compression and the yield pressure, respectively, were used to understand the compressibility of the powder. Analysis of the Kawakita plots had R^2 values (table 4) close to 1 in the order Aa>Va>Mp, all indicating high linearity and significant correlation between the parameters. This level of correlation in the model suggests plastic deformation is the main mechanism of powder compaction [43]. The same trend was also seen with the total degree of volume reduction constant *a* (Aa>Va>Mp). Higher values signify better densification and consolidation at infinite compression pressures [44]. Therefore, volume reduction followed the trend indicated. The reciprocal of *b* (P_k) was lowest for Va, followed by Aa and Mp. This pressure parameter P_k refers to the pressure that causes a 50 % reduction in the volume of the powder material on compression, and the lower this value, the higher the degree of plastic deformation. Therefore, considering the general low values of the Pk for all the powder samples, plastic deformation may occur for all of them. Powders that undergo plastic deformation on compression are desirable for producing elegant tablets or compacts with sufficient hardness or tensile strength.

The tabletability of the powder samples was determined using the tabletability model, which is a quantitative approach utilising the area under the curve (AUC) of the plot of tensile strength (TS) of powder compacts against compression pressure (P), as presented in fig. 8B.

The model is based on the principle that the tensile strength of a tablet or a compact increases with pressure. The ability of a material to form tablets with sufficient strength is usually best assessed using the AUC of the plot–the higher the value, the greater the tabletability [44, 19]. In agreement with the Kawakita model, the AUC for Aa was highest, followed by Va, and Mp (table 4). This could be attributed to a higher level of plastic deformation, as reported earlier by the Kawakita plot. Analysis of the plots (fig. 8B) indicates that Aa still showed an exponential increase in TS at the highest pressure (P3) compared to Va and Mp, which flattened after P1 and P2 pressures, respectively. This indicates that the two powder samples undergo fragmentation at these pressures, and there is a need to add other binders in a tablet formulation with Va and Mp as the main active to obtain stronger tablets that can withstand distribution and transportation stress conditions.

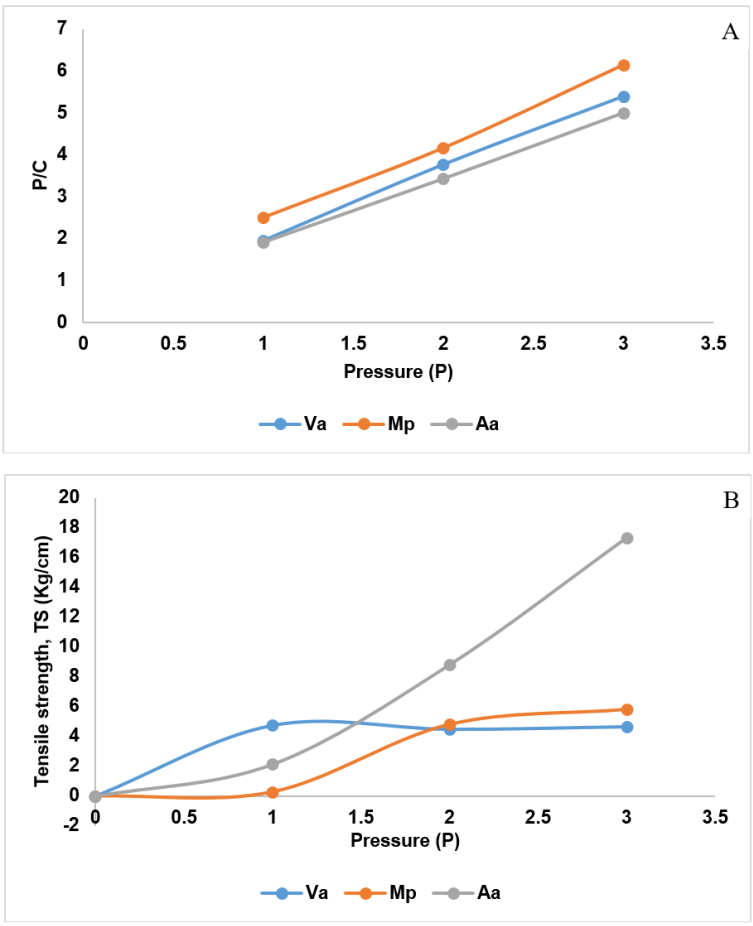

Fig. 8: Showing Kawakita plots A and tablet ability plots B for the different extract powders or their powder mix. Data plotted as Mean, n=3. P/C is a Kawita constant which measure change in compact density with respect to pressure (P), Va–*Vernonia amydalina*, Mp–*Microglossa pyrifolia*, and Aa–*Artemisia annua*

Table 4: Constants of kawakita and tablet ability plots

Constants	Aa	Va	Mp
a	0.649	0.583	0.55
b	4.16	6.258	2.857
P_k	0.240385	0.159795	0.350018
R^2	0.9999	0.999	0.9974
AUC	18.50234	9.175	7.854544

Aa–*Artemisia annua*, Va–*Vernonia amydalina*, Mp–*Microglossa pyrifolia*, a–Kawakita constant relating to total degree of powder volume reduction, b–Kawakita constant relating to yield strength of particles, P_k–1/b, R^2–correlation coefficient for Kawakita plot, AUC–Area under the curve for tabletability plot.

CONCLUSION

Applying preformulation profiling to herbal crude extracts yields equally invaluable physicochemical information that can guide formulation decisions, just like synthetic new chemical entities (NCEs). Therefore, the Aa, Va, and Mp aqueous extracts can be chemically standardised based on the selected antimalarial chemical markers and their profiled chemical and physical properties utilised for the development of stable and modern herbal drug delivery systems or dosage forms.

ABBREVIATIONS

Aa-*Artemisia annua*, ANOVA-Analysis of variance, API-Active pharmaceutical ingredient, AUC-Area under the curve, CI-Carr's index or compressibility index, CS-Crushing strength, HPLC-High-performance liquid chromatography, HR-Hausner's Ratio, Mp-Microglossa pyrifolia, NCE-New chemical entity, TLC-Thin layer chromatography, TS-Tensile strength, Va-*Vernonia amygdalina,* WHO-World Health Organization

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AUTHORS CONTRIBUTIONS

JRA conceived the research idea, participated in the experiments and data analysis, and drafted the manuscript. CA participated in the experiments, especially the HPLC quantification of chemical markers, and reviewed the manuscript. JT and NCN participated in conceptualisation, provided technical guidance and mentorship throughout the study, and reviewed the manuscript.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests

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