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Pharmacognostic and phytochemical studies as an invaluable approach for correct identification of medicinal plants: The case of *Artemisia vulgaris* L. substituted for *Artemisia annua* L. in Western Uganda

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Competing interests

The authors declare no conflicts of interest.

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Abbreviations

A. Vulgaris, *Artemisia vulgaris* L.; *A. annua*, *Artemisia annua* L.; Rf, retention factor; HPLC, High-performance liquid chromatography; TFC, total flavonoid content; TLC, Thin layer chromatography.

Citation

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Abstract

Background: Different parts of *Artemisia vulgaris* L. (*A. vulgaris*) are ethno-medicinally used as an emmenagogue and for the treatment of ailments such as malaria fever, ulcers, and cancer. However, anecdotal evidence shows that the plant is often substituted for *Artemisia annua* L. (*A. annua*) by herbalists in Western Uganda due to similarities in their morphology. Misidentification of medicinal plants and mislabelling of herbal products have been incriminated in toxicity and adverse health outcomes in traditional medicine practise. Because safety continues to be a major issue with the use of herbal remedies, it becomes imperative therefore that medicinal plants should be correctly identified. **Methods:** This study focused on investigating the macroscopic, microscopic, physicochemical characteristics and phytochemical composition of *A. vulgaris* leaves compared to *A. annua* to ease its correct identification. **Results:** The results showed that there are some colour differences between the leaves of the two species, with a close arrangement of microscopic features but different leaf constants. The leaves of the two Artemisia species had similar tastes, but their shapes and colours (greenish-yellow for *A. annua* and dark green for *A. vulgaris*) can be used by the local community to distinguish between them. The artemisinin content was higher in *A. vulgaris* leaves (1.72 %) than in *A. annua* (1.43%), but the reverse was observed for the total flavonoid content. **Conclusion:** This observation could justify the change in the use of *A. vulgaris* by the indigenous community in western Uganda. Further studies should consider the pharmacognostic comparison of *A. annua* with other species in the genus Artemisia and the use of molecular techniques such as DNA barcoding.

Keywords: Artemisinin; traditional medicine; misidentification; microscopic studies

Highlights

For the first time, pharmacognostic and phytochemical studies has been applied for correct identification of the medicinal plant Artemisia vulgaris which is usually substituted for Artemisia annua in Western Uganda traditional medicine.

Medical history of objective

Artemisia vulgaris L. (*A. vulgaris*) is a medicinal plant widely used as an emmenagogue and for the treatment of ailments such as malaria fever, ulcers, and cancer. However, anecdotal evidence shows that the plant is often substituted for *Artemisia annua* L. (*A. annua*) by herbalists in Western Uganda due to similarities in their morphology. Such misidentification and subsequent substitution can lead to adverse outcomes in traditional medicine practice.

Graphical abstract (Figure 1)

Introduction

Artemisia L. is the largest heterogeneous genus of the Asteraceae Martynov (synonym: Compositae Giseke) in the main group of flowering plants [1]. It is also the largest genus of the Anthemideae Cass and subtribe Artemisiinae Less. The genus takes its name from the Greek goddess *Artemis*, which means Diana [2, 3]. They are widely distributed in temperate areas of Europe, Asia and North America with marked preference for the Northern Hemisphere and laxity in colonising the Southern Hemisphere [1]. The genus consists of more than 500 acclimatized perennials, biennials, and annual herbs or small shrubs [4]. Species from this genus (*Artemisia vulgaris*, *A. dracunculus*, *A. absinthium,* and *A. annua*) are revered in both traditional and contemporary medicine. This is primarily due to the presence of bitter antimalarial sesquiterpenoid lactones (such as arglabin and artemisinin) and essential oils [2, 5].

One of the most important species in the *Artemisia* genus is *A. vulgaris,* which is (with the exception of Antarctica) distributed throughout the world in natural habitats, specifically in Southeast Asia, Australia, South America (mainly Brazil), North America, the Pacific islands and Africa [6–9]. It is commonly known as mugwort. In folk and traditional medicine, different parts of *A. vulgaris* are used to treat a variety of ailments, such as stomach ulcers, worm infestations, epilepsy, vomiting, cholera, indigestion, liver disorders, leprosy, restlessness, insomnia, and amenorrhoea [9–11]. Artemisinin is known to be the principal compound and biomarker in most *Artemisia* species, which is responsible for its antimalarial and other biological activities [12]. On the other hand, *Artemisia annua* L. (common name: annual absinthe) is a renowned annual herbaceous herb, hence its specific name "*annua*" [13]. The species is cultivated in Africa, Asia, Europe and America where it is used as a dietary condiment and an antimalarial herb [14, 15].

In Kamwenge district of western Uganda, anecdotal evidence suggests that native communities have generally substituted *A. vulgaris* for *A. annua* for the preparation of antimalarial infusions that can also be used to other ailments. This is because *A. vulgaris* grows abundantly as a weed with a morphology almost similar to *A. annua* [16]. A similar misidentification and possible substitution of *A. apiacea hance* (*Qing hao*) with *A. annua* (*Huang hua hao*) was cited in China [17, 18]. Misidentification of medicinal plants and mislabelled herbal products can lead to reduced effectiveness of active ingredients or adverse health events in traditional medicine [19, 20]. Because safety continues to be an important issue with the use of herbal remedies, it is imperative, therefore, that medicinal plants are correctly identified [21].

Various diagnostic approaches used for the correct identification of medicinal plants. These include pharmacognostic and phytochemical studies, DNA barcoding, and genomics [22]. Classical taxonomic techniques based on morphology and anatomy are simple and complement molecular techniques to accurately identifying morphologically similar and closely related and similar morphologically plant taxa [22]. Advanced molecular techniques (especially DNA barcoding) are important for the commercial identification of medicinal plants and their products, where they may be in the form of dried or powdered plant organs. This can contribute to their safe use and prevent adulteration [23, 24].

Currently, there are no data on diagnostic features for *A. vulgaris*; therefore, the present study was designed to carry out a pharmacognostic investigation of features such as physicochemical and phytochemical characterisation of *A. vulgaris* to differentiate it from *A. annua*. This knowledge, basing on simple features is useful for the local community, ethnobotanists and phytochemists who may not be familiar with more advanced techniques of identifying medicinal plants.

Methods

Collection and preparation

Fresh leaves of *A. vulgaris* were harvested from Kamwenge district, while those of *A. annua* were obtained from Fort Portal city in December 2021, during their flowering stages. The leaves were identified and authenticated by a taxonomist and assigned voucher numbers; IK-001 and IK-002 for *A. annua* and *A. vulgaris*, respectively, at the Makerere University Herbarium, Kampala, Uganda.

The fresh leaves were separately air dried at room temperature for two weeks and then crushed with a pestle and mortar to obtain the coarse powders. The powdered samples were labelled, stored in tightly closed containers, and kept in a dry place until the beginning of the analysis.

Macroscopic and organoleptic studies

Fresh leaves of *A. annua* and *A. vulgaris* were subjected to examinations of macroscopic characteristics, including leaf arrangement, leaf type, leaf margin, leaflet venation, leaflet shape, leaflet size, leaflet apex, and base. In addition, their organoleptic properties, namely taste, odor, texture, and colour were determined [25].

Microscopic studies on leaves

Surface preparations were made by peeling off the upper and lower epidermis of the fresh leaves. Transverse sections of the leaves were also prepared with the aid of razor blades and cleared in sodium hypochlorite solution. The cleared samples were then stained with safranin O. The samples were then mounted with diluted glycerol and

examined with a microscope [26].

Microscopic features of these plants, such as the type, nature, and size of epidermal cells, trichomes, and stomata, were observed and measurements were made using a calibrated eyepiece micrometre [27].

Quantitative-leaf microscopic studies

Palisade Ratio
Using an objective microscope with a camera, we examined sections of the upper epidermis that had been cleansed with a diluted sodium hypochlorite solution, placed them on a clean microscope slide with diluted glycerol, and traced the epidermal cells and palisade cells. To draw attention to clusters of epidermal cells, we sketched and connected the outlines of groups of four cells. The palisade cells were isolated, traced and counted. Those who had more than 50% of their body covered in epidermal cells were considered. The palisade ratio for that set was calculated by taking the result and dividing it by four [25, 26].

Stomatal Number
Sections of the upper and lower epidermis of the leaves of the plants were cleared with sodium hypochlorite solution and mounted on microscopic slides with diluted glycerol. The stomata were traced and counted in the fields in a single section of the leaves of each of the two plants with the aid of a microscope connected to a camera, and the average number of stomata per mm^2 of the epidermis was calculated [26].

Stomatal index
Sections of the epidermal portion of the leaves were mounted and examined as described above. Both stomata and epidermal cells were counted in the viewed field. The stomatal index was calculated following the procedures outlined by Evans [26].

Vein–islet Number
The number of vein islets (the minute area of photosynthetic tissues enclosed by the ultimate division of conducting strands) per mm^2 was calculated in four contagious millimetre squares in the central part of the leaf of the lamina of the two species midway between the midrib and the margin. It was determined by boiling pieces of leaves of the plants in a test tube containing a 70% chloral hydrate solution. This was followed by hydrochloric acid (10% v/v) to remove calcium oxalate crystals, thus improving visibility. A camera connected to a microscope was used to estimate an area of 1 mm2 using an X10 objective to view cleared leaf preparations, and the veins were traced in 4 contagious squares. Each vein was traced and the areas in which the veins were completely enclosed were counted and those not entirely enclosed were excluded [26].

Vein-let termination number
The number of vein-let terminations per mm^2 of the leaf surface of the two plants was determined. A camera connected to a laptop was used to view the leaf surface, divided into squares of mm^2, and vein-let terminations in each square were counted square to get the vein-let termination number [26].

Physicochemical parameters
The leaf powder was analyzed to assess its physicochemical properties, including its moisture content, total ash, acid-insoluble ash, water-soluble ash, sulfated ash, and water-soluble and alcohol (ethanol) soluble extractive values [25].

Moisture content
For each powdered leaf sample, 3 g was weighed in a conditioned moisture dish, then heated for 1 hour at 105 °C, cooled in a desiccator, and reweighed. This procedure was carried out five times to maintain steady weight. The proportion of moisture in each plant was then determined.

Total ash
Different amounts (3 g) of powdered leaf samples were weighed in a crucible. The crucibles were then slowly heated to 600 °C, at which point the contents were burnt. After being heated to remove any carbon, the samples were allowed to cool before being weighed. The total ash content was calculated.

Acid-insoluble ash
Quantitatively and carefully, the crucible and total ash obtained above for the two powdered leaf samples were poured into a beaker containing 25 mL of 2M hydrochloric acid. After boiling for 5 minutes, the mixture was filtered through ash-free paper to remove the insoluble ash. After that, hot water was used to clean the beaker that had held the acid and the crucible. The washes were continuously filtered through ash-free filter paper until the acid was completely removed. Last but not least, the residue and filter paper was dried in an oven, then heated to 450 degrees Celsius in a tarred crucible for 90 minutes, let to cool and finally weighed. Each leaf sample had its acid-insoluble ash value calculated as a percentage.

Water-soluble ash
The total ash obtained was then boiled for 5 minutes with 25 mL of distilled water; ashes-free filter paper was used to collect the insoluble material before burning at a constant weight of 450 degrees Celsius for 15 minutes. The amount of ash that could be dissolved in water was calculated by subtracting the mass of insoluble materials from the total mass of the ash. For both powdered leaf samples, we calculated the percentage of water-soluble ash.

Sulphated ash
After heating a crucible to redness for 10 minutes, we weighed it after it had cooled in a desiccator. Each powdered leaf sample was 3 grams in weight and then it was added to the crucible. Then, slowly starting to burn, the crucible was set aflame until the stuff inside was completely burnt. After cooling the residue and moistening with 1 mL of concentrated sulphuric acid, it was heated gradually until no white fumes emerged and then burned at 800 °C until all black particles had vanished. As the crucible cooled, a few drops of strong sulfuric acid were added and brought to a boil. The mixture was repeatedly ignited, cooled, and weighed. The percentages of sulfated ash were calculated for both powdered leaf samples.

Alcohol-soluble extractive value
Using a flask shaker, we macerated 4 g of powdered leaf samples in 100 mL of ethanol for 24 hours, shaking the flasks frequently for the first 6 hours. After filtering the mixture, 25 mL of the filtrate was dried to a constant weight in a boiling water bath in a beaker. Then they were weighed after being dried in a 105 °C oven for 6 hours, cooled in a desiccator for 30 minutes and transferred. Each leaf sample had its extractive value in alcohol calculated as a percentage.

Water-soluble extractive value
On the first day, we macerated 4 g of powdered leaf samples in 100 mL of 0.5% chloroform water for 24 hours, shaking the flasks frequently for the first six hours. For each sample, 25 mL of the filtrate was taken, dried to constant weight in a boiling water bath, then baked at 105 °C for 6 hours, chilled in a desiccator for 30 minutes, and weighed immediately afterwards. Each leaf was tested to see what proportion of its extractive value could be dissolved in water.

Extraction of powdered leaf samples
For maceration, 20 g of each powdered leaf sample was placed in a 250 mL conical flask and macerated with 200 mL of analytical grade methanol, hexane, and diethyl ether at room temperature with regular shaking for 48 hours. Each extract was filtered through muslin cloth and then by Whatman No.1 filter paper individually. The surplus solvent was evaporated using a low pressure rotary evaporator. The extracts were dried in the oven, weighed and stored in airtight containers at 4 °C.

Phytochemical screening of leaf extracts

Test for alkaloids
Approximately 0.5 g of extracts of methanol, hexane, and diethyl ether were weighed out and placed in their own test tubes from leaf samples. Then we added 5 mL of acetic acid in ethanol (10% v/v) and left it alone for 4 hours. The filtrated extracts were concentrated in a water bath until only a fifth of the original volume remained. A 28% ammonium hydroxide solution was added to a small volume of concentrated filtrates from extracts from the two plants to raise the pH to 9.4. Subsequently, 1 mL of chloroform was added, and the mixture was gently stirred to create distinct layers. Mayer's and Wagner's reagents were used to detect the presence of alkaloids in the aqueous layer, while the chloroform layer was discarded. Mayer's reagent was added dropwise to 1 mL of each filtrate, and the presence of a cream-colored precipitate indicated a favourable result. The Wagner's test was positive when a brownish-red precipitate formed after adding 0.5 mL of Wagner's reagent dropwise to the second 1 mL of each filtrate [25].

Test for carbohydrates

Molisch's test
Measured 1 g of extracts were separately dissolved in distilled water in separate test tubes and filtered. Four drops of Molisch's reagent were added, followed by three drops of concentrated Sulphuric acid by the side of the test tube. The appearance of a reddish-violet or purple ring at the junction of the two layers indicated the presence of carbohydrates [27].

Fehling's test
To 1 g of extracts in separate test tubes, 1 mL of distilled water was added. The test tubes were then heated in a water bath; equal volumes of Fehling's solutions were added drop by drop to the test tubes. The appearance of brick-red precipitation was considered positive for the presence of reducing sugars [25].

Test for flavonoids

Sodium hydroxide test
An equal volume of the leaf extract samples was reconstituted with water and filtered. Exactly 5 mL of each solution was taken, and 10% NaOH solution was added separately. A yellow solution that became colourless with the addition of dilute HCl was considered positive for flavonoids [27].

Lead acetate test
To 5 mL of extracts of two leaf extract samples separately, a lead acetate solution was added. A yellow precipitate indicated the presence of flavonoids [28].

Ferric chloride test
To 1 g from extract of two leaf extracts samples, separately in a test tube, 8 mL of distilled water and five drops of 10% ferric chloride solution were added together. A green or blue precipitate indicated the presence of a phenolic nucleus [28].

Test for glycosides (Killer Killani's test)
1 g of the extracts was dissolved in 2 mL of glacial acetic acid that had been spiked with a single drop of ferric chloride solution in each test tube. A lower layer was then made painstakingly by adding 1 mL of pure sulphuric acid. Glycosides were detected by the formation of a brown ring at contact [25].

Test for sterols and triterpenoids

Salkowaski's test
To 1 g of each extract in a test tube, 2 mL of chloroform was added to dissolve it and then filtered. Measured 3 mL of concentrated sulphuric acid measured was then carefully added to the filtrate to form a layer. A reddish-brown coloration at the interface was considered positive for a steroidal ring [29].

Lieberman-Burchard's test
To 1 g of extracts of extracts in separate test tubes, 2 mL acetic anhydride and 2 mL chloroform were added gently and stirred. Exactly 1 mL of concentrated sulphuric acid was added at the bottom and the formation of a reddish-green or violet brown ring was considered positive for the presence of steroid/terpenoids [25].

Test for Tannins

Ferric chloride test
The leaf extracts (1 g) were stirred separately with 10 mL of distilled water and filtered. A few drops of 1% ferric chloride solution were added to 2 mL of the filtrates. The occurrence of a precipitate of blue-black (hydrolyzable tannins) or brownish green (condensed tannins) was considered positive for tannins [25].

Bromine solution test
To 1 g of extracts in different test tubes, three drops of bromine water were added, the formation of a blue-coloured precipitate was considered positive for tannins [25].

Saponin test (frothing test)
To 1 g of extracts in different test tubes, 5 mL of water was added and stirred for 15 seconds. The appearance of a frothing column that remained for at least 15 minutes was considered evidence of the presence of saponins [29].

Test for Anthraquinones (Borntrager's test)
After adding 10 mL of benzene and stirring for 5 minutes, filtering, and adding 5 mL of 10% ammonia solution to the filtrates, 1 gram of extracts was used. After shaking the containers, the existence of free anthraquinones was confirmed by the appearance of a pink, red, or violet hue in the ammonia (below) [25].

Test for proteins and amino acids

Biuret test
Two or three drops of copper (II) sulfate solution were added to the alkaline filtrate of the extracts (a 0.5% w/v solution of the test residue). The presence of proteins and free amino acids was suggested by the presence of a reddish-violet hue.

Xanthoproteic test
Extracts of 2 mL were given 0.5 mL of strong nitric acid through a small hole on the side of the test tube. The presence of yellow indicated the existence of proteins and amino acids.

Ninhydrin test
After warming the extracts, three drops of Ninhydrin reagent were applied. Due to the presence of amino acids, a violet or purple hue has been generated [30].

Thin-layer chromatography of leaf extracts
Measured 0.5 g of the extracts from the two leaf samples were separately dissolved in their respective solvents (methanol, hexane, and diethyl ether), and then spotted on thin layer chromatographic plates precoated with silica gel (TLC silica gel 60 F254, 20 × 20 cm). Spots were allowed to dry and developed with the Hexane: Ethyl acetate (3:1, v/v) solvent system that was established by optimizing the solvent ratios. Visualization of the chromatograms was carried out under normal light, UV light (254 nm and 365 nm) and *p*-anisaldehyde sulphuric acid reagent. The retention factor (Rf) values were then calculated.

Extraction and quantification of artemisinin in the crude extracts

Extraction procedure

Measured 20 g of the dried powder of *A. vulgaris* leaf samples were exhaustively extracted at room temperature by maceration with 200 mL of diethyl ether for 24 hours. The extracts were then filtered, and the residues were rinsed with more diethyl ether. The filtrates were then concentrated using a rotary evaporator, and 100 mg of the extracts obtained were dissolved in 10 ml of 100% acetonitrile of high performance liquid chromatography grade.

Preparation of standard solutions of artemisinin

Ten mg of artemisinin standard (purity, 98%), purchased from Sigma-Aldrich, Inc. Germany) were dissolved in 10 mL of high-purity methanol for high-performance liquid chromatography (HPLC). The calibration curve for artemisinin was then generated by preparing serial dilutions of 10, 40, 80, and 100 g/mL in the mobile phase (methanol: acetonitrile: distilled water, 2:5:3, v/v).

Separation, detection, and quantification of artemisinin

We used a Shimadzu Model HPLC system (Shimadzu, Tokyo, Japan) for the separation; it had an LC-20AD pump, SPD-20 Aphotodiode array detector, CBM-20A system controller, and a rheodyne injection valve with a 20 l injection loop.

Both the artemisinin standard and the samples were pipetted into a 10 L container and injected at 30 ˚C into a Hyper Clone BDS C18 column (5 m; 250 mm × 4.6 mm) using SIL-20AC HT autosamplers equipped with thermostat column compartments.

This study used a mobile phase consisting of 2 volumes of methanol, 5 volumes of acetonitrile, and 3 volumes of distilled water, flowing at a rate of 0.8 mL/min, with a detection wavelength of 220 nm. By comparing sample peak regions with those of the standard curve, quantitative analyses were carried out using external standardization.

Equation 1 was used to calculate the artemisinin concentration as a function of dry extract weight.

$$A = \left(\frac{C \times V}{W(1-M)}\right) \times 100 \qquad (1)$$

Where C is the artemisinin concentration in the sample (mg/L), V is the final makeup volume (L) of the sample, W is the weight of the dry extract of the sample used (mg) and M is the moisture content of the dry powdered leaves.

Determination of the total flavonoid content in leaf samples

The total flavonoid content was determined by modifying the aluminium chloride colorimetric assay reported by Chang et al. [31]. Quercetin (10 mg) purchased from Sigma-Aldrich, Inc. Germany was dissolved in methanol and further diluted at concentrations of 10, 25, 50, 75, and 100 g/mL using ethanol. Ten milligrams of each extract (methanol and diethyl ether) were dissolved in 5 mL of 95% ethanol, and the resulting solution was transferred into a 10 mL volumetric flask and filled to the line. In addition, a 1M sodium acetate solution and a 10% aluminium chloride solution, both in a volume of 0.1 mL, were made using distilled water.

Extract stock solutions and standard quercetin dilutions were withdrawn in amounts of 1 mL and deposited in individual test tubes. The following were added to each test tube and thoroughly combined: 1.5 mL of 95% ethanol, 0.1 mL of aluminium chloride solution, 0.1 mL of sodium acetate solution, and 2.8 mL of distilled water. Both blank samples and the leaf extracts were produced in the same manner for both sets of standards.

Before measuring absorbance, all solutions were filtered with Whatman filter paper No. 1. The quercetin calibration curve was created by measuring the absorbance of quercetin solutions at 415 nm with a UV-Vis spectrophotometer (JENWAY, UK). The concentrations of the extracts were calculated using absorbance values and a calibration curve. Applying Equation 2, we were able to determine the total flavonoid concentration (TFC) in micrograms of quercetin equivalents per milligram of dry extract.

$$TFC = \frac{cV}{m} \qquad (2)$$

Where c = concentration of quercetin obtained from the calibration curve (µg/ml), V= volume of extract (mL) and m = mass of extract (g).

Results

Macroscopic and organoleptic characters

Macroscopically, the leaves had similar arrangement, shape, margin, and type. A difference was observed, especially in colour and taste (Table 1).

Table 1 Comparative macroscopic and organoleptic characteristics of *A. annua* and *A. vulgaris*

Parameter	Observations	
	A. annua	*A. vulgaris*
Leaf arrangement	Alternate	Alternate
Leaf type	Simple	Simple
Leaflet margin	Lobed	Lobed, serrate
Leaflet venation	Pinnate	Pinnate
Leaflet shape	Elliptical and lanceolate	Elliptical and oblong
Leaflet size	3.1 cm (long) and 2.2 cm (wide)	6.2 cm (long) and 3.1 cm (wide)
Leaflet apex	3-Toothed	4-Toothed
Leaf base	Wide olivaeform	Wide olivaeform
Color	Green-yellowish	Dark green with some hair on the adaxial surface and the lower surface has some white color
Odor	Distinct, strong, and aromatic	Sweet, strong, and aromatic
Taste	Sweet, bitter, and astringent	Pleasant, tangy, and Bitter
Texture	Smooth	Smooth

Anatomical characters on fresh leaves

The microscopic features identified in *A. annua* were: anomocytic stomata (92.8–93.2–94.7 μm of length and 63.8–70.2–72.6 μm of width) on the surfaces of the abaxial and adaxial leaf surfaces. Epidermal cells with smooth, wavy cuticle layers and also with sinuous anticlinal walls were found to be present. Unicellular and uniseriate multicellular glandular trichomes and T-shaped nonglandular trichomes were both found present (Figure 2).

Microscopic features identified in the leaves of *A. vulgaris* include amphistomatic with Anomocytic stomata type (35.6–36.8–39.1 μm of length and 20.5–33.1–34.3 μm of width). Epidermal cells were found to have a thick cuticle layer surrounded by sinuous anticlinal walls. Multiseriate unicellular glandular trichomes with abrupt ending and nonglandular unicellular T-shaped trichomes were observed (Figure 2).

Quantitative microscopic features of *A. annua* and *A. vulgaris* leaves

The quantitative microscopy of the two leaves showed that all the constants were different with *A. vulgaris* having higher values of the palisade ratio and vein-termination number. On the other hand, *A. annua* had higher values of the stomatal index (upper and lower surfaces) and vein-islet number as shown in Table 2.

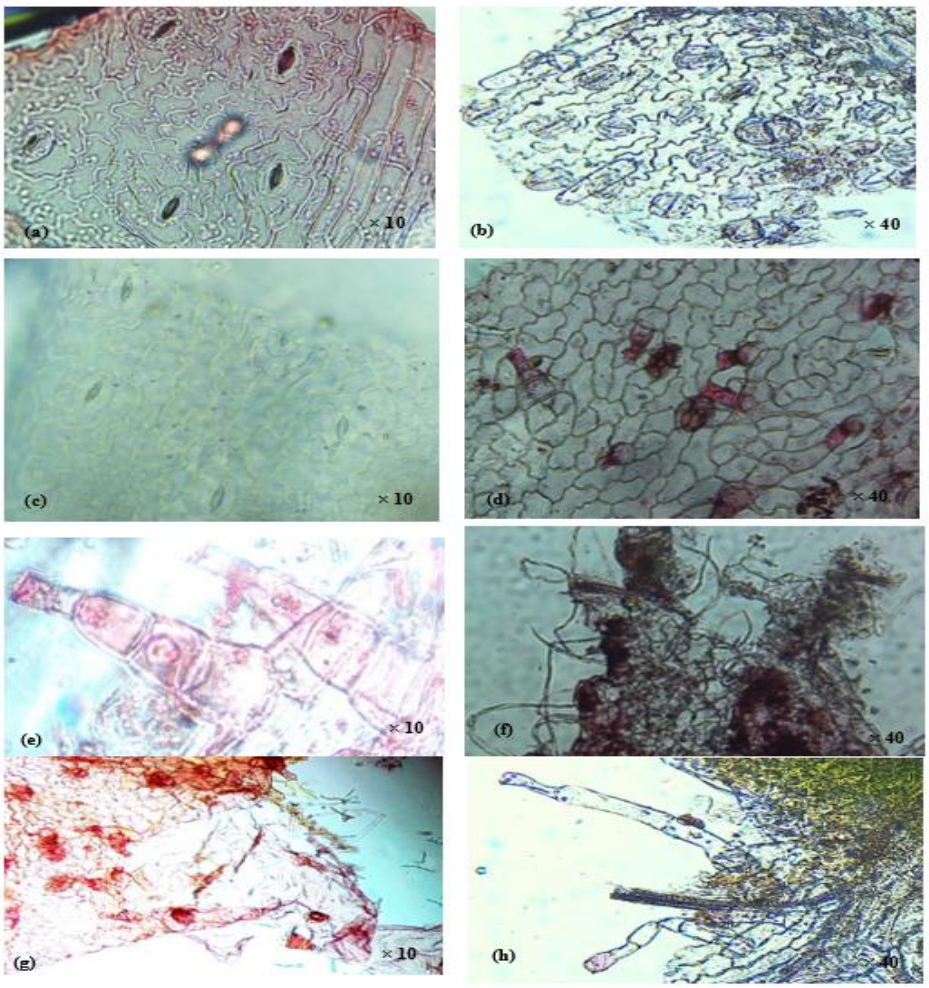


Figure 2 Some anatomical characteristics of fresh leaves. a&b shows lower epidermis; c&d shows upper epidermis; e&f, g&h shows trichomes of *A. annua* and *A. vulgaris* respectively.

Table 2 Comparative quantitative microscopic features of *A. annua* and *A. vulgaris* leaves

Part	Features	*A. annua*	*A. vulgaris*
Upper epidermis	Palisade ratio	4.2–6.4–8.0	6.8–7.8–10.0
	Stomatal number	9.0–12.0–15.0	16.0–17.0–18.0
	Stomatal index	31.0–38.7–45.4	30.8–33.3–34.0
Lower epidermis	Stomatal number	13.0–15.0–17.0	8.0–10.0–11.0
	Stomatal index	41.9–42.9–44.7	29.6–32.6–33.3
	Vein-islet number	19.0–22.0–25.0	12.0–15.0–17.0
	Veinlet termination number	10.0–15.0–22.0	13.0–17.0–23.0

Physicochemical parameters

The leaf powders of the two samples were tested for physicochemical characteristics. As a result, variables including moisture content, total ash values, acid-insoluble ash, sulphated ash and alcohol-soluble extractive values were determined (Table 3).

Table 3 Physicochemical parameters of *A. annua* and *A. vulgaris* leaves

Parameter	*A. annua*	*A. vulgaris*
Moisture content	8.10 ± 0.115	6.93 ± 0.186
Total ash	10.3 ± 0.0667	8.37 ± 0.186
Acid insoluble ash	1.60 ± 0.0884	0.59 ± 0.010
Water soluble ash	5.02 ± 0.142	4.50 ± 0.404
Sulphated ash	13.9 ± 0.238	10.7 ± 0.170
Alcohol-soluble extractive value	9.73 ± 0.120	18.1 ± 0.0233
Water-soluble extractive value	19.9 ± 0.376	27.5 ± 0.3210

Values are means ± standard error (%w/w) of triplicates.

Phytochemical screening results

The results of the qualitative phytochemical analysis are presented in Table 4. Qualitative phytochemical analysis of all solvent extracts revealed the presence of tannins, glycosides, carbohydrates, alkaloids, sterols, and triterpenoids, flavonoids, glycosides, proteins, and amino acids, while saponins and anthraquinones were absent in all extracts.

Thin layer chromatography (TLC) fingerprinting of the leaf extracts

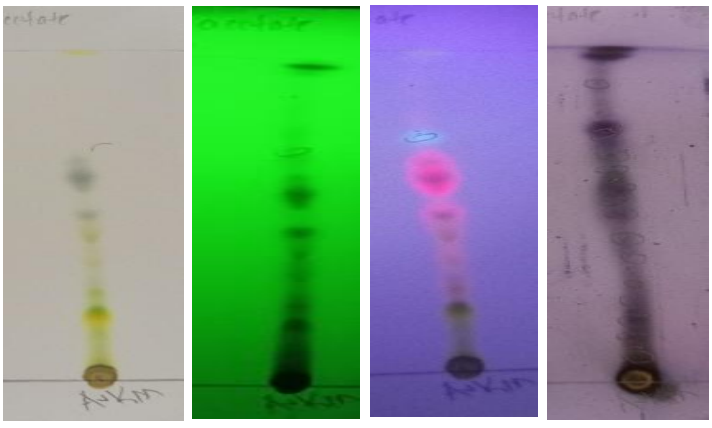
The TLC fingerprinting of the crude methanol, hexane, and diethyl ether of leaf samples from *A. vulgaris* revealed the presence of different chemical constituents. Visualization of the developed TLC plates of all extracts under normal light showed a similarity of spots (Supplementary Tables S1–S6) and Figures 3–8.

The colour of the artemisinin spot was pink and its Rf values were 0.54 for the methanol extracts, 0.46 for the *A. annua* diethyl ether extract, 0.36 for the *A. vulgaris* diethyl ether extract, and 0.42 for the hexane extracts for the two *Artemisia* species. This implies that all the extracts possess artemisinin. Similar Rf values were detected in all extracts at different detection wavelengths for the two species of *Artemisia*, which justifies the presence of other compounds in addition to artemisinin.

Table 4 Secondary metabolites identified in different solvent extracts of leaves of *A. annua* and *A. vulgaris* leaves

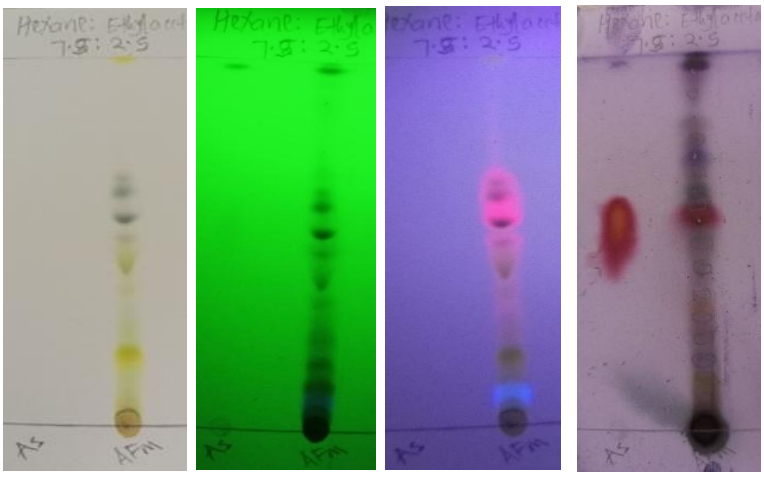
Sample	Extract	Alkaloids	Flavonoids	Glycosides	Tannins	Sterols & triterpenoids	Anthraquinones	Carbohydrates	Proteins & amino acids	Saponins
A. annua	Methanol	+	+	+	+	+	-	+	+	-
	Hexane	-	-	+	-	+	-	+	-	-
	Diethyl ether	+	+	+	+	+	-	+	+	-
A. vulgaris	Methanol	+	+	+	+	+	-	+	+	-
	Hexane	-	-	+	-	+	-	+	-	-
	Diethyl ether	+	+	+	+	+	-	+	+	-

+, present; -+-, absent.



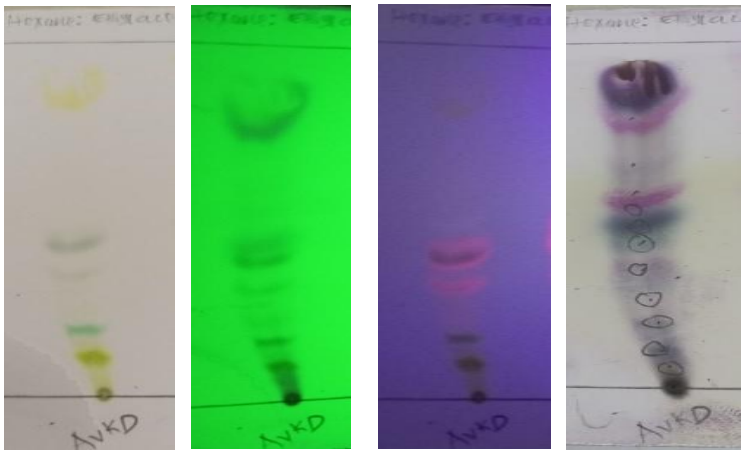
(a) (b) (c) (d)

Figure 3 TLC fingerprints for methanol extracts of *A. vulgaris* in (a) normal light, (b) 254 nm, (c) 365 nm, and (d) after spraying with *p*-anisaldehyde sulphuric acid. AVKM, *Artemisia vulgaris*-Kamwenge-methanol extract; *A. Vulgaris*, *Artemisia vulgaris* L.



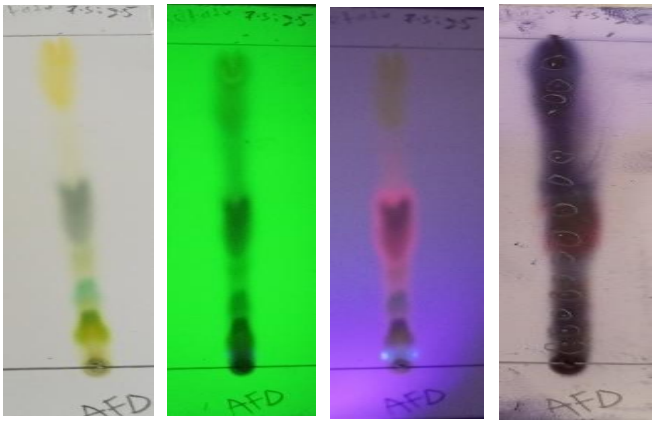
(a) (b) (c) (d)

Figure 4 TLC fingerprints for *A. annua* methanol extracts at (a) normal light, (b) 254 nm, (c) 365 nm, and (d) after spraying with *p*-anisaldehyde sulphuric acid, respectively. AFM, *Artemisia annua*-Fort Portal-methanol extract; *A. annua*, *Artemisia annua* L.



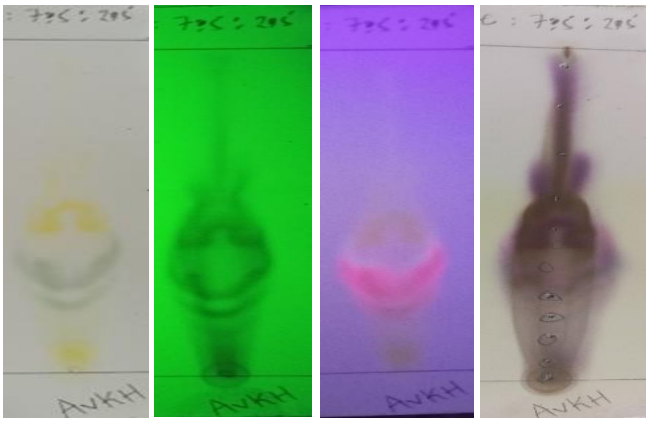
(a) (b) (c) (d)

Figure 5 TLC fingerprints extracts of *A. vulgaris* diethyl ether in (a) normal light, (b) 254 nm (c) 365 nm, and (d) after spraying with *p*-anisaldehyde sulphuric acid. AvKD, *Artemisia*; *A. Vulgaris*, *Artemisia vulgaris* L.



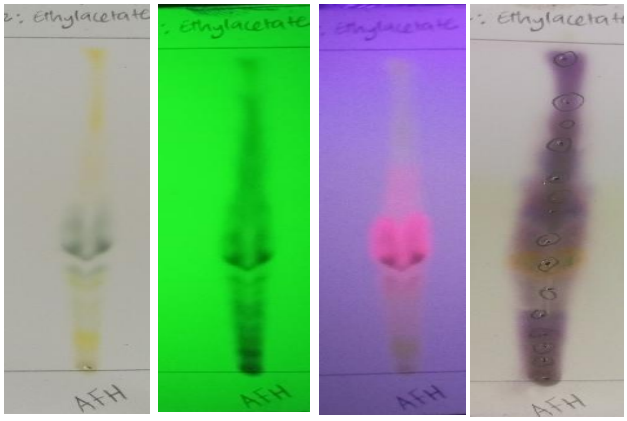
(a) (b) (c) (d)

Figure 6 TLC fingerprints for extracts of *A. annua* diethyl ether at (a) normal light, (b) 254 nm (c) 365 nm, and (d) after spraying with p-anisaldehyde sulphuric acid. AFD, *Artemisia annua*-Fort Portal – diethyl ether extract; *A. annua*, *Artemisia annua* L.



(a) (b) (c) (d)

Figure 7 TLC fingerprints for *A. vulgaris* hexane extracts in (a) normal light, (b) 254 nm, (c) 365 nm, and (d) after spraying with *p*-anisaldehyde acid. AvKH, *Artemisia vulgaris*-Kamwenge-Hexane extract; *A. Vulgaris*, *Artemisia vulgaris* L.



(a) (b) (c) (d)

Figure 8 TLC fingerprints for *A. annua* hexane extracts at (a) normal light, (b) 254 nm (c) 365 nm, and (d) after spraying with *p*-anisaldehyde sulphuric acid. AFH, *Artemisia annua* -Fort Portal-Hexane extract; *A. annua*, *Artemisia annua* L.

HPLC quantification of artemisinin from leaves of *A. vulgaris* and *A. annua*

Figure 9 shows the calibration curve used for artemisinin quantification. Linear regression was used to establish the calibration curve. The good linearity of artemisinin was found within the range of r^2 = 0.9993242 which is a good standard curve since the value is greater than 0.95. The regression equation and the correlation coefficient were determined from y = 114.8256x–128.525.

Chromatograms of diethyl ether leaves extracts showed many resolved peaks (Figures 10, 11). Artemisinin peaks were identified by comparison of their retention times with that of standard artemisinin (Figure 9). The artemisinin peaks had retention times of 10.247 (*A. annua)* and 10.467 (*A. vulgaris*). The content of artemisinin per dry weight of diethyl ether extracts was highest in *A. vulgaris* (1.72%) than in *A. annua* (1.43%).

Total flavonoid content of *Artemisia* leaf extracts

Quantification results revealed that both diethyl ether and methanol extracts from *A. annua* leaves contained higher amounts of flavonoids (82.0 ± 1.4 and 63.0 ± 0.0 µg QE/g dw) than leaves of *A. vulgaris* (10.0 ± 0.27 µg QE/g dw and 8.4 ± 0.0 µg QE/g dw), respectively.

Discussion

Physicochemical parameters of the two *Artemisia* species in Table 3 revealed that the moisture content (8.10%), total ash (10.3%), acid insoluble ash (1.6%), and sulphated ash (13.9%) was found to be higher in *A. vulgaris* than in *A. annua*. The high moisture content of the leaf samples of *A. vulgaris* could be due to the presence of an excessive amount of free water [32]. The moisture content is a requirement of the European Agency for the evaluation of medicinal products, as the influence of the water content on the stability and safety of crude drugs is significant [32]. Insufficient drying of plant material may lead to degradation of the phytoconstituents of the drug during storage [33]. The high values of the ash content imply that *A. vulgaris* contained both physiological and non-physiological ash and other extraneous matter inherent to it, therefore suggesting it to be slightly less pure compared to the other species. High ash values are likely to be caused by the presence of a high contents of nitrogen in the leaves of *A. vulgaris* [34]. The alcohol-soluble and water-soluble extractive values in *A. annua* were found to be higher than those of *A. vulgaris* as shown in Table 1, which signifies that alcohol (ethanol) and water are better extracting solvents, indicating the presence of more amounts of alcohol and water-soluble contents in the leaf samples. Alcohol and water-soluble extractive values are important for the identification of exhausted crude herbal drugs. These extractive values also measure the amount of extract that the drug yields in a solvent.

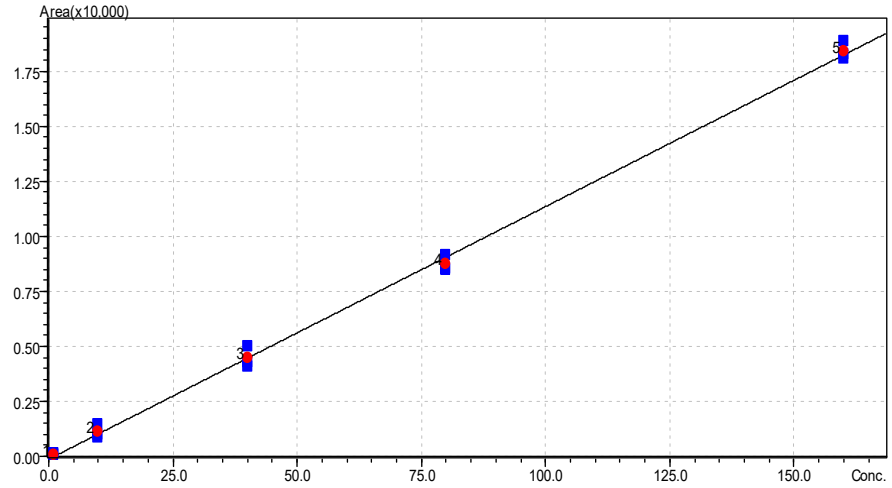


Figure 9 Calibration curve for artemisinin quantification in *A. vulgaris* and *A. annua* leaf extracts. *A. Vulgaris*, *Artemisia vulgaris* L.; *A. annua*, *Artemisia annua* L.

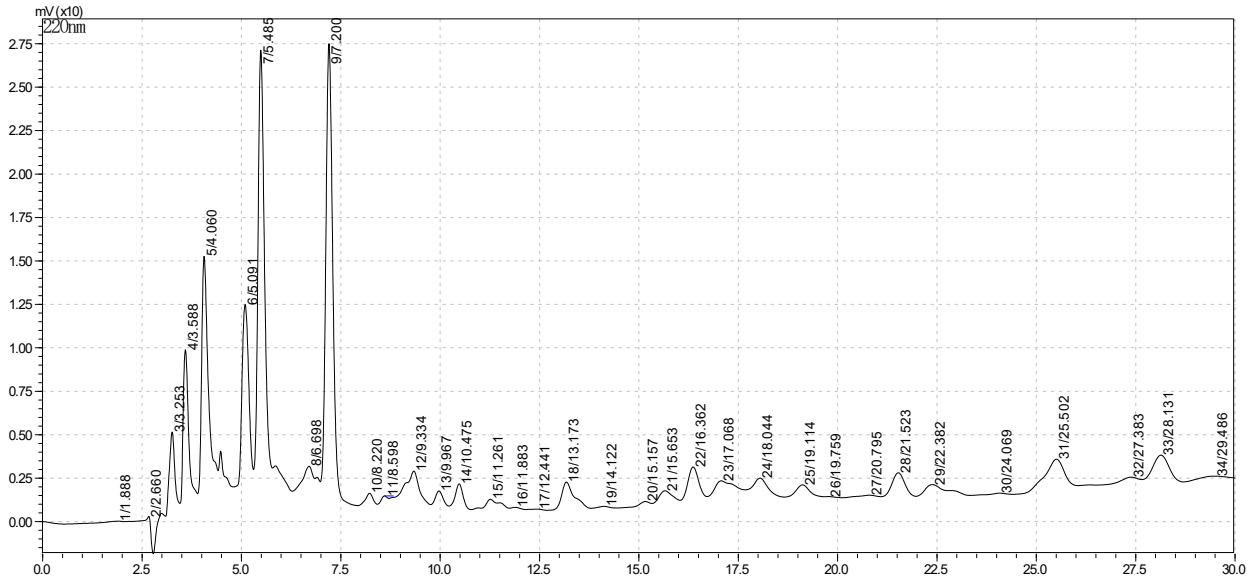


Figure 10 HPLC chromatogram of artemisinin in diethyl ether extract of *A. vulgaris* leaves. *A. Vulgaris*, *Artemisia vulgaris* L.; HPLC, High-performance liquid chromatography.

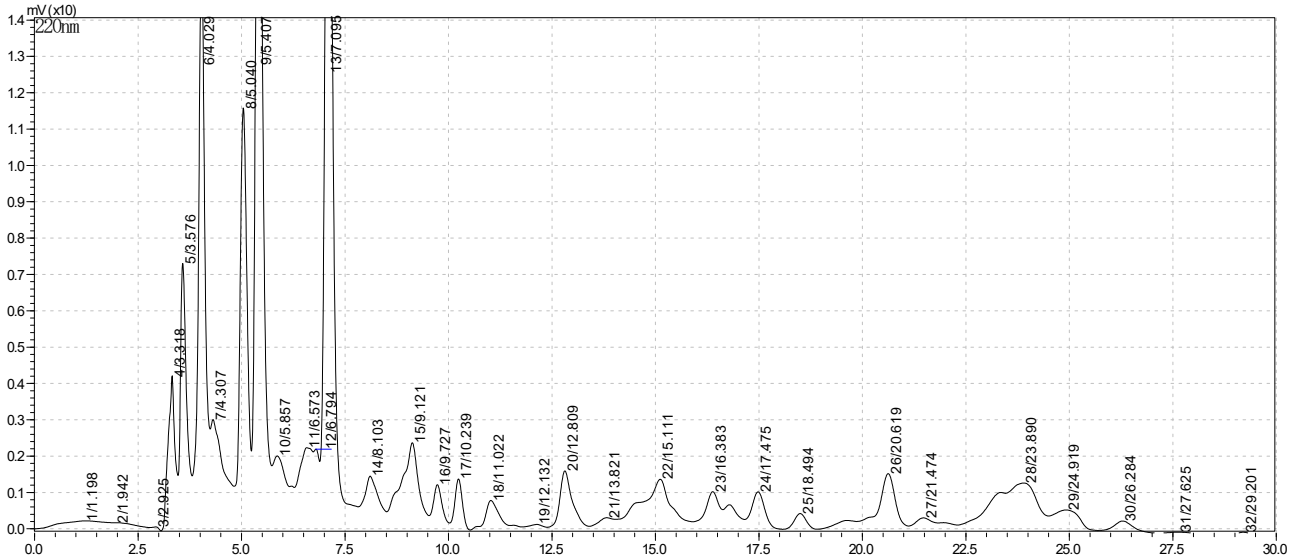


Figure 11 HPLC chromatogram for artemisinin in the diethyl ether extract of leaves of *A. annua.* HPLC, high-performance liquid chromatography; *A. annua*, *Artemisia annua* L.

The results of the phytochemical screening showed that all extracts had the same chemical constituents (alkaloids, flavonoids, carbohydrates, glycosides, sterols and triterpenoids, tannins and protein and amino acids), except saponins and anthraquinones, as shown in Table 4. A similar recent study on phytochemical screening by Thangjam et al. showed the presence of saponins, glycosides, flavonoids, proteins, and triterpenoids in the leaf extract of *A. vulgaris* [35]. The presence of phytosterols, carbohydrates, proteins and amino acids, and flavonoids in methanol extracts of *A. vulgaris* was similar to a study by Ashok and Kumud [36]. Alkaloids were detected in all extracts of the two *Artemisia* species in this study, which is not consistent with previous reports. It is possible that the alkaloids in the two species exist as free alkaloidal salts, as previously reported by Engeu et al. for *A. annua* leaves from various regions of Uganda [37].

The variation in the chemical composition of the TLC profile in the two leaf samples of *A. vulgaris* may be due to the different environmental conditions to which they are exposed during growth [38]. According to Misra et al., seasons, genetic factors, temperature, and nutrient composition affect the presence of artemisinin in *Artemisia* species [39].

TLC is important for identifying compounds present in crude herbal drugs that contain a large number of complex constituents that may have areas that overlap with more or less UV-vis. It is also useful for the detection of potential adulteration of different crude drugs [40, 41]. Furthermore, most pharmacopeias, such as American Herbal Pharmacopoeia herbal drug monographs, utilize TLC in the analysis of herbal medicines [42].

Recent studies have revealed that the artemisinin content of *A. vulgaris* in flowers, stems, leaves, and roots ranges from 0.002–1.4% and 0.002–1.8%, respectively [8]. Adjogblé et al. reported a relatively low artemisinin content (0.009%) in *A. annua* leaves from Togo [43]. Previous studies in China showed that the artemisinin yield from the aerial parts of *A. annua* ranged from 0.01% to 0.5% [44]. In general, the artemisinin content of *A. annua* leaves is at least 0.7% [45]. Previous studies have reported that artemisinin content in dried leaves of the other species of genus *Artemisia* such as *A. annua,* is mainly affected by geographical locations, agricultural practices, time of harvesting, and other environmental factors. For example, with respect to availability and temperature, it is observed that the amount of artemisinin ranges between 0.01 and 2 % w/w of dried leaf material [46]. The high content of artemisinin in *A. vulgaris* may be due to the presence of low temperatures and less humid climatic conditions. It is also noted that artemisinin levels in *Artemisia* species depend on the age of the plant, the levels rise steadily during growth, reach their peak just at the beginning of blooming, and then drop sharply as the flowers age [47].

Artemisia annua was suggested to be potent in the treatment of malaria, which could justify the change in the use of *A. vulgaris* by the indigenous community of western Uganda [46]. This is interesting because this study has confirmed that it had a higher artemisinin content than *A. vulgaris*, which is also known to exhibit antimalarial activity [16].

The TFC is in agreement with the report by Ivanescu et al. who found that the aerial parts of *A. annua* contained 13.88 mg QE/g dw which was higher than that of *A. vulgaris* 10.11 mg QE/g dw [48]. Another study investigating hairy roots of *A. vulgaris* reported a TFC of 49.4 ± .0 mg Rutin Equivalent / g of dw [49].

Flavonoids are divided into many groups, for example, based on their skeleton, they are classified into; flavones, flavanones, isoflavones, flavones, isoflavones, anthocyanins, chalcones, and flavonolignans [50]. These flavonoids exhibit several pharmacological activities, for example, the luteolin and apigenin found in *A. vulgaris* have demonstrated antimalarial, anti-inflammatory, antioxidant, and anticancer activities [10].

In conclusion, species of the *Artemisia* genus appear similar in morphology and ethnobotanical applications. Therefore, proper validation and identification is important. This study considered two *Artemisia* species whose leaves were shown to have similar tastes. To distinguish between the two *Artemisia* species, the local community can use their leaf shapes and colours (greenish-yellow for *A. annua* and dark green for *A. vulgaris*) can be used by the local community. There was an observation in the similarity of the chemical composition. However, the concentration of artemisinin and total flavonoids can be used as a distinguishing parameter during phytochemical analysis. Further studies should perform pharmacodynamic and phytochemical comparison of *A. annua* with other species in the same genus and apply molecular techniques such as DNA barcoding for their identification. Comparison of bioactivities of the two species should be done, especially antimalarial activity for which they are commonly used.

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