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Phytochemical Analysis and Characterisation of Extract of African Rosemallow (*Hibiscus acetosella*) Leaves from Kisii County, Kenya

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ABSTRACT

Colour significantly improves the environment in which we live. Dyes are compounds that add colour to objects by absorbing and emitting light only in certain wavelengths. It is estimated that 7×10^7 tonnes of synthetic dyes, which are thought to be persistent pollutants, are produced and used annually. Natural dyes are generally non-toxic and non-allergenic. The *Hibiscus acetosella* plant is a potential source of natural dyes. In this research, *H. acetosella* leaf samples were collected from Kisii County, Kenya. Cold and Soxhlet extraction methods were used upon sample preparation. The extract was characterised using physical tests such as LC-MS, GC-MS and FT-IR and chemical tests. The yield of the dye obtained using cold extraction method (80% ethanol and 0.1% HCl) was 65.44 ± 1.53 mg/g. Phytochemical analysis of the extracts indicated the presence of anthocyanins, terpenoids, flavonoids, phlobatannins, steroids, tannins, glycosides and saponins. The main anthocyanin found in *H. acetosella* leaves was Cyanidin-3-O-glucoside which eluted at a retention time 22.52 min and with a *m/z* at 449.0. Besides, some non-polar compounds present were recorded based on the GC-MS data obtained. The present study provides reliable information on dye extraction, percentage yield and phytochemical constituents of *H. acetosella* leaves.

1. Introduction

Our environment is substantially improved by colour. The colourful variations in everyday objects we use such as textiles, paints, plastics, papers and food are enticing. Around us, nature also offers a kaleidoscope of colours. Man has been looking for materials to beautify himself and his surroundings since the dawn of time. The most appealing colouring materials have always been those that were made by simply powdering minerals or putting aquatic life, insects, plants, berries, roots, lichens in water [1].

Dyes are compounds that add colour by absorbing or emitting light only in certain wavelengths. Dyes are soluble in water and/or organic solvents and are used to colour substrates to which they have affinity. Coloured, colourless or fluorescent pigments are small, finely split organic or inorganic solids that are often insoluble in water and/or organic solvents and essentially unaffected chemically by the media in which they are used. On the other hand, the interaction between light and substance is what causes colour, which is greatly influenced by the chemical and physical characteristics of matter [2]. The plant kingdom has historically been the largest source of dyes, particularly roots, berries, bark, leaves and wood. However, very few have ever seen widespread commercial application.

H. acetosella is a native species of western tropical Africa and comes in red and green varieties. It is also referred to as African rosemallow and belongs to the Malvaceae family and genus Hibiscus, which has over 200 species. This plant Although it can be utilised as a staple vegetable, it is primarily planted as an annual ornamental for the beauty of its beautiful burgundy red, maple-like leaves. *H. acetosella* is known to be resistant to nematodes and insects [3]. It is an annual crop but can be regarded perennial as well. In one season, it can grow as a shrub-subshrub. This plant is self-pollinating and bisexual. The leaf colour is lavender-red or maroon, containing some patches of red or green appearance. The leaves are also dicot and have a variation in shape from unlobed to 3-5-lobed and normally the size and division varies in the upper leaves as the plant grows [4]. Furthermore, *H. acetosella* extract can serve as a pH indicator [5]. It changes basic solutions to green and acidic solutions to magenta or dark pink. In folk medicine, various parts of this plant are used to treat a variety of conditions including fever, anaemia, headache, rheumatism, inflammations, conjunctivitis, haemorrhoids, tumours, ringworms, sores and abscesses. It is also used as an intestinal antiseptic, diuretic, sedative and to stimulate lactation in nursing mothers [6].

According to research, *H. acetosella* plant contains anthocyanins responsible for the pink, red, blue and purple colours in plant parts; leaves, fruits, flowers. Anthocyanins are the chromophores; colour bearing groups in this plant. They are responsible for the lavender red colour in the leaves of *H. acetosella* plant [7]. Anthocyanins undergo a change in colour based on pH and as a result, they can be used as pH indicators. The colour change is caused by adding or removing hydrogen ions from the molecular structure of the pigment which in turn changes its electronic properties. This alters the light wavelengths that are absorbed by the pigment . Their stability is affected by pH, structure, temperature, light, oxygen and co-pigments. The stability is higher at lower pH and temperature, and in the presence of co-pigments and/or metal ion complexes and in the dark [8].

Utilisation of natural dyes faces challenges despite their non-toxic and non-persistent nature. Some of those used as food pigments, for example, have been found to be unstable towards light exposure, temperature and pH among other factors. Such shortcomings have necessitated further research on natural dyes due to their importance in pharmaceutical, food and textile industries [9]. In this study, the extraction of the anthocyanins from *H. acetosella* leaves, their stabilisation and characterisation is reported. This work highlights the significance of this natural dye extract, which can find application in different fields.

2. Experimental Methods

2.1. Materials, Collection and Preparation

The *H. acetosella* leaf samples were collected from Kisii County, Kenya using random sampling technique. The leaf samples were washed with distilled water and allowed to dry under shade for 2 weeks. Upon drying, they were crushed using a blender in order to increase the surface area for extraction. Solvents used in this study (except distilled water) were purchased from Kopian Limited, a subsidiary branch of Sigma Aldrich, and were used without further purification. All extraction experiments were carried out in triplicates.

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2.2 Extraction

2.2.1 Cold Extraction

The procedure for extraction of the dye from crushed *H. acetosella* leaf samples was carried out as follows. Exactly 10 g of the sample was measured and placed in a conical flask (500 mL by volume). A volume of 250 mL of 80% ethanol solvent was added to a beaker followed by the addition of 0.1% HCl. The extraction was allowed to take place for 5 days at room temperature and in the dark. After the extraction, the dye was filtered using Whatman filter papers of size 460 x 570 mm and a filter funnel. Separation of polar and non-polar compounds was carried out using a separating funnel and hexane solvent. The pH of the dye was measured and the solvent evaporated using a rotary evaporator at 50 °C and the yield was determined. The results were subjected to analysis and interpretations [10]. This procedure was repeated for 80% ethanol and 2% acetic acid as the acidifying medium.

2.2.2 Soxhlet Extraction

Exactly 10 g of ground *H. acetosella* leaves was measured and placed in a thimble and thereafter loaded into the main chamber of the Soxhlet extractor. A volume of 250 mL of 80% ethanol solvent was added to a 500 mL round bottomed flask followed by addition of 0.1% HCl acid . The extraction was allowed to reflux for 4 hours. After the extraction, the contents were allowed to cool before separation of polar and non-polar compounds carried out using a separating funnel and hexane solvent. The pH of the dye was measured and then the solvent evaporated using a rotary evaporator. This procedure was repeated for 80% ethanol containing 2% acetic acid as acidifying medium [11].

2.3 Chemical Tests for Phytochemicals

All phytochemical tests were carried out as per the standard procedures provided in literature [12].

2.4 Identification of Polar Bioactive Compounds in H. acetosella Extract

A 95% LC-MS grade acetonitrile (1 mL, Sigma–Aldrich, St. Louis, MO, USA) was added to ethanol extract (300 ng/µL). The mixture was then vortexed for 10 seconds, sonicated for 30 minutes, and centrifuged for 5 minutes at 14,000 rpm, all in accordance with the procedures outlined. After LC-MS analysis of the filtrate (5 µL), the supernatant was filtered using Whatman filter paper No. 32. In order to create the chromatographic separation, a single quadrupole analyser (Agilent Technologies, Palo Alto, California) and an Agilent 1260 Infinity LC system were used. Utilising a ZORBAX SB-C18, 4.6 × 250 mm, 3.5 µm column, the experiment was conducted at 40 °C. The mobile phases used were acetonitrile (B) and methanol (A), each containing 0.01% formic acid. The following was the gradient system that was used: 30-35 minutes, 100% B; 35-37 minutes, 100-5% B; 37-42 minutes, B; 0.01 minutes, 5% B; 5-10 minutes, 5-20% B; 10-5 minutes, 20% B; 15-20 minutes, 20-80% B; 20-25 minutes, 80% B. A 3 µL injection volume and a 0.5 mL/min flow rate were maintained. The LC was interfaced to a quadruple mass spectrometer that functioned in ESI both negative and positive mode and had a mass scan range of *m/z* 100-2000. There was a 50 ms residence period for each ion. Other settings for the mass spectrometer were as follows: 400 L/h nitrogen gas flow; 110 °C source temperature; 380 °C nitrogen gas temperature for desolvation; 3.0 kV capillary voltage; 30 V cone voltage; 5 V extract voltage; and 0.5 V RF voltage [13]

2.5 Profiling of the Non-Polar Compounds

A gas chromatograph-7890A (Agilent Technologies, Inc., Santa Clara, CA, USA) coupled to a mass selective detector-5975C (Agilent Technologies, Inc., Santa Clara, CA, USA) was utilised for the study of non-polar chemicals. The non-polar compounds were subjected to GC-MS analysis while maintaining the following temperature parameters: 270 °C for the intake, 280 °C for the transfer line, and a temperature programmed between 35 °C and 285 °C. After holding the initial temperature for five minutes, the temperature was raised to 280 °C at 10 °C/min and maintained for fifty minutes. The GC was fitted with an HP-5 MS low bleed capillary column (30 m × 0.25 mm and diameter, 0.25 µm) from J & W, Folsom, CA, USA . Helium was used as the carrier gas, flowing at a rate of 1.25 mL/min. For the mass selective detector, the quadrupole temperature was maintained at 180 °C and the ion source temperature at 230 °C. The elevation energy at which Electron Impact (EI) mass spectra were obtained was 70 eV. An auto sampler 7683 (Agilent Technologies, Inc., Beijing, China) was used to inject a 1.0 µL splitless aliquot of sample. The entire scan mode, including the mass range of 40-950 *m/z*, was used to analyse the fragment ions. A filament delay of three and a half minutes was selected. By comparing the retention durations and mass spectra of the acquired components to those from the WILEY 09 and NIST 11 mass spectral databases, the chemical composition of the components was determined [14].

2.6 FT-IR Analysis

The FT-IR spectrometer was used to identify the functional groups in the sample obtained upon extraction. Two drops of each sample were positioned in contact with Attenuated Total Reflectance (ATR) plate and then loaded to the FT-IR machine for analysis. The spectra were collected at 4000-400 cm^{-1} and at a resolution of 4 cm^{-1}. The number of scans were set to 32. All spectra were subtracted from an air spectra background. The infrared light was partially absorbed since it only entered the sample a short distance after reflecting from the crystal's interior surface. A fresh reference of the air background spectra was taken after each scan. It was feasible to dry the ATR plate by thoroughly cleaning it twice with 70% isopropyl alcohol, followed by soft tissue drying and filling it with the subsequent sample. Printouts of the resulting FT-IR spectra were made and the peaks were identified and interpreted [15].

3. Results and Discussion

3.1 Extraction of the Dye

The pH of the dye was 3.20 ± 0.02 for 80% ethanol and 2% acetic acid extract, and 3.18 ± 0.01 for the 80% ethanol and 0.1% HCl extract. The extracted dye was lavender red in colour upon extraction and separation of polar and non-polar components using a separating funnel and hexane solvent. The extraction was carried out using both cold extraction and Soxhlet method of extraction. The yield of the dye (80% ethanol and 0.1% HCl) obtained using cold extraction method was 65.44 ± 1.53 mg/g of sample while that obtained using Soxhlet method of extraction was 56.27 ± 0.38 mg/g. The yield of the dye (80% ethanol and 2% acetic acid) obtained using cold extraction method was 60.20 ± 0.74 mg/g of sample while that obtained using Soxhlet extraction was 51.24 ± 0.35 mg/g.

According to research on Roselle calyces (*H. sabdariffa*), an extraction using ethanol acidified with HCl demonstrated the maximum output of anthocyanins at 13.86 mg/g dry weight while water acidified with citric acid at a 2% concentration had a yield of 10.63 mg/g. Therefore, the former was the most preferred method. The differences in the yield may be due to the difference in plant species and controlled parameters during extraction [16].

3.2 Chemical Tests for Phytochemicals

The results obtained upon the chemical tests of some phytochemicals in the two dye extracts are shown in Table 1. In the chemical tests for the phytochemicals in the extracts; A pink-red solution that turned blue-violet upon addition of NH_4OH indicated the presence of anthocyanins, a blue-green colour indicated the presence of tannins in the test for tannins, the formation of a white precipitate in Mayer's test indicated the presence of alkaloids, a grey coloured solution indicated the presence of terpenoids. The formation of a green-coloured solution indicated the presence of quinones in the test of quinones as outlined. A yellow coloured solution gave an inference that flavonoids where present upon using the alkaline reagent test and a green precipitate when ferric chloride test was used. A red precipitate indicated the presence of phlobatannins and the presence of saponins was detected by the presence of a honeycomb foam. Finally, Steroids were present as a reddish-brown ring formed at the interface and glycosides were present as a pink colour formed upon carrying out Borntrager's test [12].

Table 1 Chemical tests for phytochemicals

No.	Phytochemical	Test	*H. acetosella* leaves' extracts	
			80% Ethanol + 0.1% HCl	80% Ethanol + 2% Acetic acid
1.	Alkaloids	Mayer's	+	+
2.	Terpenoids	Salkowski's	+	+
3.	Quinones	Concentrated HCl	–	–
4.	Flavonoids	Alkaline Reagent	+	+
		Ferric Chloride	+	+
5.	Phlobatannins	HCl	+	+
6.	Steroids	Salkowski's	+	+
7.	Anthocyanins	HCl	+	+
8.	Tannins	Braymer's	+	+
9.	Glycosides	Borntrager's	+	+
10.	Saponins	Frothing/Foam	+	+

Note: + indicates presence while – indicates absence

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These findings are in agreement with literature, which indicates that the following phytochemicals; saponins, steroids, flavonoids, tannins, anthocyanins, and bioactive compounds such as organic acids, phytosterols and polyphenols are present in *H. acetosella* plant and other *Hibiscus* species. Prior research has demonstrated that the plant's leaves are rich in flavonoids, terpenoids, glycosides, tannins, and kaempferol, and that the petals contain anthocyanins, quercetin, and kaempferol [17].

3.3 Liquid Chromatography Mass Spectroscopy (LC-MS)

A total of 17 compounds were identified by LC-MS in the ethanolic extract of *H. acetosella* leaves as shown in Table 2. These compounds were identified and proposed to be contained in the extract with the aid of the MS tool and research on the fragmentation patterns, retention times, molecular mass and molecular formula of the compounds. The compounds were observed to elute at different retention times based on their polarity and molecular mass which contributed to their adherence to both the stationary and mobile phase in LC column. These compounds were classified based on their phytochemical class and among them were; flavonoids, anthocyanins, steroids, alkaloids, saponins, amino acids, phenolic acids, phenyl urea, triterpenoids, lactones and glucuronic acid derivatives.

The main anthocyanin identified in the extract was Cyanidin-3-O-glucoside (Cy3G) which eluted at a retention time of 22.52 min and had a fragmentation pattern (449.0, 205.0, 287.1) in MS as shown in Fig. 1. The fragmentation at 449.0 was attributed to M^+ (Cyanidin-3-glucoside, $C_{21}H_{21}O_{11}$), 287.1 (Cyanidin). Another anthocyanin which had the same molecular weight as Cy3G was Cyanidin-3-O-galactoside (Cy3Ga). The two anthocyanins had the same M^+ and *m/z*. Any variations in the anthocyanins can be attributed to the different glycosidic sugars attached to the anthocyanidin through glycosidic linkages or bonds. An MS/MS analysis of Cy3G and Cy3Ga exhibited a fragmentation (*m/z*) 287.1 and in MS analysis (*m/z*) 205.0 and a M^+, 449.1 [18].

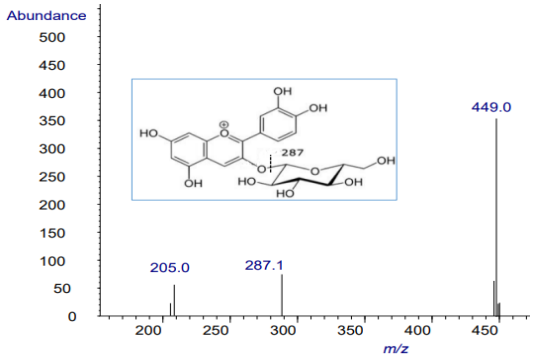

Fig. 1 MS Fragmentation of CyG

Table 2 LC-MS analysis of some of the compounds present in *H. acetosella* leaves' polar extract

S. No.	RT (min)	Class	Compound	MW (g/mol)	MF	*m/z* $[M-H]^-$/ $[M+H]^+$	Fragments	Peak Area (%)
1.	5.23	Lactones	δ-hexanolactone	114.1	$C_6H_{10}O_2$	113.1	55.0, 153.1	10.34±0.46
2.	6.63	Phenolic acids	4-hydroxybenzoic acid	138.1	$C_7H_6O_3$	138.1	92.1, 121.1, 191.1, 225.1	2.03±0.04
3.	21.12	Phenylpropenes	Anethole	148.2	$C_{10}H_{12}O$	148.1	134.1, 140.0, 157.0, 187.1, 352.3, 494.9, 626.5, 855.6	11.05±0.49
4.	21.25	Amino acid	Histidine	155.1	$C_6H_9N_3O_2$	157.1	141.2, 169.1, 187.1, 205.1	48.17±0.44
5.	21.75	Saponins	Hederagenin	472.7	$C_{30}H_{48}O_4$	471.7	494.7, 437.9, 449.0, 487.2, 795.0	0.77±0.05
6.	22.23	Triterpenoids (Triterpene acid)	Bassic acid	486.7	$C_{30}H_{46}O_5$	487.7	465.1, 504.0, 525.2, 545.3, 582.4	0.80±0.02
7.	22.28	Alkaloids	Norisoliensinine	597.3	$C_{36}H_{40}N_2O_6$	597.3	566.3, 525.2 534.3, 489.2, 475.2, 296.1,192.1	2.90±0.03
8.	22.34	Alkaloids	Liensinine	611.3	$C_{37}H_{42}N_2O_6$	613.3	580.3, 568.3, 503.3, 489.2, 446.2, 487.2, 595.3,192.1	0.46±0.03
9.	22.50	Flavonoids	Luteloin-7-O- glucoside	448.4	$C_{21}H_{20}O_{11}$	447.4	287.4, 449.1, 551.4, 611.2,	4.84±0.07
10.	22.50	Flavonoids	Kaempferol-7-O- glucoside	448.4	$C_{21}H_{20}O_{11}$	447.4	285.4, 449.1, 551.4, 611.2	4.84±0.07
11.	22.52	Anthocyanins	Cyanidin-3-O-glucoside	484.8	$C_{21}H_{21}ClO_{11}$	449.0	205.0, 287.1	0.43±0.05
12.	22.53	Anthocyanins	Cyanidin-3-O-galactoside	484.8	$C_{21}H_{21}ClO_{11}$	449.0	287.1, 205.1	0.43±0.05
13.	22.60	Flavonoids	Quercetin-3-(6''-galloylglucoside)	616.1	$C_{28}H_{24}O_{16}$	615.1	471.1, 462.2, 302.1, 141.1	0.59±0.05
14.	27.44	Steroids	Finasteride (4-azasteroid)	372.5	$C_{23}H_{36}N_2O_2$	187.1	236.1, 286.3, 309.1, 325.2, 351.2, 373.2, 511.1, 529.1	4.41±0.03
15.	30.11	Glucuronic acid derivatives	Glucaric acid	210.1	$C_6H_{10}O_8$	209.1	86.9, 100.9,	0.59±0.05
16.	33.15	Phenylurea	Monolinuron	215.1	$C_9H_{11}ClN_2O_2$	214.1	217.1, 429.1, 431.1, 429.1	0.20±0.03
17.	33.67	Flavonoids (Phenolics)	Daidzein	254.2	$C_{15}H_{10}O_4$	237.2	142.0, 167.2, 277.2, 293.2, 391.2	7.14±0.05

Table 3 GC-MS profile of the phytochemicals in *H. acetosella* leaves' hexane extracts

S. No.	RT (Min)	Library/ID	CAS NO.	Quality	MW (g/mol)	MF	Peak Area	Area (%)
1.	5.25	Toluene	000108-88-3	91	92.14	C_7H_8	3652401	7.11 ± 1.46
2.	7.94	*p*-Xylene	000106-42-3	83	106.08	C_8H_{10}	4274734	8.33 ± 3.34
3.	8.14	*m*-Xylene	000108-38-3	97	106.08	C_8H_{10}	10253385	19.97 ± 1.48
4.	8.73	*o*-Xylene	000095-47-6	60	106.08	C_8H_{10}	3906030	7.61 ± 1.55
5.	9.64	Pinene –α	000080-56-8	95	136.13	$C_{10}H_{16}$	7212520	14.05 ± 0.93
6.	10.54	Phellandrene –β	000555-10-2	91	136.13	$C_{10}H_{16}$	2827384	5.51 ± 0.42
7.	10.56	Pinene –β	000127-91-3	87	136.13	$C_{10}H_{16}$	2439002	4.75 ± 0.64
8.	11.23	Carene δ-3	013466-78-9	95	136.13	$C_{10}H_{16}$	4043258	7.88 ± 0.41
9.	11.24	Tricyclene	000508-32-7	86	136.13	$C_{10}H_{16}$	4455989	8.68 ± 1.12
10.	24.61	D-Alanyl-D-serine	003062-19-9	43	176.17	$C_6H_{12}N_2O_4$	148422	0.29 ± 0.08
11.	25.01	Epinephrine	000051-43-4	50	183.21	$C_9H_{13}NO_3$	95501	0.19 ± 0.03
12.	25.14	Phytol	000150-86-7	62	296.54	$C_{20}H_{40}O$	1883467	3.67 ± 1.43
13.	30.97	Squalene	000111-02-4	98	410.73	$C_{30}H_{50}$	6144413	11.97 ± 2.54

According to research, Cy3G is the main anthocyanin responsible for the lavender red colour in *H. acetosella* leaves. Each species contains a different anthocyanin and two anthocyanins (delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside) were identified from *H. sabdariffa* (Roselle) calyx . The Roselle calyx's colour is determined by the amount of anthocyanins it contains; 60-80% of this variance in colour is due to genetic factors. Roselles with a dark-red calyx colour have particular anthocyanin levels and better antioxidant activity than light-red roselles and white cultivars lacking red pigments. It was also discovered that certain post-harvest and environmental variables, such as the dry season, high CO_2 environments, and low temperature and airflow velocity drying processes, were associated with increased amounts of specific anthocyanin compounds [19].

The majority of anthocyanins are glycosylated or acylated on their aglycone unit by various sugars and aromatic or aliphatic acids; free anthocyanins are exceedingly uncommon. The Cy3G anthocyanin is the most prevalent kind. Several investigations have demonstrated that the predominant anthocyanin in the indicated berries and cereals is Cy3G, and that these anthocyanins are more stable than others in the presence of heat, light, and acid. Numerous studies have demonstrated the anti-aging, anti-cancer, and liver and kidney protecting properties of Cy3G. Consequently, eight plants (*Rhubus fruticosus L., Lonicera caerulea L., Ribes*

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nigrum L., Morus alba L., Zea mays L. seed, Z. mays L. cob, Brassica oleracea L., and Dioscorea alata L.) had their Cy3G content (Cy3GC) measured using HPLC [20]. Cy3G has been characterised in *Zea mays L.* [21] and in blackberries [22]. With the use of narrow bore HPLC columns and HPLC/ESI/MS, there was the ability of determining the amount of blackberry (Rubus species) juice and show that Cy3G makes up around 80% of the total anthocyanin contents in blackberry extract [23].

The amino acid (Histidine) which eluted at a retention time of 21.25 min and *m/z* = 157.1 had been identified in *H. cannabinus L.* (Kenaf) and performs different important functions including; antioxidant, anti-secretory and anti-inflammatory within the body. It also serves as a requirement for the growth and development of plants. Research on the pharmacological properties of Kenaf has shown that this edible plant has a wide range of potential medical uses, including as anti-inflammatory, hepatoprotective, antioxidant, anti-bacterial, anti-tyrosinase, anti-cancer, and anti-hyperlipidemia properties. Furthermore, *H. sabdariffa* (Roselle) and Kenaf had similar pharmacological, nutritional, and phytochemical characteristics. *H. acetosella* was observed to contain anti-oxidants, anti-bacterial, anti-cancer, anti-inflammatory, anti-anaemic effects and other health benefits which can be attributed to the presence of flavonoids such as Quercetin-3-(6''- galloylglucoside) and anthocyanins in the extract. In addition, Daidzein which eluted a retention time of 33.67 minutes contributes to anti-diabetic, anti-fibrotic, anti-hyperglycemic and treats menopausal symptoms, thrombosis, hypertension vertigo and deafness [24].

This present study agrees with literature data whereby flavonoids; Quercetin-3-(6''- galloylglucoside), Luteloin-7-O-glucoside (LuT7G), Kaempferol-7-O-glucoside (KeP7G) and 4-hydroxybenzoic acid were characterised in the extracts of leaves and flowers of *Paeonia rockii* using UHPLC-Q-Exactive in negative mode [25]. LuT7G has been identified in *H. syriacus* [26] and found to increase cytotoxic effects, anti-inflammatory, anti-cancer and anti-oxidant activities. Moreover, LUT7G regulates cell cycles and triggers apoptosis [27]. KeP7G (isomer of LuT7G) was identified in *H. rosa-sinensis* using UHPLC-ESI$^+$-Obitrap-MS. The $[M+H]^+$ = 447.4 was characterised as KeP7G, $[M-H]^-$ = 285.4 as Kaempferol unit and $[M+H]^+$ = 287.4 as Luteloin. The flavonoids that were found included flavan-3-ols like epicatechin and catechin, glycosylated flavonols from quercetin and Kaempferol, and anthocyanins from cyanidin [28].

Many plants contain Hederagenin (HG), a pentacyclic triterpenoid, in the form of sapogenins or saponins. HG was characterised in *H. acetosella* leaves' extract at a retention time of 21.75 min and $[M-H]^-$ = 471.7 which was achieved by deprotonation of the molecule. This triterpenoid saponin has the ability to stop the cell cycle and reduce the growth of tumour cells. HG has a multitude of pharmacological properties, such as antiviral, anticancer, anti-inflammatory, antidepressant, anti-neurodegenerative, antihyperlipidemic, and antihyperlipidemic properties [29]. Utilising MS spectra in the negative ion mode, the mass spectrum derived from the Hederagenin signal at 91.7 min and from conventional yeast extracts was further examined. Yeast expressing CYP72A397 had a *m/z* ratio of 471.3334 $[M-H]^-$ in its MS fragmentation pattern. The Hederagenin signal's MS^1 spectrum and the MS^2 spectrum derived from $[M-H]^-$, Hederagenin's precursor ion, were the same as those of real Hederagenin. It exhibits particularly promising results in the field of anti-tumour therapy and it is speculated that it contributes to mechanisms that reduce plasma cholesterol [30].

More than 500 negative-ion MS-MS spectra were obtained from three libraries and used in systematic toxicological investigation as well as general unknown screening in toxicology. Chemical and medicinal classes were used to categorise the compounds under research. The MS-MS spectra were manually analysed, and the scientific literature was searched for pertinent interpretation information. The protonated molecules $[M+H]^+$ undergo characteristic fragmentation through two competing fragmentation events that produce two complementary fragment ions. The *m/z* of $[M+H]^+$ +1 is equal to the total of the *m/z* values of these fragments. For the deprotonated molecules $[M-H]^-$ of the chemicals in the library collection, this kind of fragmentation was not as widely detected. When it was noticed, the *m/z* of $[M-H]^-$ -1 is equal to the total of the *m/z* values of the two pieces. There were a few negative-ion MS–MS spectra available, including those for labetolol, acebutolol, carvedilol, and isoproterenol. For these five molecules, hardly any common fragmentation pathways could be found. Three different drugs: celiprolol ($[M-H]^-$ with *m/z* 378), carvedilol ($[M-H]^-$ with *m/z* 405), and acebutolol ($[M-H]^-$ with *m/z* 335) [31].

The alkaloids; Norisoliensinine and Liensinine were characterised at a retention time of 22.28 min and 22.34 min, M^+ = 597.3 and $[M+2H]^{2+}$ = 613.3, respectively. As opposed to isoliensinine, Norisoliensinine had one fewer methyl group. Their MS/MS analysis revealed that they had the identical isoliensinine ion (*m/z* = 192.1, $C_{11}H_{14}NO_2$). Demethylation may have occurred as a result. Further evidence of their resemblance to isoliensinine was provided by detailed fragments. All the fragments were characterised as outlined in research [32]. This ionisation is possible as the molecule (M) gains two protons (H^+) during the ionisation process in the MS hence the resultant $[M+2H]^{2+}$ ion. The instrument can then use a variety of fragmentation paths to fragment this doubly charged ion. A method for identifying microcystins made of known amino acids was proposed: in the case of microcystins that give abundant $[M+2H]^{2+}$ ions, adding bases containing nitrogen to the aqueous sample solution can effectively increase the intensity of $[M+H]^+$ ions; in the case of microcystins that give weak $[M+2H]^{2+}$ ions, adding Lewis acids containing nitrogen can increase the abundances of $[M+2H]^{2+}$ ions [33].

3.4 Gas Chromatography Mass Spectroscopy (GC-MS)

The non-polar compounds found in *H. acetosella* leaves were extracted using hexane solvent and analysed using GC-MS. The compounds eluted at different retention times from the column based on their interactions with the stationary phase as shown in Fig. 2. They were later sorted into their *m/z* ratio using the MS. Phytochemical screening of the hexane fractions of *H. acetosella* leaves revealed the presence of some phytochemicals which have been observed to have antibacterial, antioxidant and other activities in other plant species. The most dominant phytochemicals observed included monoterpenes, triterpenes, catecholamines such as epinephrine which is both a neurotransmitter and a hormone, and acylic hydrogenated diterpene alcohol such as phytol which was eluted at a retention time of 25.14 minutes [34]. With the aid of NIST library, Mass Spectrometry and research, 13 compounds were able to be identified in the hexane extracts as shown in Table 3.

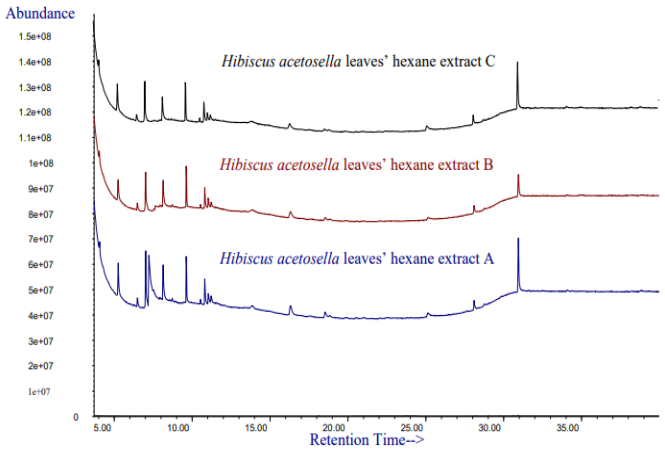

Fig. 2 GC-MS chromatogram data for the *H. acetosella* leaves' hexane extracts

The elution profile can be explained based on polarity, volatility, boiling point, density and molecular weight of the bioactive compounds in the extracts [35]. Toluene, which was eluted at 5.25 minutes, had the lowest molecular weight while Squalene, eluted at 30.97 minutes, had the highest molecular weight. In the GC column, compounds with the lowest molecular weight are eluted first. This is because their smaller size and lower boiling points cause them to interact less with the stationary phase in the column. They therefore move through the column more quickly and interact with the stationary phase for shorter periods of time, emerging ahead of heavier compounds.

In terms of boiling point, compounds with a lower boiling point are more vaporisable and volatile, which enables them to pass through the column more quickly. This explains why different isomers such as *p*-Xylene, *m*-Xylene and *o*-Xylene were eluted at different retention times besides them having the same molecular weight. The compound *p*-Xylene was eluted at 7.94 minutes and its boiling point is 138 °C, *m*-Xylene and *o*-Xylene were eluted at 8.14 and 8.73 minutes and their boiling points are 139.1 °C and 144.4 °C respectively. The relative densities of the three compounds are 0.8611, 0.8642 and 0.8802 g/mL respectively [36].

Generally, triterpenes are the largest in terms of molecular weight and the most polar compared to diterpenes and monoterpenes. The general elution programme of these terpenes indicates that monoterpenes such as Pinene –α, Pinene –β elute first followed by diterpenes such as phytol and finally triterpenes such as squalene. This was observed in the elution of such compounds from the GC column. According to research, the boiling points and relative densities were observed to affect the elution of the compounds between 9.64 and 11.24 minutes. Tricyclene was eluted last and has a relative density of 0.8668 g/mL at 25 °C and a boiling point of 185.55 °C while Pinene –α was eluted first and has a density of 0.850 g/mL and a boiling point of 155 °C. The other three compounds have the

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following relative densities and boiling points; Phellandrene –β, 0.852 g/mL and 165 °C, Pinene –β, 0.859 g/mL and 172 °C and Carene δ-3, 0.862 g/mL and 175 °C [37].

The polarity of the stationary phase is another factor that affected elution in a GC column. The relative polarities of a compound and the stationary phase determine how they interact. Stronger interactions and a slower rate of compound elution can result from a match in polarity [38]. This explains the elution profile of the compounds present in the extract where, for example, Phytol was eluted at 25.14 minutes and Carene δ-3 at 11.23 minutes. This indicated that Phytol is more polar compared to Carene δ-3 but less than Squalene which eluted at 30.97 minutes [39].

3.5 Fourier Transform-Infra Red (FT-IR) Analysis of the Polar Extracts

The crude extracts were analysed using FT-IR as shown in Figs. 3 and 4 and exhibited strong absorption bands which belonged to hydrocarbon groups (2942.84 cm^{-1} corresponding to a methyl group – stretching alkane and 2835.81 cm^{-1} corresponding to C-H_2 – stretching alkane). The strong, broad peak at 3342.03 cm^{-1} was attributed to O-H stretching alcohol (intermolecular bonded). This shows that anthocyanins extracted from *H. acetosella* can undergo intermolecular interactions to enhance their stability. The medium peak at 1648.84 cm^{-1} was attributed to C=C stretching and corresponded to a cis-disubstituted and conjugated alkene. Moreover, bands corresponding to stretching vibration of the aromatic rings were observed in the skeleton of Quercetin-3-(6''-galloylglucoside), LuT7G and Kep7G flavonoids and anthocyanins; Cy3G and Cy3Ga. The medium peaks at 1410.67 cm^{-1} and 1108.87 cm^{-1} were attributed to O-H bending of an alcohol in Daidzein and 4-hydroxybenzoic acid (polyphenols') plane deformation and C-O stretching of a secondary alcohol. The hydroxyl and C-O groups can also be observed in the structure of Cy3G and Cy3Ga anthocyanins.

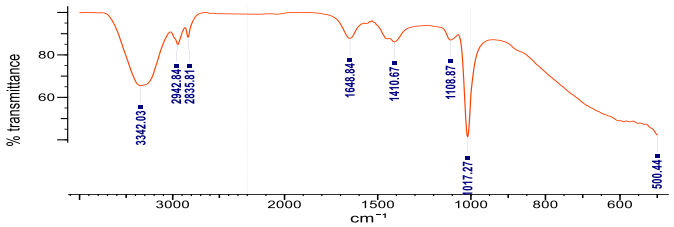

Fig. 3 FT-IR spectrum of Ethanol + 0.1% HCl extract

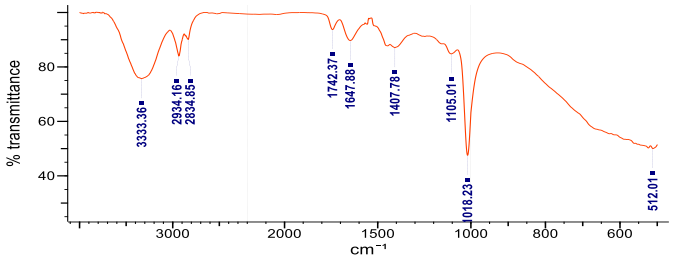

Fig. 4 FT-IR spectrum of Ethanol + 2% Acetic acid extract

The crude extracts were analysed using FT-IR as shown in Figs. 3 and 4 and exhibited strong absorption bands which belonged to hydrocarbon groups (2942.84 cm^{-1} corresponding to a methyl group – stretching alkane and 2835.81 cm^{-1} corresponding to C-H_2 – stretching alkane). The strong, broad peak at 3342.03 cm^{-1} was attributed to O-H stretching alcohol (intermolecular bonded). This shows that anthocyanins extracted from *H. acetosella* can undergo intermolecular interactions to enhance their stability. The medium peak at 1648.84 cm^{-1} was attributed to C=C stretching and corresponded to a cis-disubstituted and conjugated alkene. Moreover, bands corresponding to stretching vibration of the aromatic rings were observed in the skeleton of Quercetin-3-(6''-galloylglucoside), LuT7G and Kep7G flavonoids and anthocyanins; Cy3G and Cy3Ga. The medium peaks at 1410.67 cm^{-1} and 1108.87 cm^{-1} were attributed to O-H bending of an alcohol in Daidzein and 4-hydroxybenzoic acid (polyphenols') plane deformation and C-O stretching of a secondary alcohol. The hydroxyl and C-O groups can also be observed in the structure of Cy3G and Cy3Ga anthocyanins.

Finally, the strong absorption peak at 1017.27 cm^{-1} was attributed to C-O-C in the athocyanidin structure of Cyanidin, Daidzein polyphenol and flavonoid structures of Luteloin, Kaempferol and quercetin. The "fingerprint" region, which spans the infrared spectrum from 1542 to 965 cm^{-1}, is known for containing a variety of IR bands, including those that represent the vibration of the C-O, C-C, C-H, and C-N bonds. Important details about organic substances like glucoside and galactoside sugars, alcohols, and organic acids that are most likely present in the extract can be found in this section. Moreover, there was an additional peak in the ethanol + 2% acetic acid extract (Fig. 4) at 1742.37 cm^{-1} which was attributed to Carbonyl (C=O) stretch of a carboxylic acid and may have been detected in the acetic acid used [40].

4. Conclusion

The present study provided reliable information on the percentage yield and phytochemical constituents of *H. acetosella* leaves. It has been observed that in comparison with similar work from literature conducted on other species of *Hibiscus* genus, the identified compounds with the aid of chemical tests, GC-MS, LC-MS and FT-IR are very rich in antibacterial, anti-oxidant, anti-inflammatory, pharmacological importance and the extract can be used in textile dyeing. Therefore, it is recommended that further research could be conducted on their isolation and structure elucidation for such uses and other industrial applications.

Author contributions

Bernard W. Makau: Conceptualisation; methodology; formal analysis; validation; investigation; visualisation; software; data curation; writing - original draft; writing - review and editing. *Benson G. Ongarora:* Conceptualisation; supervision; funding acquisition; methodology; resources; project administration; writing - review and editing. *Rose Tanui:* Conceptualisation; supervision; resources; writing - review and editing; methodology. *Raja Kannan:* Technical assistance; resources; writing - review and editing; methodology. All authors have read and agreed to the published version of the manuscript.

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Competing interests

The authors declare that they have no competing interests.

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