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Phytochemical screening and antioxidant activity of *Ipomoea hederifolia* stems: A potential medicinal plant

MD. MAHMUD HOSSAIN[1], MOHAMMAD SAROWAR UDDIN[1,♥], PRODIP KUMAR BARAL[1], MAHMUDA FERDUS[1], SUTAPA BHOWMIK[2]
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Manuscript received: 2 July 2022. Revision accepted: 13 August 2022.

Abstract. *Hossain MM, Uddin MS, Baral PK, Ferdus M, Bhowmik S. 2022. Phytochemical screening and antioxidant activity of* Ipomoea hederifolia *stems: A potential medicinal plant. Asian J Nat Prod Biochem 20: 41-47. Ipomoea hederifolia* L., a plant of the Convolvulaceae family, popularly known as morning glory, possesses numerous medicinal values. The present study aimed to explore the antioxidant activity and bioactive compounds of *I. hederifolia* stems (IHS). The IHS was soaked in methanol for 21 days. The filtrate was concentrated using a rotary evaporator after filtration to obtain IHS extract, which is subjected to phytochemical screening using various tests. Antioxidant activity was determined using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and reducing power activity (RPA) methods. The phytochemical screenings revealed that IHS extract possesses carbohydrates, tannins, flavonoids, phenols, saponins, alkaloids, steroids, anthraquinones, and cardiac glycosides. The antioxidant activity of IHS extract in DPPH model was moderate (IC_{50} :174.08 µg/ml) in comparison with standards (IC_{50}: 102.28 µg/mL AA; IC_{50}: 88.52 µg/mL for BHT). It indicates that IHS exerts free radical scavenging power in a dose-dependent fashion. The RPA model also produced moderate antioxidant potential (EC_{50}: 279.58 µg/mL for IHS; EC_{50}: 23.121 µg/mL for AA; EC_{50}: 50.84 µg/mL for BHT) depending on increasing order of dose. Based on the findings of this investigation, we can conclude that IHS extract possesses various bioactive compounds and moderate antioxidant potentials, which may be a path to the discovery of traditional medicines and remedies for many critical diseases.

Keywords: Antioxidant activity, DPPH, *Ipomoea hederifolia*, phytochemical screening, RPA

Abbreviations: AA: Ascorbic acid; BHT: butylated hydroxyl toluene; DPPH: 1,1-Diphenyl-2-picrylhydrazyl; EC_{50}: Half maximal effective concentration; IC_{50}: Half maximal inhibitory concentration; IHS: *Ipomoea hederifolia* stems; RPA: Reducing power activity; WHO: World Health Organization

INTRODUCTION

Nature has been regarded as the greatest source of medicinal agents for thousands of years (Uddin et al. 2019). A huge number of modern drugs have been isolated from natural sources based on their use in traditional medicine (Millat et al. 2019; Kurian et al. 2022). Many important pharmaceuticals that indigenous people currently use originated from plant sources (Balick et al. 1996). The developing countries mostly rely on traditional plants and focus on healthcare applications (Allameh et al. 2002). Traditional medicine involves using different plant extracts or bioactive constitutions (Davis et al. 1994).

Several edible plants play a vital role in different ethnic groups by providing fresh food, nutrition, and medicines. Various research has been conducted to record ethnobotanical knowledge of wild edible plant species (Mallick et al. 2020). Many plant species have demonstrated significant pharmacological activities in animal and human studies, such as antioxidant, cytotoxic, anti-inflammatory, antiproliferative, analgesic, immunomodulatory, antimicrobial, hepatoprotective, hypoglycemic, hypotensive, hypolipidemic, diuretic, etc. (Nugroho et al. 2020; Putra et al. 2020; Ray and Saini 2021). In addition, these plants have abundant flavonoids, alkaloids, phenols, saponins, anthraquinone, tannins, cardiac glycosides, steroids, etc. These bioactive compounds and therapeutic actions verify many folk medicinal claims on edible plant species.

About 64% of the global population remains dependent on traditional medicines and medicinal plants to provide for their healthcare needs (Cotton 1996). According to a study by WHO, practitioners are prescribing traditional medicine to about 80% of patients in India, 85% in Burma, and 90% in Bangladesh. The writings indicate that the therapeutic use of plants is as old as 4000-5000 BC, and the Chinese first used natural herbal preparation as medicines. Also, modern pharmacopeia still contains at least 25% of drugs derived from plants and many other synthetic compounds isolated from plants (Tewari 2000). Since disease, decay, and death have always co-existed with life, early man had to think about disease and its treatment. Thus, the human race started using plants to treat diseases and injuries from the early days of civilization on earth. Its long journey from ancient to modern times has successfully been an effective therapeutic tool for fitting against diseases and various health hazards (Ghani 1998).

The human body possesses both enzymatic and non-enzymatic antioxidant defenses to combat free radicals and other oxidants (Alam et al. 2013). Free radicals cause cancers, Parkinson's, cardiovascular disease, Alzheimer's, neural disorders, mild cognitive impairment, ulcerative colitis, and alcohol-induced liver disease (Alam et al. 2013). Antioxidants boost protection against free radicals. Substantial data suggest that antioxidant-rich diets may be important in disease prevention. Scientists agree that a mixture of antioxidants may be more beneficial for long-term immunity boosting. Synthetic antioxidants have been seriously explored for animal use to enhance health, performance, and product quality. Possibly these substances might cause harm; thus, their innocuousness must be questioned. Many phytochemicals have been found to benefit animals in terms of improved performance and quality (Ansari et al. 2012; Lee et al. 2013), as well as an improved endogenous antioxidant system (Aggarwall and Shishodi 2006), potentially by impacting particular molecular targets directly or indirectly through stabilized conjugates that influence metabolic pathways. Therefore, scientists are searching for new antioxidant compounds to address various diseases.

Ipomoea hederifolia L. (Convolvulaceae), known as morning glory, is an annual twiner with a thin and frail stem (Flora of Bangladesh 2022). It is typically found in the untamed areas of the Chattogram Hill Tracts in Bangladesh. According to India's indigenous medicine system, it belongs to anti-psychotic, antioxidant, anti-cancer, antimicrobial, oxytocic, and anti-inflammatory activities. However, no ethnopharmacological study has been conducted on its stem. Therefore, the present study aimed to explore the antioxidant activity and bioactive compounds of *I. hederifolia* stems (IHS).

MATERIALS AND METHODS

Plant collection

IHS was collected from the Baroiyadhala National Park in Chittagong, Bangladesh. First, the stems were handpicked from healthy plants and washed with distilled water. Then stems were subjected to sun drying for two weeks. Finally, dried stems were powdered into a fine powder and stored in an airtight container.

Extract preparation

About 200 gm of powdered stems were soaked for 21 days in 1000 mL methanol to dissolve the bioactive compounds in a solvent. Then the soaked powder was filtrated with Whatman filter paper. Finally, the filtrate was collected and subjected to a rotary evaporator to obtain a concentrated extract (Millat et al. 2019; Naznin et al. 2019).

Phytochemical screening

The preliminary phytochemical tests determine the presence of several chemical groups in the extract. A tiny amount of methanol extract of IHS was subjected to preliminary qualitative phytochemical evaluation using recognized methodologies for detecting phytochemicals such as alkaloids, cardiac glycosides, anthraquinone, steroids, and saponins (Uddin et al. 2020; Talukder et al. 2022). The detection was based on visual observations of a color change or precipitate formation after adding specific reagents.

Carbohydrate screening

Benedict's test: In test tubes, a few drops of Benedict's reagent were added to 1 mL of extract and heated for a few minutes in a water bath. The presence of carbohydrates in the extracts is indicated by the formation of a reddish-brown precipitate in the tube. Molisch's Test: 1 mL of extract was mixed with 1 mL of Molisch's reagent and a few drops of concentrated H_2SO_4. The presence of carbohydrates in the extracts is shown by the formation of a purple or red color in the tube (Sheela Rani et al. 2013).

Tannin screening

In tubes, 1 mL of the extract was mixed with 2 mL of 5% ferric chloride. The presence of tannins in the extract is shown by the formation of greenish-black or dark blue in the tube. The extract was dissolved in 10 mL of distilled water and then filtered. About 1% aqueous solution of $FeCl_3$ was also added. The presence of tannins in the extracts is indicated by the formation of strong green, purple, blue, or black in the tube (Ögenler et al. 2018).

Saponins screening

Two (2) mL of distilled water were added to the 2 mL extract and thoroughly mixed for 15 minutes by shaking lengthwise in a graduated cylinder. The presence of saponins in the extracts is shown by forming a 1 cm layer of foam (Sofowora et al. 2013).

Flavonoid screening

A total of 0.5 g of extract was thoroughly mixed with petroleum ether (lipid layer) to eliminate the fatty components. Then, the defatted residue was filtered after being dissolved in 20 mL of 80% ethanol. The filtrate was used in the following experiments:

In a test tube, 3 mL of the filtrate was combined with 4 mL of 1% aluminum chloride in methanol. The formation of yellow color indicates the presence of flavonols, flavones, and chalcones in the extracts. Dilute ammonia solution (5 mL) was added to a fraction of each plant extract's aqueous filtrate, followed by adding conc. H_2SO_4. The presence of flavonoids in the extracts is shown by the formation of yellow color (Yusuf et al. 2014).

Shinoda's Test: The extract was first dissolved in alcohol, and then a fraction of magnesium was combined with conc. HCl, which was applied dropwise. The presence of flavonoids in the extracts is shown by the formation of a magenta color (Hossain et al. 2013).

Alkaloid screening

Mayer's test: 2 mL of the stem extract was mixed with 2 mL of concentrated hydrochloric acid (HCl). A few drops of Mayer's reagent were then added. The presence of

alkaloids in the extracts is indicated by forming a green or white precipitate (Polyium and Phinthida 2018).

Wagner's Test: Filtrates were subjected to Wagner's reagent treatment (Iodine in Potassium iodide). The formation of brown/reddish precipitate revealed alkaloids' presence.

Dragendroff's Test: Dragendroff's reagent was used to treat the filtrates (solution of Potassium Bismuth Iodide). The formation of an orange-red precipitate showed the presence of alkaloids.

Quinine screening

An alcoholic potassium hydroxide solution was added to 1 mL of various extracts. The color change shows the presence of quinines in the extracts from red to blue. One (1) mL of conc. H_2SO_4 was added to 1 mL of the various extracts. The presence of quinones in the extracts is shown by the formation of a red hue in the tube (Zohra et al. 2012).

Tarpinoid screening

Salkowski Test: Solvent extract (5 mL) was combined with chloroform (2 mL) and saturated H_2SO_4 (3 mL). A layer of reddish brown color appeared at the tube interface, indicating the presence of terpenoids in the extracts (Zohra et al. 2012).

Glycoside screening

Keller Killiani Test: The extract was treated with a few drops of glacial acetic acid and a ferric chloride solution. The tube was well mixed by vigorous shaking, and then conc. H_2SO_4 was added. In the tube, two layers can be seen: the reddish brown layer in the lower section and the acetic acid layer in the upper part. Subsequently, the presence of glycosides in the extracts shows the solution turning bluish-green (Mulla and Paramjyothi 2010).

Bromine water test: A few drops of bromine water were added to the extract. The presence of glycosides in the extracts is shown by forming a yellow precipitate in the test tube (Mulla and Paramjyothi 2010).

Anthraquinone glycosides

Borntrager's test: Plant extract of 100 mg was mixed in 5 mL of chloroform and filtered. The filtrate was then thoroughly agitated with an equal amount of ammonia solution (10% NH_4OH). The presence of anthraquinones is indicated by the emergence of a pink-violet or red color in the ammoniacal layer (Zohra et al. 2012).

Triterpenoid screening

Liebermann Burchard test: A few drops of acetic anhydride were added to the extract, and the mixture was heated and cooled. Along the walls of the test tube, conc. H_2SO_4 was added. The presence of triterpenoids in the extracts is indicated by the formation of a brown ring at the intersection of two layers, deep red color in the bottom half and green color in the upper part (Francis and Suseem 2016).

Phenol screening

Ferric chloride test: 50 mg extract was thoroughly mixed with distilled water before adding a few drops of 5% $FeCl_3$ solution. The presence of phenols is indicated by the formation of blue, green, and violet colors.

Gelatin test: A little amount of extract was thoroughly mixed with distilled water, and 2 mL of a 1% gelatin solution containing 10% NaCl was added and mixed thoroughly. The presence of phenols in the extracts is shown by forming a white precipitate (Sheela Rani et al. 2013).

Cardiac glycoside screening

In a test tube, 2 mL of the mother solution of extracts was mixed with a few drops of weak hydrochloric acid, 2 mL of Sodium Nitroprusside in pyridine, and 2 mL of Sodium Hydroxide solution. The presence of cardiac glycosides in the extracts is indicated by the formation of a pink to blood-red color (Zohra et al. 2012).

Steroid screening

Salkowski's test: 1 mL of each extract was mixed with 1mL chloroform and a few drops of concentrated H_2SO_4. The presence of steroids in the extracts is shown by forming a brown ring (Oyinlade 2014).

Liebermann Burchard's test: Chloroform extracts were treated with acidic anhydride, heated, cooled, and then treated with strong sulfuric acid. The appearance of a transitory greenish tint indicates the presence of steroids (Raaman 2006).

Antioxidant assay

DPPH method

The antioxidant activity of the methanol extract of IHS was determined using the scavenging activity of the stable 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical, as described by Khanapur et al. (2014) with minor alterations. At first, a fresh 0.2 mM DPPH solution in methanol was prepared. 2 mL of each extract, Ascorbic acid, and Butylated hydroxytoluene solution were pipetted into separate test tubes, and 3 mL of 0.2 mM DPPH solution was added to each sample to initiate the reaction. All of the samples were mixed and kept in a dark place for 30 minutes. After 30 minutes of incubation, the absorbance was measured using a UV-VIS Spectrophotometer at 517 nm. Methanol was utilized as a control, while a 0.2 mM DPPH solution was used as a blank. All extracts and standards were analyzed in triplicate. The antioxidant activity of each extract was measured by estimating the percentage of antioxidant activity using the following formula based on the reduction of DPPH absorbance.

$$\text{Percentage (\%) of free radical DPPH scavenging} = (A_o - A1)/A_o \times 100.$$

Where; A_0 is the absorbance of the control solution, which contains all reagents except plant extracts, and A1 is the absorbance of the DPPH solution, which contains plant extracts. Finally, the concentration of sample necessary to scavenge 50% of the DPPH free radical (IC_{50}) was

computed using the plot of the percentage of inhibition versus extract concentration. For this experiment, ascorbic acid and BHT were utilized as standards.

Reducing power activity (RPA)

The reducing power of the plant extracts, ascorbic acid, and BHT was determined using the method reported by Kubola and Siriamornpun (2008) with minor modifications. Ascorbic acid and BHT were employed as standards in this study. A variety of ascorbic acid, BHT, and IHS solutions was created. An aliquot of the varying concentrations of the standard and sample solution reagents was added, followed by 2.5 mL of sodium phosphate buffer and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated in a water bath at 50°C for 20 minutes before being cooled and terminated (reaction) with 2.5 mL of 10% (w/v) trichloroacetic acid. After centrifuging the mixture at 3000 rpm (930 x g) for 10 minutes, 2.5 mL of the supernatant was combined with 2.5 mL of distilled water and 0.5 mL of freshly made 0.1% (w/v) anhydrous ferric chloride ($FeCl_3$) solution. A UV-1601 Shimadzu UV-Vis spectrophotometer was used to measure absorbance at 700 nm. A linear connection was created after graphing the absorbance against the concentration, which was utilized as a standard curve for determining the RPA of the test samples. The results were presented as effective concentration (EC_{50}) values, and the RPA was calculated using a standard curve.

Statistical analysis

In this investigation, the findings were summarized using the mean value and the standard deviation of at least three independent samples. Statistical analyses were conducted utilizing IBM's Statistical Package for the Social Sciences (SPSS, Version 24).

RESULTS AND DISCUSSION

The phytochemical screening of IHS showed the presence of alkaloids, cardiac glycosides, flavonoids, steroids, saponin, diterpenes, tannins, carbohydrates, and phenols, as exhibited in Table 1. IHS extract was subjected to assessment for antioxidant activity based on these results. The study showed that the plant could be used for various purposes.

The free radical scavenging and antioxidant activity of medicinal plants are linked with the remedy of several diseases (Prathapan et al. 2011). The DPPH test demonstrates a substance's antioxidant activity by causing a color change when placed in an environment containing a free radical scavenger (Naznin et al. 2019). The reaction that causes the color to change from purple to yellow indicates the process by which the DPPH radical acts as the oxidizing radical that the antioxidant must reduce. The presence of an odd electron in DPPH is responsible for the color transformation and absorbance that can be seen at 517 nm (Naznin et al. 2019). The antioxidant activity of IHS was shown by its methanolic extract with DPPH, which reduced DPPH-H. The degree of discoloration that occurred was used to measure the antioxidant activity. The extracts showed a proclivity to eliminate DPPH free radicals in this study, with an IC50 value higher than the reference standards AA and BHT (Table 2). The IC_{50} values of AA, BHT, and IHS were 102.28, 88.52, and 174.08 µg/mL, respectively. The maximum % of inhibition for AA, BHT and IHS were 91.77%, 88.30 % and 88.82% at 500 µg/mL where the minimum % of inhibition were 19.28%, 18.12% and 6.43% at 0.977 µg/mL, respectively. That indicates that the DPPH radical activity of methanol IHS extracts increased dose-dependent (Figure 1). These results collectively reported that IHS possesses a strong scavenging activity compared to standards.

Another important method for the evaluation of antioxidant activity is the RPA method. A compound's reducing capability may indicate its antioxidant potential (Meir et al. 1995). The reduction of Fe_3^+ is widely employed as an indication of electron-donating activity, where the solution's yellow color changes to blue and green depending on a compound's ability to reduce free radicals. Phenols convert ferric–ferricyanide to prussian blue ferrocyanide. In this study, the maximum absorbance for BHT, AA, and IHS were 2.523, 4.12, and 0.775 at 500 µg/mL, whereas the minimum absorbances were 0.192, 0.183, and 0.175 at 1.953 µg/mL respectively (Table 3). Again, the EC_{50} value for BHT, AA, IHS were 50.84, 23.12, and 279.58 µg/mL respectively. That indicates that IHS possesses moderate antioxidant capacity as compared to standards.

Table 1. Bioactive compounds analysis of methanol extract of IHS

Constituents	Tests	Methanol extract of IHS
Alkaloids	Mayer's reagent	+
	Wagner's reagent	+
	Dragendoff's reagent	+
Cardiac glycosides	Keller-Killiani Test	+
Steroids	Salkowski's Test	+
	Lieberman-Burchard test	+
Saponins	Frothing test	+
Anthraquinones	Borntrager's test	-
Carbohydrates	Benedict's test	+
	Molisch's Test	+
Tannin		+
Flavonoid	Elimination of the fatty components	+
	Shinoda's Test	+
Tarpinoid	Salkowski Test	-
Triterpenoid	Lieberman-Burchard test	-
Phenols	Ferric chloride test	+
	Gelatin test	+

Note: (+) Indicates the presence and (-) indicates the absence of a chemical compound in the sample

Table 2. DPPH free radical scavenging (SCV) activity of methanol extract of the IHS, ascorbic acid (AA), and butylated hydroxyl toluene (BHT)

Conc. (µg/mL)	Avg. absorbance			% of inhibition			IC_{50} (µg/mL)		
	AA	BHT	IHS	AA	BHT	IHS	AA	BHT	IHS
0	0.778±0.002	0.778±0.005	0.778±0.016	0.0	0.0	0.0	102.28	88.52	174.08
0.97656	0.628±0.042	0.637±0.015	0.728±0.005	19.28	18.12	6.43			
1.9531	0.619±0.088	0.603±0.002	0.712±0.002	20.44	22.49	8.48			
3.90625	0.589±0.032	0.561±0.003	0.689±0.012	24.29	27.89	11.44			
7.8125	0.536±0.025	0.509±0.008	0.675±0.007	31.11	34.56	13.24			
15.625	0.449±0.032	0.418±0.014	0.628±0.004	42.29	46.27	19.28			
31.25	0.321±0.015	0.266±0.018	0.562±0.016	58.74	65.81	27.76			
62.5	0.206±0.077	0.159±0.003	0.387±0.008	73.52	79.56	50.26			
125	0.145±0.006	0.125±0.012	0.193±0.014	81.36	83.93	75.19			
250	0.095±0.003	0.104±0.025	0.135±0.024	87.78	86.63	82.65			
500	0.064±0.012	0.091±0.032	0.087±0.005	91.77	88.30	88.82			

Table 3. Reducing power activity of IHS, AA, and BHT

Concentration (µg/mL)	Avg. absorbance			EC_{50} (µg/mL)		
	BHT	AA	IHS	BHT	AA	IHS
1.953125	0.192±0.004	0.183±0.024	0.175±0.001	50.84	23.121	279.58
3.90625	0.204±0.026	0.222±0.002	0.185±0.022			
7.8125	0.258±0.034	0.299±0.008	0.192±0.014			
15.625	0.286±0.008	0.393±0.012	0.205±0.001			
31.25	0.41±0.043	0.576±0.004	0.226±0.005			
62.5	0.668±0.052	0.84±0.001	0.295±0.024			
125	1.247±0.038	1.82±0.043	0.344±0.012			
250	1.597±0.004	2.996±0.028	0.514±0.008			
500	2.523±0.006	4.12±0.001	0.755±0.004			

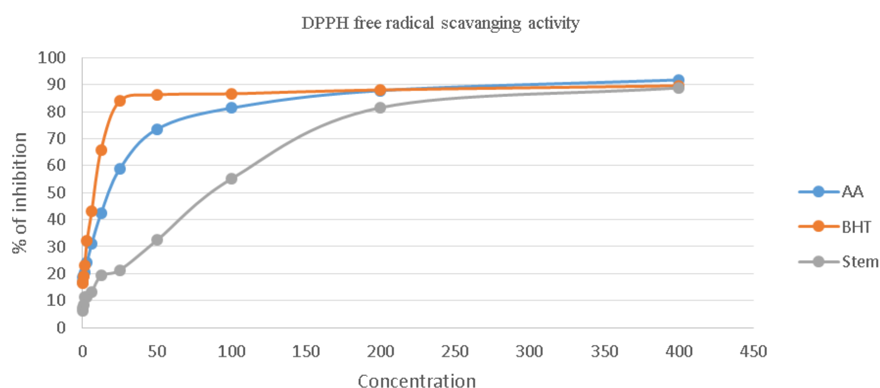

Figure 1. DPPH free radical scavenging activity assay of methanol extract of IHS in comparison with ascorbic acid and butylated hydroxyl toluene

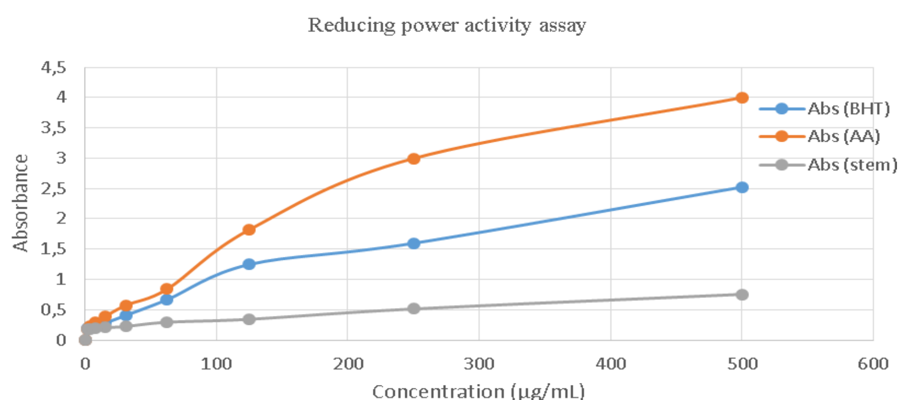

Figure 2. Reducing power activity assay of methanol extract of IHS in comparison with AA and BHT as standards

Since the dawn of civilization, people have relied on plants as a source of medicine to treat a wide variety of illnesses. Currently, the study of phytopharmacology has opened up a new field for discovering plant-derived pharmaceuticals that are helpful for the treatment of certain disorders and attract the attention of those working in the field of herbal medicine. Around thirty percent of medications are believed to be generated from plant compounds (Uddin et al. 2020). Our study has identified the presence of bioactive compounds such as alkaloids, cardiac glycosides, flavonoids, steroids, saponins, tannins, and phenols in the *I. hederifolia* stem. These bioactive compounds are responsible for curing tons of diseases. Several studies revealed that phenols and flavonoids are phytochemicals that exert strong antioxidant activity (Shi et al. 2003; Brenes et al. 2008; Lee et al. 2017). Our preliminary phytochemical screening showed the presence of phenols and flavonoids in the *I. hederifolia* stem. Therefore, we can claim that the *I. hederifolia* stem belongs to good antioxidant activity. We hope researchers will isolate new pharmacologically active compounds based on our current investigation.

In conclusion, the findings of this research demonstrate that IHS contains alkaloids, cardiac glycosides, flavonoids, steroids, saponin, diterpenes, tannins, carbohydrates, and phenols, which are powerful phytochemicals. Moreover, the current investigation also found that IHS had modest antioxidant activity compared to a reference standard. Therefore, a high concentration of phenolic compounds, as well as other phytochemicals, might be linked with the plant's antioxidant effects. So, extracting the plant's bioactive components might be a viable alternative to synthetic antioxidants.

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Comparative phytochemical analysis of mature mango leaves from nineteen cultivars of Murshidabad district, India

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Abstract. *Ghosh B, Majumder S, Acharyya S, Ghosh A, Saha S, Sarkar S, Chakraborty S, Bhattacharya M. 2022. Comparative phytochemical analysis of mature mango leaves from nineteen cultivars of Murshidabad district, India. Asian J Nat Prod Biochem 20: 48-55.* Mango (*Mangifera indica* L.), "the king of fruits," is one of the most popular fruits in tropical regions. This research aimed to qualitatively and quantitatively screen major phytochemical groups present in the leaves of nineteen cultivars of mango tree extracted with three different solvents (petroleum benzene, acetone, methanol) belonging to different polarities and to determine their antibacterial activity against two Gram-positive (*Bacillus subtilis, Staphylococcus aureus*) and two Gram-negative (*Escherichia coli, Klebsiella pneumoniae*) bacteria. The antiradical scavenging activity was performed using a DPPH assay. In addition, total phenol and flavonoids in the leaf extracts were also quantitatively analyzed. Phytochemical investigations showed that mango leaf extracts contain phenol, tannin, protein, coumarin, terpenoid, alkaloid, steroid, and cardiac glycoside. The extracts also contain a variety of total phenols and flavonoids. Antiradical scavenging activity showed that polar solvents (methanol, acetone) are more potent than nonpolar (petroleum benzene) extracts. Mango leaf extracts inhibit the growth of *E. coli, K. pneumoniae,* and *S. aureus,* but no inhibition zone against *B. subtilis.* Based on the phytochemical compounds and significant antioxidant and antibacterial properties of mango leaf extracts, mango leaves might be a potential source for developing pharmaceutical formulations and drugs.

Keywords: Antibacterial, antioxidant, mango, morphology, phytochemicals

INTRODUCTION

Mango (*Mangifera indica* L), belonging to the family Anacardiaceae, is one among sixty genera and six hundred species (Hannan et al. 2013). It is one of the economically important tropical fruits (Rymbai et al. 2013; Kumar et al. 2021). Mango plantations globally occupy an area of about 4.37 million hectares, and current mango production in the world is nearly 31.5 million metric tons (Rymbai et al. 2013). Mangoes are grown commercially in many countries (more than 111 countries), where India ranks first in both production and plantation area (Rymbai et al. 2013). Other well-known mango-producing countries include China, Thailand, Indonesia, Pakistan, Mexico, Brazil, Bangladesh, Nigeria, and the Philippines (Kumar et al. 2021). Besides fruit, mango leaves can also be used as a food supplement and a good fodder as it has a good amount of minerals, vitamins, and protein (Kumar et al. 2021; Jhaumeer et al. 2018).

Murshidabad is the producer and exporter of mango var. Himsagar. Mango var. Langra originated from Varanasi, Uttar Pradesh. Mango var. Amrapali is grown throughout India. It is a hybrid variety, a cross-breed between Dasher and Neelam mangoes, indigenous breeds. The Fazli variety is also indigenous to Malda (West Bengal) and Bihar. Gulabkhas variety is most abundant in Bihar, West Bengal, and Jharkhand (https://www.holidify.com). Mango varieties of Anaras, Sarenga, Molamjam, Champa, and Chandankosa are generally produced in Murshidabad (https://www.getbengal.com). Mango varieties of Rani, Champa, and Bhabani are also Murshidabad varieties. The Rani variety was named by Raja Prasanna Narayan Deb, Dewan of Nizamat Qilla, Murshidabad (Lahiri 2018). The Saranga variety was dedicated to the musicians who played the sarangi in Nawab's haveli. Gulabkhas, as the name suggests, has a mild taste of rose (Gulab), while the Anaras variety has a pineapple flavor; and Mohanbhog was named by the worshipers of Lord Krishna for some religious belief (https://sundayfarmer.wordpress.com/2020/05/26/). These are some of the examples which may not provide scientific data regarding the origin of mangos but signifies their nomenclature history.

Traditionally, the mango plant has been used to treat gastrointestinal tract infections, diarrhea, dysentery, mouth infection in children, typhoid, sore throat, and scurvy (Hannan et al. 2013). In addition, as a good source of vitamin A, mango can be used to treat blood disorders (Hannan et al. 2013). Mango is an important dietary source of many bioactive compounds responsible for its color and flavor. In addition, the fruit has been reported to possess antioxidant activity and could overcome oxidative stress by neutralizing the overproduction of oxidant species. The collaborative and interdependent effects of the complex

mixture of phytochemicals present in mango cannot be accomplished through micronutrient supplements (Abbasi et al. 2015).

Some parts of the mango plant have been known to have a pharmacological effect. Ground seeds and leaves of mango plants are generally used to treat diabetes. The stem bark of mango has been demonstrated to possess anti-allergic properties. Glucoside-rich mango leaves have been analyzed and contain mangiferin, a potent antiviral and antibacterial agent (Hannan et al. 2013). Mangiferin is a natural miracle biologically active compound against lifestyle-related disorders. Some studies have shown that mangiferin treats COVID-19 (Umar et al. 2021). Mangiferin has a binding affinity for the Mpro of COVID-19. Mpro is a key enzyme that plays a vital role in viral replication and transcription. Therefore, mangiferin can inhibit viral replication and transcription (Umar et al. 2021). Among the many bioactive compounds of mango leaf and fruit, polyphenols are abundant antioxidant compounds that act as anti-mutagenic, antiradical, and anti-carcinogenic agents (Rymbai et al. 2013; Abbasi et al. 2015). They reduce the risk of many chronic diseases by exhibiting varied biological activities due to one or more hydroxyl groups. The high amounts of phenolic acids are present in ripe mangoes, enabling them to play an important role in quenching and neutralizing free radicals to improve human health. Gallic acid, known as an anticancer agent, has also been reported to be in mango (Rymbai et al. 2013; Abbasi et al. 2015). Flavonoids (a group of potent bioactive polyphenols) in mango have been reported to act as an antioxidant and antibacterial agent (Rymbai et al. 2013).

Although several research studies have been conducted to find different uses of mango fruits, peels, juice, and stem bark, there are limited reports on the importance of *Mangifera indica* L. leaves and their uses. Therefore, in this present study, the objective was to determine the presence of bioactive phytochemicals in the leaves of different mango cultivars and their antibacterial activity.

MATERIALS AND METHODS

Sample collection and extraction

Healthy, disease-free leaves of nineteen different cultivars (Table 1) of mango plants were collected from mango plantations of Lalbagh (24.19°N, 88.28°E), a small town in West Bengal's Murshidabad district. Freshly collected leaves were brought to the laboratory and washed with tap water. Leaves were kept in Petri dishes and left at room temperature for drying. Then, the dried leaves were crushed in a mortar pestle using liquid nitrogen. Leaf extracts (100 mg/ml concentration) were prepared by dissolving 1 g of each sample (crushed leaves) into 10 ml of each different solvent (petroleum benzene, acetone, and methanol) for 48 hrs. Solvents were chosen based on a wide range of polarity, i.e., petroleum benzene (hydrocarbon-based, highly nonpolar), acetone (polar aprotic solvent), and methanol (most polar and protic solvent).

Morphological characteristics

Morphology is a set of important parameters used to study the shape and structure of organisms or organs (in this case, mango leaf) and their specific structural features. Length (cm), width (cm), petiole length (cm), weight (g), length: width ratio, and surface area (cm^2) of leaves were determined to study the morphology of different leaves. A measuring scale in centimeters was used to determine the length and width. Millimeter chart sheets were used for leaf area measurements. Twenty mature leaves of each variety were selected randomly, and the mean ± SD was calculated.

Qualitative Biochemical Tests

Qualitative tests for the detection of bioactive groups of molecules (tannin, protein, coumarin, terpenoid, alkaloid, steroid, and cardiac glycosides) in methanolic extracts were conducted following the protocols of Ghosh et al. (2020) and Majumder et al. (2021a).

Antioxidant activity (DPPH assay)

The free radical scavenging capacity of mango leaf extract was determined by the DPPH assay performed on petroleum benzene, acetone, and methanol leaf extract of all nineteen samples, following the protocol of Majumder et al. (2022a) and Ghosh et al. (2020). Results were expressed as the percentage of DPPH inhibition (%) that occurred due to the exposure of samples.

Quantification of total phenol content (TPC)

Quantifying total phenol content was performed by the Folin-Ciocalteu method described by Majumder et al. (2022a) and Sarkar et al. (2021). TPC was measured against the gallic acid standard curve, using the standard curve equation of $R^2=0.9975$; $y=0.0043x-0.1672$. Results were expressed as gallic acid equivalent (mg GAE/g).

Table 1. List of *Mangifera indica* varieties used in this study

Name of the variety	Sample code
Mangifera indica L. var Himsagar	S1
Mangifera indica L. var Langra	S2
Mangifera indica L. var Molamjam	S3
Mangifera indica L. var Sorikhas	S4
Mangifera indica L. var Champa	S5
Mangifera indica L. var Gulabkhas	S6
Mangifera indica L. var Rani	S7
Mangifera indica L. var Borosaheb	S8
Mangifera indica L. var Fazli	S9
Mangifera indica L. var Asina-Fazli	S10
Mangifera indica L. var Mohon-bhog	S11
Mangifera indica L. var Amrapali	S12
Mangifera indica L. var Chandankosa	S13
Mangifera indica L. var Daudi	S14
Mangifera indica L. var Bhabani	S15
Mangifera indica L. var Saranga	S16
Mangifera indica L. var Anaras	S17
Mangifera indica L. var Michriganj	S18
Mangifera indica L. var Tarabi	S19

Quantification of flavonoid content

Total flavonoid content was determined following the aluminum chloride method described by Ghosh et al. (2020) and Majumder et al. (2022a). Total flavonoid content was measured against the quercetin standard curve, using the standard curve equation of R^2=0.962; y=0.207x-0.204. Results were expressed as quercetin equivalent (mg QE/g).

Antibacterial test

Antibacterial potentials of each leaf sample against actively growing broth cultures of two Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis)* and two Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacteria were tested by well diffusion of the method following Ghosh et al. (2020) and Majumder et al. (2022b). A nutrient agar (NA) medium was used for this test. Following the pour plate method, 100 µL of bacterial broth culture was first poured into each sterile Petri dish and then sterilized. The plates were left for a few minutes at room temperature for the media to be solidified. Following solidification, a sterile steel cork borer was used to cut out circular wells (8 mm in diameter). Next, 200 µL of each leaf extract sample was poured into the well. This process was done under the sterile condition in a laminar airflow cabinet. The plates were then incubated at 37°C for 24 hours in a non-inverted position.

RESULTS AND DISCUSSION

Morphological characteristics

Mangifera indica L. is a large evergreen tree with heavy branches from a stout trunk having dense dome-shaped foliage. The mango tree grows up to a height of 10-45 m. Young leaves are reddish and thinly flaccid and release an aromatic odor when crushed, turning shiny dark green on the upper surface when they mature. The mean results of the morphological characteristics of the leaves are presented in Table 2. These results refer that the morphological characteristics of mango leaf do not show significant variation to distinguish one cultivar from the others; rather, they exhibit low morphological variation among all the varieties. Table 2 shows that leaf length ranges from 10.86±2.23 cm (S13) to 26.72±3.82 cm (S9). Data for leaf breadth in Table 2 ranged from 3.14±0.29 cm to 7.98±1.35 cm. The highest value was recorded for S9 (7.98±1.35 cm), and the lowest leaf breadth was for S13 (3.14±0.29 cm). The highest petiole length was in S6 (5.35±1.08 cm) and the lowest in S7 (2.13±0.45 cm). The heaviest leaf weight was 2.76±0.85 g for S9, followed by S12 (1.72±0.47 g) and S6 (1.53±0.6 g), while the lightest value was 0.54±0.58 g recorded for S13. The leaf surface area in different mango cultivars demonstrated that the S17 has the lowest leaf surface area of 20 cm². In contrast, S6 exhibited the highest leaf surface area with 111.5 cm², followed by S9 (104.5 cm²).

Qualitative analysis of the leaf extract

Results of qualitative analysis of mango leaf extract for tannin, cardiac glycosides, protein, coumarin, terpenoid, steroid, and alkaloid are depicted by a heat map in Figure 1. These phytochemical groups showed their various presence in different mango leaf extracts. A noticeable amount of protein was found in the leaf extract of S17. In contrast, the leaf extract of S1, S3, S6, S8, and S16 showed no indication of the presence of protein. The presence of coumarin, terpenoids, and steroids was indicated in the mango leaf extracts of all nineteen cultivars. Alkaloids tested positive in all the samples except the leaf extracts of S1, S4, S11, S12, and S19.

Antioxidant activity (DPPH assay)

Responsible reducing agents act as proton donors when free radicals are scavenged. In the DPPH assay, this antiradical activity is calculated from the decreasing absorbance that occurred in the DPPH solution (Majumder et al. 2021b). Nonpolar (petroleum benzene) and polar (acetone and methanol) solvent extracts of nineteen different varieties of mango tree leaf extracts showed the variable potential of DPPH scavenging activity. Leaf extracts of S1 (92.22±1.49%), S7 (91.91±1.30%), S8 (90.76±3.99%), S16 (91.05±1.91%), S18 (90.81±1.55%) showed a significantly high level of DPPH scavenging activity compared to others in their methanol extracts. Whereas acetone extracts of S1 (89.77±1.27%), S4 (90.99±1.38%), S7 (90.38±0.25%), S11 (90.25±1.59%), S18 (91.22±1.13%) also showed a high level of DPPH scavenging activity. So, among the high DPPH scavengers, leaf extracts of S1, S7, and S18 in methanol and acetone showed maximum DPPH scavenging activity. On the other hand, the lowest levels of scavenging activities were observed in petroleum benzene extracts. At the same time, polar solvents (methanol and acetone) showed moderate, high, or very high DPPH scavenging activity. The results of all nineteen samples are presented in Figure 2.

Quantification of total phenol content (TPC)

Quantifying total phenol was done in petroleum benzene, acetone, and methanol extracts. This quantitative analysis of total phenol revealed that the content of total phenol varies from plant to plant. The quantities of phenols were detected and converted to gallic acid equivalent, represented in Figure 3. The highest concentration of total phenol was quantified in methanol extract of S14 (20.24±1.35 mg GAE/g sample), S18 (20.07±1.07 mg GAE/g sample), S10 (19.11±0.26 mg GAE/g sample), S7 (18.51±0.85 mg GAE/g sample), S4 (17.91±0.85 mg GAE/g sample) and acetone extracts of S8 (18.20±0.14 mg GAE/g sample), S13 (18.17±0.11 mg GAE/g sample), S10 (18.16±0.2 mg GAE/g sample), S9 (16.96±0.2 mg GAE/g sample), S17 (16.77±0.12 mg GAE/g sample). In contrast, all nineteen samples recorded the lowest total phenol concentration in petroleum benzene extracts.

Table 2. Morphological analysis of mango leaves of nineteen different cultivars

Sample	Leaf length (cm)	Leaf breadth (cm)	Petiole length (cm)	Weight (g)	Length: breadth (mean)	Mean surface area (cm^2)
S1	19.7±2.45	5.71±0.11	4.4±0.45	1.53±0.21	3.45	76.5
S2	21±1.94	5.04±0.14	3.2±1.22	1.13±0.27	4.16	41.5
S3	17.46±1.24	4.04±0.1	3.3±0.79	0.81±0.12	4.31	35.5
S4	21.8±1.47	5.08±0.13	4.2±0.63	1.43±0.21	4.29	77
S5	20±3.3	4.79±0.23	3.42±0.82	0.99±0.35	4.17	69
S6	26.4±4.69	6.15±0.25	5.35±1.08	1.53±0.6	4.29	111.5
S7	12.81±2.75	5.04±0.12	2.13±0.45	0.62±0.28	2.54	55.5
S8	21±3.43	5.08±0.18	4.35±0.57	1.16±0.48	4.13	66.5
S9	26.72±3.82	7.8±1.35	5.68±1.03	2.76±0.85	3.34	104.5
S10	17.1±4.67	4.66±0.26	4±1.33	1.39±0.6	3.66	37.5
S11	16.2±3.96	6.15±0.2	3.1±0.66	1.45±0.6	2.63	50.5
S12	24.37±3.06	4.43±0.13	4.25±0.88	1.72±0.47	5.49	56
S13	10.86±2.23	3.14±0.29	2.2±0.36	0.54±0.58	3.45	31
S14	20.6±5.14	4.52±1.03	3.5±1.35	1.33±0.46	4.55	48
S15	17±1.98	5.25±0.29	3.82±0.73	0.99±0.18	3.23	69
S16	19.81±4.42	4.43±0.18	3.09±1.44	1.05±0.47	4.46	47.5
S17	17.37±3.73	3.25±0.16	3.25±1.03	1.12±0.41	5.34	20
S18	16.4±3.37	4.53±0.19	2.3±0.63	1.07±0.38	3.62	27.5
S19	17.5±1.71	5.68±0.37	3.3±0.82	0.81±0.13	3.08	55.5

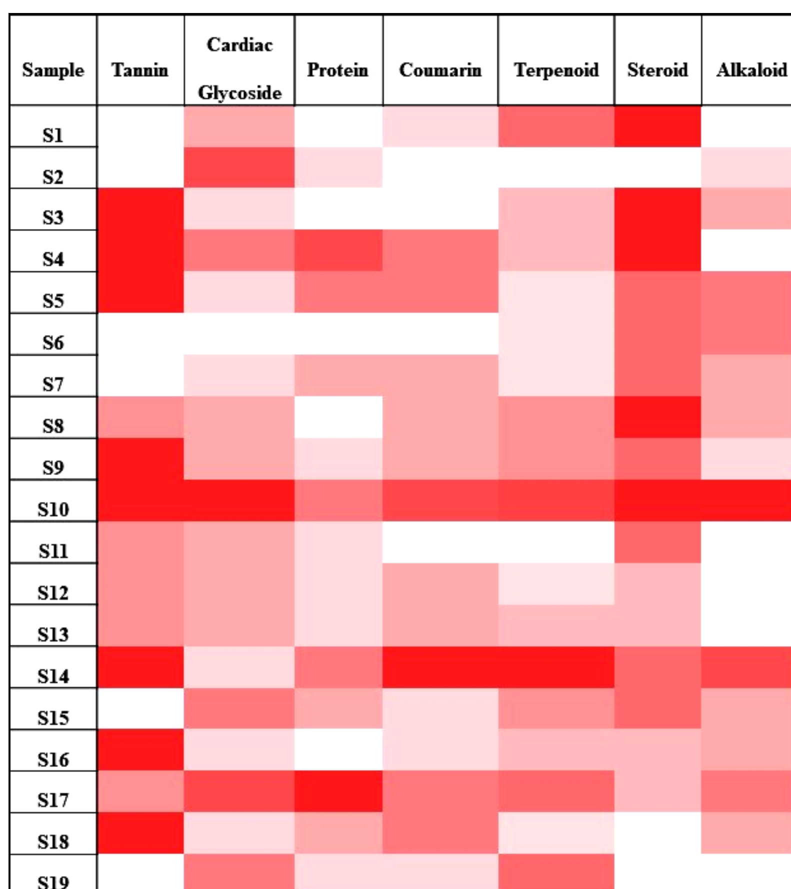

Figure 1. Heat map (red- white = high-low) showing results of phytochemical groups in mango leaf extract

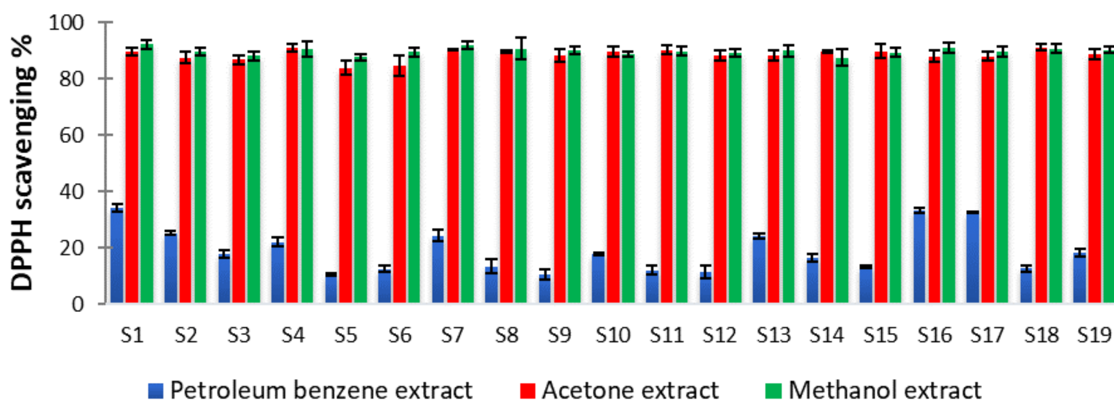

Figure 2. DPPH free radical scavenging (%) activity shown by *Mangifera indica* leaf extracts

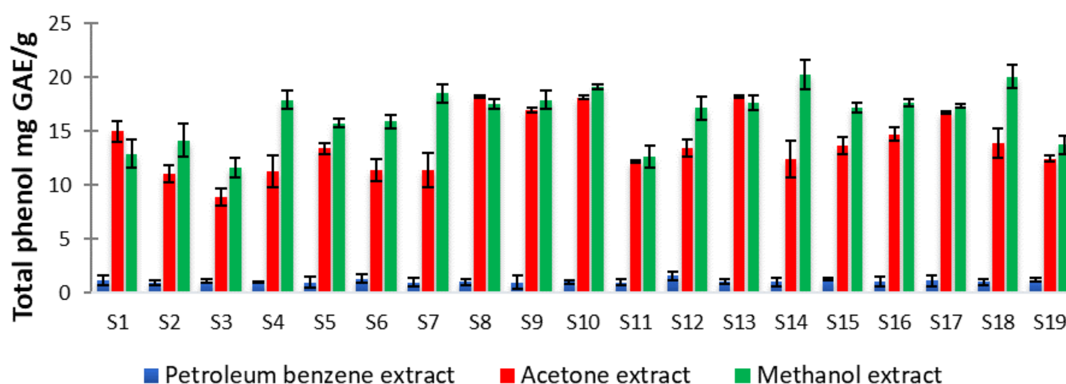

Figure 3. Total phenol content (TPC) as Gallic acid equivalent detected in solvents extracts of different leaves

Quantification of total flavonoid content

Quantifying flavonoids was conducted in petroleum benzene, acetone, and methanol extracts of all the nineteen varieties of a mango leaf. Determination of total flavonoid content was expressed as mg Quercetin equivalent (QE)/g fresh leaves of mango plant extracted by three (petroleum benzene, acetone, methanol) different solvents. The quantities of flavonoids were detected and converted to quercetin equivalent. The results are represented graphically in Figure 4. Methanol leaf extracts of S11 (3.14±0.13 mg QE/g sample), S8 (2.74±0.09 mg QE/g sample), S3 (2.29±0.24 mg QE/g sample), S14 (2.09±0.21 mg QE/g sample), S18 (2.04±0.19 mg QE/g sample) showed the maximum quantity of flavonoids. At the same time, mango leaf extracts in acetone also showed a promising result. Acetone leaf extracts of S1 (3.09±0.07 mg QE/g sample), S5 (2.53±0.17 mg QE/g sample), S11 (2.3±0.25 mg QE/g sample), S2 (2.24±0.19 mg QE/g sample), S13 (2.18±0.08 mg QE/g sample), also exhibited a significant quantity of flavonoids. In contrast, petroleum benzene leaf extracts of all nineteen samples showed minimum quantities of flavonoids.

Antibacterial activity

Mango leaf extracts showed potent antibacterial activity against Gram-positive and Gram-negative bacteria. Methanol extract of S5 and acetone extract of S7 showed an inhibition zone of 2.5 cm and 2 cm, respectively, against *E. coli*. An inhibition zone of 1.5 cm was also observed against *K. pneumoniae* by acetone extract of S7. There was no zone of inhibition observed against *B. subtilis*. In contrast, acetone extract of S18, S7, and S17 showed a wide inhibition zone of 1.5 cm, 1.2 cm, and 1 cm, respectively, against *S. aureus*.

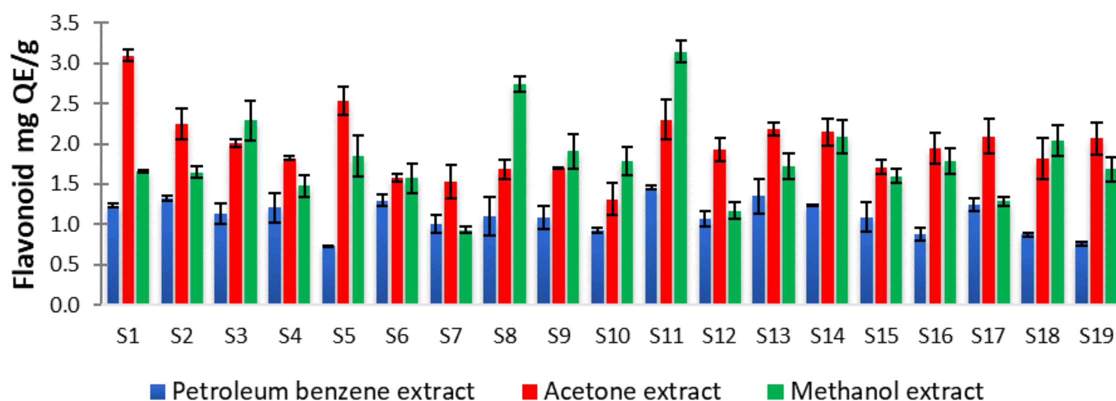

Figure 4. Total flavonoid content as quercetin equivalent detected solvents extracts of different leaves

Discussion

Murshidabad district was once the capital of exotic mango varieties, but nowadays, Murshidabad is missing out on past honors as the district is facing massive genetic erosion of the mango germplasm (Pal 2017; De et al. 2014). Around 250 varieties of mango have been grown in this district (Pal 2017). The origin of mango cultivars used in this research was studied from the available literature. The nawabs of Murshidabad had purchased different mango cultivars from different parts of the country and took the initiative to create new varieties through grafting. Till the 1970s and 1980s, some rare varieties were found on the mango plantation of Lalbagh of Murshidabad. Due to climate change and inadequate preservation facilities, farmers are losing interest in producing some varieties (Pal 2017). However, a few exotic mango tree species are still present in the orchards of Lalbagh. Therefore, Lalbagh was chosen for sample collection for this study.

The leaves characters are spirally arranged on branches, lanceolate-elliptical, pointed at both ends, and borne on 1-12.5 cm long petioles. Leaves are mostly about 16-30 cm long and 3-8 cm wide on flowering branches and up to 50cm long on sterile branches (Shah et al. 2010; Igbari et al. 2019). So, the values of morphological analysis for mango leaves of all the nineteen different cultivars depicted in Table 2 fitted perfectly with the ranges mentioned above.

Mangifera indica has attracted to be studied to determine new biomolecules from different parts of plants like fruits, leaves, stems, and seed kernels. Its therapeutic importance is well established and has been used as a traditional remedy for treating several diseases (Jhaumeer et al. 2018). For example, a previous study by Kumar et al. (2021) showed that the leaf extract had cytotoxic activity and oxidative effects on breast cancer cells and was non-invasive against non-carcinogenic cells (Kumar et al. 2021). In addition, several studies have reported that the different parts of mango plants, such as leaves, bark, stems, and seeds, exhibited antimicrobial activity against some multi-drug resistant (MDR) microbes (Kumar et al. 2021; Hannan et al. 2013).

Mango leaves have been reported to contain several beneficial chemical compounds that can be used to treat various diseases, such as diabetes, hepatitis, and wound healing. Kumar et al. (2021) stated the major phytochemicals responsible for the antimicrobial activity were phenolics, alkaloids, glycosides, tannins, terpenes, and saponins. Some compounds from mango can be used in the pharmaceutical industry, such as steroids, gallic acid that has antifungal and antiviral activity, flavonoids that have antioxidant activity, and tannin as a diarrhea remedy (Ali et al. 2020). Tannin is an astringent and biosynthesized in plants and can be pharmacologically useful against various diseases (Okuda et al. 1992). In addition, tannin acts as an antibacterial (Awosika 1991) by inhibiting bacterial proliferation through the denaturation of enzymes involved in microbial metabolism; further, it precipitates proteins in tissues that are beneficial for wound healing (Tyler et al. 1998). In this research, methanol extract was chosen for the qualitative biochemical tests, as methanol showed a favorable result during DPPH antioxidant assay. Tannin was found in all the samples in moderate quantity. Cardiac glycosides were present in all samples, while a significant amount was found in S2, S10, and S17. Cardiac glycosides are used to treat congestive heart failure and cardiac arrhythmia. Therefore, mango leaf extract might be used for cardiac ailments.

Free radical DPPH scavenger activity indicates the ability of plant extracts to scavenge free radicals and is considered an indicator of antioxidant activity. All leaf extracts showed promising DPPH scavenging activity, although S1, S7, and S18 (methanol and acetone extracts) have higher DPPH scavenging activity. However, their DPPH scavenging activity did not differ significantly from other extracts. (Figure 2). .Mango leaves have been reported to have antioxidant activity mostly from phenolic compounds (Ali et al. 2020). Phenols generally protect plants and human cells from oxidative damage (Ali et al. 2020). Therefore, S7, and S18, which showed the noticeable potential of DPPH antioxidant activity, were also rich in total phenol content. Flavonoids are polyphenols, a group of natural biomolecules with variable phenolic structures available in almost all plant parts. They primarily confer antioxidant properties (Panche et al. 2016) and have antimicrobial, anti-inflammatory, anti-allergic,

and anticancer activity (Balch and Balch 2000). Therefore, the mango leaf is also a good flavonoid source (Ali et al. 2020). S1 and S18 are good DPPH scavengers and exhibit high flavonoid content. This research showed that methanol and acetone leaf extracts possess good phenol and flavonoid content (Figures 3 and 4).

The phytochemical content in the mango leaf extracts is responsible for the antibacterial, anti-inflammatory, and antioxidant activity (Jhaumeer et al. 2018). A previous study by Jhaumeer et al. (2018) showed that crude extracts of mango leave exhibited moderate to good antibacterial activity against some Gram-positive bacteria (*Pseudomonas aeruginosa*, *Bacillus cereus,* and *S. aureus*) and Gram-negative bacteria (*Staphylococcus epidermidis*, *K. pneumoniae,* and *E. coli*). Interestingly, the essential oil of five different Egyptian mango cultivars also showed potential antimicrobial activity (Kumar et al. 2021). This study showed that leaf extract of S5, S7, S17, and S18 showed effective antimicrobial activity. However, there was no inhibitory zone against *B. subtilis*.

Furthermore, several extracts that contain several phytochemical compounds (S10, S14, S1, and S11) were not active as antibacterial. Therefore, further analysis (fractionation and separation of phytochemical) is needed to study the bioactivities in depth. The detailed qualitative phytochemical profiling suggested that mango leaves are a good resource of phytochemicals like flavonoids, tannins, protein, coumarin, terpenoids, alkaloids, steroids, cardiac glycosides, and phenols. Methanol extracts of all mango varieties had good DPPH scavenging activity, total phenol, and flavonoid content. Furthermore, varieties such as Rani, Anaras, Michriganj, and Champa are effective against *E. coli, K. pneumoniae,* and *S. aureus*.

Furthermore, mango leaves exhibit exceptional antioxidant and antibacterial properties. Therefore, the extract of mango leaves might be utilized by pharmaceutical industries to benefit humankind. Results suggest the presence of phytochemicals (cardiac glycosides, polyphenols, tannin, terpenoids, coumarin, etc.) in mango leaf extracts are responsible for their antioxidant and antimicrobial activity. Moreover, variations in results among the cultivars had been considered for the comparative analysis, which will help to choose promising samples for further chromatographic studies to evaluate their bioactivities.

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Assessment of phytochemical compositions, antibacterial effects and DPPH scavenging activities of ethanolic root extracts of *Pterocarpus erinaceus*

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Abstract. *Okoli EC, Emaru IJ, Olawale O. 2022. Assessment of phytochemical compositions, antibacterial effects and DPPH scavenging activities of ethanolic root extracts of* Pterocarpus erinaceus. *Asian J Nat Prod Biochem 20: 56-62. Pterocarpus erinaceus* Poir. serve as a medicinal plant to many populations of Nigeria and West Africa. The stem bark, leaves, and roots have been studied for their antioxidant, antimalaria, antiulcerogenic, and antibacterial properties. This study aimed to determine the phytochemical compositions, antibacterial effect and antioxidant activity of the root bark of *P. erinaceus*. A gas chromatography flame ionization detector (GC-FID) instrument was used for the analysis and quantification of phytochemicals present in the ethanolic root extract of *P. erinaceus*. The antibacterial test was carried out using the agar well method against standard bacteria: *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa* and *Escherichia coli* while the antioxidant activity was carried out by evaluating the DPPH scavenging activity. Phytochemicals found in the extracts include: flavonoids (44.50%), alkaloids (18.87%), steroids (4.38%), tannins (1.73%), and anti-nutrients (15.12%). The ethanolic root extracts of *P. erinaceus* exhibited significant antibacterial activity against *E. coli* and *S. aureus* but exhibited no growth inhibitory effect on *B. subtilis* and *P. aeruginosa* at all tested concentrations (100 µg/mL, 150 µg/mL, and 200 µg/mL). DPPH scavenging activity was significantly high, with 62.91% at a concentration of 1000 µg/mL and 36.48% at 40 µg/mL. This study revealed that the crude ethanolic extract of *P. erinaceus* root bark contained pharmacologically active compounds and exhibited significant antibacterial activity and DPPH free radical scavenging activity in a concentration-dependent manner.

Keywords: Antioxidant, ethanolic extract, GC-FID, phytochemicals, *Pterocarpus erinaceus*

INTRODUCTION

One-third of all deaths worldwide are due to infectious diseases. Drug resistance is the current cause of this increase and is more common in less developed countries, where one in two people die prematurely from the disease, than in developed countries. With limited access to quality medicines, people in many developing countries still use plants to treat common ailments (Tittikpina et al. 2018). About 64% of the world population still depends on traditional medicines and medicinal plants to meet their health needs (Hossain et al. 2022). According to a WHO survey, traditional medicines are prescribed for about 80% of patients in India, 85% in Burma, and 90% in Bangladesh (Hossain et al. 2022). Records indicate that the medicinal uses of the plant date back to 4000-5000 BC. AD and the Chinese were the first to use natural herbal preparations as medicine. Even modern pharmacopeias still contain at least 25% of pharmaceutical products of plant origin and, more often, synthetic compounds isolated from plants (Hossain et al. 2022). As a result, much research has been done to uncover the active ingredients in these plants. The most famous example of this approach is the discovery of artemisinin from *Artemisia annua* (a treatment for malaria), but artemisinin is not the only promising plant-based compound. Accordingly, many authors from Asia and Africa have studied the activity of plant extracts, the purified chemicals in the extracts, against various microorganisms associated with bacterial and fungal diseases (Tittikpina et al. 2018).

Pterocarpus erinaceus Poir. (Leguminosae, Papilinoideae) is a species of deciduous tree in the legume family, native to the savannas and dry forests of Africa. The tree is 12-15 m high and 1.2 m in diameter. The bark is dark gray and scaly, the leaves are feathery up to 30 cm long. It flowers in yellow. The fruit has developed pink spots. Seeds are kidney-shaped to oblong, often oblong and curved at the level of small telium. Also known as African rosewood. The leaves and seeds are edible after thorough cooking. We produce the finest wood from the country of origin. At the end of the dry season, the leaves and young bark are often cut for fodder for sheep, goats, cattle and horses (Ahmed et al. 2017). According to the ethnographic findings of Saslis-Lagoudakis et al. (2011). The leaves, bark, and roots of the plant *P. erinaceus* Poir. are used in traditional Burkinabe medicine to treat inflammatory diseases such as fever, bacterial infections, malaria, ulcers, rheumatism, and inflammation. In addition, the roots of *P. erinaceus* are used to treat inflammation, ulcers, and gastric diseases (Noufou et al. 2016).

The bark, leaves, and roots of *P. erinaceus* have been studied for their anti-inflammatory, analgesic, and anti-inflammatory properties (Noufou et al. 2016), antidiarrheal, antiemetic, antimalarial, antioxidant, antifungal (Karou et

al. 2003; Ezeja et al. 2012; Olaleye et al. 2013; Tittikpina et al. 2019). The evidence has confirmed the importance of this plant.

Although it is widely used in traditional medicine, little is known about its phytochemicals. Therefore, the objective of this study was to evaluate the phytochemical components and antibacterial and antioxidant activities of the ethanolic extract from the root bark of *P. erinaceus.*

MATERIALS AND METHODS

Study area

This study was carried out at Federal University, Wukari, Taraba State, Nigeria, between February 2022 to July 2022. Wukari town is the headquarters of Wukari Local Government Area in Taraba State, Nigeria. It lies between latitude 7.9303°N and longitude 9.8125°E of the equator.

Materials

The root bark samples of *P. erinaceus* were collected from uncultivated farmland of Federal University Wukari, Wukari Local Government Area of Taraba State, Nigeria. The plant was taxonomically identified and authenticated in the Department of Plant Science of Modibbo Adama University of Technology, Yola, Nigeria.

Root extraction

The root samples were rinsed with distilled water before being air-dried over a period of thirty days. It was then ground into powder by grinding in a mortar and pestle. One hundred and fifty grams of the powdered root were cold macerated in 500 mL of ethanol inside an Erlenmeyer flask shaken at an interval of an hour and then allowed at room temperature to stand for 48hours, and filtered using Whatman's filter paper No. 1. The extract was then concentrated to dryness using a rotary evaporator. It was then stored under a frozen condition until required.

GC-FID identification and quantification of phytochemical constituents

For the GC-FID (Gas Chromatograph/Flame Ionization Detector) analysis, 1 g of root extract of *P. erinaceus* was weighed and transferred into a test tube. Fifteen (15) mL of ethanol and 10 mL of 50% w/v potassium hydroxide were added to the crushed root bark in the test tube. The test tube was allowed to stand in a water bath at 60°C for 60 minutes. Then the content of the test tube was carefully transferred into a separatory funnel and the tube was rinsed into the same funnel with 10 mL of cold water, 10 mL of hot water, 20 mL of ethanol, and 3 mL of hexane. The extract in the test tube was washed three times with 10 mL of 10% v/v ethanol solution. The extract solution was then dried with anhydrous sodium sulfate and the solvent was evaporated. A sample of the extract was then made soluble in 100 µL of pyridine of which 20 µL was transferred into a vial on the Gas Chromatography machine for phytochemical analysis.

The GC-FID phytochemical analysis was performed on a BUCK M910 Gas Chromatograph (GC) (BUCK Scientific, USA), equipped with a flame ionization detector (FID). A RESTEK 15-meter MXT^{-1} column (15 m x 250 µm x 0.15 µm) was used. The injector temperature was 280°C with a splitless injection of 2 µL of sample and a linear velocity of 30 cms^{-1}, Helium 5.0 Pas was the carrier gas with a flow rate of 40 mLmin^{-1}. The oven operated initially at 200°C, it was heated to 330°C at a rate of 3°Cmin^{-1} and was kept at the temperature of 320°C. Phytochemicals were determined by the ratio between the area and mass of the internal standard and the area of the identified phytochemicals (Ugoeze et al. 2020).

Antibacterial assay

The antibacterial activity of ethanolic root bark extract of *P. erinaceus* was determined by the agar well diffusion method as adopted by Umaru et al. (2022). The bacterial cultures were purchased from the National Veterinary Research Institute, Vom, Plateau State, Nigeria. Twenty (20) mL of molten nutrient agar was poured into each of the Petri dishes and allowed to solidify. The 0.5 McFarland standardized bacterial broth was spread on the dry nutrient agar with a spreader pre-sterilized in ethanol and flame overnight. With the aid of a sterile cork-borer, four wells of 6 mm depth each and about 5 cm apart were made in the nutrient agar. Three wells were filled with 500 µL of the *P. erinaceus* root bark extract dissolved in sterile distilled water, one with the water only (the negative control) and the last with 1% standard antibiotic, gentamicin. The positive control was dispensed into the wells in triplicates. After incubating for 24 at 37°C, the antibacterial activities were determined by measuring the diameter of the inhibition zone. The zones of inhibition observed with the extract were compared with that of the standard antibiotic, gentamicin. The experiment was carried out in three sets. The measured gentamicin inhibition zones' diameters were subsequently matched with the respective standard zones' diameters for *Escherichia coli* (Gram -ve), *Staphylococcus aureus* (Gram +ve), *Bacillus subtilis* (Gram +ve), and *Pseudomonas aeruginosa* (Gram -ve). The *P. erinaceus* zone of inhibition from 9-14 mm in diameter was taken as a positive antibacterial activity based on the growth inhibition standard (Umaru et al. 2022).

DPPH scavenging activities

Different concentrations (40-1000 µg/mL) of the root bark ethanolic extracts were taken in different test tubes. The volume was adjusted to 250 µL by adding MeOH. Two milliliters of a 0.18 mM (0.005%) methanolic solution of DPPH (2,2, -diphenyl-1-picrylhydrazyl) were added to these tubes and shaken vigorously. The tubes were allowed to stand in the dark at room temperature for 30 min (Singh et al. 2002). The control was prepared as above without any extract, and MeOH was used for the baseline correction. Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

% Scavenging Activity = [(A_{517} Control - A_{517} treatment)/ A_{517} Control] X 100

RESULTS AND DISCUSSIONS

The result of GC-FID identification of phytochemicals in the root extract is presented in Figure 1 and Table 1. Studies have reported that medicinal plants are particularly abundant in various bioactive chemicals (Lewis and Ausubel 2006).

Flavonoids are the most abundant phytochemical in the extract (44.50%). Flavonoids are natural substances with different phenol groups found mainly in vegetables, some grains, stems, and flowers. They are well known for their valuable health benefits, especially their antioxidant, anti-mutagenic, anti-inflammatory, anti-cancer and enzyme-regulating properties. Flavonoids in the extract include: naringenin, proanthocyanin, flavone, flavan-3-ol, catechin, epicatechin, and kaempferol.

Epicatechin was the flavonoid with the highest concentration (38.09 µg/mL or 21.98%), while flavonoid with the lowest concentration is proanthocyanin (2.04 µg/mL or 1.18%) (Table 1). Catechin and epicatechin are also among the flavonoids present in the ethanolic extract of the root of *P. erinaceus*. While catechins are found in various foods and herbs such as apples, grapes, berries, and tea, epicatechin is mainly found in green and black tea, with the highest levels of epicatechin found in cocoa (Isemura 2019; Prakash et al. 2019). Catechin possesses great health benefits such as anti-obesity, anti-cancer, hepatoprotective, anti-diabetic and neuroprotective effects, while epicatechin is known to have cardioprotective, anti-inflammatory activities. antioxidant, anti-diabetic and anti-cancer. Epicatechin-rich green tea has also been shown to have antiplatelet effects in vivo and increase insulin sensitivity (Ugoeze et al. 2020).

Because of their powerful antioxidant activity, proanthocyanin and anthocyanin are widely distributed pigments in land plants, where they function as stress suppressors and health-promoting components (Ugoeze et al. 2020). Naringenin is found mostly in citrus fruits and tomatoes and is effective in treating cancer, cardiovascular disease, and osteoporosis (Galluzzo et al. 2008; Ugoeze et al. 2020). In addition, it has recently been proven to generate a considerable reduction in collagen fiber formation in rats with liver injury. Other positive features of naringenin include its capacity to minimize oxidative stress, as well as its anti-inflammatory, anti-diabetic, anti-hyperlipidemia, antioxidant, and antidepressant properties (Ugoeze et al. 2020).

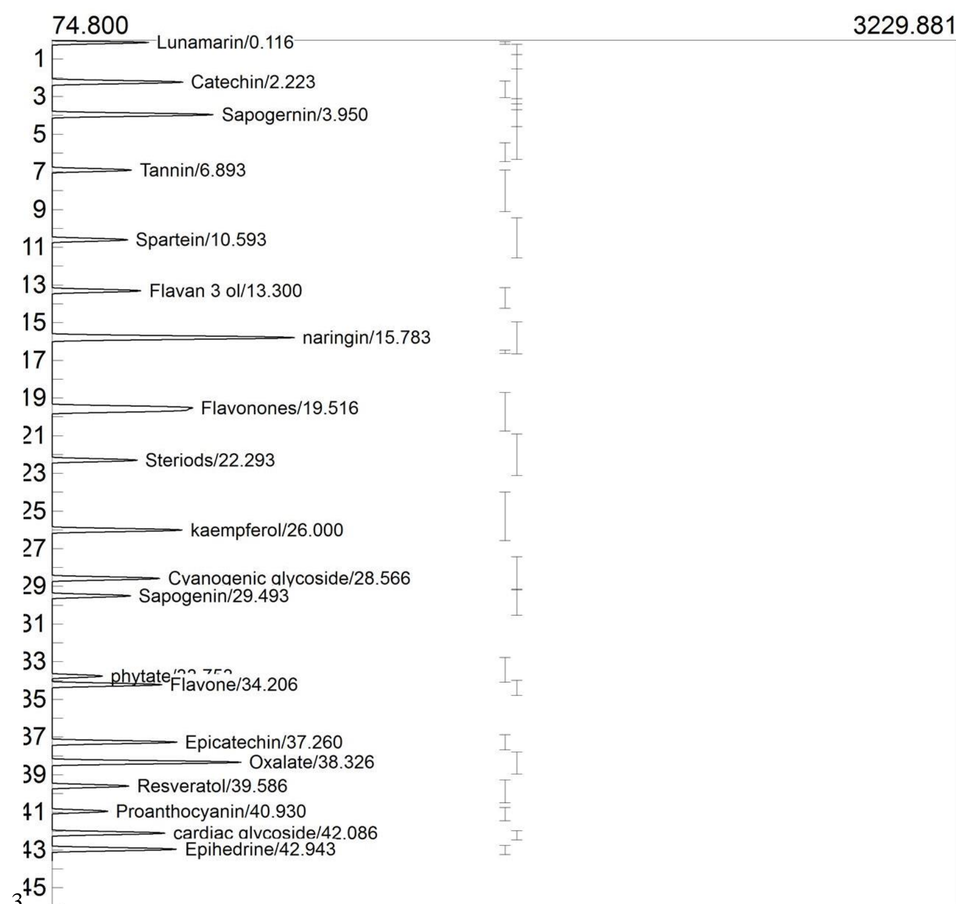

Figure 1. Chromatogram showing the phytochemical constituents of ethanolic root extract of *Pterocarpus erinaceus*

Table 1. Phytochemical components identified in root extract of *Pterocarpus erinaceus* by GC-FID

Type of phytochemical	Phytochemical constituents	RT	Area	Height	Conc. (µg/mL)	% Composition
Flavonoids (44.50%)	Catechin	2.22	6793.22	529.00	5.01	2.89
	Flavone	34.26	5932.53	458.61	4.42	2.55
	Epicatechin	37.26	6525.25	508.86	38.09	21.98
	Proanthocyanin	40.93	3451.57	270.98	2.04	1.18
	Flavan-3-ol	13.30	4918.61	385.00	2.91	1.68
	Kaempferol	26.00	6833.38	529.58	6.15	3.55
	Naringin	15.78	12794.39	919.80	10.56	6.09
Alkaloids (18.87%)	Lunamarin	0.12	3681.83	411.03	9.30	5.37
	Spartein	10.59	4339.04	337.68	1.83	1.06
	Epihedrine	42.94	6524.26	510.97	21.57	12.45
Saponins (6.52%)	Sapogernin	3.95	8180.04	637.04	8.17	4.72
	Sapogenin	29.49	4459.40	349.79	3.12	1.80
Tanins (1.73%)	Tannin	6.89	4491.19	350.85	3.00	1.73
Steroids (4.38)	Steroids	22.29	4749.76	372.51	7.60	4.38
Other phenols (1.45%)	Resveratol	39.59	4412.40	345.67	2.51	1.45
Anti-Nutrients (15.12%)	Oxalate	38.326	9393.732	732.257	21.985	12.69
	Phytate	33.753	3207.273	252.912	4.203	2.43
Glycosides (7.70%)	Cardiac glycoside	42.09	6000.13	470.14	7.94	4.58
	Cyanogenic glycoside	28.57	5744.95	450.31	5.41	3.12

Flavones and flavanones are other important forms of flavonoids. Flavones are found mainly in leaves, flowers, fruits, celery, parsley, and red peppers, while flavanones are found in all citrus fruits, such as lemons, grapes, and oranges (Ugoeze et al. 2020). Flavones can interact with proteins and bind to human serum albumin to facilitate plasma-mediated transport (Jiang et al. 2016). On the other hand, flavanones have antioxidant, antihyperlipidemic, and anti-inflammatory effects (Ugoeze et al. 2020). Kaempferol is another flavonoid commonly found in many other vegetables and plants, such as grapes, green tea, potatoes, onions, and cucumbers. Like other flavonoids, they may have anti-diabetic, antitumor, and anti-inflammatory activities (Calderon-Montano et al. 2011). In addition, it has been reported to regulate several key factors of cell signaling pathways involved in apoptosis, angiogenesis, inflammation and metastasis, thereby potentially inhibiting the growth of cancer cells and angiogenesis by inducing cancer cell apoptosis (Chen and Chen 2013).

These protective effects of flavonoids in organic systems are generally attributed to their ability to donate electrons to free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce α-tocopherol radicals, and prevent oxidases (Ugoeze et al. 2020). Oxidative stress and inflammation are frequent response that contributes to tumor development by stimulating defective cells to promote and progress tumors, initiating direct damage to genomic nucleic acids, initiating aberrant cell proliferation, and altering intracellular signaling of the normal reaction to do (Bhattacharyya et al. 2014). The rich flavonoid content of ethanolic extract of the root of *P. erinaceus* has given it many pharmacological activities such as anti-inflammatory, antipyretic, hypoglycemic, antifungal, antibacterial, antitumor, and wound healing properties.

Plant alkaloids are one of the largest groups of natural products, composed of structurally distinct and biogenetically unrelated molecules (Ugoeze et al. 2020). They have a wide range of pharmacological activities and have been used as components of many herbal remedies (Alves de Almeida et al. 2017). These include narcotic analgesics, morphine, and codeine. They have also been shown to have potent antimalarial, antibacterial and antiprotozoal properties (Franck et al. 2004). The results of the present study indicate that ethanol extracts of *P. erinaceus* root contain significant amounts of alkaloids (23.96%), with epihedrine (Figure 2) in the extract (21.57 µg/mL or 6.09%) having the highest concentration, followed by lunamarin (9.30 µg/mL or 5.37%), then spartein (1.83 µg/mL or 1.06%) (Table 1). Lunamarin has been reported to have free radical scavenging properties (Ugoeze et al. 2020). In addition, lunamarins have anti-cancer, immunomodulatory, antiestrogenic, and antiamoebic properties8. These alkaloid levels may be attributed to some pharmacological properties of *P. erinaceus* root extract.

Saponins comprise a group of structurally related natural compounds that include either steroidal or triterpenoid aglycones (sapogenins) and are found primarily in plants and other lower marine animals (including some bacteria). They are found in a wide range of plants and crops, with triterpenoid saponins being more common as they are found in many legumes such as soybeans, beans and peas. Pharmacological effects attributed to saponins include immunomodulatory, anti-inflammatory, antifungal, antiviral, antibacterial, hypercholesterolemic, and anticarcinogenic properties. Saponins accounted for 6.52% of the total phytochemicals obtained from the ethanol extract of *P. erinaceus* root (Table 1). Sapogenins are known for many beneficial properties, but other harmful properties have also been documented. For example, their hemolytic and cytotoxic effects have been observed. It has also been observed to significantly impair protein digestion and vitamin and mineral absorption in the small intestine, causing hypoglycemia (Ugoeze et al. 2020).

Tannins are found in tea, cocoa, vegetables, legumes, and some immature fruits (Sharma et al. 2021). The extract of *P. erinaceus* root was found to contain low levels of tannins (1.73%), accounting for 1.73% of total phytochemicals (Table 1). Tannins play an important role in traditional Asian medicine, and tannin-rich plant extracts are used as astringents and diuretics. It is also used to treat diarrhea, gastrointestinal ulcers, and tumors. It also has anti-inflammatory and antioxidant properties (Ugoeze et al. 2020). However, tannin-rich diets have been reported to be responsible for reduced feed intake and efficiency in experimental animals and are, therefore, usually considered low nutrient density. In addition, it is thought to inhibit their conversion into bodily substances (Chung et al. 1998; Ertop and Bektas 2018). The formation of tannin-protein complexes can lead to the inactivation of digestive enzymes and reduced protein digestibility caused by interactions with protein substrates and ionized iron (Ugoeze et al. 2020).

Anti-nutrients are not considered beneficial because they interfere with mineral absorption. Blockage of nutrient absorption is known to cause headaches, rashes, nausea, bloating, and malnutrition (Popova and Mihaylova 2019). Anti-nutrients are mostly organic or synthetic structures that are highly reactive and thus can have toxic effects. Phytates and oxalates are anti-nutrients found in the ethanolic extract of *P. erinaceus* root (Figure 2). Phytic acid (myo-inositol hexaphosphate) is found in various foods, including nuts, seeds, and whole grains. It also contains significant amounts of roots and tubers. Phosphorylated inositol, especially phytic acid, has been suggested to be involved in insulin secretion by pancreatic beta cells (Ugoeze et al. 2020). It has also been suggested that phytic acid inhibits plaque development and lowers serum cholesterol and triglycerides (Schlemmer et al. 2009; Ugoeze et al. 2020). Oxalates, on the other hand, are known to interfere with the absorption and utilization of calcium by forming calcium oxalate crystals that lead to the formation of kidney stones. They also irritate and swelling in the mouth and throat (Ugoeze et al. 2020).

The ethanolic stem bark extract of *P. erinaceus* exhibited different levels of antibacterial activity against the tested bacterial strains. The bacterial strains used were clinical and laboratory isolates. All these bacterial species are known to cause serious human infections. From a clinical point of view, *E. coli* causes septicemias and can infect the gall bladder, surgical wounds, skin lesions and the lungs (Seeram et al. 2002). The *S. aureus* causes dermatitis and sialadenitis (Mastroeni 2002). The *B. subtilis* is known to cause disease in severely immune-compromised patients (Seeram et al. 2002), and *P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns, wounds and also causes other blood infections. All assayed bacteria were sensitive to the extract.

The results shown in Table 2 depict that the ethanolic root extract of *P. erinaceus* exhibited significant growth inhibition of *E. coli* and *S. aureus* but exhibited no growth inhibition of *B. subtilis* and *P. aeruginosa*.

The extract showed an increasing inhibitory activity across all concentrations (100 µg/mL, 150 µg/mL, and 200 µg/mL) in a dose-dependent manner in the two bacteria in which it was found active. The highest inhibition was observed in *S. aureus* with a mean growth inhibition zone of 6.10 mm at a concentration of 200 µg/mL, while the lowest inhibition was observed in *E. coli* with a mean growth inhibition zone of 2.23 mm at 100 µg/mL concentration.

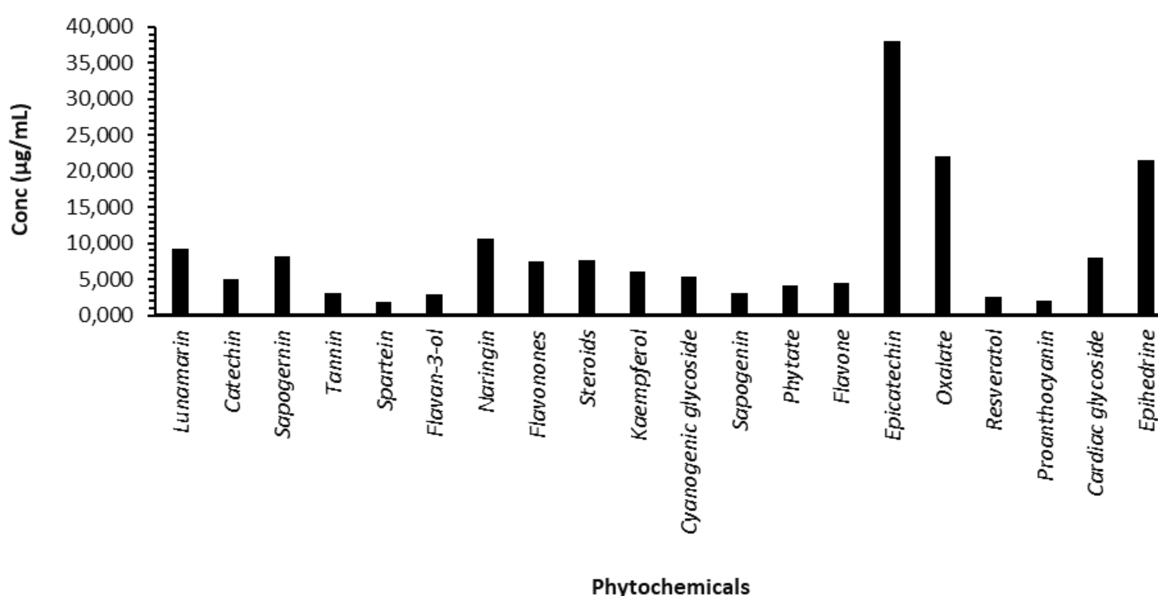

Figure 2. Concentrations of phytochemicals in ethanolic root extract of *Pterocarpus erinaceus* identified by GC-FID

Table 2. Effect of ethanol root extract of *Pterocarpus erinaceus* on bacteria

Extract (µg/mL)	*Bacillus subtilis* (Gram +ve) Inhibition zone (mm)	*Escherichia coli* (Gram -ve) Inhibition zone (mm)	*Pseudomonas aeruginosa* (Gram -ve) Inhibition zone (mm)	*Staphylococcus aureus* (Gram +ve) Inhibition zone (mm)
Gentamicin (µg/mL)	20.67 ± 0.31^{a}	20.47 ± 0.21^{a}	30.20 ± 0.26^{a}	23.90 ± 0.17^{d}
100	-	2.23 ± 0.25^{a}	-	3.13 ± 0.15^{a}
150	-	4.03 ± 0.15^{b}	-	4.40 ± 0.36^{b}
200	-	6.06 ± 0.15^{c}	-	6.10 ± 0.10^{c}

Note: Result is Mean ± SD. Value with same superscript within a column are statistically not significant, while values with different superscripts within a column are statistically significant (P<0.05)

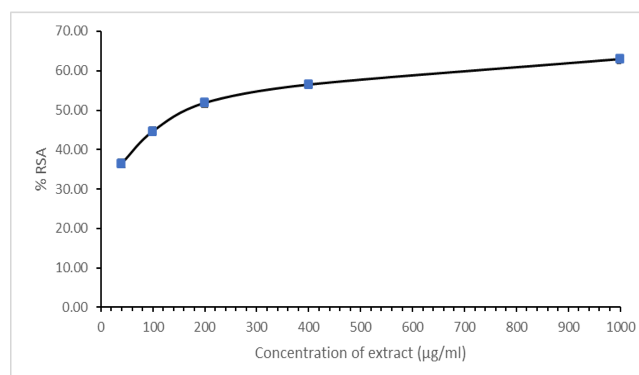

Figure 3. % RSA of the ethanolic extract of *Pterocarpus erinaceus* root at different concentrations

When compared to the standard drug's mean inhibition zone, the values of the two organisms (*E. coli* and *S. aureus*) are found to be statistically significant when compared to the mean inhibition zone of the standard drug (gentamicin). This result is not in trend with the study of Tittikpina et al. (2018), who reported that the methanolic-dichloromethane extracts of *P. erinaceus* root exhibited inhibitory effects ranging from 42-77% against all selected bacteria strains (*Enterococcus faecalis, S. aureus, P. aeruginosa, Acinetobacter baumannii, E. coli* and *Klebsiella pneumoniae)* at the concentrations of 256 µg/mL. However, they obtained MICs (minimum inhibitory concentrations) with individual fractions (Butanol, petroleum ether, ethyl acetate, dichloromethane, and water) against *E. faecalis*, *S. aureus* and *P. aeruginosa*. The MICs values obtained were ranging from 64 to 256 µg/mL, depending on the individual fraction and the bacteria tested.

Studies conducted on the free radical scavenging activity of medicinal plants have shown that the efficiency of each plant species differs depending on the particular assay methodology, reflecting the complexity of mechanisms involved in total antioxidant capacity. The DPPH method was used to study the antioxidant activity of the root extract of *P. erinaceus*. Revealed that *P. erinaceus* has a relatively strong radical scavenging activity, which is dose-dependent. For example, the highest activity of 62.91% at a concentration of 1000 µg/mL (Figure 3), while the lowest activity of 36.48% at a concentration of 40 µg/mL.

In conclusion, this plant part could represent a potential source of lead molecules with pharmacological activities for developing new novel pharmaceutical products for treating malaria and other diseases. Also, the presence of compounds with biological activities justifies the traditional use of the root of *P. erinaceus* for treating malaria and other diseases. However, further studies are needed into the isolation and identification of the individual bioactive compounds responsible for their therapeutic activity and elucidating their mechanism(s) of action.

Not all bacterial strains used were sensitive to the root extract of *P. erinaceus.* Hence the root is not a promising therapeutic agent that can be used in combating infectious diseases caused by drug-resistant microorganisms. Furthermore, the root extract of *P. erinaceus* proved to have a moderate DPPH-scavenging antioxidant potential. Further study is needed to isolate and structurally characterize the pure compounds and evaluate their antimicrobial activity against multidrug-resistant microbial strains.

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The phytochemical profiles and growth of *Prunus africana* in Kenya

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Abstract. *Nyamai DW, Burugu MW, Ng'ang'a MM, Muchugi AN. 2022. The phytochemical profiles and growth of* Prunus africana *in Kenya. Asian J Nat Prod Biochem 20: 63-74.* African mountain ranges are home to the evergreen tree *Prunus africana* (Hook.f.) Kalkman. Benign prostate hyperplasia can be treated with the bark or extracts from the bark of this plant. The primary goal of this research was to compare the domesticated stand at Muguga (Kenya) to samples taken from a wild stand in Kobujoi and a remnant on-farm stand in Karuri in terms of their growth characteristics and phytochemical profile. Various solvents, including water, hexane, dichloromethane, and methanol, were used in the compound extraction process. The phytochemical analysis was performed with Liquid Chromatography and Gas Chromatography-mass spectrometry. The GC Chemstation version 11 program was used to examine the data from the GC-MS. The trees in the Muguga tamed stand varied in height from 3 meters to 14 meters, with a corresponding range in circumference at breast height from 0.9 centimeters to 104.5 centimeters. A total of 273 trees were planted in the orchard, and 92 (33%) produced fruit when the data was collected. When comparing the raw organic extract yields of the three groups, there was no statistically significant difference (p>0.05). Myristic acid, linoleic acid, lauric acid, methyl myristate, methyl laurate, and methyl linoleate made up the bulk of the essential oils in the bark samples across all three sources. For men with benign prostatic hyperplasia (BPH), these substances reduce cholesterol in the prostate. It was shown that dichloromethane and hexane extracts of the three populations included Campesterol, β-sitosterol, lup-20(29)-en- 3-one, palmitic acid, β-sitostenone, (3.β., 5.α)- stigmast-7-en-3-ol, stigmastan-3,5-diene, and α-tocopherol. Increased urine output and decreased prostaglandin synthesis in the prostate are effects of (3.β., 5.α)-stigmast-7-en-3-ol, β-sitosterol, and β-sitostenone. Cyanidin-o-galactoside, cyanidin-3-o-rutinoside, procyanidin B5, and robinetinidol-(4-α-8) catechin-(6,4-α) robinetinol are thought to have anticancer effects by inhibiting cell proliferation and scavenging free radicals in cancer cells. Evidence suggests that ursolic acid can reduce inflammation, prevent cell damage, and slow the growth of BPH. The essential oils of the Karuri people had noticeably (p<0.05) more myristic and lauric acids than any other population. Myristic acid, linoleic acid, methyl myristate, and α-tocopherol concentrations all varied significantly (p<0.05) between the Muguga population and the Karuri and Kobujoi populations. The findings show that the phytochemical composition of *P. africana* is not significantly changed by domestication (p>0.05), allowing for its cultivation in agricultural settings. Strategies for the sustainable collection, management, and conservation of this species through cultivation can be derived from the morphological and phytochemical data, which has crucial significance for these areas.

Keywords: Growth, phytochemical profile, *Prunus africana*

INTRODUCTION

In addition to its impressive height (more than 40 meters) and trunk diameter (up to 1 meter), the evergreen *Prunus africana* (Hook.f.) Kalkman also impresses with its broad crown (Gachie et al. 2012). Its leaves are glossy, its bark is dark brown, and its blossoms are either green or white. Although *P. africana* has a wide distribution, it is confined to the higher elevations (more than 1,500 m asl.) of the Afromontane forest islands of Africa (Somalia, Tanzania, Sudan, Uganda, Malawi, South Africa, Kenya, Ethiopia, Zaire, Zimbabwe, Angola, Cameroon, and Malawi) and underlying islands like Sao Tome' and Madagascar (Hall et al. 2000). The species prefers moist, tropical, or subtropical environments. The tree has several African uses, including construction materials and traditional and modern medicine.

Phytochemicals found in the stem and root bark extracts have been shown to have anticancer, anti-inflammatory, and antiviral properties (Vincenti et al. 2013; Tamasi et al. 2021). Prostate cancer patients who take bark extracts benefit from an apoptotic and antiproliferative action on the prostate, reducing their urologic symptoms (Kadu et al. 2012). Traditional practitioners have used the bark to cure various ailments, including heart problems, urinary tract infections, stomach discomfort, renal disorders, and malaria. Chewing or grinding the bark into a powder to make tea are two common consumption methods (Stewart 2003). In response to distinct geographical patterns, plant species exhibit wide ranges in chemical composition due to environmental changes.

Research conducted in Cameroon, Madagascar, and Zaire (R.D. Congo) revealed that the chemical makeup of *P. africana* extracts varies by habitat (Gachie et al. 2012). Analysis of nuclear and chloroplast DNA markers in *P. africana* reveals five unique African zones, reflecting the continent's diverse human population (Kadu et al. 2011). Only the volcanic highlands and mountains of "High Africa," an area spanning 34°S to 12°N, are home to this species. Its range in equatorial Africa spans from about 1,000 to 3,500 meters in altitude, where annual precipitation averages more than 3,000 millimeters (mm) at lower elevations and between 500 and 700 mm (mm) at higher ones (Kadu et al. 2011). Below the montane zone,

this species is mostly found in rocky places and along drainage lines (Hall et al. 2000).

Because of the strong demand for *P. africana* bark extracts, the species' wild population is in significant danger (Cunningham et al. 1997). Herbal remedies for treating BPH are manufactured with more than 3,000 tons of bark and bark extracts exported annually to Europe (Cunningham et al. 1997). The wild population of *P. africana*, the primary bark source, suffers greatly due to the increasing demand. As a result, several countries, including Kenya, are moving toward *P. africana* cultivation. Regeneration of *P. africana's* bark is possible so long as it is removed without damaging the tree's vascular cambium (Cunningham and Mbenkum 1993).

The *P. africana* is surprisingly resistant to debarking. However, large-scale debarking strains the tree even when full re-growth occurs, and in arid locations, bark re-growth begins, even limited. *P. africana* faces a greater threat due to its restricted distribution to a few Afromontane islands and the increased removal of land for agricultural purposes. Due to rising demand on a global scale, the species was included in Appendix II of CITES during its ninth meeting, making it legally protected as an endangered species (Betti 2008). Furthermore, the IUCN Red List has placed this species in a vulnerable conservation position.

Even though there are still control concerns and enforcement issues, it is necessary to find and implement conservation and domestication strategies as part of a sustainable management plan. Knowledge of regional variation is crucial for maximizing the sustainable conservation of *P. africana*. This research set out to compare the growth rates, phytochemical profiles, and yields of *P. africana* bark samples collected in Muguga, Kobujoi, and Karuri. Finding no statistically significant variations in these features, the results of this study will aid in the sustainable management of domesticated species. This information will also be useful for determining when it is best to gather bark and develop conservation plans for *P. africana* in the wild and on farms.

MATERIALS AND METHODS

Study site

Located in Muguga, Kenya, the *P. africana* stand was planted by the World Agroforestry Centre. The coordinates (10°14′ S 360°38′ E) for the Muguga Regional Research Centre in Kiambu County are as follows: The elevation of Muguga is around 2,150 m asl., and the town receives about 1,200 millimeters of rain a year. The Muguga population is a cultivated stand of *P. africana* maintained through regular trimming and undergrowth removal. An on-farm remnant stand of *P. africana*, the Karuri population, is intercropped with food crops. Kiambu county is home to the Karuri and Muguga sites. Hence they have similar climates. On a farm, Karuri trees were interplanted with edible ones. The *P. africana* population in Kobujoi is located in a natural forest in northern Nandi, where it coexists with many other plant species.

Sample collection

The *P. africana* forest in Muguga provided samples of stem bark for phytochemical study. At the height of 1.3 meters off the ground, debarking was accomplished with the help of a panga that had a serrated edge. Stem bark samples were also taken as a standard from Kobujoi, Nandi, and Karuri, all of which are remnant forests on farms. Five trees were chosen randomly from each population for the debarking process. The direction of the bark removal was randomized across four cardinal directions (East, West, North, and South) to prevent any potential for bias. The study did not include trees with a breast height diameter of fewer than 20 centimeters. Labels were placed on each sample after the wet weight was determined with an automated weighing scale.

Stem form, Diameter at Breast Height (DBH), tree height, and fruit set were all considered. Trees were measured in meters in height and scored on a scale from 1 to 5 for their stem shapes. In order to evaluate the growth rate of domesticated *P. africana*, we measured the diameter of the trees both at breast height and full height. We used the ring method to calculate tree ages by measuring ring widths using the TSAP-WinTm program. A DBH meter was used to measure the tree's circumference at chest height, and the results were recorded in cm. The DBH refers to a tree's circumference at a height of 1.3 to 2 meters. The DBH measurements in this investigation were performed from a height of 1.3 meters and above. A Suunto optical height meter was utilized to measure the height of individual trees. Tree heights served as a categorization key for the morphological data. It was assumed that all of the trees in the Muguga stand were planted simultaneously.

Preparation of tree rings for age determination

Cross-sections of trees that had been chopped down as part of a thinning effort in the Muguga forest were analyzed for their annual growth rings. All ring samples were labeled, and cross sections were cut with a chain saw at the height of 1.3 meters. Because the number of tree rings decreases with increasing height, the optimal height for ring counting was set at 1.3 meters. After the samples were cured, they were prepared for inspection using an orbital power sander to remove scratches and smooth the surface. After that, the surfaces were hand-sanded with increasingly finer grits of sandpaper (80, 120, 180, 240, and 360) to make the growth rings more apparent. Each ring's boundary was identified, and the rings were counted and marked starting from the outermost. The ring's width was observed using a pair of binocular microscopes and then measured with a computer program called TSAP-WinTm. To prevent under or over-estimation of ring width, samples were continuously modified to guarantee ring width was measured perpendicularly. The ring's breadth was measured at two different radii.

Visual cross dating

Rings can be dated to the exact year of their creation through a procedure called "*cross-dating*" (Stokes and Smiley 1968; Schweingruber 2007). The environmental conditions' effect on the growth pattern of trees can be seen

by accurate cross-dating (Worbes 1995). Using the skeleton plot method, we could visually cross-date samples by comparing the patterns of narrow and wide rings (Stokes and Smiley 1968). On the skeleton plot, the width was used to assign values between 1 and 10 to the rings, where 1 represents the narrowest ring, and 10 is the widest. Then, the rings were compared, and the narrowest ones, most indicative of the dry season, were given values close to 10. In this study, we only scored the widest rings (with a B) and ignored the average ones (Stokes and Smiley, 1968). The wide and narrow widths patterns must coincide with obtaining the composite skeleton map that enables dating the rings to the year they were produced (Stokes and Smiley 1968). After measuring ring width and performing visual cross-dating, the resulting data was saved on the computer in Raw picture format.

Determination of tree density

Randomly selected trees in Muguga had cross-sections taken at their DBH to collect samples for the density calculation. First, each sample's raw weight was determined with the help of an electronic balance. Then, the displacement technique was used to calculate the wet volume using water as the liquid. After drying the samples for 24 hours at 100°C, their dry weight and volume were immediately calculated. Next, the weight of the wood sample was determined by placing it in a water-filled measuring cylinder and then calculating the volume of water displaced. Finally, the density was calculated using Archimedes' principle based on the samples' dry weight and volume.

Reagents and reference compounds

Unless otherwise specified, all chemicals and solvents used were acquired from Sigma Aldrich Chemical Company limited in California, USA. Unless otherwise specified, all chemicals and reagents used were of a purity between 95% and 100% and were obtained from Sigma Aldrich Chemical Co. Ltd. Sigma Aldrich Chemical Co., Ltd. was sourced for both the crisofulvin and 1-heptene standards. Lupeol standard utilized was isolated from *Fagara tessmanii* by Ivan Addae-Mensah (University of Ghana).

Sample preparation

After being harvested, the bark samples were dried in a dark place for a month to reduce their moisture content. Next, the dried samples were sliced into smaller pieces and powdered using a fine grinder. Powdered bark from the Muguga, Karuri, and Kobujoi trees weighed 400 g and was soaked in hexane, dichloromethane, and methanol in that order for 24 hours. The extraction process required 700 ml of each solvent. After that, Whatman filter no. 1 was used with a vacuum suck pump to filter the extracts. Then, the filtrate was concentrated using a rotary evaporator in a vacuum at 40°C and a decreased pressure. Next, each extract was steeped in distilled water before being boiled in a water bath at 60°C for 5 hours after the organic solvent extraction. Next, the aqueous extracts were filtered through a No. 1 Whatman filter and concentrated in an SP Scientific AdVantage 2.0 benchtop lyophilizer.

Extraction of essential oils

Each powdered bark sample was weighed at 300 g and placed in a distillation flask. Each bark sample was boiled with 1.5 liters of distilled water. After boiling, the heat was reduced to 70°C and kept at that level for two hours. Next, to help dissolve the essential oils as they condense, 5 mL of hexane was added to the Clevenger apparatus. The condenser was cooled to between -15 and 15°C. The samples were dissolved in hexane and then placed in vials. The materials were distilled, concentrated using a short path distillation device, and extracted using one milliliter of dichloromethane.

Preparation of extracts for GC-MS analysis

Samples were weighed at 1 mg and placed in a 1.5 mL Eppendorf tube. Each hexane and dichloromethane extract was dissolved in 1 mL of dichloromethane and vortexed for 30 seconds. After centrifuging the samples at room temperature for five minutes at 1,300 rpm, they were sonicated with a Branson 2510E- DTE sonicator. The materials were then placed in sample vials with a capacity of 2 mL.

Instrumentation and chromatographic conditions

Gas chromatography research entails crucial optimization steps like (i) introducing sample extract into the GC column, (ii) separating components on an analytical column, and (iii) detecting target analysis utilizing a mass spectrometer detector. Using an HP-5 phenyl methyl siloxane capillary column that was 30 meters long, 320 µm in diameter, and 0.25 µm in film thickness, GC-FID analysis on a GC-FID (Model: 7890B Agilent) that consisted of an auto-sampler and gas chromatograph interfaced to a Flame Ionization Detector was performed. An electron ionization device with an ionization energy of 70Ev was employed for GC-FID detection. Hydrogen (99.99%) was used as the carrier gas at a constant flow rate of 30 mL/min; the injector temperature and mass transfer line temperature were set to 270 and 280°C respectively; an injection volume of 1 µL was used (splitless); and the oven temperature was programmed from 35°C (isothermal for 5 min), with an incremental of 10°C/min to 280°C for 5.4 min, and of 50°C/min to 285°C for 35 min.

Gas chromatography – Mass Spectrometry analysis

Lupeol was utilized as an internal standard to determine the concentration of hexane and dichloromethane extracts in the GC-MS. Using 1-heptene as an internal standard, GC-MS also identified and quantified essential oils. Before evaluating the extract using Gas Chromatography and Mass Spectrometry, the oven temperature, gas flow rate, and electron gun were initially programmed. Then, gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed using a GC-MS (7683 Agilent Technologies, Inc., Beijing, China) instrument with a gas chromatograph interfaced to a mass spectrometer and an HP-5 MS (5% phenyl methyl siloxane) of 30 m length,

0.25 mm diameter, and 0.25 μm film thickness low bleed capillary column. An electron ionization device with an ionization energy of 70Ev was employed for GC-MS detection. For the carrier gas at a constant flow rate of 1.25 mL/min, this experiment utilized helium (99.99 %), the injector temperature was set to 250°C, the mass transfer line temperature was set to 200°C, and the injection volume was 1 μL (splitless mode). The oven was set to preheat to 35°C for 5 minutes, rise by 10°C per minute to 280°C for 10.5 minutes, and finally, rise by 50°C per minute to 285°C for 29.9 minutes for a total run time of 70 minutes.

The following were the MS's settings: Interface temperature was 250°C; ionization energy was 70eV; ion source temperature was 230°C; solvent cut time was 3.3 minutes; relative detector gain mode; scan speed was 1666 μ/sec, and the scan range was 40-550 m/z. A total of 70 minutes were devoted to GC-MS operation. First, the extract's relative percentage was calculated from peak area normalization. Next, to verify the accuracy of the data, it was compared to the compounds in a small library of chemicals maintained by the National Institute of Standards and Technology.

Identification of components

The computed fragments, molecular mass, and molecular structure were all used for identification. An explanation of the Mass Spectrum NIST's database of more than 62,000 patterns was used for the GC-MS analysis. First, the test materials' constituents, molecular weights, and chemical structures were identified. Next, library-based mass spectrometry queries were conducted on the NIST 05, Chemecol.L, NIST 11, and Adams 2.l databases. Next, the NIST mass spectral search programmed version 2.0 was employed to support the GC-MS data system's characterization. Finally, quantification was performed by adding an internal standard to the region.

Liquid Chromatography-Mass Spectrometry analysis

Preparation of samples for LC-MS

Sample extracts were weighed in milligrams after being placed in a 1 mL Eppendorf with methanol and water. Each sample extracted with methanol had one milliliter of the methanol added to it to be re-dissolved. Dissolving aqueous extracts in a mixture of 95% methanol and 5% distilled water. After 30 seconds, the samples were vortexed again. Five minutes of sonication were performed using a Branson 2510E-DTE sonicator. Afterward, the samples were centrifuged at room temperature for 5 minutes at a speed of 1,300 rpm before being poured into 1.5 mL vials. Liquid chromatography and mass spectrometry were used to examine a methanol and water extract.

LC-MS analysis

An HP 1100 capillary system equipped with an auto-sampler and a micro-pump (Agilent Technologies, Incorporation, Beijing, China) was used for the HPLC separations. In LC-MS, chemical concentrations were determined using griseofulvin as an internal standard. The chemicals were separated utilizing a Waters symmetry column, 100 μm 2.1 mm, 3.5 μm. All analyses were performed with an injection volume of 2 μL and a temperature compartment of the auto-ampler set to 4°C. Water (component A) and methanol (component B) made up the mobile phase. The solvent gradient was started at 10% B and held for 30 minutes, next programmed to 50% in 3 minutes and held for 5 minutes, and next to 100% and held for 10 minutes at a flow rate of 200 L/min. Next, to avoid contaminating the ESI source, the LC's effluent should send from the first 5 minutes before analysis to the drain. The data was analyzed using ESI-MS with the positive ion mode. Moreover, to determine the concentration of griseofulvin as an internal standard and to identify any pentacyclic triterpenoids in the extracts, the data were consulted in the METLIN metabolite database and reviewed the relevant literature.

Statistical analysis

Before combining the growth curves of the trees into a chronology, the ring width data were analyzed with COFECHA software to identify the issue segments and verify cross-dating (Grissino-Mayer 2001). Winks version 7 software was used to examine data gathered from both wild and domesticated populations. The average chemical compounds of the five trees in each group were computed to provide a context for their interactions. Tukey's student-centered honestly significant difference (at the 5% significance level) was used to distinguish between the means. Variation was standardized by calculating the coefficients of variation within the population. The relationships between the various components, as well as those between the components and the tree's size and the surrounding environment, were also determined. The population differences were examined using an ANOVA test. Finally, the association between tree age and growth rates, wood density and DBH, ring number, and DBH was calculated using a Pearson's correlation analysis.

RESULTS AND DISCUSSION

Morphological characterization of *Prunus africana* trees at Muguga stand

Table 1 displays the results of an analysis conducted on the morphological data of *P. africana* trees in the Muguga stand based on categorizing tree heights. That the trees were all the same age was based on the assumption that they had been planted simultaneously. Only two trees out of the whole population were less than 5.5 meters. In total, 146 trees range from 5.6 meters to 10.5 meters tall. The third cluster included 124 trees, ranging in height from 11.6 to 15 meters. There was a statistically significant ($p>0.05$) difference between group one and the other height groups in the Muguga stand, and the stems from each group looked identical. The average DBH of the three groups increased with increasing height. Both DBH and height can be used as indicators of development.

The average DBH across all three categories was between 24 and 140 cm. The mean diameter at breast height was substantially smaller in group one compared to the other two groups ($p<0.05$). The percentage of trees in a

given group that had reached fruition was reported. Both of the Group 1 trees were still too young to bear fruit. Group 2 consisted of 146 trees, 40 of which (26.4%) were in the fruiting stage and 106 (72.6%) without fruits. Refer to appendix 43 for a blueprint of the Muguga *P. africana* display, where the placement of trees in the stand is depicted here. On the West side of the plantation, trees near the *Grevillea robusta* border were subjected to longer times of shade than those near the grass border.

Visual and statistical cross dating

First, this investigation used the skeleton plot technique for visual cross-dating (Stokes and Smiley, 1968). Wedging ring detection was achieved by utilizing two radii on the cross sections. Fifteen of the trees out of twenty-three were correctly cross-dated, and the results showed an age range of fifteen to twenty-six years.

Relationship between age and growth rate in Muguga population

Table 2 displays the ages and growth rates (in millimeters) of seven randomly selected trees in Muguga. A non-significant relationship between age and average growth rate was found using Pearson's correlation analysis for trees randomly selected from the Muguga population (t = -0.916, DF= 16, r2=0.0498, p>0.05). There was also a negative relationship between age and development rate (r = -0.223).

Relationship between ring number and DBH of trees from Muguga population

The link between the number of rings in a tree's trunk cross section and its DBH was not statistically significant in a regression and Pearson's correlation study of trees randomly sampled from the Muguga population (DF= 14, r2=0.0858, p>0.05) (Figure 1). However, a positive correlation (r = 0.293) was found between the number of growth rings in the trunk cross-section and the DBH of the chosen trees.

Relationship between wood density and the DBH of trees from the Muguga population

Trees were randomly sampled from the Muguga population, and their wood density and DBH were analyzed for correlation and regression. However, the results indicated no statistical significance (DF=16, r2=0.0196, p>0.05) (Figure 2). There was a negative relationship between the density of wood section samples and tree DBH in the Muguga population (r = -0.140).

Relationship between wood density (g/cm^3) and the growth rate (mm)

Wood sample density and the growth rate of trees randomly selected from the Muguga population did not correlate significantly (DF=14, r2=0.0279, p>0.05) (Figure 3), according to a Pearson correlation and regression analysis. Muguga population tree growth rate was favorably linked with wood density (r = 0.167).

Crude yield extracts of individual populations

Hexane, dichloromethane (DCM), methanol, and water were used to extract bark samples from all three populations. Table 3 details the organic solvent yields. The crude bark extracts from methanol, DCM, and hexane did not differ significantly (p>0.05) amongst the three groups. Methanol crude extract yield was highest for the Karuri population and lowest for the Kobujoi population. The crude extract yields of DCM and hexane were highest for the Kobujoi population, whereas the Karuri population yielded the least. All three populations obtained their maximum yield from methanol extract.

Table 2. Relationship between age (years) and growth rate (mm)

Age (years)	Growth rate (mm)	t value	DF	r^2
1	3.53±1.69	-0.916	16	0.0498
2	3.42±1.96			
3	2.38±1.98			
4	1.82±1.14			
5	2.67±2.05			
6	1.38±0.71			
7	1.06±0.27			
8	2.07±1.84			
9	1.69±0.99			
10	4.78±3.08			
11	2.26±1.37			
12	2.36±1.13			
13	3.18±1.36			
14	1.80±0.73			
15	3.38±1.98			
16	2.00±0.75			
17	1.22±0.70			
18	1.56±0.68			

Note: Values are expressed as Mean ± SD (n=7).

Table 1. Growth characteristics of trees at Muguga (Kenya) *Prunus africana* stand

Groups (height)	No. of trees	Height (m)	Stem shape rating	DBH(cm)	Fruiting	
					Yes	No
Group 1 (0-5)	2	$4.20±1.70^a$	$1.50±0.71^a$	$9.45±12.09^b$	0	2 (100)
Group 2 (6-10)	146	$9.22±1.16^b$	$1.53±0.60^a$	$40.17±12.19^a$	40 (27.40)	106 (72.60)
Group 3 (11-15)	124	$11.67±0.80^b$	$1.63±0.64^a$	$54.85±16.26^a$	52 (41.94)	72 (58.06)

Note: Values are expressed as Mean ± SD (n=3). Values followed by the same superscript in the same columns are not significantly different (*p*>0.05). Values in parenthesis after groups show the tree heights in each group. Values in parentheses in the fruiting column are expressed as percentages

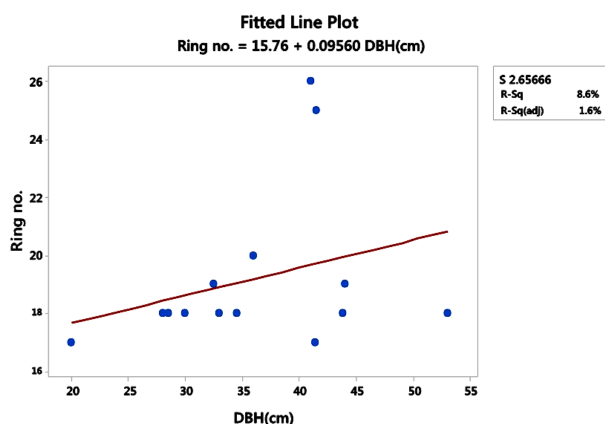

Figure 1. A fitted line plot of ring number versus DBH (cm)

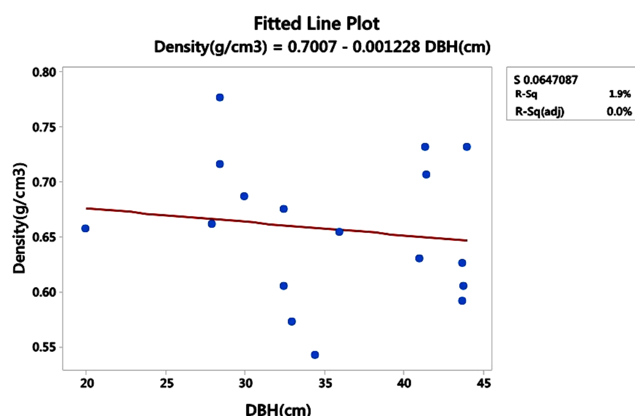

Figure 2. A fitted line plot of wood density (g/cm^3) versus DBH (cm)

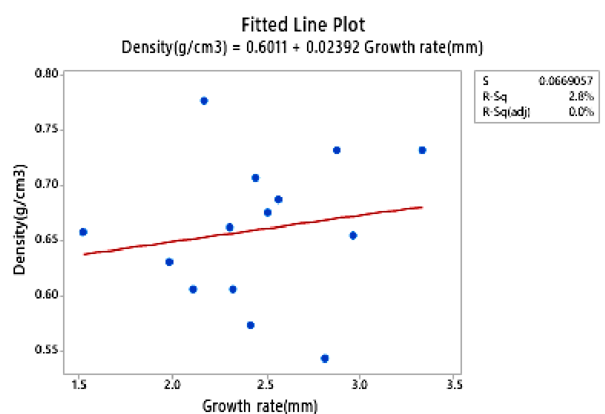

Figure 3. A fitted line plot of density (g/cm^3) versus growth rate (mm)

Table 3. Crude yields (g) for organic extracts

Population	Methanol	DCM	Hexane
Muguga	3.58±2.38^a	0.59±0.55^a	0.56±0.08^a
Karuri	4.94±0.50^a	0.88±0.41^a	0.46±0.11^a
Kobujoi	2.79±2.97^a	0.90±0.47^a	0.82±0.74^a

Note: Values are expressed as Mean ± SD (n=3). Values followed by the same superscript in the same column are not significantly different (*p*>0.05)

Phytochemical yields in the three populations

Total essential oils yields in Muguga, Karuri, and Kobujoi populations

Essential oils from each of the three groups were analyzed for their phytochemical composition using GC-MS. The analysis showed that all three groups contained linoleic acid, methyl laurate, methyl linoleate, lauric acid, methyl myristate, and myristic acid. Table 4 provides a breakdown of polyunsaturated fatty acids and their methyl esters. The essential oil composition of the Muguga showed the highest content of linoleic acid, methyl linoleate, and methyl myristate. Essential oil composition showed the highest quantities of myristic acid and lauric acid in the Karuri people and the highest concentration of methyl laurate in the Kobujoi population.

Essential oil samples from Muguga, a domesticated stand, had a considerably higher linoleic acid content than those from Karuri and Kobujoi (p<0.05); Kobujoi has the lowest linoleic acid content, and Muguga has the greatest. There was a statistically significant difference (p<0.05) between the lauric acid concentration in the Karuri samples and that in Kobujoi and Muguga samples. Lauric acid was found at the highest concentration in Karuri (1152.14) and the lowest concentration in Kobujoi (4.12). There was no statistically significant difference (p>0.05) between the methyl laurate concentrations of the three groups. However, there were statistically significant differences (p<0.05) in the methyl linoleate concentrations of the three groups. There was much more methyl myristate in Muguga than in Karuri or Kobujoi (p<0.05). The levels of myristic acid varied greatly between the three populations, with Karuri having the highest.

Hexane extract yields of Muguga, Karuri, and Kobujoi populations

Hexane extracts from all three populations were analyzed for their phytochemical content using GC-MS, and the results showed the presence of campesterol, β-sitosterol, palmitic acid, β-sitostenone, (3.β.,5.α)- stigmast-7-en-3-ol, lup-20(29)-en-3-one, stigmastan-3,5-diene, and α-tocopherol compounds. Table 5 provides an analysis of these chemicals. The Muguga people possessed the highest hexane-extracted chemical concentrations such as campesterol, lup-20(29)-en-3- one, palmitic acid, squalene, β-sitostenone, 3β,5.α-stigmast-7-en-3-ol, stigmastan-3,5-diene, myristic acid, and α-tocopherol compounds. Both lauric acid and beta-sitosterol were found in maximum quantities in Karuri communities. campesterol, β-sitosterol, squalene, lup-20(29)-en-3- one, lauric acid, β-sitostenone, stigmastan-3,5-diene, 3β,5.α-stigmast-7-en-3-ol, palmitic acid, and α-tocopherol contents in hexane extracts from the three groups were not statistically different (p>0.05). However, the levels of α-tocopherol in the Muguga samples varied considerably (p<0.05). Since one of the replicates did not contain lauric acid or myristic acid, the standard deviation for those two chemicals was quite significant.

Table 4. The concentration of essential oils in Muguga, Karuri, and Kobujoi (mg/kg)

Compound	Muguga	Karuri	Kobujoi
Linoleic acid	196.35±3.48[b]	29.13±3.47[a]	28.93±1.98[a]
Lauric acid	382.66±2.61[a]	1152.14±315.29[b]	4.12±1.07[a]
Methyl laurate	2.54±0.41[a]	3.32±0.50[a]	3.36±0.71[a]
Methyl linoleate	27.82±1.28[a]	14.83±1.59[b]	7.51±1.29[c]
Methyl myristate	26.71±0.64[b]	4.59±0.16[a]	5.80±5.20[a]
Myristic acid	287.09±1.36[a]	554.99±22.60[b]	92.84±1.81[c]

Note: Values are expressed as Mean ± SD (n=3). Values followed by the same superscript along rows are not significantly different (p>0.05)

Table 5. Concentrations of compounds in hexane extracts of Muguga, Karuri, and Kobujoi (mg/kg)

Compound	Muguga	Karuri	Kobujoi
Campesterol	9.16±1.93[a]	4.51±3.75[a]	6.70±3.29[a]
Lauric acid	2.62±1.18[a]	4.84±7.56[a]	0.72±0.32[a]
β-Sitosterol	131.04±31.34[a]	160.05±3.91[a]	153.36±13.01[a]
Lup-20(29)-en-3-one	13.32±2.81[a]	10.04±5.29[a]	7.97±3.61[a]
Palmitic acid	82.24±30.049[a]	34.28±19.83[a]	55.13±58.46[a]
Squalene	34.25±14.99[a]	26.72±3.18[a]	28.34±19.13[a]
β-sitostenone	36.92±12.05[a]	19.79±0.49[a]	27.80±13.87[a]
3β,5α-Stigmast-7-en-3- ol	15.63±4.71[a]	10.46±4.64[a]	11.23±6.01[a]
Stigmastan-3,5-diene	35.99±11.50[a]	20.42±2.49[a]	27.21±16.94[a]
Myristic acid	7.21±0.50[a]	5.65±5.06[a]	2.02±0.09[a]
α-Tocopherol	13.44±2.71[b]	1.84±1.15[a]	4.88±1.38[a]

Note: Values are expressed as Mean ± SD (n=3). Values followed by the same superscript along rows are not significantly different (p<0.05)

DCM extract yields of Muguga, Karuri, and Kobujoi populations

Furthermore, to determine the phytochemical composition of the DCM extracts from the three populations, GC-MS analysis was performed. The results showed the presence of campesterol, palmitic acid, β-sitosterol, lup-20(29)-en-3-one, β-sitostenone, (3.β.,5.α)-stigmast-7-en-3-ol, stigmastan-3,5-diene, and α-tocopherol. Analysis of hexane extracts revealed a phytochemical profile comparable to that of DCM extracts. Although somewhat different in polarity, these two solvents are both non-polar. So they extract the same molecules, albeit at slightly different amounts, and all three populations included these chemicals. Table 6 provides a breakdown of these chemicals, which play a significant role in managing BPH.

Campesterol, palmitic acid, lup-20(29)- en-3-one, squalene, β-Sitosterol, β-sitostenone stigmastan-3,5-diene, and myristic acid were found at the highest concentrations among the Muguga community. The lauric acid content was highest in the Karuri population, while the 3-β,5α-stigmast-7-en-3-ol, and α- tocopherol content was highest in the Kobujoi group. DCM extracts from the three populations did not differ substantially (p>0.05) concerning the amounts of campesterol, lauric acid, β-sitosterol, lup-20(29)-en-3-one, β-sitostenone, 3-β,5α-stigmast- 7-en-3-ol, stigmastan-3,5-diene, squalene, palmitic acid and α-tocopherol. Compared to Karuri and Kobujoi samples, the concentration of myristic acid in DCM extracts of Muguga samples was substantially different (p<0.05).

Methanol extract yields of Muguga, Karuri, and Kobujoi populations

Using LC-MS, methanolic extracts from all three populations were analyzed for their phytochemical composition. The results showed the presence of procyanidin B5, feruloyl-quinic acid, prunetrin, robinetinidol-(4-α-8)-catechin-(6-α)-robinetinol, quercetin3,3'- dimethyl ether-4'-glucoside, cyanidin-o-galactoside, ursolic acid, chlorogenic acid, isochamaejasmin+, cinnamtannin A2, isoliquiritin, and two other substances. Cyanidin-3-o-rutinoside was exclusively found in Karuri population methanol extracts, although isoliquiritin and isochamaejasmin+ were absent. In methanol extracts, the Kobujoi population exhibited the highest quantity of feruloyl-quinic acid, chlorogenic acid, procyanidin B5, quercetin3,3'-dimethyl ether-4'-glucoside, cinnamtannin A2, and isochamaejasmin, as shown in Table 7. Ursolic acid, isoliquiritin, and unidentified chemical 1 were found in methanol extracts at their greatest concentration in the Muguga community. Prunetrin, cyanidin-o-galactoside, and robinetinidol-(4-α-8)-catechin-(6-α)-robinetinol levels in methanol extracts were highest in the Karuri community. There was no statistically significant difference (p<0.05) between the feruloyl-quinic acid, chlorogenic acid, cyanidin-o-galactoside, ursolic acid, procyanidin B5, and unidentified chemical 2 concentrations in methanolic extracts of samples from the three groups. Concentrations of prunetrin in the Karuri population were significantly different from those in the Muguga and Kobujoi populations (p<0.05). Cyanidin-3-o-rutinoside was not found in methanolic preparations of Muguga or Kobujoi. The levels of cinnamtannin A2 in Kobujoi were substantially greater than in the other samples (p<0.05). Isochamaejasmin+ concentrations were substantially different (p<0.05) amongst the three populations, with the chemical being absent from Karuri samples.

Table 6. The concentration of compounds in DCM extracts of Muguga, Karuri, and Kobujoi (mg/kg)

Compound	Muguga	Karuri	Kobujoi
Campesterol	12.55±3.75[a]	7.32±0.56[a]	8.10±2.51[a]
Lauric acid	1.19±0.768[a]	1.85±0.93[a]	1.71±1.24[a]
β-Sitosterol	130.20±72.95[a]	103.59±28.29[a]	117.16±20.85[a]
Lup-20(29)-en-3-one	14.04±1.89[a]	9.99±0.43[a]	8.30±3.502[a]
Palmitic acid	116.63±42.44[a]	65.55±23.54[a]	90.63±67.70[a]
Squalene	34.56±14.55[a]	22.23±6.35[a]	28.34±9.90[a]
β-sitostenone	43.21±15.52[a]	26.51±4.48[a]	30.62±6.73[a]
3β,5α-Stigmast-7-en-3-ol	18.39±7.69[a]	11.59±2.83[a]	19.57±13.93[a]
Stigmastan-3,5-diene	36.83±15.75[a]	26.13±3.66[a]	29.57±11.68[a]
Myristic acid	6.47±0.99[b]	2.89±1.27[a]	3.19±1.40[a]
α-Tocopherol	6.80±1.04[a]	7.67±2.12[a]	11.08±2.44[a]

Note: Values are expressed as Mean ± SD (n=3). Values followed by the same superscript along rows are not significantly different (p>0.05)

Table 7. The concentration of compounds in methanol extract from the three populations (mg/kg)

Compound	Muguga	Karuri	Kobujoi
Feruloyl-quinic acid	1.89±0.69[a]	2.21±0.67[a]	2.56±1.45[a]
Chlorogenic acid	2.05±0.82[a]	2.07±1.33[a]	2.36±0.73[a]
Isoliquiritin	7.48±0.65[a]	0.000[b]	7.48±0.18[a]
Prunetrin	1.27±0.62[a]	2.90±0.630[b]	1.20±0.52[a]
Cyanidin-*O*-galactoside	9.87±2.79[a]	10.69±0.25[a]	7.37±0.85[a]
Ursolic acid	2.39±2.04[a]	0.78±0.26[a]	1.57±0.34[a]
Unknown compound 1	16.16±4.93[a]	12.56±2.90[ab]	6.98±1.63[b]
Procyanidin B5	1.29±0.63[a]	0.82±0.33[a]	3.10±1.60[a]
Cyanidin-3-*O*-rutinoside	0.000[a]	11.74±1.74[b]	0.000[a]
Quercetin3,3'-dimethyl ether-4'- glucoside	1.14±0.35[a]	0.62±0.18[a]	20.27±0.71[b]
Robinetinidol-(4-α-8)catechin- (6,4-α)robinetinol	0.84±0.27[b]	4.81±0.35[a]	4.22±2.43[ab]
Unknown compound 2	4.31±0.66[a]	3.63±0.69[a]	6.16±2.98[a]
Cinnamtannin A2	0.67±0.14[a]	0.74±0.03[a]	2.29±0.49[b]
Isochamaejasmin+	1.14±0.39[a]	0.000[b]	17.92±0.46[c]

Note: Values are expressed as Mean ± SD (n=3). Values followed by the same superscript along rows are not significantly different (p<0.05)

Aqueous extract yields of Muguga, Karuri, and Kobujoi populations

The aqueous extracts from all three populations were analyzed for their phytochemical composition using LC-MS. The results showed the presence of procyanidin B5, robinetinidol-(4-α-8)-catechin-(6-α)- robinetinol, quercetin 3,3'-dimethyl ether-4'-glucoside, cyanidin-o-galactoside, feruloyl-quinic acid, chlorogenic acid, ursolic acid, cyanidin-3-o-rutinoside, cinnamtannin A2, isoliquiritin, prunetrin and two unknown compounds. Table 8 summarizes the results of the investigation. Only the aqueous extracts of the Muguga population contained the bioactive compounds feruloyl-quinic acid and prunetrin. Quercetin3,3'-dimethyl ether-4'-glucoside was not detected in aqueous extracts of Kobujoi.

The highest concentrations of quercetin3,3'-dimethyl ether-4'-glucoside, unidentified compound 1, and unidentified compound 2 were found in aqueous extracts from the Muguga population. Chlorogenic acid, cyanidin-o-galactoside, ursolic acid, procyanidin B5, and cinnamtannin A2 were found in the greatest concentrations in Kobujoi aqueous extracts. The robinetinidol-(4-α-8)-catechin-(6-α)-robinetinol, isoliquiritin, and cyanidin-3-o-rutinoside compounds were found in the greatest amounts in the aqueous extracts from the Karuri population. In aqueous extracts of samples from the three groups, there was no statistically significant difference (p<0.05) in the amounts of cyanidin-3-o-rutinoside, procyanidin B5, unknown compound 1, or cinnamtannin A2. In aqueous extracts, only the Muguga population indicated the presence of prunetrin and feruloyl-quinic acid.

The presence of unidentified compound 2 was not detected in aqueous extracts of Karuri. The chlorogenic acid levels in the Kobujoi samples were considerably greater than in the Muguga and Karuri samples (p<0.05). Muguga and Karuri samples had substantially different quercetin- 3, 3'-dimethyl ether-4'-glucoside concentrations (p<0.05), but Kobujoi aqueous extract did not. Cyanidin-o-galactoside concentrations in Karuri and Kobujoi aqueous extracts were not substantially different from one another (p<0.05), but they were considerably different from those in the Muguga samples.

Table 8. Concentrations of compounds in aqueous extracts from the three populations (mg/kg)

Compound	Muguga	Karuri	Kobujoi
Feruloyl-quinic acid	5.64±3.24[b]	0.000[a]	0.000[a]
Chlorogenic acid	1.93±1.714[a]	4.06±2.09[a]	10.06±2.27[b]
Isoliquiritin	18.469±4.77[a]	30.97±5.11[b]	17.61±2.56[a]
Prunetrin	1.74±0.91[b]	0.000[a]	0.000[a]
Cyanidin-*O*-galactoside	1.56±1.64[b]	3.50±0.75[ab]	5.48±0.56[a]
Ursolic acid	13.73±7.89[b]	19.31±4.17[ab]	27.72±0.84[a]
Unknown compound 1	13.53±6.26[a]	11.75±2.39[a]	6.21±0.16[a]
Procyanidin B5	5.58±4.18[a]	6.99±1.44[a]	11.27±0.32[a]
Cyanidin-3-*O*-rutinoside	16.53±10.13[a]	33.03±5.85[a]	18.09±2.74[a]
Quercetin3,3'-dimethyl ether- 4'-glucoside	7.64±2.74[b]	3.01±1.00[a]	0.000[a]
Robinetinidol-(4-α-8)-catechin-(6,4-α)robinetinol	1.34±0.43[a]	5.605±0.47[b]	3.72±0.50[c]
Unknown compound 2	21.45±2.80[a]	0.000[b]	11.13±0.43[c]
Cinnamtannin A2	3.06±1.63[a]	3.54±1.33[a]	5.50±1.08[a]

Note: Values are expressed as Mean ± SD (n=3). Values followed by the same superscript along rows are not significantly different (p<0.05)

Discussion

The different shading times throughout the population likely caused the variations in height and DBH among the trees in the Muguga *P. africana* stand. Trees on the eastern side of the plantation were likewise found to be significantly shorter than their counterparts on the western side. This height variation may be due to the trees' competition for sunlight in areas where it is scarce. Given its importance to photosynthesis, light is an important environmental component that affects plant development (Canham et al. 1990; Valladares et al. 2003). All plants are subjected to various shades throughout their lives since light gradients can vary both within plant canopies and within the crowns of individual plants. High or low light levels might limit a plant's development ability, yet sunlight is necessary for photosynthesis (Grubb 1998).

There is no set time of year for *P. africana* flowers to bloom in the equatorial regions, and some plants bloom

nearly every month (Munjuga et al. 2000). Different fruiting patterns were seen because of the large variation in flowering times. The plant matures its fruit two to three months after flowering, in time with the onset of rain (Munjuga et al. 2000). Indicating that complex environment-plant interactions determine the growth rates of plant species. There is little to no correlation between short-term growth rates and individual environmental parameters (Berman and DeJong, 1997). The average growth rate of randomly selected *P. africana* individuals in the Muguga stand was inversely linked with age. Yoder et al. (1994) noted the same thing about *Pinus contorta* and *Pinus ponderosa*. It has been hypothesized that a species' photosynthesis slows with age, explaining the negative correlation between age and growth rate (Yoder et al. 1994).

Seasonal shifts influence cambial activity, affecting the width of tree growth rings and other phonological characteristics. Due to the lack of distinct tropical seasons (Hoadley, 1990), tree ring boundaries may be difficult to distinguish. Nevertheless, dendrochronology methods have successfully detected ring boundaries and date tropical trees. Seasonal shifts cause a widening or narrowing of the rings. The wetter seasons are represented by wider rings, while the drier ones are indicated by narrower ones (Fichtler et al. 2003; Trouet et al. 2009). In particular, P. africana does not disrupt the dormancy of cambium activity during short rains, but it reawakens cambium activity when the long rains begin (Krepkowski et al. 2011). As shown in *Podocarpus falcatus*, secondary cell enlargement and thickness can cause an increase in ring width even in the absence of cambium activity, allowing for growth even during dry periods (Deslauriers et al. 2009).

Dimensional stability, workability, and mechanical timber qualities are all aspects of wood quality that can be described in terms of wood density. It was found that the DBH of trees was inversely related to their wood density. A negative correlation was recorded in the *Picea mariana* species (Zhang et al. 1996). In the Muguga population, there was a positive correlation between the number of growth rings and the diameter of trees' breast height. A higher cambium activity is associated with maturation, and a higher wood density is associated with a faster growth rate. Earlier research has shown that dense hardwoods do not develop faster than less dense hardwoods (Zobel and van Buijtenen 1989; Zhang 1995). *Betula pendula* and *Prunus serotina* were also reported to have this characteristic (Nepveu and Velling 1983; Koch 1967). Hernandez et al. (1998) and Pliura et al. (2007) found a weak negative association between growth rate and wood density in *Populus* species, whereas other research found no correlation between the two (Debell et al. 2002; Zhang et al. 2003).

It has been found that *P. africana* bark extract helps alleviate lower urinary tract symptoms in patients with benign prostatic hyperplasia by lowering inflammation, decreasing bladder reactivity, and shrinking the enlarged prostate (Andro and Riffaud 1995; Ishani et al. 2000). Extracts are thought to reduce BPH by blocking the enzyme 5-α-reductase, reducing inflammation, lowering prolactin levels, and slowing the proliferation of prostatic fibroblasts in response to growth stimuli (Capasso et al. 2003). Each substance in this study has a role in either curing BPH or reducing its symptoms (Donovan et al. 1998; Carbin et al. 1990; Kampa et al. 2004). Phytosterols, ketones, phenolic compounds, and pentacyclic triterpenoids are all examples of such substances.

Hydro-distillation yielded an essential oil rich in polyunsaturated fatty acids and their methyl esters. Yet, hexane and DCM extracts included myristic acid and lauric acid, albeit in lower amounts than the essential oil. Since oils are volatile, some were possibly lost during the evaporation procedure used to concentrate the DCM and hexane extracts, which led to the observed disparity. Phytosterols were found in hexane and DCM extracts because they are non-polar to mid-polar molecules and are, therefore, simple to extract. Polar substances such as pentacyclic triterpenoids and phenolic compounds were found in methanol and water extracts. Management techniques that promote species production and biodiversity conservation require knowledge about phytochemical content. The delivery of crucial ecosystem services is influenced by phenotypic variety, genetic variation, and species richness, all of which are enhanced by phytochemical variation in agroforestry and cultivation (Cardinale et al. 2012). The anti-edematous and glucosyl-transferase-inhibiting properties of the pentacyclic triterpenoids found in *P. africana* bark extracts are well-known (Kokwaro, 1993; Donovan et al. 1998; Mothana et al. 2006). Researchers have shown that ursolic acid can stop the spread of cancer cells, including melanoma and prostate cancer cells (Nataraju et al. 2007).

This study had no statistically significant variance for the concentrations examined. Prohibiting prostaglandin production, phytosterols like β-sitosterol and β-sitostenone reduce inflammation and prevent prostate enlargement (Raicht et al. 1976; Carbin et al. 1990). In addition to ferulic acid esters and their derivatives, which exhibit anticancer and hypocholesterolemic effects on the prostate, the bark extracts are also contained (Kampa et al. 2004). In addition, researchers have found chemopreventive effects of phenolic compounds in *P. africana* bark on estrogen-dependent breast cancer (Noratto et al. 2009).

Compared to β-sitosterol, the concentration of β-sitostenone was lower in DCM and hexane extracts from the three populations. The lower levels of β-sitostenone in *P. africana* were also noted by Catalano et al. (1984). While analyzing DCM extracts, the highest concentration of β-sitosterol was found in Muguga (130.20 mg/kg). The chemical has been studied for its potential anticancer and cholesterol-lowering effects (Awad and Fink, 2000). Compared to several other species, *P. africana* has significantly greater quantities of β-sitosterol. Bark values in avocado trees are comparable to those in *P. africana* trees, and avocados are a major source of β-sitosterol (Duester 2001). Traditional medicine uses the diuretic effects of *Moringa oleifera* (Anwar et al. 2007) and *P. spinosa* (Wolbiš et al. 2001) due to their high levels of β-sitosterol. β-sitosterol concentrations in this investigation were shown to be independent of environmental controls. However, soybeans found a lower concentration of β-

sitosterol in seeds generated by plants grown in colder climates (Yamaya et al. 2007). The Muguga had the highest levels of cholesterol and campesterol, as well. Aside from β-sitosterol, campesterol and stigmast-7-en-3-β-ol have also been found in Hypoxis species (Pegel 1979; Moghadasian 2000). The phytosterols in question have been shown to fight cancer (Choi et al. 2003), lower cholesterol, and reduce inflammation (Quilez et al. 2003).

Compared to the Karuri and Kobujoi populations, the Muguga population had greater quantities of myristic acid and α -tocopherol in their extracts. The soil types and ambient circumstances of the three populations explain the variation in concentration. Plants' development and metabolic pathways may differ because of the range of chemical, physical, and biological characteristics in soils. Indirectly affecting DNA through the regulation of transcription of genes involved in metabolic activities, environmental variables can affect epigenetics, which in turn controls the production of secondary chemicals in plants.

Across all three groups, palmitic acid was most prevalent, followed by myristic acid and then lauric acid. The two saturated fatty acids had a lower concentration in *P. africana* than the other fatty acids, and this variation had previously been observed (Ganzera et al. 1999; Abe et al. 2009). Also found in saw palmetto and pumpkin seeds are the aforementioned fatty acids (Ganzera et al. 1999). *Artocarpus heterophyllus* (Chowdhury et al. 1997) and *P. amygdalus* (Munshi and Sukhija 1984) have been shown to contain trace amounts of lauric acid and myristic acid. Sterols and fatty acids may lower prostate growth by preventing testosterone from converting to dihydrotestosterone. These drugs inhibit the 5-α-reductase enzyme, which stops the production of dihydrotestosterone, the prostate growth regulator (Edeoga et al. 2005; Bent et al. 2006).

In the three populations, friedelin was not detected though the compound had been reported in *P. lusitanica* (Sainsbury 1970) and *P. africana* bark extracts (Catalano et al. 1984). Friedelin is a triterpenoid with anti-inflammatory activity (Antonisamy et al. 2011). Ursolic acid has been a component in traditional medicine and is also a natural pentacyclic triterpenoid in plants (Amico et al. 2009). Ursolic acid has been reported to have antiproliferative, antioxidant, and anti-inflammatory activities (Nataraju et al. 2007; Amico et al. 2009). It also serves for the biosynthesis of more potent bioactive compounds as starting material for the biosynthesis, like antitumor agents (Ma et al. 2005). Ursolic acid at up to 2,000 mg/kg concentrations has also been detected in *Eriobotrya japonica* (Zhou et al. 2011).

Despite reports of friedelin in *P. lusitanica* (Sainsbury, 1970) and *P. africana* bark extracts, this study could not identify this chemical in any of the three populations (Catalano et al. 1984). Friedelin is an anti-inflammatory triterpenoid (Antonisamy et al. 2011). Another component of traditional medicine, ursolic acid, is found naturally in plants as a pentacyclic triterpenoid (Amico et al. 2009). Some studies have found that ursolic acid possesses anti-inflammatory, antiproliferative, and antioxidant properties (Nataraju et al. 2007; Amico et al. 2009). Furthermore, it is a precursor in producing other, more powerful bioactive chemicals, such as anticancer drugs (Ma et al. 2005). *E. japonica* has also been found to contain ursolic acid at levels as high as 2,000 mg/kg (Zhou et al. 2011).

Polyphenols such as cyanidin-o-galactoside and cyanidin-3-o-rutinoside have also been found in plums (Kim et al. 2003; Usenik et al. 2008). Phenolic chemicals are employed as anticancer treatments due to their anti-oxidative activity; they also help with diabetes and cardiovascular disease (Utsunomiya et al. 2005; Belkaid et al. 2006; Noratto et al. 2009). Phytochemicals in plums include hydroxycinnamic acid analogs, such as chlorogenic acid and quercetin derivatives (Raynal et al. 1989; Kim et al. 2003). *P. domestica*, coffee, and blueberries have all been linked to chlorogenic acid (Donovan et al. 1998; Prior and Cao 2000). Robinetinidol-(4-α-8) catechin-(6,4-α) robinetinol, cyanidin-o-galactoside, cyanidin-3-o-rutinoside, and procyanidin B5are products of the flavonoid family having antiproliferative and free radical-scavenging properties (Rukunga and Waterman1996; Cai et al. 2004; Jacob et al. 2012). Inhibiting topoisomerases and protein kinases are just one of the many functions flavonoids serve; they also influence apoptosis and cell differentiation and have antioxidant activity (Kuo 1997; Pinhero and Paliyath 2001). As a result of their characteristics, flavonoids are valuable molecules in the study of cancer. Some anthocyanins in Japanese plums include cyanidin-o-galactoside and cyanidin-3-o-rutinoside (Wu and Prior 2005). Most fruits get their color from flavonoids, especially anthocyanins (Usenik et al. 2009).

Similar profiles were observed among the three populations, and there was no appreciable difference in the component concentrations between organic and aqueous extracts. However, more catechin chemicals were found in samples of tea grown in agroforests and other mixed-crop areas compared to forest samples (Ahmed et al. 2013). The various tea administration methods are responsible for these variations. A recent study found the phytochemical profile of both domesticated and wild *Amburana cereansis* comparable. Results from this study lend credence to using cultivated medicinal plants concept to produce herbal remedies (Canuto et al. 2012). It protects the native ecosystem and guarantees a steady flow of high-quality raw materials (Canuto et al. 2012).

While in Muguga, temperatures ranged from 9 to 18ºC, while in Kobujoi and Karuri, they ranged from 8.8 to 23.7ºC and 10 to 21ºC, respectively, during the time of bark harvest. The coldest location was Muguga, and the hottest was Kobujoi. Some microsites inside a forest may have significantly different temperature regimes than others (Longman and Jenik 1987). Chilling injury caused by these temperature variations may cause metabolic disruptions, toxin buildup, and plant membrane permeability increases (Gachie et al. 2012). In addition, different plants within the same population may produce different amounts and types of secondary metabolites due to intraspecific genetic variation (Kadu et al. 2012).

In conclusion, variation in tree height and DBH across trees growing in the same habitat suggests that shading periods impact the growth rate of *P. africana*. Even among

members of the same *P. africana* species growing in the same environment, there was a wide range of flowering times and hence, a wide range of fruiting times. Potentially age-related decreases in photosynthesis and increases in respiration demand explain the age-related deceleration in *P. africana's* growth rate. This study found that crude yields of bark extract from the *P. africana* species did not vary greatly with the habitat of the individual trees used for the extraction. The phytochemical profile of the methanol and aqueous extracts was identical to that of the hexane and DCM extracts. However, the quantities of the various phytochemicals differed across the solvents. High yields of the phytochemicals in *P. africana* bark require using all these solvents. The concentration of most compounds associated with BPH treatment does not significantly differ across *P. africana* trees sourced from the wild, domesticated stand, and on-farm remnant environments. In contrast, the concentration of other phytochemicals changed with habitat; therefore, whether the species is wild or cultivated when collecting bark for medicinal purposes is not very important with compounds associated with BPH treatment.

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Phytochemical research of the anticancer potential of *Aloe turkanensis*

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Abstract. *Adem FA, Yenessew A, Yusuf AO, Wanjohi JM. 2022. Phytochemical research of the anticancer potential of* Aloe turkanensis. *Asian J Nat Prod Biochem 20: 75-88.* The number of people diagnosed with cancer is rising worldwide, particularly in Sub-Saharan Africa. The search for new cancer treatments continues to benefit greatly from nature as a rich supply of promising chemicals. The anticancer effects of quinones have made them a popular medicinal class among natural chemicals (e.g., daunomycin and doxorubicin). *Aloe turkanensis* Christian, like other members of the genus *Aloe*, is a good place to get quinones. Dried and powdered *A. turkanensis* rhizomes and leaves were cold percolated in a mixture of dichloromethane and methanol (1:1). The crude extracts significantly decreased the viability of the human extrahepatic bile duct cancer cell line (TFK-1). Twelve chemicals were isolated from the crude extracts through chromatographic separations on silica gel, Sephadex LH-20, and preparative TLC. Spectroscopic techniques such as UV, ^{1}H, and ^{13}C NMR, COSY, NOESY, HMBC, and HSQC were used to determine the structures of the isolated compounds. Two naphthoquinones [3,5,8-trihydroxy-2-methylnaphthalene-1,4-dione (1) and 5,8-dihydroxy-3-methoxy-2-methylnaphthalene-1,4-dione (2)], seven anthraquinones [chrysophanol (3), aloesaponarin I (4), aloesaponarin II (5), laccaic acid D methyl ester (6) helminthosporin (8) aloe-emodin (10) and α-L-11-*O*-rhamnopyranosylaloe-emodin (11)], a pre-anthraquinone [aloesaponol I (7)] a pyrone derivative [feralolide (9)] and a benzoic acid derivative [3,4-dihydroxybenzoic acid (12)] were the chemicals that made up these substances. This study reported again that the *Aloe* genus produced the naphthoquinones [3,5,8-trihydroxy-2-methylnaphthalene-1,4-dione (1) and 5,8-dihydroxy-2-methoxy-2-methylnaphthalene-1,4-dione (2)]. In addition, this study made the first report of 3,5,8-trihydroxy-2-methylnaphthalene-1,4-dione (1) from the Asphodelaceae family. Human extrahepatic bile duct carcinoma (TFK-1) and liver cancer (HuH7) cell lines were used to test the extracted compounds for in-vitro anticancer activity. The anthraquinone aloe-emodin (10) and the naphthoquinone 5,8-dihydroxy-3-methoxy-2-methylnaphthalene-1,4-dione (2) showed significant inhibition against TFK-1 cell lines using the 3- [4,5-dimethylthiazol-2-yl]-2-,5-diphenyltetrazolium bromide (MTT) assay, with IC_{50} values of 6.0 and Aloesaponol I (7), a pre-anthraquinone, inhibited the development of TFK-1 cells with IC_{50} values of 10.0 µg/mL and HuH7 cells with 88.0 µg/mL. Both α-L-11-*O*- rhamnopyranosyl aloe-emodin (11) and aloesaponarin II (4), an anthraquinone, decreased TKF-1 cell line viability with IC_{50} values of 23.0 and 34.0 µg/mL, and HuH7 cell line viability with IC_{50} values of 47.0 and 55.0 µg/mL, respectively. IC_{50} values of 46.0 µg/mL for helminthosporin (8) showed considerable inhibition of TFK-1 cell growth, although it did not affect HuH7 cells at those concentrations. Extrahepatic bile duct (TFK-1) and liver (HuH7) cancer cell lines are sensitive to these chemicals identified here for the first time. Before these chemicals can be established as potentially effective anticancer medicines, more research on normal cell lines and their mechanism of action is required.

Keywords: *Aloe turkanensis,* anticancer potential, phytochemistry

INTRODUCTION

The human race has relied on natural remedies for thousands of years to cure and prevent disease. At least a thousand years of written evidence of ethnomedical science exist in countries like China (Chang and But 1986; Liu et al. 2014; Shen et al. 2014; Liu et al. 2015; Huang et al. 2016; Li and Xing 2016; Han et al. 2017; Gao et al. 2018; 2019) and India (Kapoor 1990; Baruah et al. 2013; Taram et al. 2020). Old plant cures are being used by many people today, especially in developing countries. Conventional medicines are either prohibitively expensive or in short supply in low-income nations. As a result, nearly 80% of the population in developing countries may use traditional medicine as their primary source of healthcare (WHO 2002).

Scientists have been refining the active ingredients responsible for curing many ailments based on ancient traditional procedures. *Artemisia annua*, from which the antimalarial ingredient artemisinin (13) was extracted, is an example of traditional Chinese medicine used to treat malaria (Dewick 2002). Moreover, thanks to developments in pharmacy and chemistry, aspirin (14), one of the first entirely synthetic medications, was created by acetylating salicylic acid (15), the pain-relieving active element in *Salix alba* (Samuelson 2004). Taxol (16), extracted from the Indian herb *Taxus brevifolia*, was the first substance developed for cancer chemotherapy. It has been demonstrated that this is useful for cancer therapy (Wani et al. 1971).

Cancer develops when the body's natural processes for dividing and eliminating cells from the population become unbalanced. The body's normal cells have a predictable life cycle of growth, division, and eventual death. Apoptosis refers to the processes of programmed cell death, the failure of which can lead to cancer development. Cancer cells, in contrast to healthy cells, do not die off naturally but keep dividing and expanding. As a result, it causes an

accumulation of aberrant cells that can spread to other body parts during their unchecked proliferation (Bright and Khar 1994). Cancer risk factors include illness, exposure to chemicals and radiation at work, and environmental variables, but one's personal lifestyle choices are by far the most influential (Buell and Dunn 1965; Kolonel and Wilkens 2006).

Natural chemicals with therapeutic potential are a constantly expanding field of scientific inquiry. The process of isolating and characterizing active ingredients from medicinal plants is ongoing. For example, Vinca alkaloids, such as vinblastin (17) and vincristine (18) from the Madagascar Catharanthus roseus (Apocynaceae), are effective cancer chemotherapeutic drugs (Stefania et al. 2009). Recently, a bi-cyclic peptide named romidepsin (19) was identified from the bacteria Chromo bacterium violaceum strain 968 and found to be cytotoxic against multiple human cancer cell lines. Currently, it is being tested in clinical studies for cancer treatment (Haigentz et al. 2012).

Among naturally occurring chemicals, those containing the quinone moiety are among the most well-known for their cancer-fighting effects. For example, streptomyces peucetius var. caesius was the source for daunomycin (20), and its derivative doxorubicin (21) is a quinone moiety drug used to treat various solid tumors and acute myeloid leukemia (Octavia et al. 2012).

The availability of molecules containing the quinone moiety suggests that the genus *Aloe*, which includes *Aloe turkanensis* Christian, may prospectively present in the search for new anticancer medicinal medicines.

Cancer is a leading cause of death worldwide, accounting for over 13% of all fatalities from the disease. An estimated 12.2 million people were diagnosed with cancer in 2008. In addition, there was an uptick in cancer incidence in developing nations (Ferlay et al. 2013). Furthermore, 80% of the world's population relies on medicinal plants whose safety and efficacy are not well recognized or documented and where cancer rates are predicted to rise by 2020 (Murray and López 1996). Therefore, research into new anticancer medications and plant preparations that are both effective and reasonably priced is essential.

MATERIALS AND METHODS

General

Instrumentation

The NMR spectra were acquired by the Department of Pharmaceutical Biology at Saarland University, Germany, using a Bruker Avance (500 MHz) spectrometer and a reference of residual solvent signals. Standard Bruker software was used to acquire data in the fields of homonuclear Correlation Spectroscopy (COSY), Heteronuclear Single Quantum Correlation (HSQC), and Heteronuclear Multiple Bond Correlation (HMBC) (Top spin 3.0 pl 3). In addition, Saarland University in Germany used TECAN sunrise software XFluor4 to measure the absorbance of a purple formazan solution in living cells at 550 nm and 690 nm. Furthermore, silica gel (70-23 mesh) and Sephadex LH-20 were employed in column chromatography (CC) for chemical purification. In addition, pre-coated silica gel 60 F254 plates are used in analytical thin-layer chromatography (Merck).

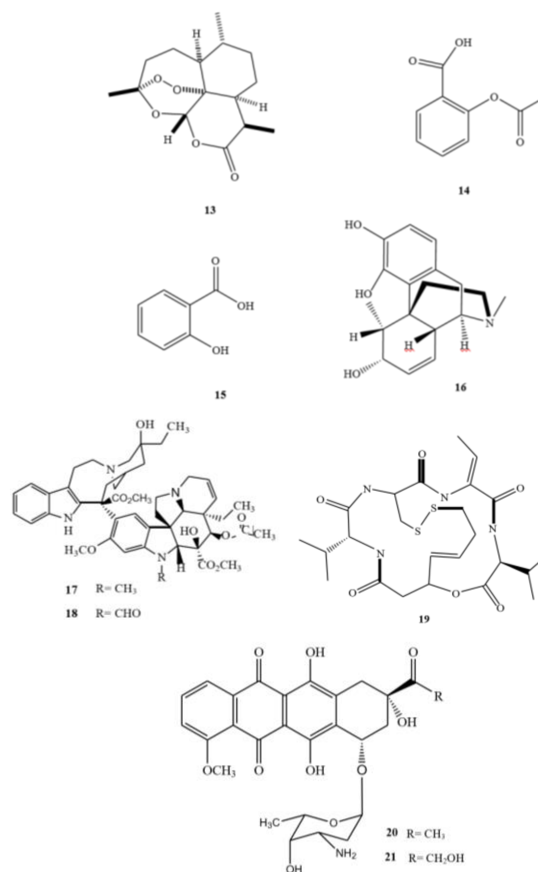
Plant material

Marigat in Indao, Kenya, provided the *A. turkanensis* rhizome and leaves for this study in June 2012. Mr. Simon Mathenge of the University of Nairobi's Botany Department School of Biological Science positively recognized this plant. Therefore, a voucher specimen was deposited with the deposit number FAA 2012/001.

Extraction and isolation

Extraction and isolation from rhizomes of Aloe turkanensis

The *A. turkanensis* rhizomes were sun-dried, then ground into a powder. Exhaustive cold percolation with a 1:1 mixture of CH_2Cl_2 and MeOH was used to extract 2 kilograms of powdered plant material. After combining the extract, it was filtered and concentrated under low pressure to obtain 30 g of crude extract. Ethyl acetate and water were used to separate the extract. A rotary evaporator concentrated the ethyl acetate layer into a crude extract weighing 20 grams. Column chromatography on oxalic acid deactivated silica gel (400 g) eluting with n-hexane containing increasing levels of ethyl acetate yielded 250 fractions of ca. 250 mL each from a 15 g sample of the ethyl acetate extract. Based on their TLC profiles, these were merged to form 21 different fractions.



Using CC on Sephadex LH-20 (eluting with CH2Cl2/MeOH, 1:1), fraction 2 (1% ethyl acetate in n-hexane) was further separated to provide 3,5,8-trihydroxy-2-methyl naphthalene1,4-dione (2, 4.0 mg) and 5,8-dihydroxy-3-methoxy-2-methylnaphthalene-1,4-dione (2, 4.0 mg) (1, 3.0 mg). Chrysophanol (3, 6 mg) was obtained by collecting fraction 3 (3% ethyl acetate in n-hexane) as a yellow solution, then concentrating the resulting yellow precipitate, filtering, and washing with n-hexane. By filtering and washing with n-hexane, fraction 6 (10% ethyl acetate in n-hexane) was converted to aloesaponarin I (4.25 mg). The filtrate from fraction 6 was purified using Sephadex LH-20 (eluted with CH2Cl2/MeOH; 1:1) to yield aloesaponarin II (5.20 mg). 6.4 milligrams of laccaic acid D-methyl ester were isolated from a brown solution of fraction 12 (30% ethyl acetate in n-hexane) using CC on Sephadex LH 20 (eluting with CH2Cl2/MeOH; 1:1). Aloesaponol I was isolated from fraction 16 (50% ethyl acetate in n-hexane), which precipitated as a colorless solid after being filtered and washed with an n-hexane/acetone combination (7.20 mg).

Extraction and isolation from the leaves of Aloe turkanensis

The *A. turkanensis* leaf powder (2 kg) was extracted and concentrated as described above to get 31 grams of crude extract. After separating the extract into ethyl acetate and water, 25 g was obtained after evaporating the organic solvent. On oxalic acid-impregnated silica gel (400 g) eluting with n-hexane containing increasing quantities of ethyl acetate, CC was performed on 20 g of the ethyl acetate extract. The collected 250 mL was divided into 20 equal parts. Further purification by CC over Sephadex LH-20 (eluent: CH_2Cl_2/MeOH; 1:1) yielded helminthosporin (8.3 mg) and 3,5,8-trihydroxy-2-methylnaphthalene1,4-dione (14 mg). Fraction 2 was eluted with 1% ethyl acetate in n-hexane (1, 2.5 mg). Chrysophanol was produced using a similar purification process using fraction 3 (3 percent ethyl acetate in *n*-hexane) (3, 5 mg). Purification of fraction 6 (7% ethyl acetate in *n*-hexane) using Sephadex LH-20 (CH_2Cl_2/MeOH; 1:1) yielded aloesaponarin I (4, 15 mg) and aloesaponarin II (11, 30 mg) as dark green solutions (5, 10 mg). Two blue fluorescence spots were observed in fraction 8 (15% ethyl acetate in *n*-hexane) and were resolved by CC over Sephadex LH-20 (elution: CH_2Cl_2/MeOH; 1:1), yielding feralolide (9, 15 mg) and 3,4-dihydroxybenzoic acid (12, 12 mg).

From fraction 13 (40% ethyl acetate in *n*-hexane) and fraction 16 (60% ethyl acetate in *n*-hexane), crystals of aloe-emodin (10, 10 mg) and *α* -L-11-*O*-rhamnosyl aloe-emodin (11, 8.5 mg) were produced.

Physical and spectroscopic properties of isolated compounds

3,5,8-Trihydroxy-2-methyl naphthalene-1,4-dione (1)

Red amorphous solid. UV λ_{max} 300, 420, 480 nm. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 12.81 (1H, *s*, 5-OH), 11.48 (1H, *s*, 8-OH), 7.29 (1H, *d*, *J* = 9.5 Hz, H-6), 7.18 (1H, *d*, *J* = 9.5 Hz, H-7), 2.11(3H, *s*, 2-CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 188.8 (C-1), 182.0 (C-4), 157.6 (C-5), 157.1 (C-8), 153.8 (C-3), 134.1 (C-6), 127.4 (C-7), 121.6 (C-2), 110.7 (C-5a), 110.1 (C-8a), 8.3 (2-CH_3).

5,8-Dihydroxy-3-methoxy-2-methyl naphthalene-1,4-dione (2)

Red amorphous solid. UV λ_{max} at 300, 480 nm. EIMS *m/z* $[M]^+$ 234.9. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 12.72 (1H, *s*, 8-OH), 12.32 (1H, *s*, 5-OH), 7.24 (1H, *d*, *J* = 10.0 Hz, H-7), 7.22 (1H, *d*, *J* = 10.0 Hz, H-6), 4.14 (1H, *s*, 3-OCH_3), 2.11 (3H, *s*, 2-CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 188.4 (C-1), 183.5 (C-4), 158.3 (C-3), 158.3 (C-5), 157.5 (C-8), 133.2 (C-2), 129.9 (C-7), 128.6 (C-6), 111.5 (C-8a), 111.1 (C-5a), 61.4 (OCH_3), 9.0 (CH_3).

Chrysophanol (3)

Orange needles. UV λ_{max} 300, 420 nm. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 12.11 (1H, *s*, H-8), 12.00 (1H, *s*, H-1), 7.82 (1H, *dd*, *J* = 1.0, 8.5 Hz, H-5), 7.68 (1H, *t*, *J* = 7.5 Hz, H-6), 7.64 (1H, *bs*, H-2), 7.29 (1H, *dd*, *J* = 1.0, 8.5 Hz, H-7), 7.09 (1H, *bs*, H-4), 2.46 (3H, *s*). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 192.5 (C-10), 181.9 (C-9), 162.7 (C-8), 162.4 (C-1), 149.3 (C-3), 136.9 (C-6), 133.6 (C-5a), 133.2 (C-4a), 124.5 (C-7), 124.3 (C-4), 121.3 (C-2), 119.9 (C-5), 115.8 (C-8a), 113.7 (C-1a), 22.2 (CH_3).

Aloesaponarin II (4)

Orange crystals. UV λ_{max} 260, 290, 400 nm. EIMS *m/z* $[M]^+$ 254.58. ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H 12.94 (1H, *s*, 1-OH), 7.68 (1H, *t*, *J* = 7.5 Hz, H-3), 7.58 (1H, *dd*, *J* = 7.5, 1.5 Hz, H-2), 7.41 (1H, *d*, *J* = 3.0 Hz, H-5), 7.29 (1H, *dd*, *J* = 8.5, 1.0 Hz, H-4), 6.99 (1H, *d*, *J* = 2.5 Hz, H-7), 2.66 (3-H, *s*, 8-CH_3). ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 189.2 (C-9), 182.1 (C-10), 162.2 (C-1), 161.4 (C-6), 145.2 (C-8), 136.7 (C-5a), 135.8 (C-3), 132.4 (C-4a), 116.3 (C-1a), 124.4 (C-7), 124.1 (C-2), 122.2 (C-8a), 118.1 (C-4), 111.9 (C-5), 23.5 (CH_3).

Aloesaponarin I (5)

Orange crystals. UV λ_{max} 260, 300, 410 nm.^{1}H NMR (acetone-d_6, 500 MHz): δ_H 12.87 (1H, *s*, 1-OH), 7.70 (1H, *dd*, *J* = 5.5, 2.5 Hz, H-4), 7.73 (1H, *s*, H-5), 7.68 (1H, *t*, *J* = 1.9, H-3), 7.29 (1H, *dd*, *J* = 7.5, 1.0 Hz, H-2), 3.95 (3H, *s*, OCH_3), 2.70 (3-H, *s*, 8-CH_3). ^{13}C NMR (acetone-d_6, 125 MHz): δ_C 190.4 (C-9), 182.3 (C-10), 167.9 (C=O), 162.9 (C-1), 159.5 (C-6), 142.9 (C-7), 136.9 (C-5), 133.8 (C-5a), 133.6 (C-4a), 130.8 (C-8), 125.2 (C-8a), 124.4 (C-2), 119.2 (C-3), 117.9 (C-1a), 113.0 (C-4), 52.7 (OCH_3), 20.3 (8-CH_3).

Laccaic acid D methyl ester (6)

Orange crystals. UV λ_{max} 300, 410 nm. EIMS *m/z* $[M]^+$ 328.71. ^{1}H NMR (acetone-d_6, 500 MHz): δ_H 13.16 (1H, *s*, 1-OH), 7.72 (1H, *s*, H-5), 7.18 (1H, *d*, *J* = 2.5 Hz, H-4), 6.65 (1H, *d*, *J* = 2.5 Hz,H-2), 3.93 (3H, *s*, OCH_3), 2.70 (3-H, s). ^{13}C NMR (acetone-d$_6$, 125 MHz): δ_C 189.3 (C-9), 182.7 (C-10), 168.1 (C=O), 166.2 (C-1), 165.1 (C-3), 159.3 (C-6), 142.3 (C-7), 138.0 (C-5a), 135.5 (C-4a), 130.9 (C-8), 109.2 (C-2), 108.1 (C-4), 113.1 (C-5), 111.7 (C-1a), 52.6 (OCH_3), 20.3 (CH_3).

Aloesaponol I (7)

Colorless solid. The TLC showed blue fluorescence under UV light (366 nm). UV λ_{max} 300, 380 nm. EIMS *m/z* $[M]^+$ 316.90. ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H 15.27 (1H, *s*, 9-OH), 6.95 (1H, *s*, H-5), 6.92 (1H, *s*, H-10), 4.24 (1H, *m*, H-3), 3.83 (3H, *s*, OCH_3), 3.14 (1H, *dd*, *J* = 3.3,15.8 Hz, H-4), 2.96 (1H, *dd*, *J* = 3.3, 17.1 Hz, H-2), 2.90 (1H, *dd*, *J* = 6.8, 15.6 Hz, H-4), 2.70 (1H, *dd*, *J* = 1.8, 5.4 Hz, H-2), 2.70 (3H, *s*). ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 203.7 (C-1), 168.2 (C=O), 165.9 (C-9), 140.8 (C-7), 137.2 (C-8), 136.6 (9a), 155.1(C-6), 125.4 (C-8a), 116.6 (C-5), 107.5 (C-10), 110.2 (C-1 0a), 64.4 (C-3), 52.1 (OCH_3), 46.4 (C-4), 37.5 (C-2), 20.8 (CH_3).

Helminthosporin (8)

Red needle. UV λ_{max} at 500, 580 nm. ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H 12.83 (1H, *s*, 5-OH), 12.03 (2H, *s*, 1-OH and 8-OH), 7.65 (1H, *brs*, H-4), 7.44 (1H, *d*, H-7), 7.44 (1H, *d,* H-6), 7.26 (1H, *brs*, H-2), 2.50 (3H, *s*,3-CH_3). ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 189.9 (C-9), 186.3 (C-10), 161.7 (C-1), 157.1 (C-5), 156.4 (C-8), 149.1 (C-3), 132.8 (C-4a), 129.6 (C-7), 129.4 (C-6), 113.8 (C-1a), 124.3 (C-2), 120.2 (C-4), 112.6 (C-8a), 112.5 (C-5a), 22.1 (CH_3).

Feralolide (9)

Brown solid. UV λ_{max} 310 nm. EIMS *m/z* $[M]^+$ 344.75. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 11.21 (1H, *s*, OH-8), 6.42 (1H, *d*, *J* = 1.9 Hz, H-7'), 6.37 (1H, *d*, *J* = 2.5 Hz, H-5'), 6.31 (1H, *d*, *J* = 1.5, H-5), 6.26 (1H, *d, J* = 1.9, H-7), 4.80 (1H, *m*, H-3), 3.08 (1H, *dd*, *J* = 5.5,14Hz, H-1'), 3.22 (1H, *dd*, *J* = 6.9,13.5 H-1‘), 2.94 (1H, *dd*, H-4), 2.92 (1H, *dd*, H-4), 2.57 (1H, *s*, CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 203.9 ($COCH_3$), 170.3 (C-1), 165.2 (C-8), 165.0 (C-6), 160.3 (C-6'), 160.1 (C-4'), 142.9 (C-4a), 139.4 (C-2'),121.0 (C-3'), 111.7 (C-7'), 107.6 (C-5), 102.5 (C-5'), 101.9 (C-7), 101.8 (C-8a), 80.5 (C-3), 39.5 (C-1'), 33.1 (CH_3), 32.8 (C-4).

Aloe-emodin (10)

Orange crystals. UV λ_{max} at 260, 300, 420 nm. ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H 11.93 (2H, *s*, 1-OH, 8-OH), 7.81 (1H, *t*, *J* = 8.0 Hz, H-6), 7.71 (1H, *dd*, *J* = 7.5, 1.1 Hz, H-5), 7.68 (1H, *d*, *J* =1.6 Hz, H-4), 7.38 (1H, *dd*, *J* = 9.0, 1.1 Hz, H-7), 7.28 (1H, *d*, *J* = 1.6 Hz, H-2), 4.62 (2H, *s*,H-11). ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 191.6 (C-9), 181.4 (C-10), 161.6 (C-1), 161.3 (C-8), 153.7 (C-3), 137.3 (C-6), 133.3 (C-4a), 133.1 (C-5a), 124.4 (C-7), 120.6 (C-2), 119.3 (C-5), 115.9 (C-8a), 114.4 (C-1a), 107.1 (C-4), 62.07 (C-11).

α-L-11-O-Rhamnopyranosyl aloe-emodin (11)

Orange crystals. UV λ_{max} 260, 300, 430 nm. EIMS *m/z* $[M]^+$ 416. ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H 7.82 (1H, *st*, *J* = 8.4 Hz, H-6), 7.73 (1H, *dd*, *J* = 7.5, 1.1 Hz, H-7), 7.68 (1H, *d*, *J* =1.6 Hz, H-4), 7.41 (1H, *dd*, *J* = 8.4, 1.1 Hz, H-5), 7.32 (1H, *d*, *J* = 1.6 Hz, H-2), 4.75 (1H, *d*, *J* = 14.0 Hz, H-11), 4.71 (1H, *d*, *J* = 1.6 Hz, H-1'), 4.61 (1H, *d*, *J* = 13.9 Hz, H-11), 3.73 (2H, *dd*, *J* = 3.4, 1.7 Hz, H-2'), 3.62-3.28 (33H, *m*, H-3'), 3.23 (2H, *s*, H-4'), 1.16 (3H, *d*, *J* = 6.2 Hz, CH_3). ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 191.6 (C-9), 181.4 (C-10), 161.4 (C-1), 161.3 (C-8), 148.8 (C-3), 137.4 (C-6), 133.4 (C-4a), 133.3 (C-5a), 124.4 (C-7), 121.8 (C-2), 119.3 (C-5), 117.7 (C-4), 115.9 (C-8a), 115.3 (C-1a), 99.9 (C-1'), 71.8 (C-4'), 70.4 (C-2'), 69.0 (C-3'), 66.9 (C-11), 70.7 (C-5'), 17.9 (CH_3).

3,4-Dihydroxybenzoic acid (12)

Brown solid. ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 7.53 (1H, *d*, *J* = 2.0 Hz, H-2), 7.47 (1H, *dd*, *J* = 6.5, 2.0 Hz, H-6), 6.89 (1H, *d*, *J* = 4.9 Hz, H-5). ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 168.7 (C=O), 167.5 (C-3), 150.6 (C-4), 146.2 (C-1), 123.6 (C-6), 117.4 (C-2), 115.6 (C-5).

Biological tests

In vitro anticancer activities

The cytotoxicity test was conducted in Germany at Saarland University's Department of Pharmaceutical Biology using the colorimetric assay, also known as the MTT assay, per the methods described by Heo et al. (1990).

Cell culture

DMEM (#42460-025, Gibco, Germany) supplemented with 20% Fetal Calf Serum (FCS) and 1% penicillin/streptomycin (10.000 units/mL/10mg/mL) was used to cultivate the human extrahepatic bile duct cancer cell line TFK-1. In addition, cultures of the human hepatocellular carcinoma cell line HuH7 were maintained in RPMI 1640 (#R8757, Sigma, Germany) supplemented with 20% fetal calf serum (FCS) and 1% penicillin/streptomycin (10.000 units/mL/10mg/mL). The cell line was grown in a humidified incubator at 37 ° C with 5% carbon dioxide (Heo et al. 1990).

MTT assay

The cells were tallied, and then 1 x 104 cells were plated into each well of a 96-well plate. Both cancer cell lines were exposed to isolated chemicals dissolved in DMSO and diluted with culture media (1-100 g/mL) for 48 hours. First, cell proliferation was measured using 3-[4, 5-dimethylthiazol-2- yl]-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL) in Phosphate-Buffered Saline (PBS) after incubation. Next, MTT solution (150 L/well) was added after incubating for an hour, and the incubation media containing the MTT was aspirated to dissolve the formazan crystals. Then 80 L of DMSO was added to each well. The formazan concentration was determined with the help of TECAN's dawn software XFluor4, using 550 and 690 nm as reference wavelengths (Figure 1).

Statistical analysis

Microsoft Office Excel was used for all data analysis. The data were shown as means ± SEM. The t-test for independent samples, two-sample-size-estimation, was used to calculate the differences' significance. The significance level for a difference was set at a p-value of less than 0.05 (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

RESULTS AND DISCUSSION

Preliminary test

The cell viability of the TFK-1 cell line was significantly decreased in preliminary tests using a crude

extract from the rhizomes and leaves of *A. turkanensis*. In addition, TLC detected colored spots that absorb UV light in the crude extracts (254 and 366 nm). Scientists believe these to be quinone derivatives as the yellow dots became crimson when exposed to ammonia vapor. Chromatographic separation was used to obtain the chemicals. Finally, the extracted compounds were put through anticancer testing using HuH7 hepatoma carcinoma and TFK-1 extrahepatic bile duct carcinoma cell lines. Compounds identified from A. turkanensis rhizomes and leaves are discussed, along with their pharmacological profiles and cancer-fighting properties below.

Characterization of compounds from the rhizomes of *Aloe turkanensis*

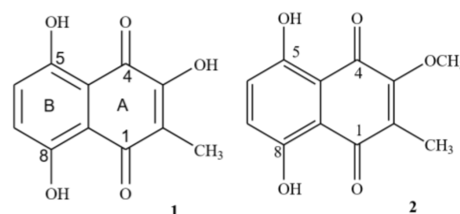
Following a cold percolation extraction with dichloromethane/ methanol (1:1), the rhizomes of A. turkanensis were partitioned between ethyl acetate and water. Two naphthoquinones, four anthraquinones, a pre-anthraquinone, a chromone derivative, and a benzoic acid derivative were separated chromatographically from the ethyl acetate extract. As will be seen in a moment, spectroscopic methods were used to determine the precise structures of the compounds.

3,5,8-Trihydroxy-2-methylnaphthalene-1,4-dione (1)

Isolated as a red amorphous solid, compound 1 possesses the UV spectral features typical of 3,5,8-trihydroxy substituted naphthoquinones (λ_{max} 300, 420, 480 nm) (Bringmann et al. 2011). The ^{13}C NMR spectra corroborated this finding by revealing three oxygenated carbon atoms at δ_C 153.8, 157.1, and 157.6 for C-3, C-8, and C-5, respectively.

Eleven carbon signals were found in the ^{13}C NMR spectrum (Table 1), both indicative of carbonyl (at δ_C 182.0 and 188.8, respectively, for C-4 and C-1). NMR spectra further revealed that the naphthoquinone structure contained a methyl carbon at δ_C 8.3 (δ_H 2.11) and two chelated hydroxyl substituents at δ_H 11.48 and 12.81. Additionally, two ortho-coupled aromatic protons were detected in ring B of 1's ^1H NMR spectra (Table 1), which are replaced at C-5 and C-8 with hydroxyl groups. The ^1H NMR spectra (Table 1) of 1 also showed the existence of two *ortho*-coupled aromatic protons in ring B, which are exchanged at C-5 and C-8 with hydroxyl groups [at δ_H 7.18 (J = 9.5 Hz, δ_C 127.4) and 7. 29 (J = 9.5 Hz, δ_C 131.4].

The aromatic proton at δ_H 7.18 in the HMBC spectrum was assigned to H-7 due to its Correlation with C-8 and C-8a, and the signal at δ_H 7.29 was assigned to H-6 due to its Correlation with C-5 and C-5a. The HMBC Correlation with C-1, C-2, and C-3 supports the position of the C-2 methyl group at δ_H 2.11.1. Therefore, the structure of 1 was identified as 3,5,8-trihydroxy-2-methyl-1,4-naphthoquinone, also known as 8- hydroxydroserone, a somewhat unimaginative term. It is the first report of compound 1 from the family Asphodelaceae, which has before been isolated from Droseraceae and Nepenthaceae families (Macbeth and Winzor 1935); it was also reported from lyophilized cell culture of *Triphyophyllum peltatum* (Bringmann et al. 2011).



5, 8-Dihydroxy-3-methoxy-2-methylnaphthalene-1,4-dione (2)

EIMS showed a molecular ion [M]+ peak at m/z 234.91, which corresponds to the molecular formula of $C_{12}H_{10}O_5$; this compound was separated as a red amorphous solid. The ultraviolet (UV) spectrum (maximum wavelengths of 300 and 480 nm) is indicative of a 1,4-naphthoquinone structure (Bringmann et al. 2008). The ^{13}C NMR spectra of 1,4-naphthoquinone matched these measurements, revealing two carbonyl signals at δ_C 183.5 and 188.4 for carbon atoms C-4 and C-1, respectively. It appears that 2 is a methyl ether derivative of 1 based on its ^1H and ^{13}C NMR spectra (Table 1), which are nearly identical to those of 1, except for the presence of a methoxy group at C-2 (δ_H 4.14, δ_C 61.4). As a result, twelve carbon signals, including methyl carbon at δ_C 9.01 (δ_H 2.11), were observed in the ^{13}C NMR spectrum (Table 1). There were also two chelated hydroxyl proton signals in the ^1H NMR spectra, at δ_H 12.32 and δ_H 12.72 for 5-OH and 8-OH, respectively. Assigned proton energies of δ_H 7.24 (δ_C 129.9) and δ_H 7.22 (δ_C 128.6) (J = 10.0 Hz) for the aromatic protons H-6 and H-7 in ring B, respectively. HMBC spectrum corroborated the substitution pattern in ring B (Table 1).

Accordingly, the chemical was 5,8-dihydroxy-3-methoxy-2-methylnaphthalene-1,4-dione (2), a recently described novel compound from the roots of *A. secundiflora* (Induli et al. 2012). On the contrary, it is the second instance of compound 2 found in *Aloe*.

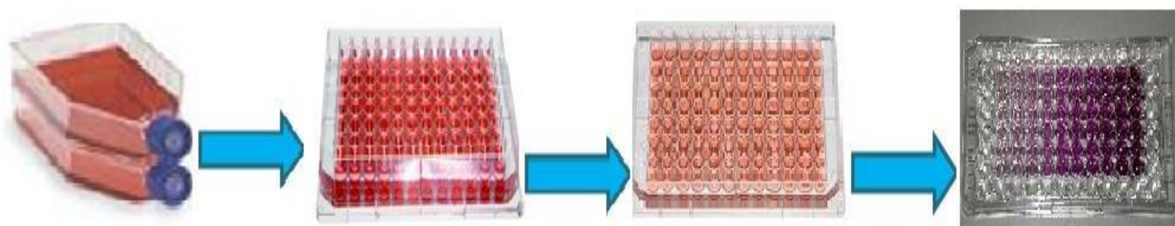


Figure 1. Picture of MTT assay

Chrysophanol (3)

Isolated in the form of orange needles, compound 3 exhibits the signature UV absorption of 1,8-dihydroxyanthraquinones at λ_{max} 300 and 420 nm (Dagne et al. 1994). Furthermore, two chelated hydroxyl protons, at δ_H 12.00 and 12.11 for 1-OH and 8-OH, were found in this molecule. In addition, one methyl, five methines, nine quaternary carbons (two of which are oxygenated, at (δ_C 162.4 and 162.7), and two carbonyls (δ_C 181.9 and 192.5) were detected in the ^{13}C NMR spectrum (Table 2).

On the ^{1}H NMR spectrum, the biogenetically predicted methyl group was located at C-3 (δ_H 2.46, δ_C 22.6). In addition, there were two broad singlet aromatic protons at δ_H 7.64 and 7.09, both ascribed to H-2 and H-4 of ring A. Protons in the ring C show an AMX pattern at 7.82 (1H, *dd*, J = 1.0, 8.5 Hz, H-5), 7.68 (1H, *t*, J = 8.5 Hz, H-6), and 7.29 (1H, *dd*, J = 1.0, 8.5 Hz, H-7).

Therefore, 1,8-hydroxy-3-methylanthraquinone (commonly known as chrysophanol) was determined to be the correct name for this substance (3). In addition, there is evidence that additional genera of the Asphodelaceae family also contain compound 3, which was originally isolated from Aloe roots (Yenesew et al. 1988).

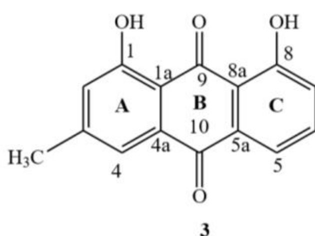


Table 1. ^{1}H (500 MHz), ^{13}C (125 MHz), and HMBC spectral data of compounds 1 and 2 ($CDCl_3$)

Carbon no.	Compound 1			Compound 2		
	^{1}H δ_H (*m*, *J* in Hz)	**^{13}C**	**HMBC**	**^{1}H δ_H (*m*, *J* in Hz)**	**^{13}C**	**HMBC**
1	-	188.8		-	188.4	
2	-	121.6		-	133.2	
3	-	153.8		-	158.3	
4	-	182.0		-	183.5	
5	-	157.6		-	158.3	
5a	-	110.7		-	111.5	
6	7.29 (*d*, 9.5)	127.4	C-5, C-5a	7.22 (*d*,10.0)	128.6	C-5, C-5a, C-8
7	7.18 (*d*, 9.5)	131.4	C-8, C-8a	7.24 (*d*,10.0)	129.9	C-8, C-8a, C-5
8	-	157.1		-	157.5	
8a	-	110.1		-	111.1	
2-CH_3	2.11 (*s*)	8.2	C-1, C-2, C-3	2.11 (*s*)	9.0	C-1, C-2, C-3
3-OCH_3				4.14 (*s*)	61.4	C-3
5-OH	12.81(*s*)	-	C-5, C-5a	12.32 (*s*)	-	C-5a, C-6, C-5
8-OH	11.48 (*s*)	-	C-8, C-8a	12.72 (*s*)	-	C-8a, C-7, C-8

Table 2. ^{1}H (500 MHz) and ^{13}C (125 MHz) spectral data of compounds 3 and 4 (DMSO-d_6)

Carbon no.	Compound 3		Compound 4	
	^{1}H δ_H (*m*, *J* in Hz)	**13C**	**^{1}H δ_H (*m*, *J* in Hz)**	**^{13}C**
1	-	162.4	-	162.2
1a	-	113.7	-	116.3
2	7.64 (*bs*)	121.3	7.29 (*dd*, J = 8.5, 1.0 Hz)	124.1
3	-	149.3	7.68 (*t*, J = 7.5 Hz)	136.7
4	7.09 (*bs*)	124.3	7.58 (*dd*, J =7.5, 1.5 Hz)	118.1
4a	-	133.2	-	132.4
5	7.82 (*dd*, J = 8.5, 1.0 Hz)	119.9	7.41 (*d*, J = 3.0 Hz)	111.9
5a	-	133.6	-	136.7
6	7.68 (*dd*, J = 7.5, 1.5 Hz)	136.9	-	161.4
7	7.29 (*dd*, J = 7.5, 1.5 Hz)	124.5	6.99 (*d*, J = 2.5 Hz	124.4
8	-	162.7	-	145.2
8a	-	115.8	-	122.6
9	-	181.9	-	189.2
10	-	192.5	-	182.1
1-OH	12.00 (*s*)	-	12.94 (*s*)	-
8-CH_3	2.46 (s)	22.6	2.66 (*s*)	23.5

Aloesaponarin II (4)

Isolated in an orange crystal, compound 4 has UV absorption at λ_{max} 260, 290, and 400 nm, the characteristic of anthraquinones (Yagi et al. 1974). The molecular formula ($C_{15}H_{10}O_4$) equated to an ESI [M]+ value of 254.58. Fifteen carbon signals, including two carbonyl signals (at δ_C 189.2 and 182.1, designated for C-9 and C-10, respectively, of an anthraquinone), two oxygenated aromatic carbon signals (at δ_C 162.2 and 161.4), and a methyl signal at δ_C 23.5, were seen in the ^{13}C NMR spectrum (Table 2). This molecule is thought to be isomeric with 3 according to its ^{1}H NMR spectra, which displays only one chelated hydroxyl proton signal at δ_H 12.94 and a down-field shifted methyl proton at δ_H 2.66.

Protons H-7 and H-5 of ring C are *meta*-coupled, with their corresponding H values being 7.41 (1H, *d*, J = 3.0 Hz) and 6.99 (1H, *d*, J = 2.5 Hz). In addition, the ^{1}H NMR demonstrated the presence of an AMX spin system with three aromatic protons resonating at δ_H 7.58 (1H, *dd*, J = 7.5, 1.5 Hz), δ_H 7.68 (1H, *t*, J = 7.5 Hz), and δ_H 7.29 (1H, *dd*, J = 8.5, 1.0 Hz) for H-4, H-3, and H-2 of ring A, respectively. Since this component was previously only found in *A. saponaria* (Yagi et al. 1974), it was given the name aloesaponarin II (4). (Yenesew et al. 1993; Dagne et al. 1994). It has also been stated that compound 4 is a metabolite produced by bacteria (Cui et al. 2006; Bartel et al. 1990; Fotso et al. 2003).

Aloesaponarin I (5)

The UV spectra of compound 5, isolated as an orange crystal, showed absorption at λmax 260, 300, and 410 nm, which is suggestive of an anthraquinone chromophore, similar to compound 4. Table 3 displays the ^{1}H and ^{13}C NMR spectra of compound 5, which are very similar to those of 4, with the addition of signals due to a methyl ester group (δ_H 3.95, δ_C 52.7 and δ_C 167.9) at C-7. Since there is only one aromatic singlet in the ^{1}H NMR spectrum of 5, it is determined to be at δ_H 7.73 and is ascribed to the H-5 on ring C. Ring A has three aromatic protons with identical H

values to ring 4; these protons are positioned at δ_H 7.70 (*dd*, *J* = 5.5, 2.5 Hz for H-4), 7.68 (*t*, *J* = 2.0 Hz, H-3), 7.29 (*dd*, *J* = 7.5, 1.0 Hz, H-2). There were 17 signals in the ^{13}C NMR spectra (Table 3). It has been determined through analysis that this substance is 1,6-dihydroxy-8-methylanthraquinone-7-carboxy methyl ester (trivial name aloesaponarin I). It has been observed in several species of *Aloe*, including *A. graminicola* (Yenesew et al. 1993; Dagne et al. 1994).

Laccaic acid D methyl ester (6)

Orange crystals of compound 6 were isolated, and its chemical formula, $C_{17}H_{12}O_7$, was determined by mass spectrometry (MS) from the presence of a molecular ion peak at *m/z* 328.71. The UV absorption spectra of compound 6 were compatible with a 9,10-anthraquinone chromophore at λ_{max} 300, 410 nm. Similarities between 5 and 6 were identified in ^{1}H and ^{13}C NMR (Tables 3 and 4), except that 6 has an extra hydroxyl group at C-3 (δ_C 165.1). In addition, two *meta*-coupled aromatic protons, at δ_H 6.65 (*d*, *J* = 2.5 Hz, H-2), 7.18 (*d*, *J* = 2.5 Hz, H-4), and a singlet aromatic proton, at 7.72 (*d*, *J* = 2.5 Hz, H-3), were detected in ring A of 6 by ^{1}H NMR spectroscopy (H-5). NMR spectra established the existence of a methyl ester (at C-6), methyl (at C-8), and three hydroxyl substituents (at C-1, C-3, and C-6) (Tables 3 and 4). Thus, laccaic acid D methyl ester (6) was determined to be the component in question; this chemical was previously found in some *Aloe* species (Yagi et al. 1974; Dagne et al. 1992; van Wyk et al. 1995).

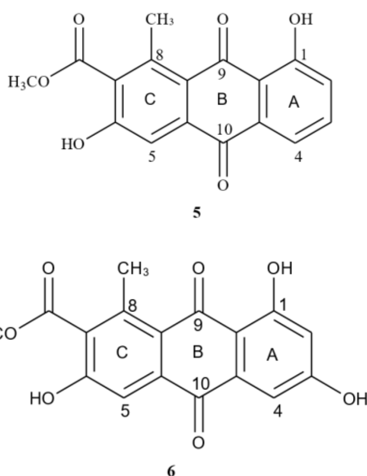


Table 3. ^{1}H (500 MHz, acetone-d_6) spectral data of compounds 5 and 6

Carbon no.	Compound ^{1}H δ_H (*m*, *J* in Hz)	
	5	6
2	7.29 (*dd*, 7.5, 1.0)	6.65 (*d*, 2.5)
3	7.68 (*t*, 1.9)	-
4	7.70 (*dd*, 5.5, 2.5)	7.18 (*d*, 2.5)
5	7.73 (*s*)	7.72 (*s*)
1-OH	12.8 (*s*)	13.16 (*s*)
8-CH3	2.70 (*s*)	2.70 (*s*)
CO-OCH3	3.95 (*s*)	3.93 (*s*)

Table 4. ^{13}C (125 MHz, acetone-d_6) spectral data of compounds 5 and 6

Carbon no.	δ_C	
	5	6
1	162.9	166.2
1a	125.3	111.7
2	124.4	109.2
3	119.2	165.1
4	113.0	108.1
4a	133.6	135.5
5	136.9	113.1
5a	133.8	138.0
6	159.5	159.3
7	142.9	142.3
8	130.8	130.9
8a	125.2	124.2
9	190.4	189.3
10	182.3	182.7
*C*O-OCH3	167.9	168.1
CO-O*C*H3	52.7	52.6
CH3	20.3	20.3

Aloesaponol I (7)

The solid form of Compound 7 was developed, and it fluoresced blue when exposed to ultraviolet light (366 nm). Pre-anthraquinone chromophores, like the one displayed by this chemical, often absorb UV light at λ_{max} 300, 380 nm (Yagi et al. 1974). Peaking at *m/z* 316.90, ESIMS detected a molecular ion indicative of the formula $C_{17}H_{15}O_6$.

Table 6 shows that the ^{1}H and ^{13}C NMR spectra of 7 of the samples were consistent with a pre-anthraquinone skeleton. A deshielded methyl group (at δ_H 2.70) was located at C-8 in the ^{1}H NMR spectrum, in addition to two singlet aromatic proton signals (at δ_H 6.95 and 6.92, respectively, for H- 5 and H-10). In addition, a methyl ester at position 3.83 (C = 52.1) and a strongly chelated hydroxyl signal at position 15.27 (^{1}H NMR) indicate that this molecule is a precursor to aloesaponarin I. The aliphatic signals in compound 7 included a multiplet for oxymethine (δ_H 4.24) at C-3 and two methylene groups [δ_H 3.14 (*dd*, *J* = 3.3, 15.8 Hz); 2.96 (*dd*, *J* = 3.3, 17.1 Hz)]; and [2.90 (*dd*, *J* = 6.8, 15.6 Hz); 2.50 (*dd*, *J* = 1.8, 5.4 Hz)], corresponding to CH_2-2 and CH_2-4.

The ^{13}C NMR spectrum (Table 5) uncovered the presence of an oxymethine carbon at a position of δ_C 64.4 (C- 3), two oxygenated sp2 hybridized carbon atoms at δ_C 155.1 for C-9 and δ_C 165.9 for C-6, and a carbonyl signal at a position of δ_C 203.7 (C-1). In addition, an ester group was detected in the ^{13}C NMR spectra (carbonyl at δ_C 168.2, methoxy at δ_C 52.1). Further, an ester group was detected in the ^{13}C NMR spectra (carbonyl at δ_C 168.2 and methoxy at δ_C 52.1). Next, this logic compound 7 was found to be a methyl ester of 3,6,9-trihydroxy-8-methyl-1-oxo-5,6,7,8-tetrahydroanthracene-2-carboxylic acid (trivial name aloesaponol I). The precise structure of this molecule at carbon position 3 is not resolved here. On the other hand, an (R) configuration at C-3 has been described (Dagne et al. 1992; Yenesew et al. 1993). The chemical was initially isolated from the underground stem of *Aloe saponaria* (Yagi et al. 1974) and subsequently isolated from other species of *Aloe* (van Wyk et al. 1995; Dagne et al. 1994).

Characterization of compounds from the Leaves of *Aloe turkanensis*

Extractions with dichloromethane/methanol (1:1) were performed using cold percolation on air-dried, powdered leaves of A. turkanensis. First, the crude extract was separated into ethyl acetate and water. Following silica gel column chromatography of the ethyl acetate layer, a naphthoquinone, five anthraquinones, a pyrone derivative, and a benzoic acid derivative were isolated. Next, roots and leaves of A. turkanensis were used to isolate compounds 1, 4, and 5, with structural elucidation reported in section 4.2. Following is a discussion of the characterization of five more compounds that could only be extracted from the leaves.

Helminthosporin (8)

The red solid compound 8 was isolated, and its UV absorption maxima were observed at 230, 250, 500, and 580 nm, all the characteristics of 1,5,8-trihydroxyanthraquinone (Yagi et al. 1977). Table 6 of the ^{1}H NMR spectra confirm this to be the case by revealing the presence of three chelated hydroxyl protons at δ_H 12.03, 12.03, and 12.83 for 1-OH, 5-OH, and 8-OH, respectively. In addition, there were fifteen carbon signals detected in the ^{13}C NMR spectrum (Table 6), two of which correspond to carbonyl groups (at δ_C 189.9 and δ_C 186.3) and one to a methyl group (at δ_C 22.1; δ_H 2.50). In addition, two broad singlet aromatic protons, corresponding to H-2 and H-4 of ring A, were observed at δ_H 7.26 (δ_C 124.3) and δ_H 7.65 (δ_C 120.2) in the ^{1}H NMR spectrum (Table 6) of compound 8. A singlet integration of two protons at δ_H 7.44 was subsequently assigned to H-6 and H-7 of ring C. Compound 8 was thus found to be 1,5,8-trihydroxy-3-methyl-9,10- anthraquinone (trivial name helminthosporin). Some species of Aloe (Yagi et al. 1977; Yenesew et al. 1993; Dagne et al. 1994) and the plants *Drechslera holmii* and *Drechslera ravenelii* have been studied for the presence of this compound (van Eijk and Roeymans 1981).

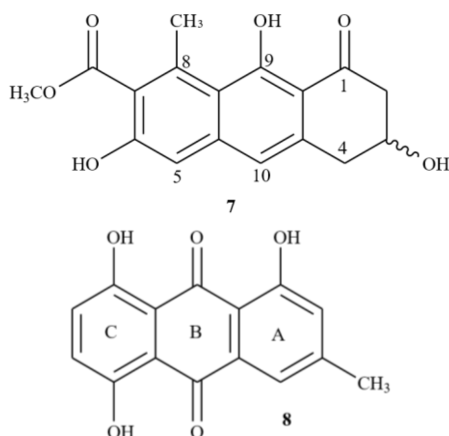


Table 5. ^{1}H (500 MHz) ^{13}C (125 MHz) data of compound **7** (DMSO-d$_6$)

Carbon no.	δ_H	δ_C
1	-	203.7
CH$_2$-2	2.70 (*dd*, *J* = 1.8, 5.4 Hz) 2.96 (*dd*, *J* = 3.3, 17.1 Hz)	46.4
3	4.24 (*m*)	64.4
CH$_2$-4	2.90 (*dd*, *J* = 6.8, 15.6 Hz) 3.14(*dd*, *J* = 3.3,15.8 Hz)	37.5
5	6.95 (*s*)	116.6
5a	-	-
6	-	155.1
7	-	140.8
8	-	137.2
8a	-	125.4
9	-	165.9
9a	-	136.6
10	6.92 (*s*)	107.5
10a	-	110.2
OCH$_3$	3.83 (*s*)	52.1
COOCH$_3$	-	168.2
CH$_3$	2.70 (*s*)	20.8
9-OH	15.27 (*s*)	-

Table 6. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of compound **8** (DMSO-*d*$_6$)

Carbon no.	^{1}H δ_H (*m*)	^{13}C
1	-	161.7
1a	-	113.8
2	7.26 (*brs*)	124.3
3	-	149.1
4	7.65 (*brs*)	120.2
4a	-	132.8
5	-	157.1
5a	-	112.5
6	7.44 (*brs*)	129.4
7	7.44 (*brs*)	129.4
8	-	156.4
8a	-	112.6
9	-	189.9
10	-	186.3
CH$_3$	2.50 (s)	22.1
OH	12.83	-
OH	12.83	-
OH	12.03	-

Table 7. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data of compound **9** (acetone-*d*$_6$)

Carbon No.	^{1}H δ_H	^{13}C δC
1	-	170.3
3	4.80 (*m*)	80.5
4	-	32.8
CH$_2$-4	2.92 (*dd*, *J* = 6.9 Hz) 2.94 (*dd*, *J* = 6.9 Hz)	32.8
4a	-	142.9
5	6.31 (*d*, *J* = 1.5 Hz)	107.6
6	-	165.0
7	6.26 (*d*, *J* = 1.9 Hz)	101.9
8	-	165.2
8a	-	101.8
1'	-	39.5
CH$_2$-1'	3.08 (*dd*, *J* = 5.5, 14 Hz) 3.22 (dd, *J* = 6.9, 13.5 Hz)	39.5
2'	-	139.4
3'	-	121.0
4'	-	160.1
5'	6.37 (*d*, *J* = 2.5 Hz)	102.5
6'	-	160.3
7'	6.42(*d*, *J* = 1.9 Hz)	111.7
*C*OCH$_3$	-	203.9
CH$_3$	2.57 (*s*)	33.1
8-OH	11.21 (*s*)	-

Feralolide (9)

The compound obtained, designated 9 in this study, is a brown solid with blue fluorescence when exposed to ultraviolet light (366 nm). The maximum UV absorption of this chemical occurs at 310 nm. [M]+ was detected at *m/z* 344.75 by ESIMS, which corresponds to a chemical formula of $C_{18}H_{16}O_7$. Based on a comparison of spectroscopic data to published records, the compound was determined to be feralolide (9) (Speranza et al. 1993, Abd-Alla et al. 2009; Elhassan et al. 2012).

Two aromatic rings, each with a pair of *meta*-coupled protons (δ_H 6.31, 6.26 for H-5, H-7, and (δ_H 6.37, 6.42 for H- 5', H-7'), were detected in the ^{1}H NMR spectrum of 9 (Table 7). Additionally, at δ_H 11.21 (8-OH), a chelated hydroxyl proton was seen in ^{1}H NMR. The ^{13}C NMR spectrum showed that oxymethine carbon (δ_C 80.5, C-3) exists, and its corresponding proton appears as a multi-plate at δ_H 4.80. Additionally, due to CH_2-4 and CH_2-1' were seen in the ^{1}H NMR spectrum. In contrast, the 13C NMR spectra displayed two methylene carbon atoms (δ_C 32.8 and 39.5) with corresponding proton signals at δ_H 2.94, 2.92, and 3.22, 3.08. Additionally, a carbonyl signal for lactones (δ_C 170.3, C-1) and a carbonyl signal for ketones (δ_C 203.9) were seen in the ^{13}C NMR spectra. It led to the determination that the compound was feralolide (9). However, it does not settle what precisely the structure of C-3 is. Nonetheless, this molecule has been described with an (R)-configuration at C-3 (Speranza et al. 1993).

Aloe-emodin (10)

In its pure form, compound 10 appears as orange crystals and exhibits the characteristic UV absorption bands of 9,10-anthraquinones (max 260, 300, 420 nm). The ^{13}C NMR spectra (Table 9) corroborated this, revealing two carbonyl signals at δ_C 191.6 and δ_C 181.4 correspond to carbon atoms 9 and 10, respectively. Three mutually linked aromatic protons were seen at δ_H 7.38 (1H, *dd*, J = 9.0, 1.1 Hz, H-7), 7.81 (1H, *t*, J = 8.0 Hz, H-6), and 7.71 (1H, *dd*, J = 7.5, 1.1 Hz, H-5) of ring C in the ^{1}H NMR spectrum (Table 8 of 10). H-2 and H-4 in ring A have been assigned broad singlet aromatic protons at δ_H 7.28 (1H, *d*, J = 1.6 Hz) and 7.68 (1H, *d*, J = 1.6 Hz), and oxymethylene (δ_H 4.61, δ_C 62.1), which must have been generated via high oxidation of the methyl in compound 3, has been ascribed to C-3. Because of this, aloe-emodin has been established as the correct name for this compound (10). Some *Aloe* species have been found to contain the compound (Reynolds 1985; Conner et al. 1990; Elhassan et al. 2012).

α-L-11-O-Rhamnosyl aloe-emodin (11)

Based on its ESI mass spectra, compound 11 is confirmed aloe-emodin glycoside (10). These compounds have UV absorption maxima of 260, 300, and 430 nm. There are two mutually linked protons at δ_H 4.75 (1H, *d, J* = 14.0 Hz) and δ_H 4.61 (1H, *d, J* = 13.9 Hz) in the ^{1}H NMR spectrum, which are absent from the spectra of 10, indicating the presence of a sugar unit connected at the oxymethylene spot. NMR spectra (Table 8) confirmed that the sugar unit was L-rhamnose, with the most prominent signal coming from the methyl group on the sugar moiety at δ_H 1.16 (d, J= 6.2 Hz) (c 17.9). In addition, the anomeric proton was detected in the ^{1}H NMR spectrum at δ_H 4.71 (1H, *d, J* = 1.6 Hz), and the equivalent ^{13}C NMR signal was found at C 99.9, showing the sugar moiety was in the *α* -configuration (Elizabeth et al. 1998). The signal at 3.73 (2H, *dd, J* = 3.4, 1.7 Hz) for H-2' in the ^{1}H NMR spectrum proved the $^1C_4$ conformation of the sugar unit.

Two carbonyls, corresponding to carbon atoms 9 and 10, were detected in the ^{13}C NMR spectrum at δ_C 191.6 and δ_C 181.4. Based on the HMBC association of the anomeric proton with C-11, C-2 ', and C-3 ', the sugar moiety was determined to be attached at the oxymethylene position (Table 8). The component was found to be -L-11-O-Rhamnosyl Aloe-emodin, which has previously been described from *Aloe rabaiensis* exudates (Conner et al. 1989).

3, 4-Dihydroxybenzoic acid (12)

The brown solid identified as compound 12 was found to be isolates. The ^{1}H NMR spectrum of 1,3,4-trisubstituted benzene displays three aromatic proton signals, each having an AXY spin system at δ_H 7.53 (*d*, J = 2.0 Hz) δ_H 6.89 (*d*, J = 4.9 Hz) and δ_H 7.48 (*dd*, J = 6.5 Hz, 2.0 Hz) corresponding to H-2 (δ_C 117.4), H-5 (δ_C 115.6) and H-6 (δ_C 123.6), respectively.

Seven carbon signals were detected in the ^{13}C NMR spectra of 12, including a carbonyl at δ_C 168.7 corresponding to the carboxylic acid substituent at C-1, and two downfield shifted signals at δ_C 167.5 and δ_C 150.6 of the two hydroxyl substituents at C-3 and C-4. 3,4-dihydroxy benzoic acid (trivial name protocatechuic acid) was found to be compound 12. It was originally obtained from the *Aloe* genus (Dagne and Alemu 1991) and later found in the Ginkgoaceae, Hypericaceae, and Rosaceae families (Ellnain-Wojtaszek 1997; Jurgenliemk and Nahrstedt 2002; Lee and Yang 1994).

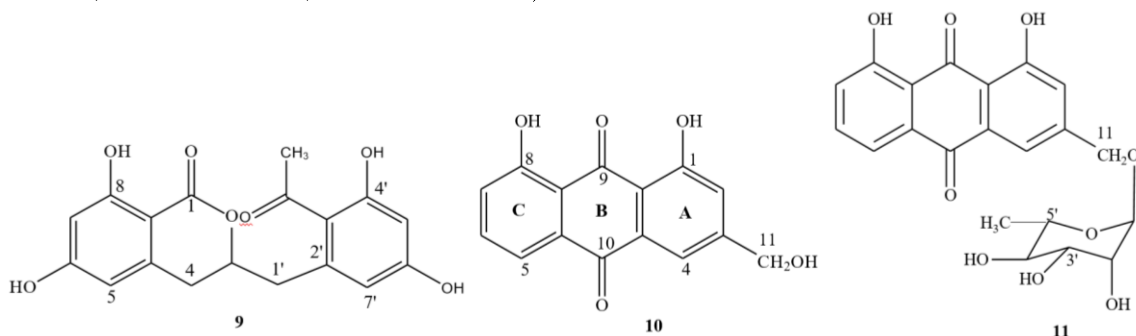


Table 8. ^{1}H (500 MHz), ^{13}C (125 MHz), and HMBC (500MHz) spectra data of compounds 10 and 11 (DMSO-d$_6$)

Carbon no.	Compound 10			Compound 11		
	^{1}H δ_H (*m*, *J* in Hz)	^{13}C	HMBC	^{1}H δ_H (*m*, *J* in Hz)	^{13}C	HMBC
1	-	161.6		-	161.4	
1a	-	114.6		-	115.3	
2	7.28 (1H, *d*, *J* = 1.6 Hz)	120.6	C-1, C-1a	7.32 (1H, *d*, *J* = 1.6 Hz)	121.8	C-1a, C-4
3	-	157.3		-	148.8	
4	7.68 (1H, *d*, *J* = 1.6 Hz)	117.1	C-1a, C-1, C-2	7.68 (1H, *d*, *J* = 1.6 Hz)	117.7	C-1a, C-2, C-4a, C-10
4a	-	133.3		-	133.4	
5	7.71 (1H, *dd*, *J* = 7.5, 1.1 Hz)	119.3	C-7, C-8a, C-8	7.41 (1H, *dd*, *J* = 8.4, 1.1 Hz)	119.3	C-8a, C-5, C-8
5a	-	133.1		-	133.3	
6	7.81 (1H, *t*, *J* = 8.0 Hz)	137.3	C-5a, C-8, C-7, C-5	7.82 (1H, *st*, *J* = 8.4 Hz)	137.4	C-5a, C-5, C-8
7	7.38 (1H, *dd*, *J* = 9.0, 1.1 Hz	124.4	C-8a, C-8, C-5	7.73 (*dd*, *J* = 5.0 Hz)	124.4	C-8a, C-7, C-8
8	-	161.3		-	161.3	
8a		115.9			115.9	
9	-	191.6		-	191.6	
10	-	181.4		-	181.4	
CH$_2$- 11	4.62 (*s*)	62.1		4.75 (1H, *d*, *J* = 14.0 Hz)	66.9	
				4.61 (1H, *d*, *J* = 13.9 Hz)		C-1', C-2, C-3, C-4
1'		-		4.71 (1H, *d*, *J* = 1.6 Hz)	99.9	C-11, C-2', C-3'
2'	-	-		3.73 (2H, *dd*, *J* = 3.4, 1.7 Hz)	70.4	C-3'
3'	-			3.62-3.28 (33H, *m*)	69.0	C-4'
4'	-	-		3.23 (2H, *s*)	71.8	CH$_3$, C-5'
5'	-	-		-	70.7	-
1-OH & 8-OH	11.93 (*s*)	-	-	-	-	-
CH$_3$	-	-	-	1.16 (*d*, *J* = 6.2 Hz)	17.9	C-5'

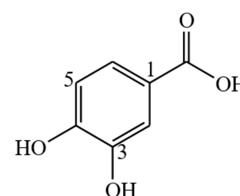
Chemotaxonomic importance of the isolated naphthoquinones

Because of its unique chemical components, Aloe has been categorized differently. The *A. secondiflora*, another member of the same plant family, also contains naphthoquinones (Induli et al. 2012). Reynolds (1996) used a chromatographic examination of leaf exudates to determine that *A. scabrifoli* and *A. turkanensis* are separate species. This study used two naphthoquinones, 3,5,8-trihydroxy-2-methylnaphthalene-1,4-dione (1) and 5,8-dihydroxy-3-methoxy-2-methylnaphthalene-1,4-dione (2), were extracted. Naphthoquinones have been found and reported in two other plant genera, and Aloe is the second. In the case of family 14 (Aloes with secund flowers), naphthoquinones may serve as a unique identifier for taxonomic purposes. It will be interesting to check if the closely related plant *A. scabrifolia* has any naphthoquinones.

In vitro anticancer activities

Effect of DMSO on cell viability

Cytotoxicity tests were performed on the extracts and the pure compounds in a DMSO solution. A series of DMSO concentrations were utilized to establish the point at which further testing would be called off due to cytotoxicity. Below 1 g/mL, DMSO has negligible effects on cell viability (Figure 2).



12

Table 9. ^{1}H (500 MHz) and ^{13}C (125 MHz) spectra data of compound 12 (acetone-*d$_6$*)

Carbon no.	δ_H (*m*, *J* in Hz)	δ_C
1	-	146.2
2	7.53 (*d*. *J* = 2.0 Hz)	117.4
3	-	167.5
4	-	150.6
5	6.89 (*d*, *J* = 4.9)	115.6
6	7.47 (*dd*, *J* = 6.5, 2.0)	123.6
COOH	-	168.7

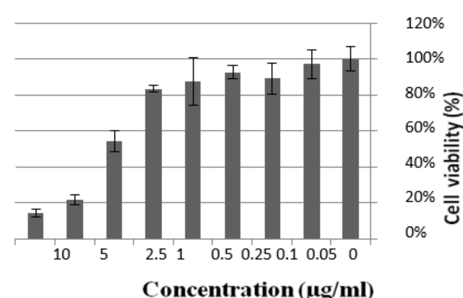


Figure 2. Effect of DMSO on TFK-1 cell viability

Anticancer test of crude extracts and compounds on TFK-1 cell line

The crude extracts and identified compounds were tested against a human extrahepatic bile duct carcinoma cell line (TFK-1). The cytotoxicity of an *A. turkanensis* rhizome extract in CH2Cl2/MeOH (1:1) against human extrahepatic bile duct cancer cells (TFK-1) is shown in Figure 3 at concentrations of 50-100 g/mL. In contrast, leaf extracts' cytotoxicity is similar at concentrations of 75 and 100 g/mL. Following the discovery of cytotoxic effects in crude extracts of *A. turkanensis* rhizomes and leaves (Figure 3), a panel of twelve compounds isolates from these extracts was tested on the human TFK-1 cell line. Compounds belonging to the anthraquinone, pre-anthraquinone, and naphthoquinone families exhibited activities.

Figure 4 shows that between 5 and 100 µg/mL ($p<0.001$), aloe-emodin (10) and its glycoside, α-L-11-*O*-rhamnopyranosyl aloe-emodin (11), reduced cell viability more than any other anthraquinone. For example, aloesaponarin II (4), which has a methyl group peri to carbonyl (at C-8), significantly decreased cell viability (10-100 µg/mL) (Figure 5). Figure 6 shows that the presence of a methyl group at C-8 (*peri* to the carbonyl) in alosaponarin II (4) is crucial for the observed cytotoxicity. In contrast, the isomeric structure chrysophanol (3), which has its methyl group at C-3, only showed significant cytotoxicity at concentrations of 2.5 and 25 µg/mL ($p<0.05$). Vis aloesaponarin I (5) and laccaic acid D methyl ester (6), two additional anthraquinones with a methyl group at C-8, likewise significantly decreased cell viability at concentrations of 10-100 g/mL and 50-100 g/mL, respectively (Figure 7). Figure 6 shows that at 5-100 g/mL concentrations, helminthosporin (8) considerably decreased cell viability, while chrysophanol (3) only did so at concentrations of 2.5 and 25 g/mL. (Figure 6). At concentrations between 5 and 100 g/mL, the pre-anthraquinone aloesaponol I (5) significantly decreases cell viability (Figure 5).

At 25-100 µg/mL doses, the naphthoquinone 5,8-dihydroxy-3-methoxy-2-methylnaphthalene-1,4-dione (2) showed substantial cytotoxicity, significantly decreasing cell viability by more than 90%. Moreover, this compound decreased cell viability significantly at doses between 5 and 15 µg/mL. Cell viability was significantly decreased at 100, 75, and 5 µg/mL concentrations for the second naphthoquinone evaluated, 3,5,8-trihydroxy-2-methylnaphthalene-1,4-dione (1) (Figure 8). However, compared to non-methylated naphthoquinone (1), methylation naphthoquinone (2) was more toxic to cells (1).

At concentrations of 25 and 50 µg/mL ($p < 0.05$), the benzoic acid derivative 3,4-dihydroxybenzoic acid (12) significantly decreased cell viability. At 5 µg/mL, feralolide (9)-a pyrone derivative was likewise significantly effective ($p < 0.05$) against the TFK-1 cell line (Figure 9). These compounds appear responsible for the reduction in TFK-1 cell viability caused by crude extracts.

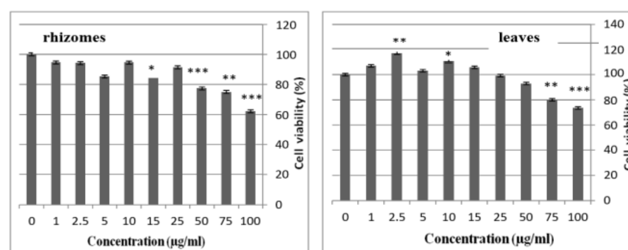


Figure 3. Effect of rhizomes and leaves extract on TFK-1 cell viability

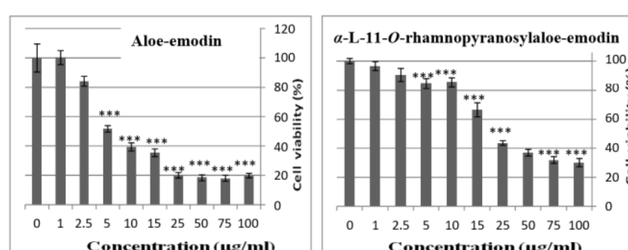


Figure 4. Effect of aloe-emodin and α-L-11-*O*-rhamnosyl aloe-emodin on TFK-1 cell viability

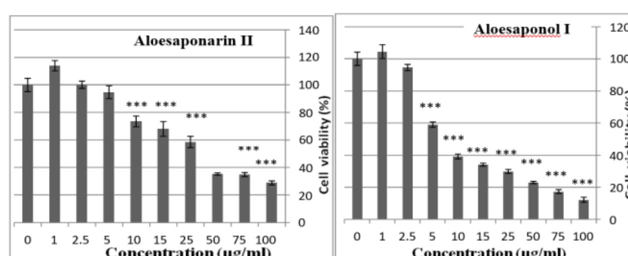


Figure 5. Effect of aloesaponarin II and aloesaponol I on TKF-1 cell viability

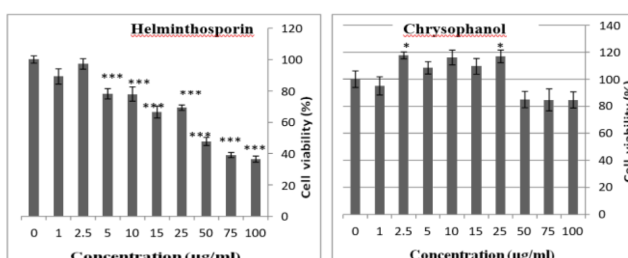


Figure 6. Effect of helminthosporin and chrysophanol on TFK-1 cell viability

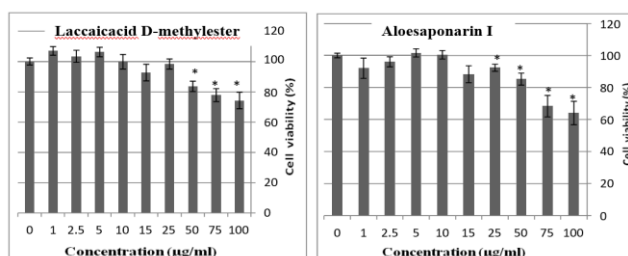


Figure 7. Effect of laccaic acid D- methyl ester and aloesaponarin I on TFK-1 cell viability

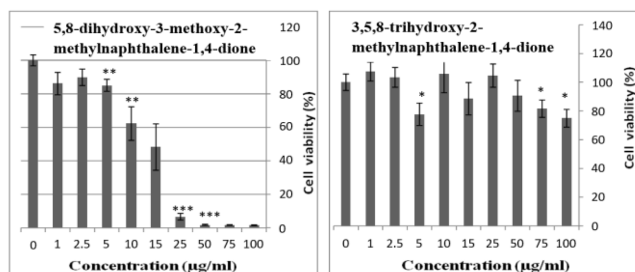


Figure 8. Effect of 5,8-dihydroxy-3-methoxy-2-methylnaphthalene-1-4-dione and 3,5,8-Tri hydroxyl-2-methylnaphthalene-1,4-dione on TFK-1 cell viability

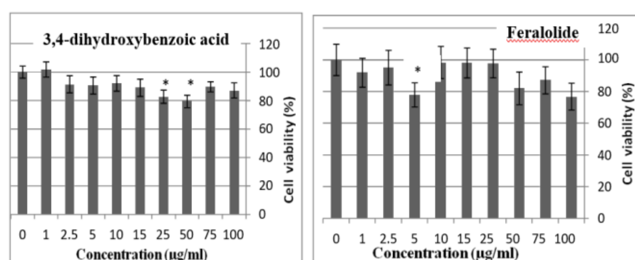


Figure 9. Effect of 3,4-dihydroxybenzoic acid and feralolide on TFK-1 cell viability

Anticancer test on selected compounds on HuH7 cell line

The human hepatoma carcinoma cell line (HuH7) was tested with the most cytotoxic compounds against the extrahepatic bile duct carcinoma cell line (TFK-1). The 25-100 µg/mL concentration range for the naphthoquinone derivative 5,8-dihydroxy-3-methoxy-2-methylnaphthalene-1,4-dione (2) resulted in the greatest decrease in cell viability (80%-97.5%). At 15 µg/mL, this compound was shown to drastically decrease cell viability (by 29.4%) (Figure 10).

At concentrations of 25-100 µg /mL, aloe-emodin (10) and its glycoside α-L-11-*O*-rhamnopyranosyl aloe-emodin (11) significantly reduced cell viability (Figure 10 and Figure 11). The higher concentrations (50-100 µg/mL) of the pre-anthraquinone aloesaponol I (7) and the anthraquinone aloesaponarin II (4) significantly decreased cell viability (Figure 12). As shown in Figure 9, the examined quantities of helminthosporin (8) had no appreciable effect on HuH7 cells.

Table 10 shows that aloe-emodin (10) is the most effective inhibitor of TFK-1 (IC_{50} = 6 µg/mL) and HuH7 (IC_{50} = 31 µg/mL) cell lines. The TFK-1 (IC_{50} values of 15 µg/mL) and HuH7 (IC_{50} values of 20 µg/mL) cell lines were likewise highly inhibited by the naphthoquinone derivative 5,8-dihydroxy-3-methoxy-2-methylnaphthalene-1,4-dione (2). With an IC_{50} value of 10 µg/mL, the pre-anthraquinone aloesaponol I (7) suppressed TFK-1 cell proliferation. Inhibition of TKF-1 cell viability was detected at IC_{50} values of 23 µg/mL and 34 µg/mL for Aloe-emodin glycoside, α-L-11-*O*-rhamnopyranosyl aloe-emodin (11) and aloesaponarin II (4), and at IC_{50} of 47 µg/mL and 55 µg/mL for HuH7 cell lines. The examined helminthosporin (8) had IC_{50} values of 46 µg/mL for inhibiting the proliferation of TFK-1 cells but did not affect the HuH7 cells at such concentrations. In contrast to their cytotoxic effects on the TFK-1 cell line, all of the chemicals tested exhibited minimal effects on the HuH7 cell line (Table 10).

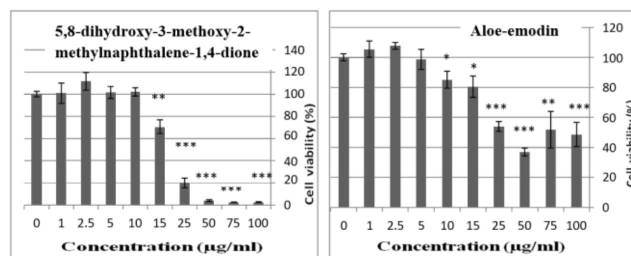


Figure 10. Effect of 5,8-dihydroxy-3-methoxy-2-methyl naphthalene-1,4-dione and aloe-emodin on HuH7 cell viability

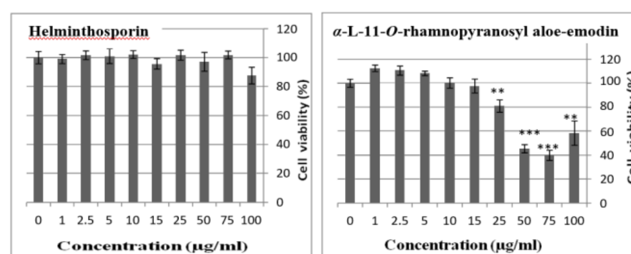


Figure 11. Effect of helminthosporin and α-L-11-*O*-rhampopyranosyl aloe-emodin on HuH7 cell viability

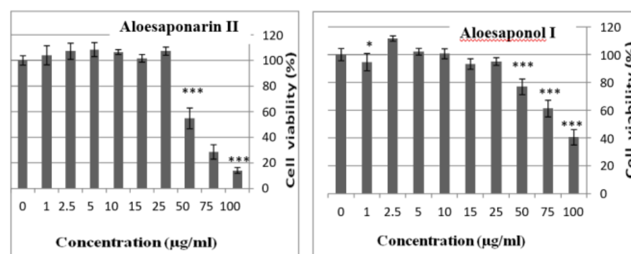


Figure 12. Effect of aloesaponarin II and aloesaponol I on HuH7 cell viability

Table 10. Cytotoxicity (IC_{50} value) of pure compounds and crude extracts from *Aloe turkanensis* against human cancer cell lines (TFK-1 and HuH7)

Compounds	IC_{50} (µg/mL)	
	TFK-1	HuH7
Crude extracts	>100	NT
3,5,8-Trihydroxy-2-methylnaphthalene-1,4-dione (1)	>100	NT
5,8-Dihydroxy-3-methoxy-2-methylnaphthalene-1,4-dione (2)	15.0	20.0
Chrysophanol (3)	>100	NT
Aloesaponarin II (4)	34.0	55.0
Aloesaponarin I (5)	>100	NT
Laccaic acid D-methyl ester (6)	>100	NT
Aloesaponol I (7)	10.0	88.0
Helminthosporin (8)	46.0	NA
Feralolide (9)	>100	NT
Aloe-emodin (10)	6.0	31.0
α-L-11-*O*-Rhamnopyranosylaloe-emodin (11)	23.0	47.0
3,4-Dihydroxybenzoic acid (12)	>100	NT

Note: NA = not active up to 100 µg/ml NT = not tested

Several studies have shown that aloe-emodin can slow the expansion of several cancer cells. For instance, aloe-emodin has demonstrated anticancer effects on the SCC-4 human tongue squamous carcinoma cells (Pecere et al. 2000) and lung squamous cell carcinoma (Lee 2001). (Chiu et al. 2009). However, aloe-reported emodin's ability to decrease mTORC2 activity and hence slow the progression of prostate cancer is relatively new (Liu et al. 2012).

Numerous synthetic and plant-based naphthoquinone structural isomers have been tested in vitro against various human cancer cell lines and in vivo against animal tumor models. Inhibition of human non-small cell lung cancer cell proliferation was seen using plumbagin (Hsu et al. 2006). Human myeloma RPMI 8226, human mammary cancer MCF-7, mouse fibroblasts LMTK, and main mouse fibroblast cell line (PMF) were all killed off by polyfluorinated 1,4- naphthoquinone derivatives (Zakharova et al. 2011). The antitumor effect of synthetic and natural naphthoquinones in several cancer cell lines was recently demonstrated by Bringmann et al. (2011). The anticancer effects of anthraquinones, pre-anthraquinone, and naphthoquinone derivatives on human extrahepatic bile duct (TFK-1) and liver cancer (HuH7) cell lines are described for the first time in this paper.

In conclusion, twelve isolates chemicals from *A. turkanensis* resulted from chromatographic separation. Among these were found to be 3,5,8-trihydroxy-2-methylnaphthalene-1,4-dione (1), 5,8-dihydroxy-2-methoxy-2-methylnaphthalene-1,4-dione (2), chrysophanol (3), aloesaponarin I (4), aloesaponarin II (5). laccaic acid D methyl ester (6) and aloesaponol I (7). The *A. turkanensis* was used to extract eight chemicals, including helminthosporin (8), feralolide (9), aloe-emodin (10), α-L-11-*O*-rhamnopyranosyl aloe-emodin (11)] and 3,4-dihydroxybenzoic acid (12). In addition, the rhizomes and leaves were analyzed for various compounds, including 3,5,8-trihydroxy-2-methylnaphthalene-1,4-dione (1), aloesaponarin I (4), aloesaponarin II (5). Herein is reported for the first time the occurrence of the naphthoquinone 3,5,8-trihydroxy-2-methylnaphthalene-1,4-dione (1) in the Asphodelaceae family. Cell viability was significantly decreased in an extrahepatic bile duct cancer cell line (TFK-1) exposed to the crude extracts. Six compounds [5,8-dihydroxy- 3-methoxy-2-methylnaphthalene-1,4-dione (2), aloesaponarin II (5), aloesaponol I (7), Helminthosporin (8), Aloe-emodin (10) and α-L-11-*O*-rhamnopyranosylaloe-emodin (11)] were found to be very strong inhibitors of the TKF-1 cell line. Higher inhibition was seen on the HuH7 cell line when exposed to the following five compounds: 5,8-dihydroxy-3-methoxy- 2-methylnaphthalene-1,4-dione (2), aloesaponarin II (5), aloesaponol I (7), Aloe-emodine (10) and α-L-11-*O*-rhamnopyranosylaloe-emodin (11). Extrahepatic (TFK-1) and liver (HuH7) cancer cell lines were used to evaluate the phytochemical and anticancer properties of the isolates chemicals, making this the first report of its kind.

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