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Variation of alkaloids in the Kenyan *Zanthoxylum gilletii* (De Wild Waterman)

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Zanthoxylum gilletii **is an African indigenous deciduous tree which is important for its medicinal use in many communities to treat a wide range of ailments. This study was conducted to identify the alkaloids present in the bark, root and leaves of the Kenyan *Z. gilletii*. The plant materials were randomly sampled, dried at room temperature, powdered and subjected to thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS) analyses for the alkaloid confirmatory tests. The bark, root and leaf extract contained peroxysimulenoline, sanguinarine, fagarine I, norchelerythrine (dihydroavicine or demethylnitidine), trans-fagaramide, 8-methylnorchelerythrine and dihydronitidine alkaloids. The distribution of the alkaloids appeared to be quite variable within different plant parts and different regions. The identified alkaloids have been documented to be useful for their medicinal value in humans and also protect the plants against predation. The medicinal value of *Z. gilletii* may be due to its contents of varied alkaloids. The information on alkaloidal variation in the species has potential value and practical applications in chemotaxonomy, toxicology and pharmacognosy. The present findings may be useful in optimizing the processing and wild-harvesting of these alkaloids.**

Key words: *Zanthoxylum gilletii,* chemotaxonomy, pharmacognosy, liquid chromatography-mass spectrometry.

INTRODUCTION

Zanthoxylum gilletii is an indigenous deciduous tree growing 10 to 35 m high, belonging to the family *Rutaceae*. The genus *Zanthoxylum* is distributed world-wide from the tropics to the temperate zones. There are over 200 species from small shrubs to large trees (USDA, 2003). In Africa, *Z. gilletii* is widely distributed in countries such as Sierra Leone to Kenya, Sudan, Angola, Malawi, Zambia and Zimbabwe. The bark is chewed and the juice swallowed for the treatment of stomachache (Kokwaro, 1993). The stem bark decoction is commonly used for back pain and externally for all urinogenital complaints including infections. The root bark or the fruit pulp is a liniment for rheumatism and all kinds of pain. The decoction of young leaves eases coughs and is said to be effective on gonorrhea and bilharzia. Several phyto-chemical studies have identified numerous compounds with medical and antioxidant potential including alkaloids, xanthophylls, phenolic acids, sapo-nins, coumarins and hydroxycinnamic acids (Islam and Ahsan, 1997). Phytochemical investigations carried out on a related species, *Zanthoxylum chalybeum* have yielded pure crystalline alkaloids (Olila and Opuda, 2001). In Nigeria, chemical investigations of *Z. gilletii* showed the presence of furoquinoline alkaloid, skim-mianine, the cinnamic acid amide, fagaramide and benzo phenanthridine alkaloids, nitidine, dihydrochelerythrine and chelerythrine alkaloids

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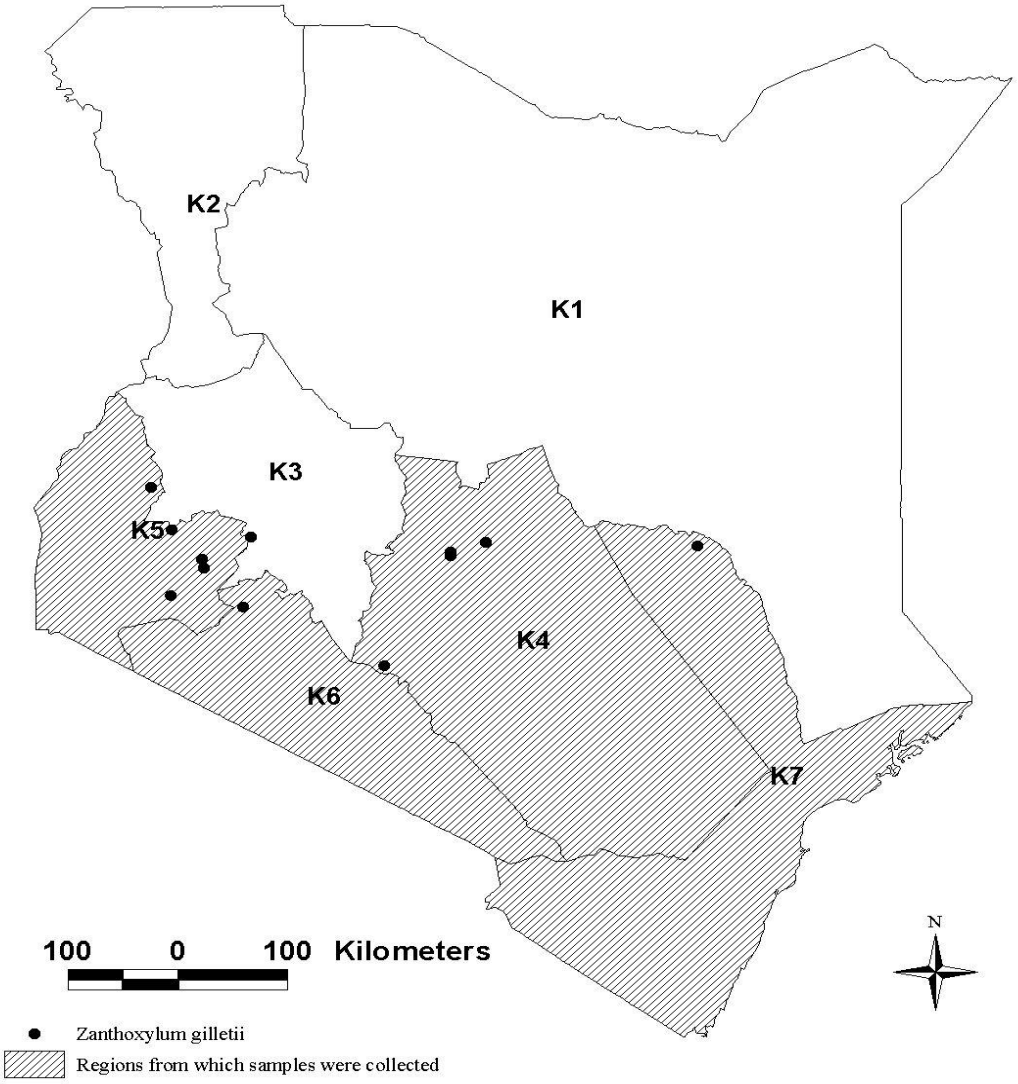

Figure 1. Geographical distribution of *Z. gilletii* in Kenya (Source: Seeds for Life Project, National Museum Kenya database).

(Adesina and Johannes, 1988). Other constituent compounds isolated included volatile oils, vanillic acid and hydroxyl benzoic acid (Adesina, 2005). Geographical variation may have some effects on the level of medicinal active ingredients of plants of the same species (Sheen et al., 1991).

Variation among plants of the same species may be due to age, climate, soil or season of the year. In Kenya, there is limited information on the active ingredients and variation of the secondary compounds in *Z. gilletii* across agro ecological zones and plant parts. Such knowledge on the chemical constituents of plants is desirable, not only for the search of therapeutic agents, but also because such information may be of value in disclosing new sources of such economic materials such as tannins, oils, gums, precursors for the synthesis of complex chemical substances. The aim of this study was to identify the presence and variation of main alkaloids in *Z. gilletii* from Kenya.

MATERIALS AND METHODS

Study site

The plant samples (leaf, root and bark) were collected from trees growing in the Kakamega forest (altitude 1649 m, 00° 20' 195 N; 034° 52' 607 E), Southern Mau forest (altitude 2340 m, 00° 47' 357 S; 035° 34' 831 E) and Mount Kenya region (1768 m, 00° 14' 115 S; 037° 35' 031 E). The records for the distribution of the species were retrieved from voucher specimens at the National Museums of Kenya. Sampling points (K4, K5 and K6) correspond to the species range in Kenya (Figure 1).

The samples were cleaned then chopped into smaller pieces before being dried in the shade at 25 ± 2°C for ten days. The dried plant samples (leaves root and bark) were then ground into fine

Table 1. Solvent systems used in TLC (butan-1-ol, acetic acid and water; MAC = methanol, ammonia and chloroform).

Solvent system	BAW- 40 ml butan-1-ol: 10 ml acetic acid: 50 ml water	MAC*- 10 ml methanol: 1 ml ammonia: 89 ml chloroform
Plates	Silica 5553	Silica 5577
Standard(s)	Papaverive Chloride	Papaverine Chloride
Spray(s) used	Dragendorffs' reagent	Dragendorffs' reagent

powder in a grinder and sieved to give particle size of 50to 150 mm. The ground powder was then packed into 1 kg and stored in labeled zip locked polythene bags at room temperature before they were transported to the Jodrell Laboratory, UK for extraction and phytochemical analysis. In the laboratory, the samples were documented and given accession database numbers in the biological interactions' (BI) accession database.

Extraction of plant material

Nine bottles were labeled with BI accession database numbers. Into each of the labeled sample bottles, 0.5 g of dry powder sample was weighed, and this was repeated for all samples. To each vial, 5 ml of 100% methanol was added and shaken well and left for three days to extract. The extracts were filtered and analyzed by thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS).

Thin layer chromatography (TLC) analysis

Solid Phase Sorbent was mounted on aluminum backed silica plates without florescent indicators F254 (sigma code 5334). Methanol extract (20 µl of each sample) was applied to the selected TLC plates (10 x 20 cm) and allowed to dry between each 5 µl application. Standard papaverine chloride (in 100% methanol) at a concentration of 2 mg/ml was then applied (5 µl), to the plate (Svendsen and Verpoorte, 1983). The solvent front was marked on the plate in pencil immediately on removal from tank and allowed to air dry in a fume cupboard. TLC-UV spectra were observed under the UV spectrophotometer (model UV- Desage) at wavelengths of 254 and 366 nm. The plates were further developed by spraying with the Dragendorffs' reagent and the retention factor (Rf) was recorded.

$$Rf = \frac{\text{Distance moved by compound}}{\text{Distance moved by solvent front}}$$

After viewing plates under the UV light, and before spraying with any reagents, the plates were scanned (a Camag TLC Scanner 3) under a range of wavelengths from 254 to 350 nm. The resulting data for different colour peaks, heights and bases of the peaks observed at 350 nm were analyzed and interpreted.

Alkaloid analysis

Each of the samples was filtered, dried and rehydrated in 10 ml of 0.5 M hydrochloric acid. Acidified extract was added to an equal volume (10 ml) of chloroform in a separating funnel, shaken and left to settle. The bottom layer (acidified chloroform) was drained into a labeled bottle and a second equal volume (10 ml) of chloroform was added to the aqueous extract, shaken and left to settle. The acidified chloroform layer was air dried in the fume cupboard while the 1 to 2 ml of aqueous layer was basified with concentrated ammonia and the pH confirmed using pH paper (Sigma). A volume of 10 ml chloroform was added to the basified sample in separating funnel, shaken and left to settle. The bottom layer was drained out into a 20 µl labeled vial and a second equal volume of chloroform added to the aqueous extract. The basified chloroform layers were allowed to dry in the fume cupboard while the aqueous layer was dried in a heat block at 40°C. The two chloroform layers (acid + basic) were rehydrated in 100% methanol. The dry aqueous layer was re-hydrated in 100% water, kept in the cold room prior to analysis. The alkaloid partition fractions were run in confirmatory alkaloid tests using TLC and LC-MS analyses (Table 1).

HPLC and LC-MS analysis

In order to remove all the particulates, the extracts were centrifuged at 10,000 rpm for 2 min and the clarified extracts were transferred into small clean labelled vials (BI 18826 - 18834 stock solutions) using a glass pipette. From the stock solution, 1 ml was placed into an HPLC vial (Chromacol) and 300 µl into a smaller LC-MS vial (Chromacol). HPLC analyses were carried out on a Waters 600 pump with a 600E controller, Waters 717plus autosampler coupled to a Waters 996 photodiode array detector controlled through a PC workstation running Empower software. Detection of alkaloids was achieved by scanning through 200 to 550 nm scans per second and data were collected for 30 min with a 10 min between injections to ensure column equilibrium between samples. Extracts were analyzed using gradient solvent programme with a flow rate of 1 ml per min with in-line degassing and an injection volume of 20 µl. Accurate mass LC-MS analyses were carried out on a ThermoScientific LTQ Orbitrap XL with an Electrospray source (ESI) operating on positive or negative mode with an Accela system (LC system). The data was analyzed using XCalibar software. Chromatography was achieved on a Phenomenex Luna C18 column 150 mm x 3 mm i.d. x 3 µm with a 0.4 ml/min flow rate and a 5µl injection volume. The samples were run in both positive and negative mode in full ms scan mode to allow data to be recorded as well as for accurate mass determination. Alkaloids present in the extracts were identified on their UV spectra and retention times (HPLC analysis) while LC-MS analyses was based on accurate mass, molecular formula and mass fragmentation pattern (µs/µs) were compared with known compounds from library of compounds based at the Jodrell Laboratory. The column used the HPLC analysis of a Phenomenex Luna C18 capillary column 250 mm x 4 mm i.d. x 5 µm. Extracts were analyzed using gradient solvent programmes (Table 2) with a flow rate of 1 ml/min with in-line degassing and an injection volume of 20 µl.

The LC-MC chromatography was achieved on a Phenomenex Luna C18 column 150 mm x 4.6 mm i.d. x 3 µm using a gradient (Table 3) with a 0.5 ml/min flow rate and injection volume of 10 µl.

RESULTS

The screening of alkaloids using papaverine chloride as

Table 2. Gradient conditions for HPLC analyses (A- methanol; B- water; C- 5% acetic acid in methanol)

Time	A	B	C
0	15	75	10
20	90	0	10
25	90	0	10
27	15	75	10
30	15	75	10

Table 3. Gradient conditions for LC-MS analyses (A- methanol; B- water; C- 1% formic acid in acetonitrile).

Time	A	B	C
0	0	90	10
20	90	0	10
25	90	0	10
27	0	90	10
37	0	90	10

the standard together with the sample extracts on TLC plate showed the best separation of the components of the extracts. On viewing the photographic illustration under UV-light at 366 nm, the colour display for leaf extracts were blue, purple and red, while the bark and root extracts showed yellow, purple, orange and blue coloration. Distinct orange coloration confirmed the presence of alkaloids in all the extracts after application of Dragendorff's reagent on the plates. Different colour bands showed the presence of different components in the root, leaf and bark extracts. The appearance of different colours in the TLC plate and formation of the various peaks viewed after the TLC scan represented the separation of the different compounds present in the extracts. As shown in the ESI - MS detector chromatograms of root bark and leaf extracts (Figures 2 to 4), there are varying peaks. The peaks represent the abundance and diversity of alkaloids in different plant parts as well as geographical locations. The detection of alkaloids in the root, leaf and bark extracts was based on retention times and peak pattern heights.

HPLC and LC-MS analysis

The HPLC and LC-MS analyses of the extracts confirmed the presence of peroxysimulenoline, sanguinarine, fagarine I, norchelerythrine (dihydroavicine or demethylnitidine), trans-fagaramide, 8-methylnorchelerythrine, dihydronitidine as alkaloids. Fagarine I and 8-methyl norchelerythrine were found to be present in all the extracts except in leaves from Kakamega and Mau forests. Norchelerythrine, dihydroavicine and demethylnitidine occurred in all plant parts but were absent in the leaves from all the regions. The retention time, molecular weights and chemical formula of the alkaloids found in *Z. gilletii* are shown in Table 4, while the molecular structures of the alkaloids are shown in Figure 5.

The occurrence of dihydronitidine and sanguinarine were restricted only to the roots and barks as opposed to trans-fagaramide that was evenly distributed in all the plant parts and across regions (Table 5).

DISCUSSION

Alkaloids are considered as the main bioactive constituents of many plant species including *Z. gilletii* (Pěnčíková et al., 2011). The extreme variation of alkaloids observed in the bark, roots and leaves should be taken into account if the plant is to be collected for medicinal purposes. Similar variation trends have also been reported in other plants in Northern Turkey (Çirak et al., 2008). The variations could be due to morphometric traits as well as other various environmental factors present at various locations along with altitudes (Kjaer et al., 2004). Other factors such as water availability, acidity or salinity could also contribute to variation in the concentrations of alkaloids in *Z. gilletii* across the studied regions in Kenya.

The restricted occurrence of dihydronitidine and sanguinarine alkaloids in the roots and bark concurs with the preference of the plant organs mostly harvested and used in traditional medicine. The presence of sanguinarine in the bark and roots confirmed in previous studies showed its usefulness in treating periodontal disease (Colombo and Bosisio, 1996). In countries like the United States derivatives such as sanguinarine chloride have even been included in commercial tooth-pastes and mouthwashes (Bruneton, 1999). These alkaloid activities validate the use of *Zanthoxylum* sp. in the traditional treatment of mouth ulcers and toothache. There is sufficient evidence that the Chinese use *Zanthoxylum armatum* (Hartley, 1966), Kenyans *Zanthoxylum chalybeum* (twigs) and Nigerians the roots of *Zanthoxylum zanthoxyloides* (Lam.) as teeth cleaning sticks. Even distribution of Fagarine I and 8-methylnorchelerythrine in the leaves, roots and bark could be due to their role as chemical defense against herbivores and other plant enemies. They could also serve as a plant natural source of insecticides and fungicides. The principal action of alkaloids is on the nervous system (Pěnčíková et al., 2011) and their high concentrations in plants protect them from grazing animals. Alkaloids such as trans-fagaramide, 8-methylnorchelerythrine and dihydronitidine have been

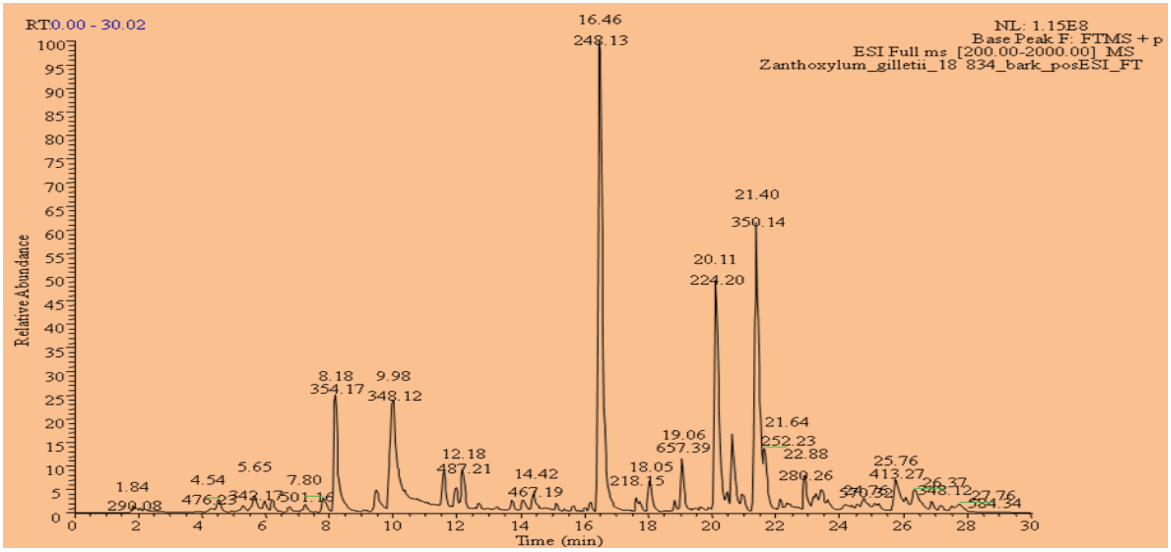

Figure 2. Liquid chromatography-mass spectrometry (LC-MS) profiles of methanolic bark extracts of *Z. gilletii*.

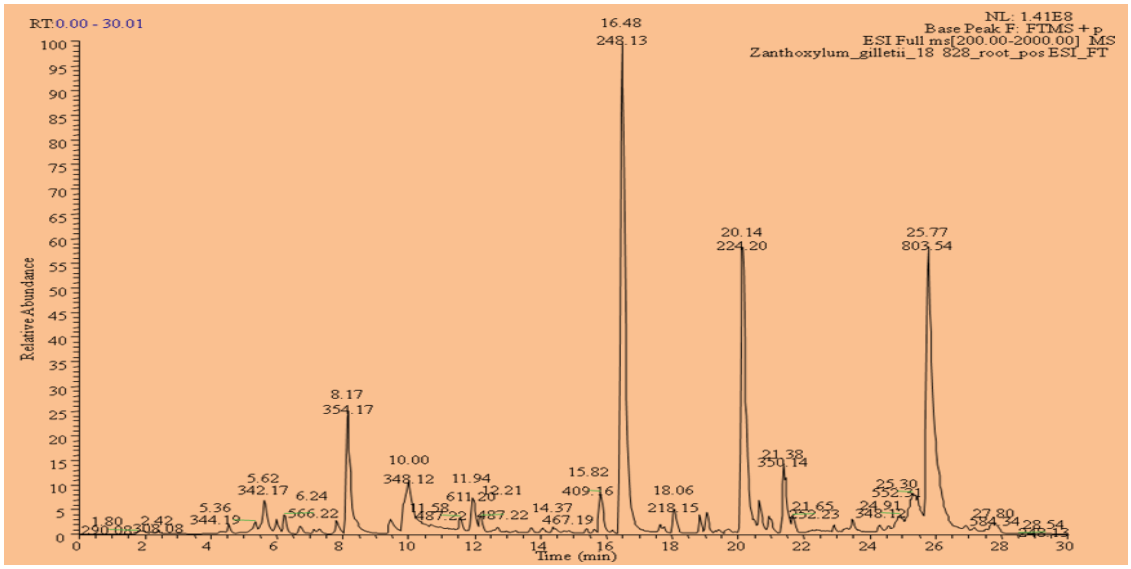

Figure 3. Liquid chromatography mass spectrometry (LC-MS) profiles of methanolic root extracts of *Z. gilletii*.

reported to help biologically in storage of waste nitrogen, cationic balancing and protection against parasites (Ting, 1982). Sanguinarine, which also shows specific toxic effects to herbivores and microbial pathogens, is proposed to function as an inducible defense compound (Schmeller et al., 1997).

In line with other African *Zanthoxylum* species such as *Zanthoxylum capense, Zanthoxylum chalybeum* and *Zanthoxylum davyi, Z. gilletii* is used for the treatment of snakebite (Mpondo in Transkei, Vhavenda in Limpopo) and severe coughs and colds (Kokwaro and Johns, 1998). The spines are used to manag e infected wounds, the leaves for chest pains, the stem bark to treat boils, pleurisy and toothache, and root preparations for mouth ulcers, sore throats and as an aphrodisiac (Mabogo, 1990). Root-bark decoctions are used by the Zulu as a tonic both for man and animals (Tarus et al., 2006) and to treat toothache.

Conclusions

Z. gilletii contains many alkaloids with antimicrobial and cytotoxic activities used to manage periodontal disease and caries in many African communities. The study has shown that presence of alkaloids should be taken

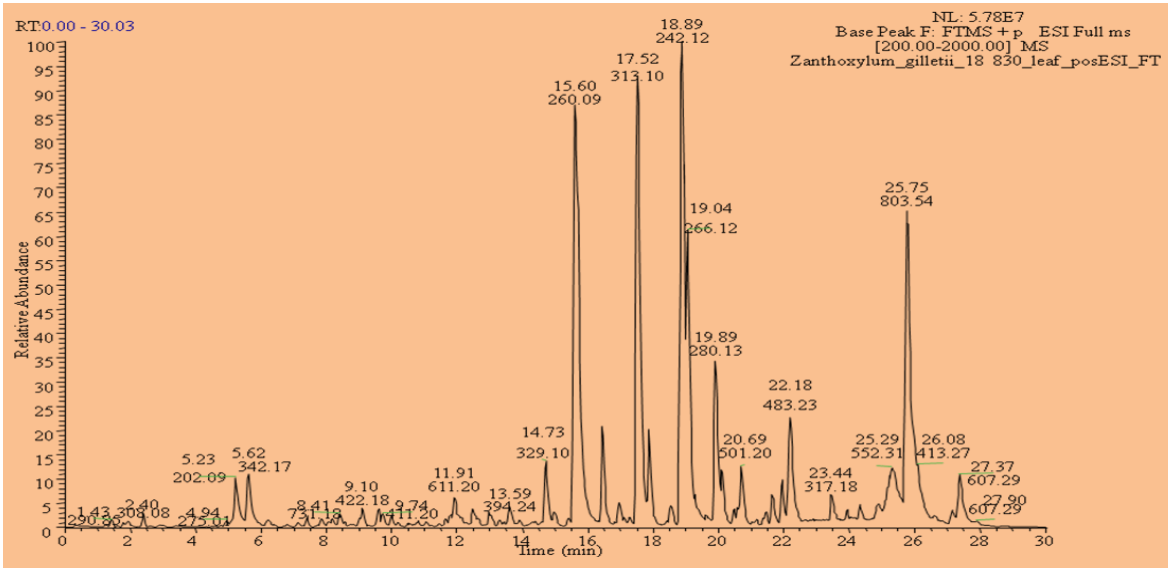

Figure 4. Liquid chromatography mass spectrometry (LC-MS) profiles of methanolic leaf extracts of *Z. gilletii*.

Table 4. Chemical formula, retention time and molecular weights of the alkaloids.

S/N	Alkaloid	Formula	Retention time	Molecular weight
1	Peroxysimulenoline	$C_{20}H_{23}NO_4$	5.60	341
2	Sanguinarine	$C_{20}H_{14}NO_4$	9.30	332
3	Fagarine I	$C_{21}H_{23}NO_5$	7.60	369
4	Norchelerythrine (dihydroavicine or demethylnitidine)	$C_{20}H_{15}NO_4$	9.48	333
5	Trans-fagaramide	$C_{14}H_{17}NO_3$	16.49	247
6	8-Methylnorchelerythrine	$C_{21}H_{17}NO_4$	10.00	347
7	Dihydronitidine	$C_{21}H_{19}NO_4$	21.4	349

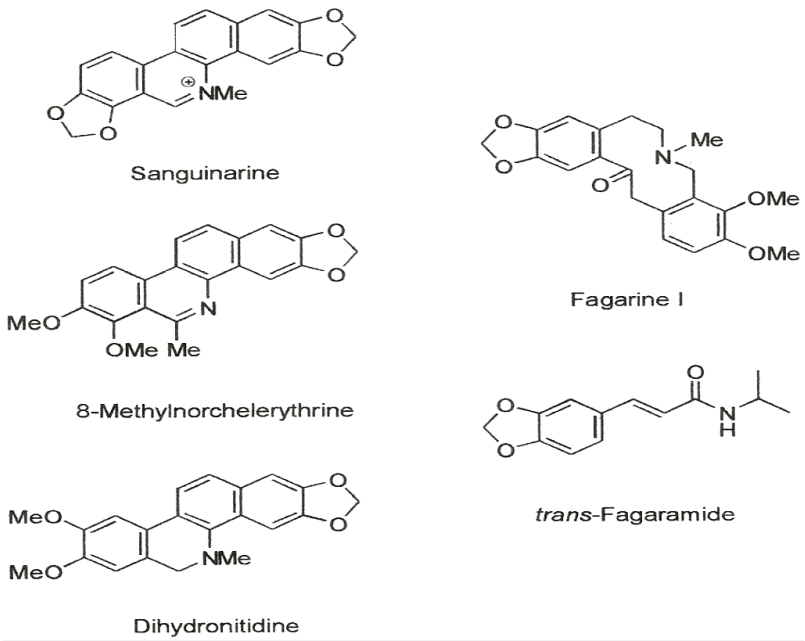

Figure 5. Structures of alkaloid isolates of *Z. gilletii* root, bark and leaf extracts.

Table 5. Alkaloid profiling in the extracts of *Z. gilletii* from the different regions and plant parts (R = root; B = bark; L = Leaf; √ = alkaloid present; X = alkaloid absent).

Alkaloid	Region								
	18826	18828	18832	18834	18829	18831	18830	18827	18833
	Kakamega Forest	Mount. Kenya	Mau Forest	Kakamega Forest	Mt. Kenya	Mau Forest	Kakamega Forest	Mount Kenya	Mau Forest
	R	R	R	B	B	B	L	L	L
Peroxysimulenoline	√	√	√	√	√	√	X	√	√
Sanguinarine	√	X	√	√	√	X	X	X	X
Fagarine I	√	√	√	√	√	√	X	√	X
Norchelerythrine	√	√	√	√	√	√	X	X	√
Trans-fagaramide	√	√	√	√	√	√	X	√	X
8-Methylnorchelerythrine	√	√	√	√	√	√	√	√	X
Dihydronitidine	√	√	√	√	√	√	X	X	X

into account if the plant is collected for medicinal purposes. It is also evidenced that the same plant species at different localities are chemically different and as a result, show variation in biological activity and potential toxicity. For use of alkaloids for chemotaxonomic purposes, it is important to take into account the plant part used, the different stages of development and genetic variation of plants and the geographical distribution. Finally, most of the medicinal uses of this species can probably be attributed to its rich diversity of alkaloids acting synergistically.

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