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Pharmacognostic investigation of *Tribulus bimucronatus* (Zygophyllaceae) grown in Saudi Arabia

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Abstract

A comprehensive botanical study of all parts of *Tribulus bimucronatus* was performed to enable the identification and quality control of this biologically active plant. The powder is characterized by the presence of anomocytic stomata, numerous non glandular unicellular trichomes with different sizes, cluster crystals of calcium oxalate, pericyclic fibers and lignified xylem vessels, in addition to sclerides of the fruits which formed from spineless or wingless mericarps. Also, the investigation of the non-polar fraction (*n*-hexane) was performed using GC/MS for identification of unsaponifiable and saponifiable matters. 93.58 % of unsaponifiable matters was identified, the major compound was found to be phytol (36.91%). While 77.23% of saponifiable matters was identified and pentadecanoic acid-14-methyl, methyl ester (29.93%) was found to constitute the major compound. This is the first record of botanical characters and the GC/MS analysis of *Tribulus bimucronatus* growing in Saudi Arabia.

Key Words

Botanical study, GC/MS, Saponifiable matter, *Tribulus bimucronatus,* Unsaponifiable Matter.

INTRODUCTION

Tribulus bimucronatus (Zygophyllaceae) is one of the *Tribulus* species found in Saudi flora [1]. In a previous study the methanolic extract of *T. bimucronatus* showed significant dose dependent (100, 200 & 400 mg/kg of body weight) anti-inflammatory activity, in addition to marked hypotensive and antinociceptive effect at the higher dose (400 mg/kg). *T. bimucronatus* is a promising plant and expected to be comparable to the famous plant; *Tribulus teresteris* [2]. *T. teresteris* was found to have stimulant, diuretic, aphrodisiac, immunomodulatory, antidiabetic, absorption enhancing, hypolipidemic, cardiotonic, hepatoprotective, anti-inflammatory, analgesic, antispasmodic, anticancer, antibacterial, anthelmintic and larvicidal, activities [3]. *T. alatus* was reported to have antioxidant [4], diuretic [5] and testosterone-increasing activities [6]. The botanical features of *T. alatus* were studied [7]. However no data was reported in the current literature describing the botanical features of *T. bimucronatus*. The macroscopic and microscopic diagnosis of leaflet, rachis, stem, root, flower and fruit are reported in this study. In addition to investigation of the non-polar fraction (n-hexane) for the identification of unsaponifiable and saponifiable matters.

MATERIALS AND METHODS

Plant material

The fresh aerial parts of *T. bimucronatus* were collected from Makkah district, Saudi Arabia, in January 2014 and authenticated by Dr. Kadry Abdel Khalik, Botany Department, Faculty of Applied Sciences, Umm Al-Qura University. A voucher specimen has been kept in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Umm Al-Qura University (UQU-2014-1). Dried aerial parts were reduced to a fine powder with a mechanical grinder and used for preparation of methanol extract.

Another sample of the same plant from the same place was collected in December 2015 for botanical study.

Preparation of *n*-hexane extract

The fine powder (500 g) was extracted on cold with methanol till exhaustion, concentrated in a rotary evaporator (Buchi Co., Switzerland), and diluted with water and fractionated using *n*- hexane. The hexane fraction was dried over anhydrous sodium sulfate and concentrated in the rotary evaporator (Percentage yield (w/ w) of 10% was obtained), the residue was subjected to saponification according to standard procedure [8]. The unsaponifiable and saponifiable matters were subjected to GC/MS analysis using a Thermo Scientific, Trace GC Ultra / ISQ Single Quadrupole MS.

Chemicals

Chloral hydrate, concentrated hydrochloric acid, glycerol, *n*-hexane, methanol, phloroglucinol, potassium hydroxide were purchased from Sigma-Aldrich Co.

Method for anatomical study

Anatomical sections of the fresh plant organs, and powdered samples were prepared by hand for the

microscopic studies. Standard laboratory methods for staining were performed [9]. The sections were cleared with aqueous chloral hydrate solution (4 g/ml), and stained with Phloroglucinol (1% in alcohol) and concentrated hydrochloric acid. Isolated elements were prepared using 5% aqueous potassium hydroxide and heated in a boiling water bath from 15- 30 minutes according to the plant organ. The results were registered by means of a Nikon digital microscope.

Method for saponifiable and unsaponifiable matters

The GC/MS analysis was performed using a TG-5MS fused silica capillary column (30m, 0.251mm, 0.1 mm film thickness). For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as the carrier gas at a constant flow rate of 1ml/min. The injector and MS transfer line temperature was set at 280^{o} C. The oven temperature was programmed at an initial temperature 50^{o} C (hold 2 min) to 150 ^{o}C at an increasing rate of 7^{o} C /min. then to 270^{o} C at an increasing rate 5^{o} C /min (hold 2 min) then to 310^{o} C as a final temperature at an increasing rate of 3.5 ^{o}C /min (hold 10 min).

The quantification of all the identified components was investigated using a percent relative peak area. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system.

RESULTS AND DISCUSSION

Macroscopic Diagnosis

T. bimucronatus is a prostrate hairy herb (Fig. 1), with cylindrical yellowish green stem. The stem size is around 30 cm, but may reach 1 meter long. It bears opposite compound paripinnate leaves with 3-6 pairs of green leaflets. Each leaf with 3 pairs of leaflets is alternating with that with 6 pairs of leaflets. The lamina is oblong to elliptical having acute apex, entire margin, asymmetric base and pinnate reticulate venation. The leaf rachis is cylindrical, pale green, hairy and measures 2-3.5 cm in length and 0.2-0.3 cm in diameter. *T. bimucronatus* has a characteristic odor and slightly bitter taste. The plant has small yellow single alternating flowers originate from the axial part of the small leaves. The flower bears pale green hairy calyx with 5 free sepals, corolla with 5 free yellow petals, androecium with 10 free stamens arranged in two worlds and gynaecium with 5 united carpels, short or no style and united stigma. The fruit is small globular in shape, pubescent, shizocarpic, separating into 5 indehescent one seeded fruitlets. The root is pale yellow cylindrical tap root may reach 30 cm long and 0.5 cm width with fine rootlets.

Microscopic Diagnosis

The leaf

The transverse section of the leaflet (Fig. 2) showed isobilateral structure with cylindrical palisade cells. The palisade layer is continuous over the vascular bundle in the midrib region as well as the terminal part of the leaflet. The epidermis is rectangular to square in shape bearing numerous unicellular non-glandular trichomes. The spongy tissue consists of nearly rounded parenchyma cells containing numerous cluster crystals of calcium oxalate. The cortical tissue of the midrib region is formed of thin parenchyma cells, the inner most layer consists of large barrel-shaped cells forming a distinct endodermis. The vascular bundle consists of radiating lignified xylem vessels situated above a narrow zone of phloem. The transverse section of the leaf-rachis (Fig. 3) is nearly rounded in shape with two lateral projections or ridges. The cortical tissue is formed of parenchyma cells with minute intercellular spaces. Several vascular bundles are present and arranged in a circle. Additional smaller vascular bundles are found under each ridge and a group of pericyclic scelerenchyma is present abutting each vascular bundle. The vascular bundles are formed of groups of thin phloem elements under radiating lignified xylem tissue.

Surface preparation of epidermal cells (Fig.4) revealed the presence of anomocytic stomata on both sides upper and lower but the trichomes are longer in lower epidermis than in upper epidermis.

The stem

The transverse section of the stem is circular in shape (Fig.5). The epidermal cells carry non-glandular trichomes with different lengths. The cortex is relatively narrow consisting of several layers of thin-walled parenchyma. The pericycle consists of patches of lignified fibers interrupted by parenchyma cells forming a circle around the stele. The stele consists of a complete ring of vascular bundles formed of groups of thin phloem elements and radiating xylem tissue. The xylem is composed of lignified vessels, parenchyma and xylem fibers. The pith is formed of a wide zone of parenchyma cells containing scattered cluster crystals of calcium oxalate.

The root

The transverse section of the root is circular in shape (Fig.6). Cork represents the outer few layers followed by cortex which is formed of few layers of thin parenchyma and a wide layer of pericyclic fibers. Phloem tissue form a complete ring surrounding the central lignified xylem.

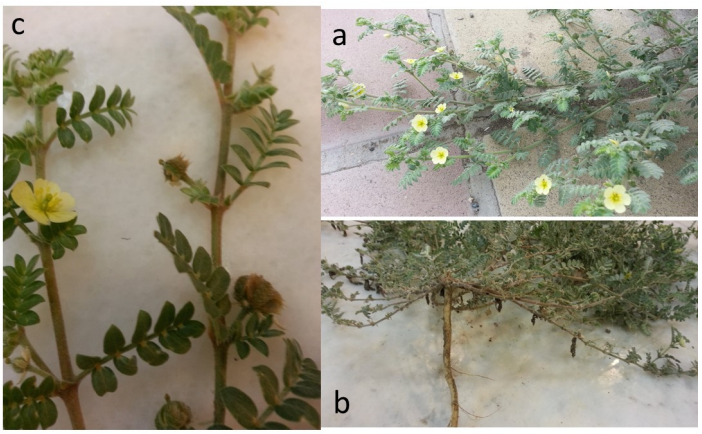

Fig. 1: Macroscopic characteristics of *Tribulus bimucronatus*.
a : the plant in its original place, b: the whole plant including the root,
c: branches of the plant showing the distribution of leaves, flowers and fruits.

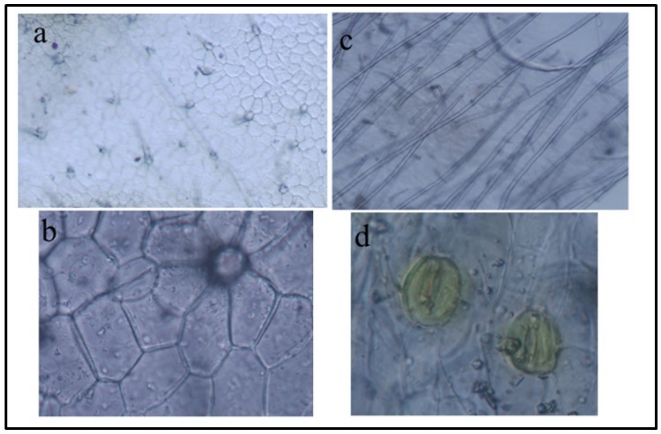

Fig. 4: Surface preparation of the *T. bimucronatus* leaflet. a: the upper epidermis showing small Hairs (X100), b: the upper epidermis showing anomocytic stomata & cicatrix (1000), c: the lower epidermis showing long hairs, d: the lower epidermis showing anomocytic stomata (X1000).

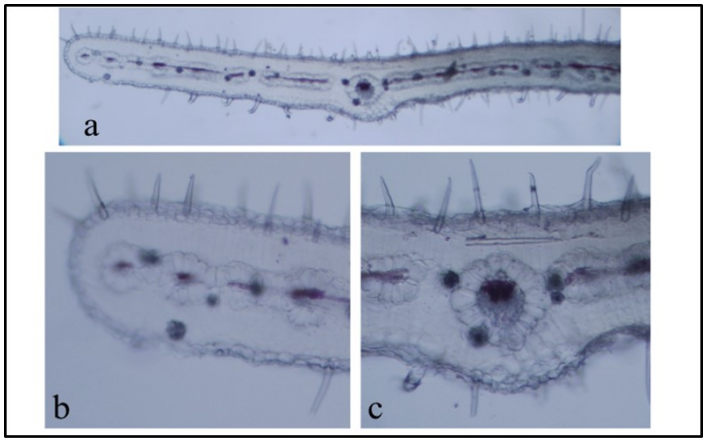

Fig. 2: Transverse section of the *T. bimucronatus* leaflet. a: T.S. of the whole leaflet (X50), b: leaf margin region (X100), c: midrib region

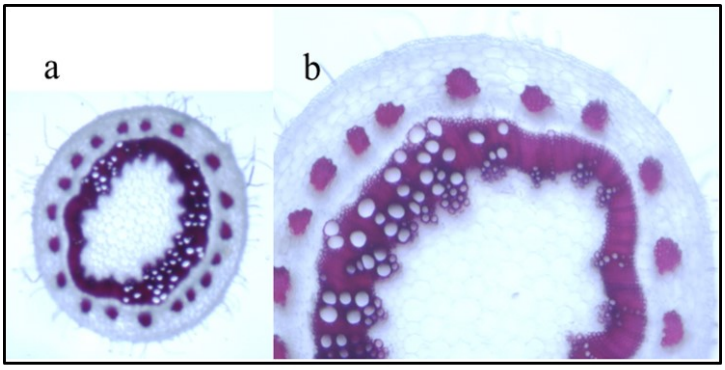

Fig. 5: Transverse section of the *T. bimucronatus* stem. a: T.S. of the whole stem (X50) b: part of the T.S. of the stem (X100).

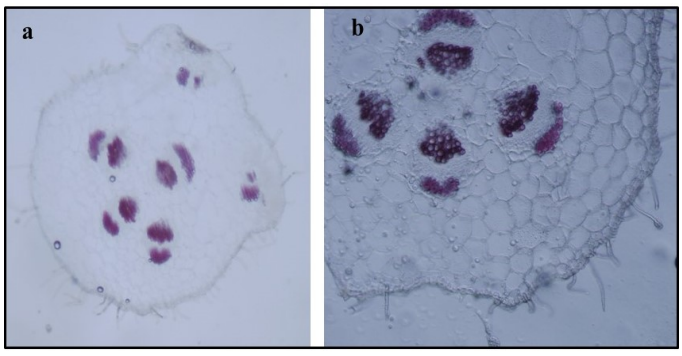

Fig. 3: Transverse section of the *T. bimucronatus* leaf rachis. a: T.S. of the whole rachis (X50), b: part of terminal cortical region and vascular bundle (X100).

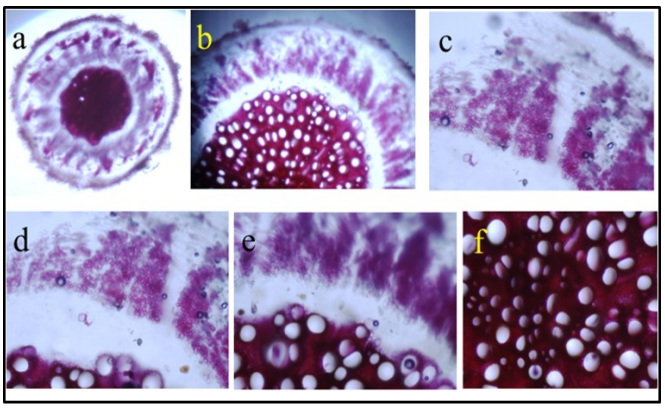

Fig. 6: Transverse section of the *T. bimucronatus* root. a: T.S. of the whole root (X50), b: part of the T.S. of the stem (X100), c: cork and cortex. d & e: cortex, phloem and xylem (X400), f: central xylem.

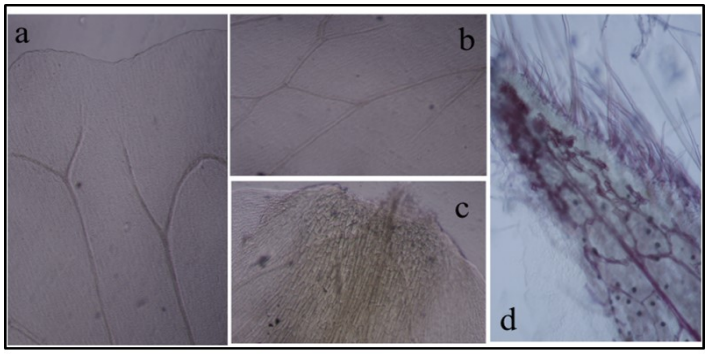

Fig. 7: Surface preparation of the *T. bimucronatus* flower's calyx & corolla
a: the tip, b: the middle, c: the base of the petal (X100), d: the sepal (X100)

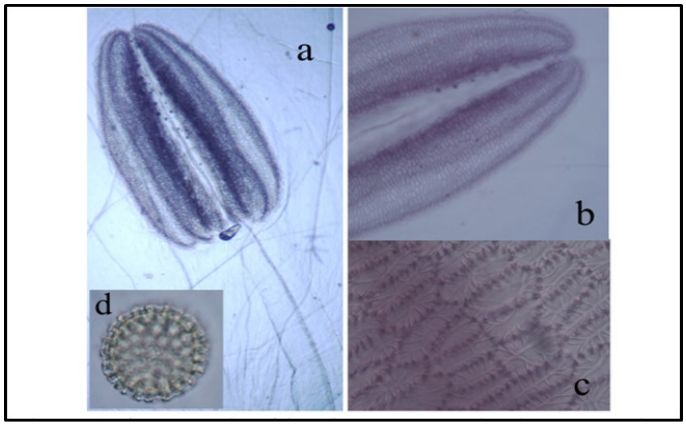

Fig. 8: Surface preparation of the *T. bimucronatus* androecium.
a: a whole stamen (X100), b: the anther lobes (X200),
c: fibrous layer of anther (X1000), d: pollen grain (X1000).

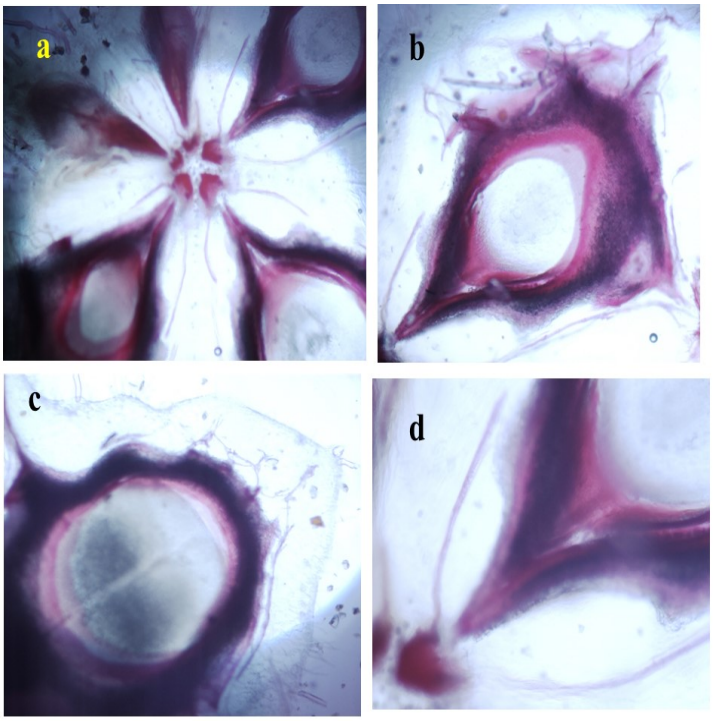

Fig. 9: Transverse section of the *T. bimucronatus* fruit. a: T.S. of the whole fruit (X50). b: T.S. of one fruitlet (X50).
c: T.S. of the terminal part of the fruitlet (X100). d: T.S. of the basal part of the fruitlet (X100).

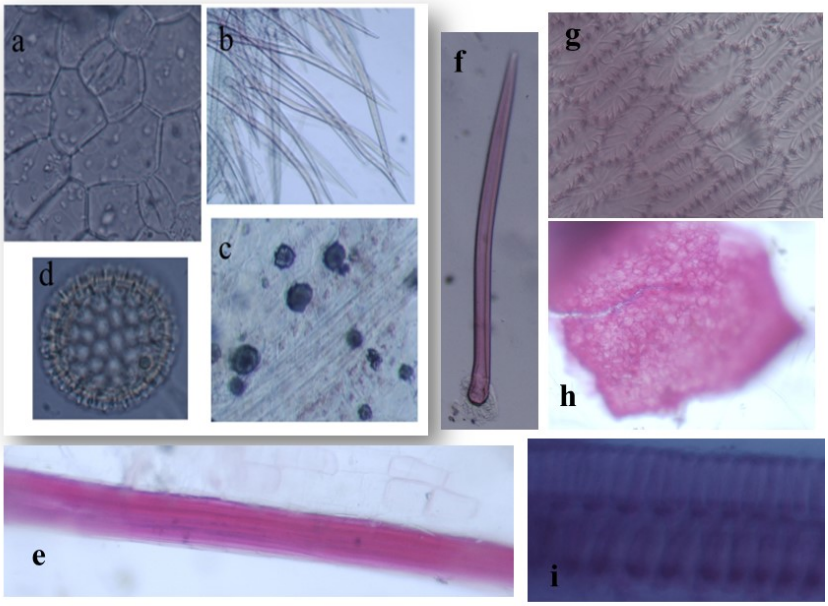

Fig. 10: Microscopic characterization of powdered aerial parts of *T. bimucronatus*,
a: epidermis showing anomocytic stomata (X1000), b: unicellular non-glandular trichomes (X100), c: cluster crystals of calcium oxalate (X100), d: spherical pollen grain (X1000),
e: lignified pericyclic fibers (X100), f: lignified trichome of the calyx (400),
g: fibrous layer of the anther (X 1000), h: sclereides of the ovary (X400),
i: spiral and annular xylem vessels (X1000).

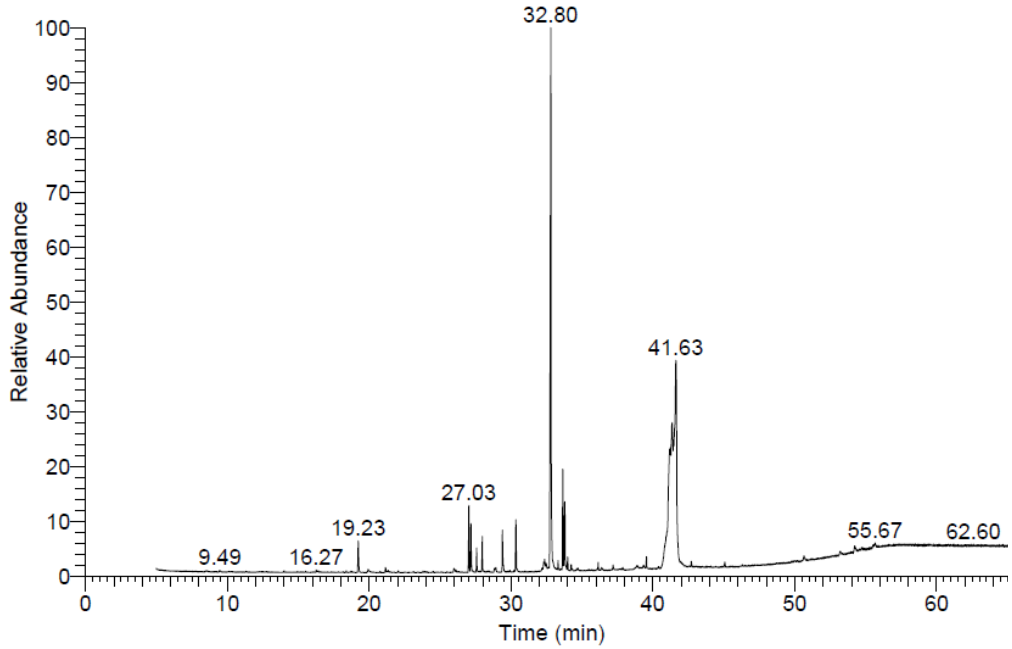

Fig.11: GC chromatogram of the unsaponifiable matter of *n*-hexane fraction of *Tribulus bimucronatus.*

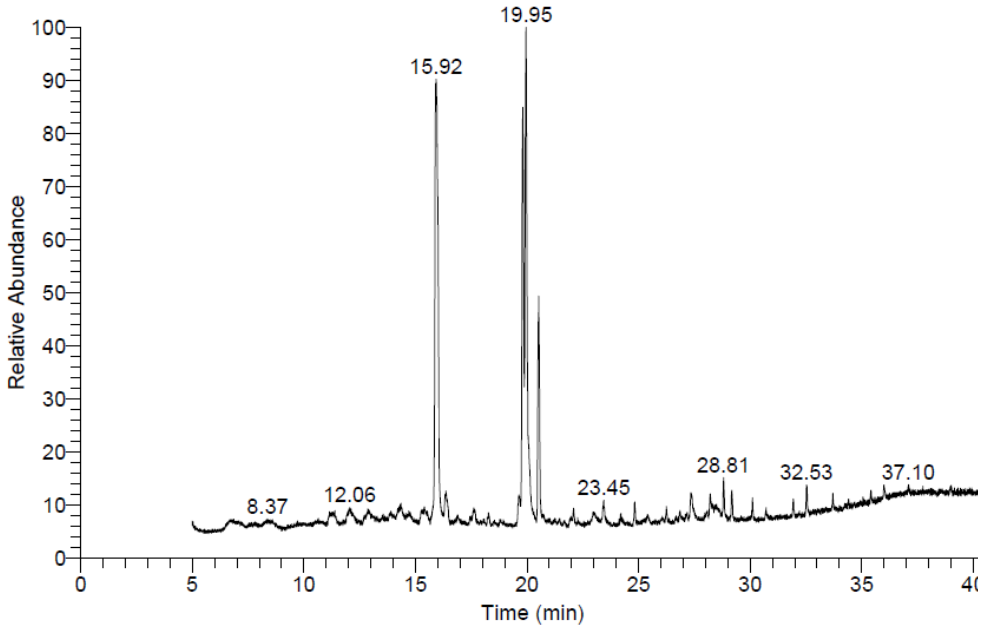

Fig.12: GC chromatogram of the saponifiable matter of *n*-hexane fraction of *Tribulus bimucronatus*

Table 1: Identified compounds of the unsaponifiable matter of *Tribulus bimucronatus*

Peak No.	Compounds	Molecular formula	Molecular weight	Rt (min)	RRt	Area %
1	Butylated Hydroxytoluene	C15H24O	276	19.23	0.58	1.80
2	**2(4H)Benzofuranone,5,6,7,7α tetrahydro 4,4,7 αtrimethyl(R)**	**C11H16O2**	**180**	**19.92**	**0.60**	**0.34**
3	1Hexadecanol	C16H34O	214	21.15	0.64	0.24
4	Neophytadiene	C20H38	278	27.02	0.82	3.45
5	2 Pentadecanone, 6,10,14trimethyl	C18H36O	268	27.17	0.83	2.68
6	Phytol, acetate	C22H42O2	338	27.97	0.85	1.93
7	Tetracosa 2,6,14,18,22 pentaene 10,11diol,2,6,10,15,19,23hexamethyl	C30H52O2	444	28.83	0.87	0.22
8	Isophytol	C20H40O	296	29.39	0.98	2.36
9	Hexadecanoic acid, ethyl ester	C18H36O2	284	30.34	0.92	2.93
10	9,12 Octadecadienoic acid, methyl ester	C19H34O2	294	32.36	0.98	0.68
11	9 Octadecenoic acid (Z),methyl ester	C19H36O2	296	32.48	0.99	0.28
12	Phytol	C20H40O	296	32.79	1.00	36.91
13	1Hexadecyn-3-ol,3,7,11,15 tetramethyl	C20H38O	294	33.32	1.01	0.48
14	Linoleic acid ethyl ester	C20H36O2	308	33.65	1.02	5.38
15	9,12,15 Octadecatrienoic acid, ethyl ester,(Z,Z,Z)	C20H34O2	306	33.78	1.03	4.81
16	Silane,[(3,7,11,15tetramethyl 2hexadecenyl)oxy]trimethyl	C23H48OSi	368	33.99	1.03	0.64
17	Octadecanoic acid, ethyl ester	C20H40O2	312	34.24	1.04	0.35
18	Nonacosane	C29H60	408	36.14	1.10	0.42
19	Cyclohexane,1,3,5trimethyl-2- octadecyl	C27H54	378	36.38	1.11	0.24
20	Cholestan-3-one,cyclic 1,2-ethanediyl acetal, (5α)	C29H50O2	430	39.34	1.19	0.26
21	(2α,3α)dihydroxy2'(3'H)oxofuro [4',5':2,3] cholestan-5α ol	C29H48O3	444	41.16	1.25	7.50
22	Lup20(29)en-3-ol,(3α)	C30H50O	426	41.33	1.26	4.59
23	4(3'Trimethylsilylethynylbenzoyl) phenyl trifluoromethane sulfonate	C19H17F3O4SSi	426	41.63	1.27	14.67
24	Squalene	C30H50	410	45.06	1.37	0.31
25	Cholest-5-en-3-ol-(3á)	C27H46O	386	50.64	1.54	0.41
26	Propanoic acid,2(3-acetoxy 4,4,14trImethylandrost-8-en-17-yl)	C27H42O4	430	55.64	1.69	0.42
Total identified compounds						93.58%
Total identified hydrocarbons (acyclic and cyclic)						4.42%
Total identified triterpenes						4.59%
Total identified steroids						8.85%
Other total identified oxygenated compounds						75.72%
Total unidentified compounds						6.42%

*RRt: Retention time relative to Phytol

Table 2: Identification of the components of the saponifiable matter of *Tribulus bimucronatus*

Peak No.	Compounds	Molecular formula	Molecular weight	Rt (min)	*RRt	Area%
1	Pentadecanoic acid, 14-methyl,methyl ester	C17H34O2	270	15.92	1.00	29.93
2	9,12 Octadecadienoic acid, methyl ester,(E,E)	C19H34O2	294	19.81	1.24	16.50
3	10 Octadecenoic acid,methyl ester	C19H36O2	296	19.95	1.25	19.11
4	Octadecanoic acid, methyl ester	C19H38O2	298	20.52	1.28	9.18
5	Eicosanoic acid,methyl ester	C21H42O2	326	24.82	1.55	0.89
6	Docosanoic acid, methyl ester	C23H46O2	354	28.81	1.80	1.13
7	Hexacosanoic acid, methyl ester	C27H54O2	410	36.00	2.26	0.63
Total identified long chain fatty acids						77.23%
Total identified saturated fatty acids						41.73%
Total identified unsaturated fatty acids						35.5%
Total unidentified compounds						22.77%

*RRt relative to Pentadecanoic acid-14-methyl, methyl ester

The flower

Surface preparation of the *T. bimucronatus* flower parts (Fig. 7& 8), the sepals are covered with lignified trichomes, while petals are free from trichomes. Each stamen of the androecium is formed of a filament and two anther lobes with characteristic lignified fibrous layer. The pollen grains are spherical with smooth exine.

The fruit

The transverse section in the fruit (Fig. 9) showed 5 indehiscent parts which are conical in shape, and each part shows one locule containing one seed. The cells of the epicarp are thin polygonal cells with straight anticlinal walls, covered with smooth cuticle and showing non glandular unicellular hairs .The mesocarp consists of several layers of polygonal thin cellulosic parenchyma cells and stone cells. Vascular strands extended through the mesocarp from the endocarp. The endocarp is formed of sclernchymatous cells which are highly thickened and lignified.

Powdered aerial parts

The powder of the aerial parts is greyish green in color with characteristic odor and slightly bitter taste. The powder (Fig. 10) is characterized microscopically by:

1. Fragments of epidermis of leaflets showing anomocytic stomata and numerous non glandular unicellular trichomes. The epidermal cells are polygonal nearly isodiametric in top view with straight anticlinal walls covered with smooth cuticle.
2. Numerous non glandular trichomes of different shapes, thicknesses and sizes (40 µm to 170 µm in length, 1-5 µm in width) which are unicellular with enlarged bases, tapering ends and covered with smooth cuticle, Hairs of the calyx are lignified.
3. Fragments of pericyclic fibers which are large fusiform with tapering ends and thick lignified walls.
4. Fragments of lignified spiral and annular xylem vessels.
5. Sclerides of the fruit endocarp and mesocarp with highly thick lignified sinuous walls.
6. Numerous cluster crystals of calcium oxalate (3-6 µm).
7. Spherical pollen grains with smooth exine (4-6 µm).

The Unsaponifiable Matter

The results of the GC/MS analysis of the unsaponifiable matter of *Tribulus bimucronatus* (Fig.11) and Table 1 revealed the presence of large number of compounds from which 26 compounds (93.58%) were identified whereas (6.42%) couldn't be identified.

The identified compounds were classified as hydrocarbons (acyclic and cyclic) (4.4%), steroids (8.85%), triterpenes (4.95%) and other oxygenated compounds (75.72%). Phytol was the major identified compound from the unsaponifiable matter (36.91%), therefore it was used for calculation of relative retention time. Phytol, which is an acyclic diterpene alcohol has antioxidant and anticancer activities [10] and functions as a precursor for vitamin E and K1.

The results of GC/MS analysis of the saponifiable matter of *Tribulus bimucronatus*

(Figure12 and Table 2) revealed the presence of a number of compounds from which 7 were identified as methyl esters of long chain fatty acids (77.23%). Total identified saturated fatty acids (41.73 %) and total identified unsaturated fatty acids (35.5%). Relative retention time was calculated relative to pentadecanoic acid-14-methyl, methyl ester (29.93%) which constitutes the major compound.

CONCLUSION

Fruits that divide at maturity into 5 indehiscent mericarps are the most significant part of this genus, *T. teresteris* is characterized by spiny mericarps [11], while *T.* alatus has winged mericarps [7, 11]. *T. bimucronatus* mericarps are spineless or wingless.

Phytol was the major identified compound from the unsaponifiable matter, while pentadecanoic acid-14-methyl, methyl ester was the major identified fatty acid methyl ester of the saponifiable matter of *n*-hexane fraction of *Tribulus bimucronatus.*

These results are reported for the first time in the species *Tribulus bimucronatus* which is a promising plant for many important pharmacological activities.

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