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African Journal of Plant Science

Full Length Research Paper

Morpho-anatomical characters of *Zehneria capillacea* (Schumach) C. Jeffrey and *Zehneria scabra* (L.F.) Sond Cucurbitaceae

Josephine Agogbua, Chimezie Ekeke* and Bosa Ebenezer Okoli

Department of Plant Science and Biotechnology, Faculty of Biological Sciences, University of Port Harcourt, P.M.B. 5323, Choba, Rivers State, Nigeria.

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Comparative studies on macro-morphology, foliar epidermis, stem and petiole anatomy of two indigenous wild cucurbits (*Zehneria capillacea* and *Zehneria scabra*) in Nigeria were carried out in order to improve the delimitation of the species based on these parameters. The morphological features of significance observed include variations in leaf and stem pubescence density, leaf shape, fruit shape and color, flower color, seed shape and coat texture. Similarities were observed in their growth habit, shape of foliar epidermal cells, stomatal features and stem anatomy. The two species evaluated have a creeping growth habit, uniseriate eglandular trichomes, anomocytic and tetracytic stomata, pentagonal shaped stem with 8 bicollateral vascular bundles. Glandular 4-celled head trichome and isotricytic stoma were only found in *Z. capillacea.* The leaves of both species are amphistomatic having adaxial and abaxial stomatal densities of 2.47±0.058 and 13.68±0.021 for *Z. capillacea* and 3.24±0.125 and 19.72±0.199 for *Z. scabra*. However, the observed petiolar vascular bundle was 5 in *Z. capillacea* and 7 in *Z. scabra*. This distinct number in the petiole provides additional distinguishing information for maintaining them as different species.

Key words: Anatomy, bicollateral vascular bundles, stomata, trichome.

INTRODUCTION

The genus *Zehneria* Endl. belongs to sub-tribe Cucumerinae of the Benincaseae together with eleven other genera (Jeffrey, 2005) and is presently restricted to those minor cucurbits with small, white or yellow, mostly monoecious flowers. There are about 35 species in the Old World tropics, extending from South Africa and Madagascar through tropical Africa and Asia to Japan, Malaysia, Australia and Polynesia (Jeffrey, 1990; De Wilde and Duyfjes, 2004). Ethno-botanical surveys showed species such as *Zehneria capillacea*, *Zehneria cordifolia*, *Zehneria minutiflora* and *Zehneria mucronata* in the Niger Delta region of Nigeria (Edwin-Wosu and Ndukwu, 2008).

In African countries, villagers generally consume leaves,

*Corresponding author. E-mail: ekeke.uc@gmail.com.

fruits and flowers of cultivated cucurbits and also harvest leaves and fruits of some wild cucurbits for consumption and medicinal use (Jansen van Rensburg et al., 2004). *Zehneria* species have enormous ethno-botanical value and are used by different tribes for food as wild edible plants and treatment of various ailments. In India, the leaves of wild cucurbits such as *Z. maysorensis* are powdered and taken with honey to kill stomach worms (Ayyanar and Ignacimuthu, 2005). The root extract of *Z. scabra* is used with milk to treat fever and diarrhea while the leaf extract is used to treat skin rashes (Anand and Jeyachandran, 2003) and has anti-bacterial and anti-inflammatory properties. Leaves of *Z. scabra* and bark and leaf of *Rumex nervosus* are pounded and rolled in cloth, and tied on swelling to reduce the effect (Amenu, 2007).

In Nigeria, roots of *Z. cordifolia* are used by herbalists to induce abortion (Chike et al., 2006), while the powdered leaves of *Z. hallii* is used for the treatment of tapeworm and as sedatives (Burkill, 1985). The leaves of *Z. capillacea* and *Z. cordifolia* are used as vegetable in soup (Edwin-Wosu and Ndukwu, 2008; Omara-Achong et al., 2012). Despite the immense economic potentials of cucurbits, information on their morphology and anatomy is either scanty or completely lacking (Okoli, 2013).

According to Stace (1980), the anatomical characterization of plants is not affected by environmental changes and the knowledge has been utilized to delimit species, genera and families in plant, this study is widely used in systematic identification, placing anomalous groups in a satisfactory position in classification and explaining patterns of relationship that may have not been clearly expressed in morphological features. Among Nigerian species of cucurbits, the uses of morphological and anatomical characters in their delimitation have been reported (Okoli 1984; Aguoru and Okoli, 2012; Agbagwa and Ndukwu, 2004; Ndukwu, 1988; Ndukwu and Okoli, 1992; Ajuru and Okoli, 2013). Also, taxonomic, cytogenetic and ethno-botanical investigations have been conducted on some of these indigenous cucurbits by researchers in the Universities and national research institutes (Okoli, 1984, 1987; Ndukwu, 2000; Ndukwu et al., 2005; Agbagwa, et al., 2007; Agbagwa and Ndukwu 2004) but reports on the anatomical features of *Zehneria* species is lacking.

This study was carried out with the aim of comparing the morphological and anatomical features of *Z. capillacea* and *Z. scabra* which are two wild cucurbits with little scientific report and to use these characters to improve the delimitation of the species.

MATERIALS AND METHODS

Experimental site

The experiment was carried out in the biosystematics and taxonomy laboratory of the Department of Plant Science Biotechnology, University of Port-Harcourt, Choba, Rivers State, Nigeria (04° 54' 29.00''; 006° 55' 02.90'').

Plant material

Fresh plant materials of *Z. capillacea* and *Z. scabra* were collected during field trips to various parts of Rivers State, Bayelsa State and the University of Port-Harcourt environment.

Morphological studies

Standard morphological descriptors for *Cucumis* germplasm prepared by The International Plant Genetic Resources Institute (IPGRI) Italy, was used to score vegetative and inflorescence characters (IPGRI, 2003). Overall morphology of the leaf, flowers and fruits were recorded by photography.

Epidermal studies

Foliar materials for epidermal studies were collected fresh from plants in the field. 5 mm – 1 cm square leaf cuttings were obtained from identical regions of each fresh leaf, generally from mid-way between the leaf base and apex of lamina including the mid-rib. The adaxial and abaxial epidermal peels were obtained using sharp pointed forceps. Peels were stained with 1% safranin or alcian blue rinsed with distilled water to remove excess stain and then mounted in a drop of pure glycerol on clean glass slides. A cover glass was placed over the drop and sealed with nail vanish to prevent dehydration (Okoli and Ndukwu, 1992). The epidermal features that were observed include: organization of the epidermis, arrangement of the epidermal cells, nature of trichomes, shape of epidermal cells and nature of the anticlinal cell wall of the leaf epidermis, stomatal types, density and index. The stomatal index (SI) was estimated based on Metcalfe and Chalk (1979) while the terminology for the stomatal type is taken after (Malvey, 2004). The mean and standard deviation were calculated using Microsoft excel 2010.

Stem and petiole anatomical studies

Specimens for anatomical analysis were obtained fresh from matured plants and fixed in FAA for 12 h. They were dehydrated, transferred to 70% ethanol and kept at room temperature until required. The stem and petiole were hand sectioned using sharp razor blades (Okoli and Ndukwu, 1992). The sections were stained in 1% safranin red for two minutes, viewed and micro-photographed using a photomicroscope.

RESULTS

The summary of the results of this study is presented in Tables 1 – 3 and Figures 1 – 4.

Habit, habitat and morphology of *Zehneria* species

The species grow in swampy, seasonal flood plain or moist soils. *Z. scabra* predominantly grows in sandy

Table 1. Morphological characteristics of *Z. capillacea* and *Z. scabra* evaluated in the wild.

Descriptors	Species	
	Z. capillaceae	***Z. scabra***
Growth habit	Indeterminate	Indeterminate
Stem pubescence	Moderate	Dense
Leaf shape	Triangular with hastate base	Triangular with cordate base
Leaf color	Dark green	Green
Fruit shape	Elliptical	Oval (Globose)
Flower color	White	Yellow
Primary fruit color	Green	Light green
Secondary fruit color	Dark green	Dark green
Ripe fruit color	Green	Red
Seed coat	Light brown	Dark brown
Seed coat texture	Smooth	Rough
Inter-node length (cm)	4 - 6	6 - 7
Petiole length (cm)	2 - 4	3 – 5

Table 2. Epidermal characteristics of *Zehneria capillaceae* and *Zehneria scabra.*

Epidermal character	*Z. capillacea*		*Z. scabra*	
	Adaxial	**Abaxial**	**Adaxial**	**Abaxial**
Shape of epidermal cell	Regular	Irregular	Regular	Irregular
Anticlinal cell wall pattern	Slightly straight or curved	Undulating but partly curved	Slightly straight or curved	Undulating
Stomata type	Anomocytic, tetracytic, isotricytic	Anomocytic, tetracytic	Anomocytic, tetracytic	Anomocytic, tetracytic
Stomata index (S.I)	2.47±0.058	13.68±0.022	3.24±0.125	19.72±0.199
Trichomes	**+**	**+**	**+**	**+**
Glandular	**+**	**+**	**-**	**-**
Eglandular	2 Type	2 Type	1 Types	1 Types

+ = Present; - = Absent.

Table 3. Anatomical characteristics of *Zehneria capillaceae* and *Zehneria scabra.*

Anatomical character	*Z. capillacea*		*Z. scabra*	
	Stem	**Petiole**	**Stem**	**Petiole**
Shape	5-angled	U-shaped	5-angled	U-shaped
No. of vascular bundles	8	5	8	7
Nature of adaxial surface	NA	U-shaped or curved	NA	V-shaped

NA = not applicable.

beaches or soils while *Z. capillacea* grows on moist soil. The qualitative descriptors scored for morphological traits in the two species are shown in Table 1. The plant habit (creeping) was similar for both species (Figure 1). However, there was phenotypic variation in the vegetative and reproductive morphology of plants evaluated. The leaf size of *Z. scabra* was visually observed to be bigger in size and more hairy than *Z. capillacea* (Figure 1a-d). The flower color is yellow in *Z. scabra* and white in *Z. capillacea*. Other observed variation include stem and leaf pubescence, leaf shape, fruit shape, fruit primary and secondary color and seed coat texture (Table 1 and Figure 1e-f).

Epidermal characters

The result of the leaf epidermal study for the two species revealed uniseriate epidermis. The two species are amphistomatic. Three stomata types were observed on the epidermal surfaces of these species. The stomata types include tetracytic (stoma completely surrounded by

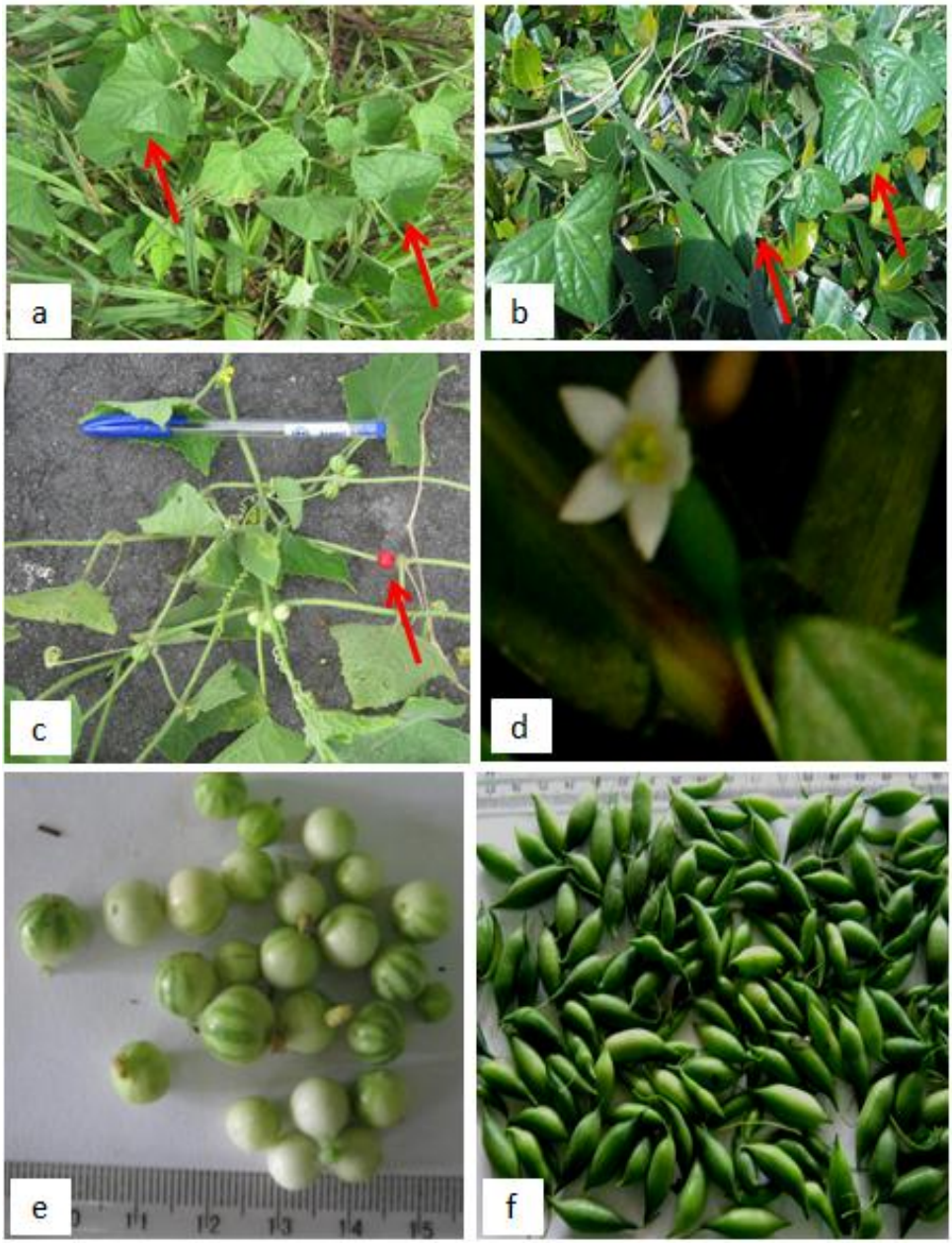

Figure 1. Habit and habitat of *Z. scabra* (a) and *Z. capillacea* (b). Arrows show the *Zehneria* species. Plate 2. Reproductive characters of *Zehneria* species: (c) *Z. scabra* showing yellow flowers, arrow shows ripe red fruit; (d) *Z. capillacea* showing white flowers; (e) Fruits of *Z. scabra* and (f) Fruits of *Z. capillacea.*

only 4 subsidiary cells, variable in size and shape, of which two are polar and two are lateral in position), anomocytic (stoma completely surrounded by only 4 or more subsidiary cells, variable in size and shape other than tetracytic and stuarocytic types) and isotricytic (stoma completely surrounded by only 3 subsidiary cells, variable in position and shape but 3 of the subsidiary cells are more or less of equal size) (Table 2). The data recorded revealed that epidermal characters such as stomatal density and stomatal index are more on the abaxial surface than the adaxial surface (Table 2). The stomatal index on the adaxial surface was 13.7±0.021 and 19.7±0.199 for *Z. capillacea* and *Z. scabra* respectively while on the abaxial surface, it was 2.47 ± 0.058

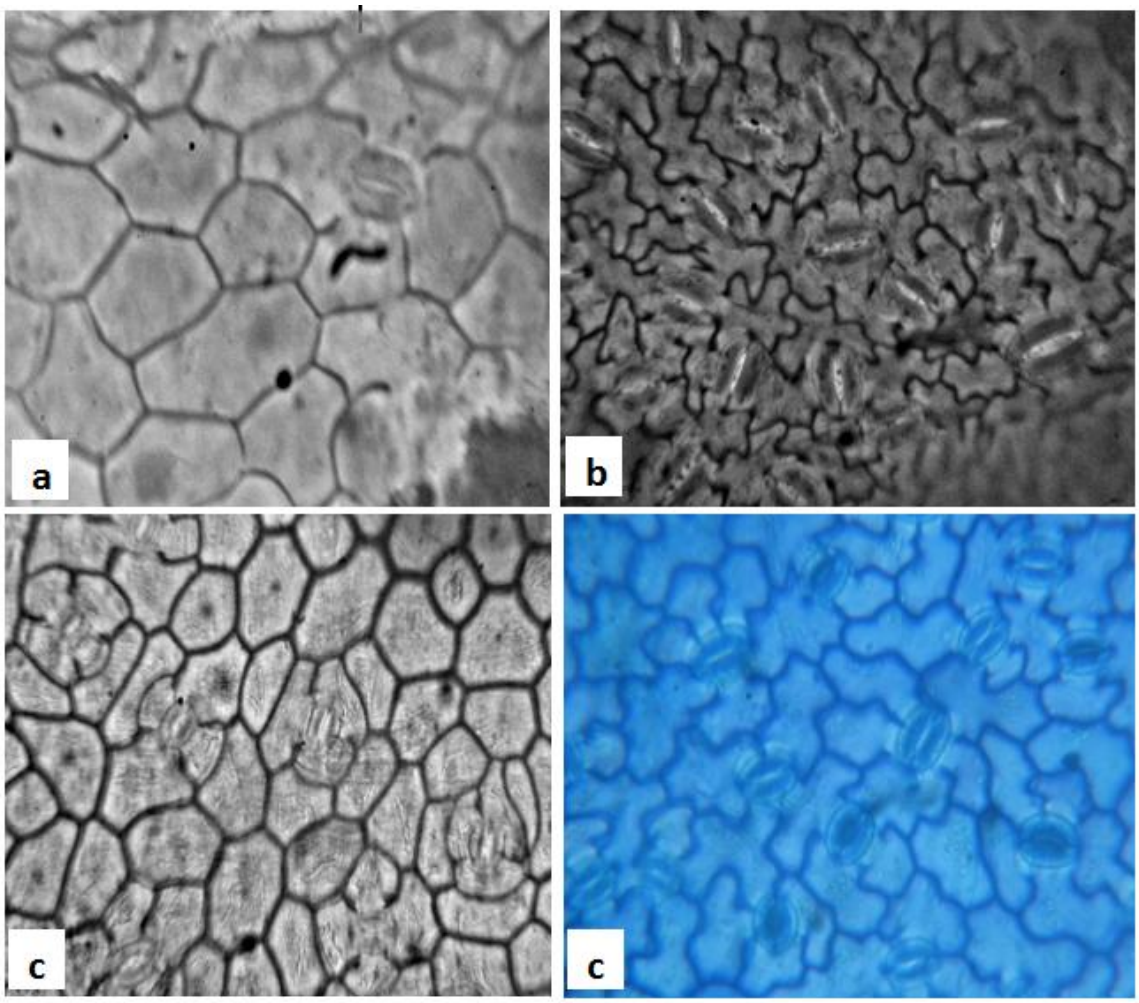

Figure 2. Epidermal features of *Zehneria* species: ((a). Adaxial surface of *Z. scabra* (x100); (b). Abaxial surface of *Z. scabra* (x100); (d). Adaxial surface of *Z. capillacea* (x40) and (e). Abaxial surface of *Z. capillacea* (x40). Note: polygonal shape of the upper epidermis and the irregular crenated shape of the lower epidermis in both species.

and 3.24 ±0.125 for *Z. capillacea* and *Z. scabra*, respectively. Variations were also observed in the shape of the epidermal cells. The abaxial cells are irregular, wavy or crenulated while the adaxial cells are more regular in shape (Figure 2).

Only uniseriate eglandular trichomes were observed in *Z. scabra* (Figure 3a) while uniseriate eglandular and multicellular glandular trichomes are present on leaf surfaces of *Z. capillacea* (Figure 3b-d). The two types of eglandular trichomes observed on the adaxial surface of *Z. capillacea* are a short thick walled eglandular trichome with an acute tip and a broad multicellular base (Figure 3c) and a short unbranched multicellular trichome (Figure 3d). The latter which is absent on the abaxial surface has 4-celled head, serrated short stalk and a spherical broad base.

Anatomical characters

The stem anatomy of the two species revealed a pentagonal-shaped or 5-angled stem with 8 bicollateral vascular bundles arranged in two major rings (Table 3 and Figure 4a-b). The five peripheral smaller bundles occur on each angle and alternate with the three bigger inner bundles which border on the pith cavity. The stem epidermal cells have uniseriate eglandular unbranched trichomes. Angular collenchyma cells are present below the epidermis and a broad band of perivascular fibres in the cortex.

Transverse section of the petiole showed single layered epidermis which consists of thin walled cells with conical uniseriate eglandular unbranched trichomes. It also revealed a free bundle vasculation pattern arranged in a

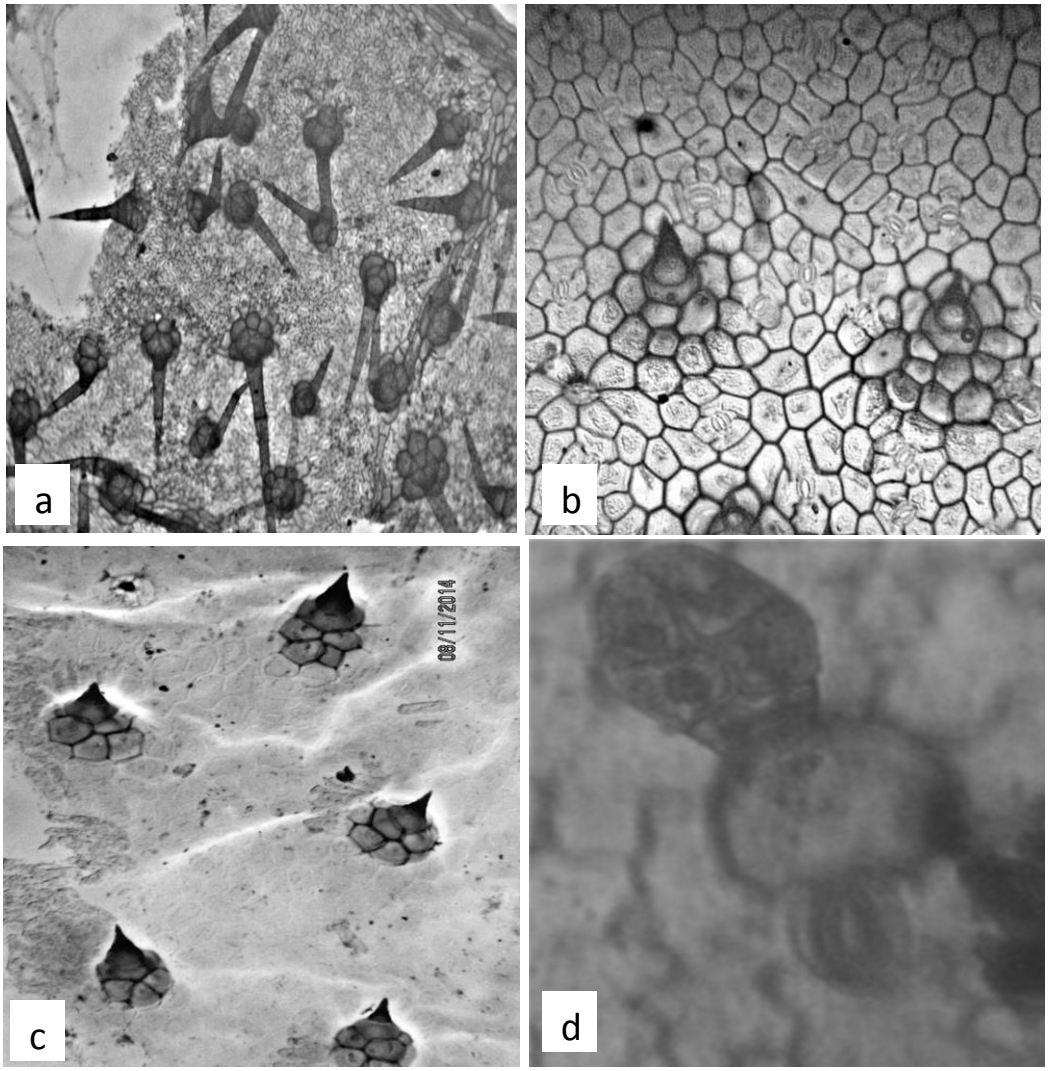

Figure 3. Trichomes in foliar epidermis of *Zehneria* species. (a) Unisariate eglandular trichome of *Z. scabra* with multicellular base; (b) Unisariate eglandular trichome of *Z. capillacea;* (c) Eglandular trichome of *Z. capillacea* with multicellular base only on adaxial surface and (d) Glandular trichome of *Z. capillacea* showing 4-celled head.

semi cylinder. There are 5 bicollateral vascular bundles present in *Z. capillacea* (Table 3 and Figure 4c) *Z. scabra* had 7 vascular bundles (Figure 4d). The abaxial surface of *Z. scabra* is V-shaped while that of *Z. capillacea* is curved or U-shaped (Figure 4c-d).

DISCUSSION

Most curcubits are dioecious (male and female flowers on different plants) and rarely monoecious (male and female flowers on the same plant). For example, *Citrullus lanatus* Thunb (watermelon) and *Cucurbita pepo* L. (pumpkin) are dioecious (Agbagwa and Ndukwu, 2004), *Cucumis sativus* L. (cucumber) are monoecious (Ndukwu, 1988). Then, there are some special plants like limon-cetriolo, lemon-cucumber which has staminate (male) flowers but also hermaphroditic (Ndukwu and Okoli, 1992). Some cultivars of watermelon have male, female and hermaphrodite flowers on the same plant (Okoli, 1984). Flowers are small, white or yellow, monoecious or dioecious, rarely hermaphroditic. Male flowers are solitary or few to many in sessile or pedunculate racemiform or umbelliform clusters. The fruit is solitary or clustered, globose, ellipsoid or fusiform, red, whitish or green, smooth, sometimes finely pitted when dry (Hutchinson and Dalziel, 1954). The seeds are small, elliptic to broadly ovate in outline, compressed or flat (Hutchinson

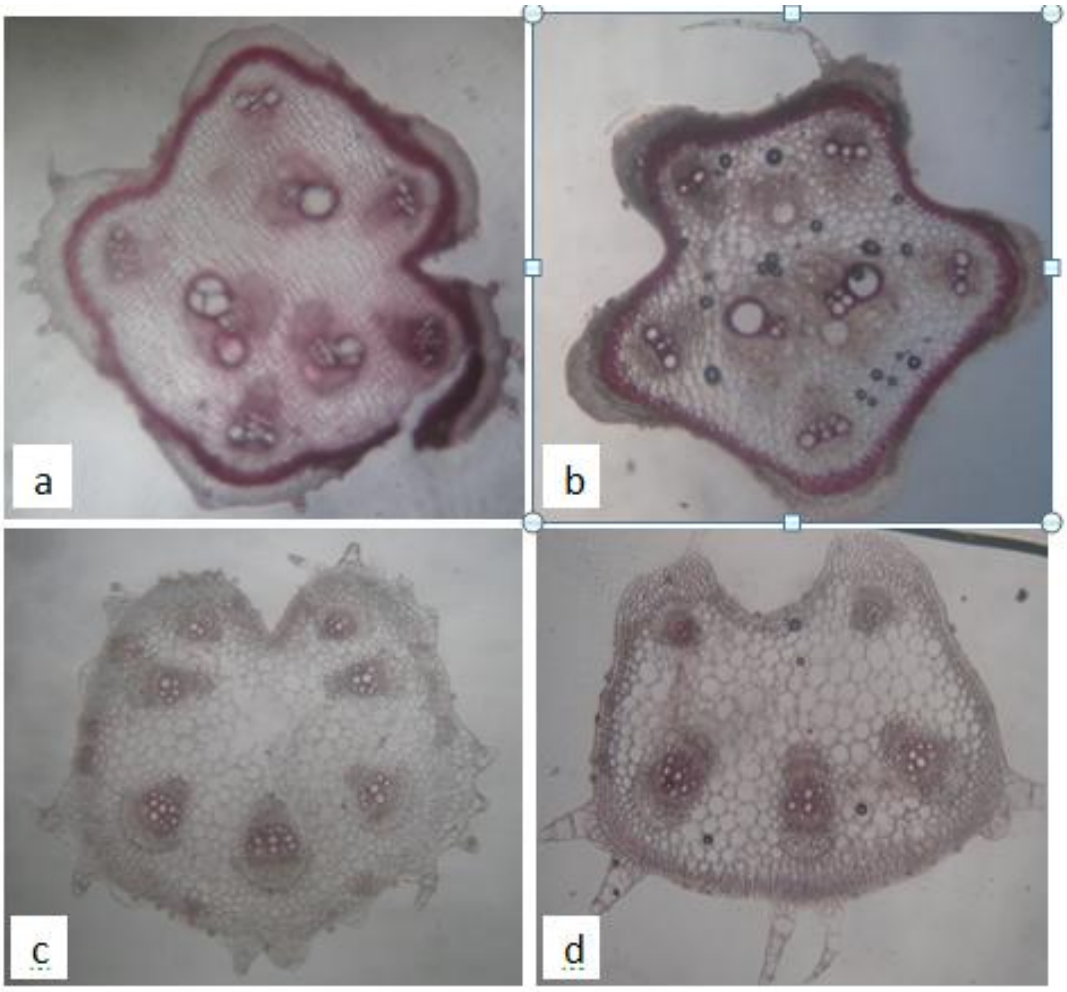

Figure 4. Anatomical features of transverse section (TS) of *Z. capillacea* and *Z. scabra*: (a) Stem of *Z. scabra*; (b) Stem of *Z. capillacea*; (c) Petiole of *Z. scabra* (V-shape of abaxial surface) and (d) Petiole of Z. *capillacea* (curved or U-shape of abaxial surface) Note: 5-angled (pentagonal) stem with similar vascular bundles (a and b) and the variation in the petiole anatomy and number of vascular bundles (c and d).

and Dalziel, 1954; Okoli 1984; Ndukwu, 1988; Ndukwu and Okoli, 1992; Jeffrey, 1990).

The variations that exist between the two species of *Zehneria* is of taxonomic value as observed in this study. The plant trailing growth habit, ecology, habitat and the vegetative morphological traits described are consistent with the description reported by Jeffrey (1990) but in contrast with the color of *Z. capillacea* fruits which was reported as red instead of green as observed in the current study. *Z. scabra* occurred predominantly in sandy soils while *Z. capillacea* can grow in any soil type.

The leaves of the species are amphistomatic with anomocytic, tetracytic and isotricytic types of stomata. Anomocytic type of stomata was the dominant stomata type observed in the species. It has been described in many plant species of the Cucurbitaceae (Okoli, 1989) and it is the most common in angiosperms. However, isotricytic stoma was found only on the adaxial epidermal surface of *Z. capillacea.* This feature is distinctive and can be used to distinguish among the species studied. Furthermore, there are more stomata on the abaxial than the adaxial surface. This is expected since is in line with reports of Adebooye et al. (2012) on *Trichosanthes cucumerina*. A type of uniseriate eglandular trichome and one type of glandular trichome with 4-celled head were identified in *Z. capillacea* while only uniseriate eglandular trichome was observed in *Z. scabra*. The occurrence and types of these trichomes could be used to distinguish the species. For instance, the 4-celled head glandular trichome was only observed on the abaxial surface of *Z. capillacea.* Also, uniseriate eglandular trichome was found in both species but the ones in *Z. capillacea* are

short and thick, while the ones in *Z. scabra* are long and thin. This is consistent with the different types of glandular and eglandular trichomes that have been studied and described in cucurbits (Okoli, 1989; Kolb and Muller, 2004; Aguru and Okoli, 2012; Agbagwa and Ndukwu, 2001).

Morphology of plants is an important factor used in making useful taxonomic conclusion about plants but it cannot be solely used. Anatomical feature is also of great importance in taxonomy since they are less affected by environmental factors. In this study, the fruit shape and size, the vascular system of the petiole, the presence of isotricytic stoma and trichome types are all diagnostic. The diagnostic features of the two species of *Zehneria* therefore as belonging to the family Cucurbitaceae include the presence of bicollateral vascular bundles and arrangement of the vacsular bundles in two rows. The observed anatomical similarities among the *Zehneria* species studied indicate phylogenetic relatedness of the taxa. The anatomical differences observed in each specie must have been as a result of evolution, conferring heritable variation that could be exploited for taxanomic purposes.

Conclusion

Two species share the same ancestral gene pool as they have most characteristics in common. Based on anatomical features, the two species of *Zehneria* studied can be distinguished from one another based on morphological traits and variation in the number vascular bundles present in the petiole. This information is useful in identification and authentication of the species.

Conflict of Interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENT

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REFERENCES

Adebooye OC, Hunsche M, Noga G, Lankes C (2012). Morphology and density of trichomes and stomata in *Trichosanthes cucumerina* (Cucurbitaceae) as affected by leaf age and salinity. Turk. J. Bot. 36:328-335.

Agbagwa IO, Ndukwu BC (2001). Epidermal Micro-morphology of *Cucurbita* L. Species in Nigeria. J. Appl. Sci. Environ. Mgt. 5(2):59-64.

Agbagwa IO, Ndukwu BC (2004). *Cucurbita* L. Species in Nigeria Under-exploited Food and Vegetable Crops. Niger Delta Biologia, 4(2):11-15.

Agbagwa IO, Ndukwu BC (2004). *Cucurbita* L. species in Nigeria: Underexploited food and vegetable crops. Niger Delta Biologia, 4(2):11-15.

Agbagwa IO, Ndukwu BC, Mensah SI (2007). Floral biology, breeding system and pollination ecology of *Cucurbita moschata* (Duch. Ex Lam) Duch. Ex Poir. Varieties (Cucurbitaceae) from parts of the Niger Delta, Nigeria. Turkey J. Bot. 31:451-458.

Agbagwa IO, Okoli BE, Ndukwu BC (2007). Comparative Anatomy of *Abrus* Adanson species in Parts of Tropical West Africa. Asian J. Plant Sci. 6:732-740.

Aguoru CU, Okoli BE (2012). Comparative stem and petiole anatomy of West African species of *Momordica* L (Cucurbitaceae). Afr. J. Plant Sci. 6(15):403-409.

Ajuru MG, Okoli BE (2013). Comparative Vegetable Anatomy of some Species of the Family Cucurbitaceae Juss in Nigeria. Res. J. Bot. 8:15-23.

Amenu E (2007). An Ethnobotanical Approach. A Thesis submited to The school of Graduate Studies, Addis Ababa Ethiopa. 104p.

Anand SP, Jeyachandran R, Xavier FT (2003). Antibacterial activities of *Zehneria scabra* root extracts. J. Trop. Med. Plants 4(2):173-179.

Ayyanar M, Ignacimuthu S (2005). Traditional knowledge of Kani tribal's in Kouthalai of Tirunelveli hills, Tamil Nadu, India. J. Ethnopharmacol. 102:246-255.

Berrie GK, Berrie A, Eze JMO (1987). Tropical Plant Science. Longman Scientific and Technical, UK. Botany 5:18-26.

Burkill HM (1985). The useful plants of West Tropical Africa. 2nd ed. Vol. 1. Families A-D. Kew, UK : Royal Botanical Gardens, p.960.

Chike CPR, Dapper DV, Odee P (2006). Possible abortifacient effect of *Zehneria cordifolia* methanol extract on female Wistar rat. Afr. J. Appl. Zool. Environ. Biol. 8:140-145.

Clive AS (1980). Plant taxonomy and Biosystematics, Paper back.

De Wilde WJJO, Duyfjes BEE (2004). *Zehneria* (Cucurbitaceae) in Thailand, with a note on the Indian *Zehneria maysorensis*. Thai For. Bull. Bot. 32:17-18.

Edwin–Wosu NL, Ndukwu BC (2008). The Ethno-Botany and utility evaluation of some Species of Cucurbits among the people of Niger Delta Nigeria. Global J. Pure Appl. Sci. 14(3):279-284.

Hutchinson J, Dalziel JM (1954). Flora of West Tropical Africa. Crown Agents, London UK.

IPGRI (2003). Descriptors for Melon (*Cucumis melo* L.). International Plant Genetic Resources Institute, Rome, Italy.

Jansen van Rensburg WS, Venter SL, Netshiluvhi TR, van den Heever E, Vorster HJ, de Ronde JA (2004). Role of indigenous leafy vegetables in combating hunger and malnutrition. South Afr. J. Bot. 70(1):52-59.

Jeffrey C (1990). An outline classification of the Cucurbitaceae. In: Bates DM, Robinson RW, Jeffrey C (eds.), Biology and utilization of the Cucurbitaceae. Cornell University Press Ithaca, NY. pp. 449-463.

Jeffrey C, (2005). A new system of Cucurbitaceae. Bot. Zhurn 90:3332-3335.

Kolb D, Müller M (2004). Light, conventional and environmental scanning electron microscopy of the trichomes of *Cucurbita pepo* subsp. *pepo* var. *styriaca* and histochemistry of glandular secretory products. Ann. Bot. 94:515-526.

Malvey P (2004). Structure, nomenclature and classification of stomata. Acta Bot. Sin. 44(2):242-252.

Metcalfe CR, Chalk L (1979). Anatomy of the Dicotyledons. 2nd Edn., Clarendon Press, Oxford, UK. P 276.

Ndukwu BC (1988). Morphological, anatomical and cytological studies on some lesser known Nigerian Cucurbits. M.Sc. Thesis, University of Port Harcourt. Nigeria.

Ndukwu BC, Obute GC, Wary-Toby IF (2005). Tracking sexual dimorphism in *Telfairia occidentalis* Hooker F. (Cucurbitaceae) with morphological and molecular markers. Afr. J. Biotechnol. 4(11): 1245-1249.

Ndukwu BC, Okoli BE (1992). Studies on Nigerian *Curcurbita moschata*. Nig. J. Bot. 5:18-26.
Okoli BE (2013). Fluted Pumpkin, Telfairia-The Under-Exploited Golden Treasure. University of Port Harcourt Press, Nigeria.
Okoli BE (1984). Wild and cultivated cucurbits in Nigeria. Econ. Bot. 38:350-357.
Okoli BE (1987). Anatomical studies in the leaf and probract of *Telferia* Hooker (Curcurbitaceae). Feddes Repertorium, 98:231-236.
Okoli BE (1989). SEM study of surface characteristics of the vegetative and reproductive organs of *Telferia* (Cucurbitaceae). Phytomorphol. 39:103-108.
Omara-Achong TE, Edwin-Wosu NL, Edu EA, Nkang AE, (2012). Survey of indigenous vegetables species in parts of Ogoja and Calabar, Cross River State, Nigeria. Eur. J. Exp. Bio. 2(4):1289-1301.

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Participatory variety selection of okra (*Abelmoschus esculentus* L.) genotypes for adaptation to the semi-arid agro-ecology of Northern Ghana

Sugri I.[1]*, Abdulai M. S.[1], Larbi A.[2], Hoeschle-Zeledon I.[3], Kusi F.[1] and Agyare R. Y.[1]

[1]CSIR-Savanna Agricultural Research Institute, P.O. Box 52, Tamale, Ghana.
[2]International Institute of Tropical Agriculture, Africa RISING Project, P. O. Box TL6, Tamale, Ghana.
[3]International Institute of Tropical Agriculture, Oyo Road, Ibadan, Oyo State, Nigeria.

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In a two-year study, participatory variety selection (PVS) was employed to evaluate the performance of 19 okra (*Abelmoschus esculentus* L.) c. v. Moench genotypes in a semi-arid agro-ecology in northern Ghana. The PVS sessions were organized at 65, 80 and 95 days after planting for 272 farmers to select the most preferred genotypes base on plant growth, maturity period and yield components. Results of this study indicate high significant differences among the genotypes (*P< 0.0001*) for most essential agronomic yield traits evaluated. Five most recurring preference traits were high fruit yield, early maturing, multiple harvest frequency, drying quality and long tender-life. Glabrous leaf, stem and fruit were important to farmers because most of the production operations are still being manually done. Genotypes with high potential included: NOKH 1004 (9.55 ton\ha), FV-Unn-manna (5.85 ton\ha), NOHK 1003 (5.17 ton\ha), NOKH 1002 (2.83 ton\ha), FV-Kpazeya (2.83 ton\ha), TZ-SMN-86 (2.30 ton\ha), AAK (2.11 ton\ha), NB-55-Srivan (1.98 ton\ha), Sasilon (1.70 ton\ha), FV-Unn (2.20 ton\ha) and Ex-Makutopora (1.27 ton\ha). Genotypes Sasilon, NOKH-1004, NB-55-Srivan and NOKH-1003 recorded the highest ranking among farmers during the PVS. High yielding and early maturing genotypes which are amendable to drying may show wide adoption rate due to the premium on dehydrated okra. These genotypes can be suggested as candidates for inspection by the National Variety Release and Technical Committee for release to farmers to increase access to improved okra varieties.

Key words: Okra genotypes, Sudan savanna, drying quality, participatory variety selection, preference traits.

INTRODUCTION

Okro (*Abelmoschus esculentus* L.) c.v. Moench is a traditional vegetable in many tropical, subtropical and Mediterranean countries (Düzyaman, 2005; Arapitsas, 2008; Saifullah and Rabbani, 2009). Every part of the

*Corresponding author. E-mail: isugri22@gmail.com. Tel: +233243850292.

plant (leaf, flower and fruit) is consumed either in the fresh or dehydrated form. The young immature pods are consumed in various forms: boiled, fried, cooked or dehydrated for future utilization. Seven-day old fresh pods have the highest concentration of nutrients (Ndunguru and Rajabu 2004; Agbo et al., 2008). The fresh pods are low in calories (20/100 g), practically no fat, high in fiber, and have several valuable nutrients such as 30% of the recommended levels of vitamin C (16-29 mg), 10-20% of foliate (46-88 mg) and about 5% of vitamin A (14-20 RAE). Both mesocarp and seeds are excellent sources of zinc (80 mg/g) as well as phenolic compounds with important biological properties like quartering derivatives, catechins, oligomers and hydroxycinnamic derivatives (Glew et al., 1997; Arapitsas, 2008). In Ghana, okra is the fourth most popular vegetable after tomato, pepper and garden egg (Oppong-Sekyere et al., 2012). It is often the vegetable of choice among rural and urban consumers, and even at food joints. National production hovers around 120,000 Mt produced on 19,500 ha of arable land with yield potential of 5.5 Mt\ha. Okra production serves an important source of cash to farmers particularly in the dry season where commercial production is carried out using smallholder irrigation schemes such as dug-outs, small dams and along river banks. On arable lands, they may appear as a sole, inter or boarder crop. In northern Ghana, okra production has a strong commercial value particularly to rural women farmers, where both fresh and dehydrated products are sold to supplement household income (Opong-Sekyere et al., 2012).

A review of okra seed systems suggests that breeding programmes' has only been carried out in a limited scale by the commercial sector (Kumar et al., 2010; Benchasri, 2012; Opong-Sekyere et al., 2012). In particular, seed programmes to enhance access to improved genotypes are not well developed in most developing countries. Most crop improvements efforts over the years have been oriented towards intensive cultivation using high yielding, short duration, pests and disease resistance as well as wide adaptation to photoperiod insensitivity (Benchasri, 2012). Genotypes characterization have been typically to identify high yielding genotypes with resistance to yellow mosaic virus, fruit borers, jassid and higher vitamins C content in species that can be utilized for the improvement of *A. esculentus* (Bisht and Bhat, 2006; Nwangburuka et al., 2011). However, some traits of aesthetic and sensory qualities of essence to end-users still require some research. Considerable effort is currently being made in breeding programmes to improve yield attributes such as seed yield, number of pods per plant, pod length, width and shape (Alam and Hossain, 2008). Although some attractive American and Indian cultivars have found their way to commercial growers in some parts of Africa, there is still enormous scope for cultivar improvement. Crossing between promising parents combined with pedigree selection or backcrossing remains the most common breeding procedure (Benchasri, 2012). However, the quest for high pod yield per plant is limited due to low genotypic variability (Ariyo, 1990; Nwangburuka et al., 2011). Up to now, molecular markers analyses have shown rather low level of genetic diversity in cultivated okra in spite of large phenotypic variability. Quiet scanty information exist on improvement using biotechnology apart from *in vitro* DNA extraction and plant regeneration from various explants and callus tissue. Thus, opening up the need to prioritize research strategies to amplify the potential of okra for food, nutrition and income security to both commercial and smallholder farmers is essential.

In most parts of Ghana, the existing okra cultivars are land races which have been recycled for many decades. Some of these landraces are late maturing and photoperiod-sensitive with low marketing and export potential; due to fruit color, shape and pubescence. However, some of the landraces have multi-purpose traits such as high yielding, good drying properties and resistance to drought, diseases and pests. Most of the improved cultivars and hybrid seed introduced to many parts of Ghana are not widely adapted by farmers due to their relative susceptibility to some biotic and abiotic stresses, and some are not also amendable to drying. Thus, requiring interventions to increase access to improved seed, and scale-up improved production to postharvest technologies to increase production, availability and utilization as well as economic returns to farmers. Selection for genotypes with good drying properties in particular could reduce current postharvest losses in okra. Accordingly, the Savanna Agricultural Research Institute (SARI) of the Council for Scientific and Industrial Research (CSIR) in collaboration with the World Vegetable Centre (AVRDC) have been providing training and information on good vegetable production practices; collecting, characterizing and establishing database of various traditional African vegetables; and developing extra-early high yielding vegetable varieties and hybrids to growers. Most of the previous okra selection studies have been conducted on-station with little involvement of the growers and end-users. This study involved the use of participatory variety selection to evaluate the agronomic performance of 19 genotypes in a semi-arid Sudan-savanna agro-ecology in northern Ghana. This approach enabled the identification of some essential traits to growers and end-users that can be incorporated into future okra improvement programmes.

MATERIALS AND METHODS

Study site

The study was carried out during the 2012 and 2013 cropping seasons at the Manga Agriculture Station (Latitude 11° - 01' N,

Table 1. Some physical and chemical properties of the surface (0-15 cm) soil at the experimental site at the Manga Agricultural Station and; weather characteristics in the years, 2012 and 2013.

Soil physical and chemical properties		Month	Average Year : 2012			Average Year: 2013		
			Rainfall (mm)	Temperature (°C)	Relative humidity (%)	Rainfall (mm)	Temperature (°C)	Relative humidity (%)
Sand (%)	80.4	June	150	27.7	79.0	128.4	29.0	75.0
Silt (%)	14	July	256.1	27.2	83.0	184.4	27.2	84.0
Clay (%)	5.6	August	214.8	26.5	73.0	161.4	26.8	84.0
Soil texture	Loamy sand	September	222.8	27.7	68.0	192.5	25.3	75.0
Soil pH (H_20)	4.22	October	22.0	26.8	52.0	42.7	27.6	73.0
Organic carbon (%)	0.47							
Total nitrogen (%)	0.06							
Available P (mg/ kg)	20.25							
Ca	0.08							
Mg	0.04							
K	27.30							
CEC [cmol (+)/ kg]	4.55							

Longitude 00° - 16° W, and elevation of 249 m above sea level) of the CSIR-SARI near Bawku in the Upper East region of Ghana. The study area is in the Sudan savanna agro-ecological zone with annual rainfall of approximately 950 mm. The rainfall distribution, which is mono-modal, peaks between August and September, and ends in October. The soils, which are of Plinthic lixisol classification and developed from granite and stones, are light, varying in texture from coarse sands to loams. Table 1 describes some physical and chemical properties of the surface (0-15 cm) soil at the experimental site at the Manga Agricultural Station and; some weather characteristics in the years of 2012 and 2013.

Morphological and agronomic data collection

Nineteen okra genotypes, consisting of 13 improved genotypes (Sasilon, P1496946, TZ-SMN-86, ML-OK-37, ML-OK-16, ML-OK-35, ML-OK-10, AAK, NOKH 1002, NOKH 1003, NOKH 1004, NB-55-SRIVAN and Ex-Makutopora) and 6 landraces, were evaluated. Planting was done on July 17, 2012 and June 30, 2013 at a spacing of 75 cm between ridges and 30 cm apart on a ridge. In both years, planting was done under rain-fed conditions, and seedlings were thinned to 1-3 plants per stand at 2 weeks after germination. Plots were not sprayed with insecticides. Agronomic data collected included: plant establishment, plant height, days to flower bud nodulation, days to 50% flowering and fruiting stages, stem diameter, branching habit and fruit yield components. Basic morphological characteristics of the genotypes were described using qualitative descriptors such as: color, shape and texture of main stem, leaf and fruits. Leaf and fruit shape characteristics were described using reference descriptors for okra (IBPGR, 1991) as shown in Figure 1.

Farmers field schools and participatory variety selection

Three sessions of farmer field schools (FFS) and participatory variety selection (PVS) were organized at 65, 80 and 95 days after planting (DAP) for a total of 272 farmers from 9 major vegetable producing communities (Table 2). During FFS, participants were taken through good vegetable production practices from nursery and field management to post-harvest and marketing operations.

During PVS, farmers were asked to select their preferred genotype using traits such as plant vigor, architecture, yield, harvesting frequency, viscosity, taste, color, drying quality and marketing potential. The PVS processes enable farmers to identify genotypes they preferred as well as traits that are critical to end-users.

Data analysis

The agronomic data were analyzed as a randomized complete block design with genotypes over years using GenStat (9^{th} Edition) statistical package. Differences between treatment means were separated by least significant difference at 5% level of probability. Pearson's correlation analyses between pairs of agronomic parameters were performed using Statistix (Edition 9.1). Data sets from the FFS and PVS sessions were analyzed

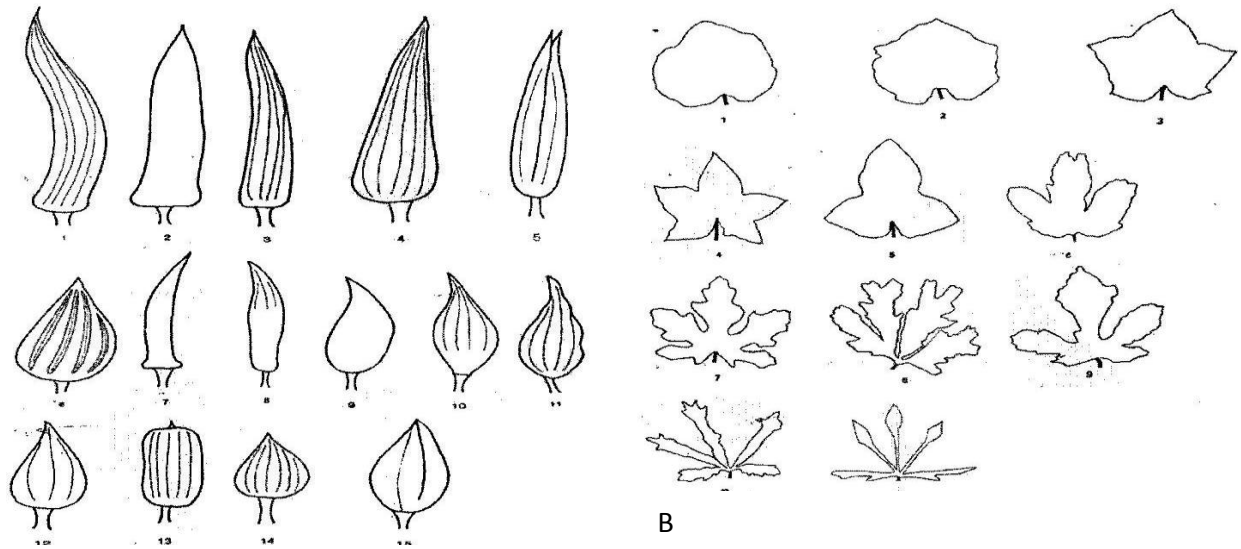

Figure 1. Reference descriptors for fruit and leaf shapes of okra genotypes. A: variability in fruit shape (IBPGR, 1991); B: variability in leave shape (IBPGR, 1991).

Table 2. Number of farmers attending the farmer field schools and participatory variety selection sessions.

District	Community	Number of farmers in attendance		
		Male	Female	Total
Binduri	Nayorko 1	5	20	25
Binduri	Boku	5	20	25
Binduri	Azum-Sapielga	5	20	25
Bawku municipal	Nyorigu	5	20	25
Bawku municipal	Mognori	10	20	30
Bawku municipal	Tampezua	10	20	30
Kassena-Nankana East	Bado	6	16	22
Kassena-Nankana East	Tekuru	15	30	45
Kassena-Nankana East	Bonia	10	25	35
Total = 3	**9**	**81**	**191**	**272**

using descriptive statistics involving frequencies and percentages.

RESULTS

Morphological and agronomic yield components

Table 3 shows some morphological characteristics of the genotypes. Most genotypes had erect growth and sparse branching habits with green stem, leaf and fruit color. Leaf and fruit shape varied widely among the genotypes. Two morphological features of interest to farmers were stem, leaf and fruit pubescence; and plant height (Figure 2). For instance, height at flowering and fruiting were of significance because tall plants with thin stems are often susceptible to lodging, which has influence on dry matter, disease incidence, fruit yield and quality. Glabrous leaf, stem and fruit pubescence were also critical to farmers as weeding and harvesting operations are still manually being done.

Plant establishment, height at harvest, days to 50%flowering and fruiting stages, and fresh fruit yield varied significantly ($P<0.0001$) among the genotypes (Table 4). Using the number of days to 50% flowering and fruiting stages as criteria for earliness, the genotypes can be grouped into three maturity classes, namely: extra-early-maturing genotypes attaining 50% flowering by 40-50 days after planting (DAP) and 50% fruiting at 55-65 DAP, e.g., genotypes NOKH 1002, NOKH 1003, NOKH 1004 and NB-55-Srivan; early-maturing genotypes attaining 50% flowering at 51-65 DAP and50% fruiting at

Table 3. Description of some morphological characteristics of the genotypes.

Variety	Growth habit	Branching habit	Stem color	Stem pubescence	Leaf color	Leaf pubescence	Leaf shape	Fruit color	Fruit pubescence	Fruit shape
Sasilon	Erect	Sparse	Red	Intermediate	Green, PV	Intermediate	3	Red, GV	Intermediate	1
TZ-SMN-86	Erect	Sparse	Red	Intermediate	Green, PV	Intermediate	7	Red, GV	Intermediate	1
P1496946	Erect	Sparse	Green	Intermediate	Green, PV	Intermediate	3	Green	Hairy	6
AAK	Erect	Sparse	Green	Hairy	Green	Hairy	9	Green	Intermediate	1
Ex-Makutopora	Erect	Sparse	Green, PV	Intermediate	Green, PV	Intermediate	9	Green, PV	Intermediate	3
NB-55-Srivan	Erect	Sparse	Green	Hairy	Green	Hairy	9	Green	Intermediate	1
ML-OK-10	Erect	Sparse	Green	Hairy	Green, PV	Intermediate	4	Green, PV	Intermediate	3
ML-OK-16	Erect	Sparse	Green, PV	Intermediate	Green	Glabrous	5	Green, PV	Intermediate	3
ML-OK-35	Erect	Sparse	Green	Hairy	Green	Intermediate	4	Green,	Intermediate	7
ML-OK-37	Erect	Sparse	Green	Intermediate	Green	Hairy	9	Ash-green	Intermediate	2
NOKH 1002	Erect	Sparse	Green	Intermediate	Green	Glabrous	9	Green	Intermediate	1
NOKH1003	Erect	Sparse	Green	Hairy	Green	Intermediate	10	Green	Intermediate	1
NOKH 1004	Erect	Sparse	Green	Hairy	Green	Intermediate	9	Green	Intermediate	1
FV-Unn	Erect	Sparse	Green	Hairy	Green	Hairy	9	Green	Hairy	4
FV-Unn manna	Erect	Sparse	Green	Hairy	Green	Hairy	3	Green	Hairy	4
FV-Kpazeya	Erect	Sparse	Green	Hairy	Green	Intermediate	3	Green	Hairy	4
FV-Kpora napong	Erect	Sparse	Green	Hairy	Green	Hairy	7	Green	Intermediate	8
FV-Kpora nasong	Erect	Sparse	Green	Hairy	Green	Hairy	5	Green	Intermediate	3
FV-Shie manna	Erect	Sparse	Green	Hairy	Green	Hairy	5	Ash-green	Intermediate	8

Description of leaf and fruit shape variability was done using reference descriptors for okra (IBPGR, 1991) as shown in Figure 1; PV: with purple vernation; GV: with green vernation.

60-70 days, e.g., genotypes Sasilon, TZ-SMN-86, AAK, ML-OK-37 and Ex-Makutopora; and the late-maturing group which attained 50% flowering 66 DAP. Genotypes with high potential included: NOKH 1004 (9.55 ton\ha), FV-Unn-manna (5.85 ton\ha), NOHK 1003 (5.17 ton\ha), NOKH 1002 (2.83 ton\ha), FV-Kpazeya (2.83 ton\ha), TZ-SMN-86 (2.30 ton\ha), AAK (2.11 ton\ha), NB-55-Srivan (1.98 ton\ha), Sasilon (1.70 ton\ha), FV-Unn (2.20 ton\ha) and Ex-Makutopora (1.27 ton\ha). Correlation analysis of some agronomic yield components of the genotypes is provided in Table 5. The correlation analyses showed significant, high ($r > 0.78$), positive correlations between fresh fruit yield and fruit counts at 60, 90 and 120 days. Thus, days to flower bud nodulation, flowering and fruiting which translate into early harvesting from 60 DAP had significant association with yield.

Consumer preference traits

Genotypes Sasilon, NOKH-1004, NB-55-Srivan and NOKH-1003 recorded the highest ranking from the different PVS sessions (Table 6). Traits influencing farmers' ranking were high yield, early maturing, harvesting frequency, drying quality and long tender-life. Other frequently discussed traits were fruit size, taste, fruit viscosity, price, late senescing and market potential. Fruit color, such as purple in Sasilon, did not influence farmers' criteria of selection. Fruit viscosity was a much subjective trait as some prefer much viscous as compared to less viscous types and vice versa. Tender-life referring to the number days the fruits maintain tender quality before becoming fibrous was stated. Most growers harvest at every 3 to 7 days; depending on mainly the market days interval

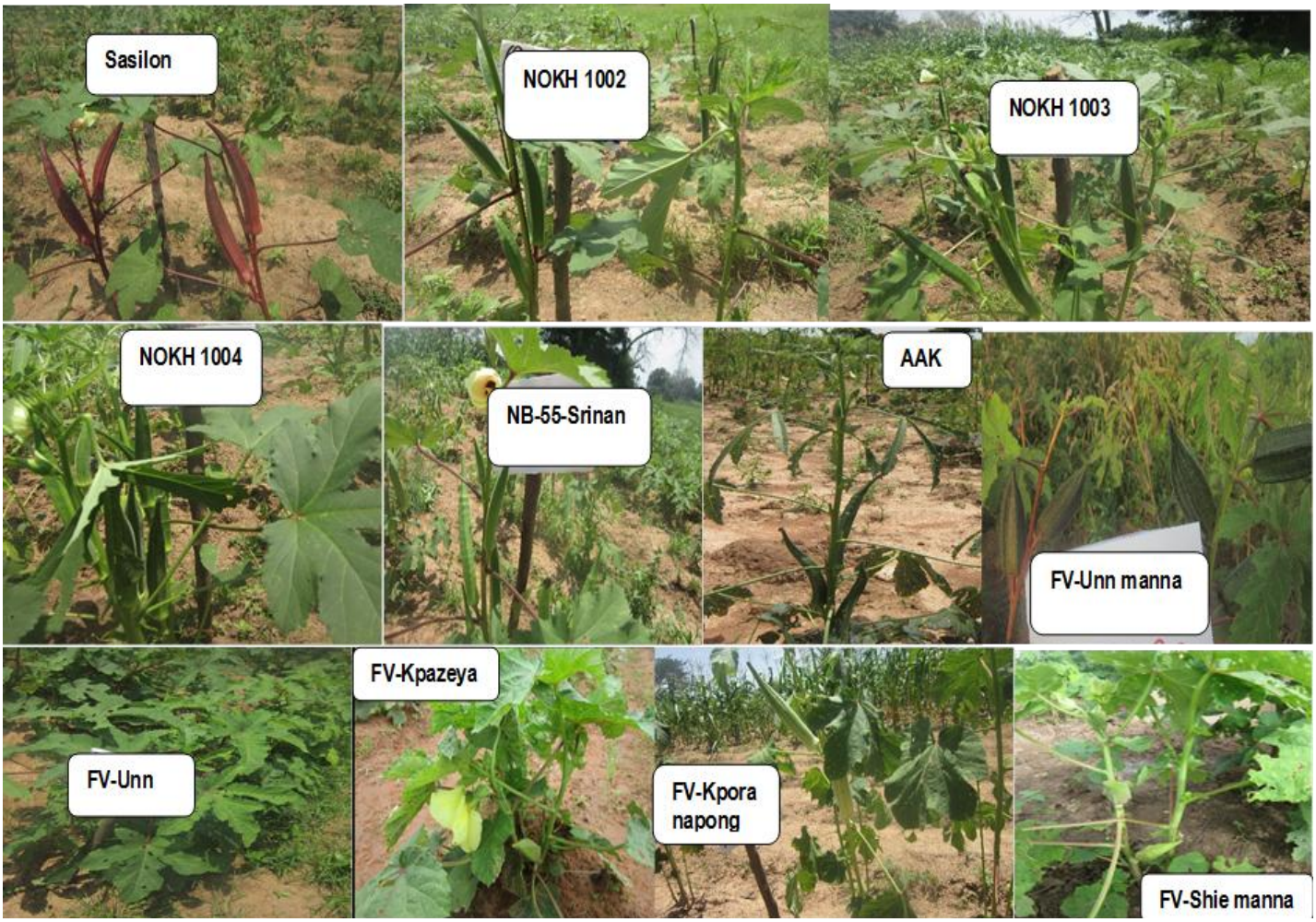

Figure 2. Features of some of the most preferred genoptypes.

of the area.

DISCUSSION

In Ghana, okra production has remained an essential component of household income across gender to urban, peri-urban and rural farm families. Farmers often grow okra as a pre-season cash crop or a second crop to take advantage of residual water after harvesting an earlier crop. In most parts of sub-Sahara Africa generally, okra is traditionally cultivated as a rainy season crop typically by women on marginalized lands which are easily accessible to them (Kumar et al., 2010). Due to rapid urbanization and population growth, the demand for vegetables such as fresh and dehydrated okra has increased over the years. Thus, requiring appropriate production strategies to meet the supply gap. This study demonstrates the superiority of 4 genotypes as compared to 6 widely grown landraces in the area. Five traits which were most critical to farmers include high yield (HY), early maturing (EM), multiple harvest harvest frequency (MHF), drying quality (DQ) and long tender-life. For production under irrigation, dry season gardens and pre-season production, EM, HY and MHF were essential traits identified at the group discussions. Late maturing genotypes with poor DQ were less preferred. The farmer varieties in this study were usually selected due to their high yield under rain-fed conditions and good drying properties. Drying quality was particu-larly critical due to seasonal gluts occurring at the peak of harvesting. The high ranking for Sasilon was of particular significance since The World Vegetable Center (AVRDC) has promoted some of these promising lines (Sasilon, Batoumambe and Safi) in Mali and The Gambia over the last 5 years (Kumar et al., 2010). In our view, any geno-type with yield potential of above 1 ton\ha may perform well at other locations due to the peculiar low soil fertility

Table 4. Summary of agronomic yield components of the genotypes.

Genotype	Plant establishment (%)	Plant height at harvest (cm)	Days to			Fruit count			Average fruit		Yield (t/ha)
			50% flower bud nodulation	50% flowering stage	50% fruiting stage	60 days	90 days	120 days	Length (cm)	Diameter (cm)	
Sasilon	75.0	43.5	48.2	60.3	69.3	17.0	32.0	50.3	18.7	7.2	1.70
TZ-SMN-86	87.2	53.2	56.8	64.2	71.2	1.2	20.0	94.5	18.7	7.4	2.30
P1496946	65.4	28.7	70.0	82.3	93.2	0.0	5.0	36.0	8.7	7.3	0.43
AAK	67.0	39.8	44.2	50.7	57.3	62.7	66.7	108.0	18.7	7.6	2.11
Ex-Makutopora	56.8	57.0	45.5	59.8	68.5	25.0	50.5	66.8	14.5	7.7	1.27
NB-55-Srivan	52.8	58.2	44.2	47.3	50.3	42.0	56.3	104.5	15.0	8.1	1.98
ML-OK-10	35.1	23.0	64.0	75.3	87.7	0.0	4.3	14.5	12.7	7.5	0.37
ML-OK-16	46.5	48.0	57.5	72.0	80.5	0.0	12.3	19.0	13.0	6.9	0.36
ML-OK-35	37.8	19.3	61.8	71.3	84.3	0.0	5.8	10.5	10.0	7.3	0.35
ML-OK-37	62.9	38.7	53.0	65.0	79.3	11.0	24.2	44.0	13.0	6.1	0.68
NOKH 1002	51.2	48.8	43.2	46.8	51.0	41.2	54.7	126.0	14.0	8.3	2.83
NOKH 1003	86.7	45.2	42.2	47.2	51.7	93.7	150.2	295.5	17.0	8.6	5.17
NOKH 1004	85.4	51.0	42.2	47.9	53.0	115.8	156.2	296.8	14.0	8.8	9.55
FV-Unn	84.4	83.8	56.3	69.5	78.2	0.0	5.7	213.5	10.3	9.5	2.20
FV-Unn manna	80.2	79.5	44.5	56	64	11.7	92.8	213.5	8.9	9.6	5.85
FV-Kpazeya	83.5	28.2	64.5	72.2	80.8	0.0	36.8	195.2	10.7	8.6	2.83
FV-Kpora napong	63.0	41.0	55.8	65.5	75.3	2.8	8.0	24.3	12.3	7.0	0.26
FV-Kpora nasong	68.2	44.3	55.8	66.2	78.2	1.6	11.7	15.2	11.1	7.3	0.45
FV-Shie manna	74.3	33.7	59.5	72.8	83.0	2	8.8	10.3	11.3	7.1	0.27
Genotype	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Year	0.064	0.047	0.020	0.071	0.343	0.004	0.204	0.001	0.955	0.917	0.295
Genotype x Year	0.894	0.232	0.578	0.607	0.001	0.010	0.097	0.001	0.895	0.970	0.345
$LSD_{(0.05)}$	16.14	15.31	9.51	4.57	5.91	9.71	25.44	20.03	3.371	1.21	0.75
CV (%)	7.7	10.2	2.3	1.2	1.2	1.8	6.9	1.7	8.1	1.8	4.5

status of the study area and late planting in both years; which was due to pre-season drought. Although, the genotype AAK was high yielding, it was probably not selected due to stem, leaf and fruit pubescence. Due to pre-season drought which led to late planting of experiments, the yield potential of the late maturing genotypes, which were mostly also photoperiod sensitive, was not fully expressed.

Most important agro-yield traits in okra generally include number of total fruits per plant, fruit size characteristics, maximum plant height, days to flower bud nodulation, stem diameter and number of fruiting internodes and branches. Many studies demonstrate that number of fruits per plant, fruit weight and total fruit production often show high variability as compared to other quantitative traits (Adeniji and Aremu, 2007; Bennet-Lartey and Oteng-Yeboah, 2008; Ahiakpa et al., 2013). It has been suggested that component breeding would be very effective when there is positive association

Table 5. Correlation analyses of agronomic yield components of the genotypes.

	DFBN	DFF	DFFR	FC120	FC60	FC90	FD	FL	PPE	PLHT	YD
DFBN	1										
DFF	0.8132**	1									
DFFR	0.7797**	0.9531**	1								
FC120	-0.4152*	-0.5265*	-0.5700**	1							
FC60	-0.6207**	-0.7486**	-0.7577**	0.7164**	1						
FC90	-0.6141**	-0.7084**	-0.7200**	0.8234**	0.8584**	1					
FD	-0.1393	-0.2465*	-0.2983*	0.6303**	0.2969*	0.3976**	1				
FL	-0.4648**	-0.4912**	-0.5141**	0.1236	0.3699*	0.3159*	-0.1797*	1			
PPE	-0.0689	-01021	-0.1558	0.5525**	0.2468*	0.3695*	0.3633*	0.0498	1		
PLHT	-0.2096 *	-2367*	-0.3167*	0.2426*	0.2023*	0.1273	0.3359*	0.1396	0.2473*	1	
YD	-0.4769**	-0.5602**	-0.5893**	0.8858**	0.7757**	0.8301**	0.5525**	0.1396**	0.4997**	0.2282*	1

Where: (*), (**) Significant at 5 and 1% levels of probability, respectively; DFBN: days to 50% flower bud nodulation stage, DFF: days to 50% flowering stage, DFFR: days to 50% fruiting stage, FC120: fruit count at 120 days, FC60: fruit count at 60 days, FC90: fruit count at 90 days, FD: fruit diameter, FL: fruit length, PPE: percent plant establishment, PLHT: plant height, YD: fresh fruit yield.

Table 6. Overall ranking and traits influencing farmers' selection of the genotypes.

Most preferred genotype	Overall preference		Traits influencing their selection	Reasons for dislike
	Frequency	%		
Sasilon	41	15.1	HY, EM, Long-bold fruits, long tender-life, viscous, good taste	Poor DQ
NOKH 1004	40	14.7	HY, EM, MHF, long fruits, good taste	Poor DQ, short tender-life
NB-55-Srivan	35	12.9	HY, EM,MHF, long fruits, viscous	Poor DQ, short tender-life
NOKH1003	24	8.8	HY, EM, MHF, Long fruits, less hairy	Poor DQ, short tender-life
FV-Kpazeya	21	7.7	HY, MHF, good DQ, high price of dried okra, viscous	Late maturing, hairy
FV-Unn manna	20	7.4	HY, MHF, good DQ, high price of dried okra, viscous	Late maturing, hairy
FV-Kpora napong	20	7.4	HY, MHF, high price, long tender-life, less viscous	Late maturing
FV-Kpora nasong	20	7.4	HY, MHF, high price, long tender-life, less viscous	Late maturing
FV-Unn	19	7.0	HY, MHF, good DQ, high price of dried okra, drought resistant, good taste	Late maturing, hairy
FV-Unn manna	20	7.4	HY, MHF, good DQ, high price of dried okra	Late maturing, hairy
AAK	16	5.9	HY, EM, Long fruits, long tender-life, viscous, good taste	Poor DQ, hairy
NOKH 1002	5	1.8	HY, EM, long fruits, less hairy	Poor DQ, short tender-life
ML-OK-16	4	1.5	HY, EM, long fruit, viscous	Poor DQ
Ex-Makutopora	4	1.5	HY, EM, less hairy, long fruits	Poor DQ, short tender-life
TZ-SMN-86	3	1.1	HY, EM, less hairy, good taste	Poor DQ , late maturing
Total	272	100		

Poor drying quality: The cultivar respond slowly to drying or become fibrous or easily grow mouldy when dried, HY= high yielding, EM=early maturity, MHF= multiple harvesting frequency, DQ= drying quality.

of major yield characters (Hazra and Basu, 2000). The correlation analyses in this study showed high (r > 0.78) correlation between fruit yield and fruit counts at 60, 90 and 120 days. Thus, days to flower bud nodula-tion, flowering and fruiting which translate into early harvesting from 60 DAP influenced fruit pro-duction. A related study showed strong positive correlation between fruit yield and first fruit pro-ducing node (r = 0.76); first fruit producing node and first flowering node (r = 0.79); and number of fruits per plant and stem diameter at base (r = 0.88) (Ahiakpa et al., 2013). They also found highly significant correlation (P < 0.01) between fruit length at maturity and total fruits per plant and suggest the possibility of selecting highly prolific fruit types with longer fruits. Adeniji and Aremu (2007) reported negative correlations among these traits. Opong-Sekyere et al. (2012) also noticed strong correlation between the first flowering node and first fruiting node (r =0.74) in evaluating fruit yield. They recommend that total fruit production, first fruit-producing node, first flowering node, number of fruits per plant and stem diameter at the base should be given more attention when selecting for higher yield and high dry matter in okra. However, the view of this study is in contrast with earlier view that early flowering is detri-mental to overall productivity of okra as the source to sink ratio will be potentially limited for effective photosynthesis (Aboagye et al., 1994). Another opinion is that wide range of flowering periods among the accessions implies varying maturity periods even on the same plant making it difficult for uniform or mechanical harvesting (Ahiakpa et al., 2013). Generally, early flowering and fruiting is particularly essential in irrigation and pre-season crop-ping systems where time, water and labor resources are limiting factors. Respondents in this study preferred genotypes that can produce at least one fruit a week for a prolonged period; apparently due to the high price varia-bility at harvest and other socio-economic considerations.

Conclusion

This study was part of broad strategies to unlock the potential of okra for food, nutrition and income security to urban, peri-urban and rural farm families through eva-luating, introducing and promoting promising high yielding genotypes which can be utilized in both rain-fed and irrigation production ecologies. The study showed that it is possible to upgrade the current okra germplasm in Ghana using the 4 most ranked genotypes (Sasilon, NOKH-1004, NB-55-Srivan and NOKH-1003). Sasilon was ranked first due to its long tender-life and bold fruits. Four other genotypes: NOKH 1002, TZ-SMN-86, Ex-Makutopora and AAK, which showed good yield potential could be added in future okra improvement programmes. These 8 genotypes have been advanced to participatory multi-location on-farm evaluations to further validate their potential. The major weaknesses of these genotypes are their poor drying quality and short tender-life. These genotypes can be suggested as candidates for inspection to the National Variety Release and Technical Committee for release to farmers in order to increase access to im-proved okra varieties. The PVS suggest that high yielding and early maturing genotypes which are amendable to drying may have wide adoption among farmers; due to the premium on dehydrated okra. Most of the farmer varieties in this study were selected due to fruit yield, multiple harvesting frequencies, ability to dry, long tender-life and late senescing.

Conflict of Interest

The authors have not declared any conflict of interest.

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REFERENCES

Aboagye LM, Isodas A, Nyima H, Takasaki Y, Yoshimura T, Ishikawa T (1994). “Plant type and dry matter production in peanut (*Arachis hypogea* L.) cultivars; varietal differences in dry matter production”, Japan J. Crop. Sci. 63(2):289-297.

Adeniji OT, Aremu CO (2007). “Interrelationships among characters and path analysis for pod yield components in West African okra (*Abelmoschus caillei* A, Chev) Stevels)”, J. Agro. 6(1):162-166.

Agbo AE, Gnakri D, Beugre GM, Fondio L, Kouame C (2008). Maturity degree of four okra fruit varieties and their nutrients composition. Elect. J. Food Plant Chem. 5:1-4.

Ahiakpa JK, Kaledzi PD, Adi EB, Peprah S, Dapaah HK (2013). “Genetic diversity, correlation and path analyses of okra (*Abelmoschus* spp. (L.) Moench) germplasm collected in Ghana”, Int. J. Dev. Sustain. 2(2):1396-1415.

Alam AKMA, Hossain MM (2008). Variability of different growth contributing parameters of some okra (*Abelmoschus esculentus* L.) accessions and their interrelation effects on yield. J. Agric. Rural Dev. 6(1,2):25-35.

Arapitsas P (2008). Identification and quantification of polyphenolic compounds from okra seeds and skins. Food Chem. 110:1041-1045.

Ariyo OJ (1990). Variation and heritability of fifteen characters on okra (*Abelmoschus esculentus* (L.) Moench). Trop. Agric. 67:213-216.

Benchasri (2012). Okra (*Abelmoschus esculentus* (L.) Moench) as a valuable vegetable of the world. Ratar. Povrt. 49:105-112.

Bennet-Lartey SO, Oteng-Yeboah AA (2008). “Ghana Country Report on the State of Plant Genetic Resources for Food and Agriculture”, CSIR, Plant Genetic Resources Research Institute, (PGRRI), Bunso, Ghana, pp. 15-33.

Bisht IS, KV Bhat (2006). Genetic resources, chromosome engineering and crop improvement series. Edited by Ram J. Singh. 3:148-162.

Düzyaman E (2005). Phenotypic diversity within a collection of distinct okra (*Abelmoschus esculentus*) cultivars derived from Turkish land

races. Genet. Resour. Crop Evol. 52:1019-1030.
Glew RH, VanderJagt DJ, Lockett C, Grivetti LE, Smith GC, Pastuszyn A, Millson M (1997). Amino acid, fatty acid, and mineral composition of 24 indigenous plants of Burkina-Faso. J. Food Comp. Anal. 10:205-217.
Hazra P, Basu D (2000). Genetic variability, correlation and path analysis in okra. Ann. Agric. Res. 21(3):452-453.
International Board for Plant Genetic Resources, IBPGR (1991). Report on International Workshop on Okra Genetic Resources, National Bureau for Plant Genetic Resources, New Delhi, India.
Kumar S, Dagnoko S, Haougui A, Ratnadass A, Pasternak D, Kouame C (2010). Okra (*Abelmoschus* spp.) in West and Central Africa: potential and progress on its improvement. Afr. J. Agric. Res. 5(25):3590-3598.
Ndunguru J, Rajabu AC (2004): Effect of okra mosaic virus disease on the above-ground morphological yield components of okra in Tanzania. Sci. Hortic. 99:225-235.
Nwangburuka CC, Kehinde OB, Ojo DK, Popoola AR, Oduwaye O, Denton OA, Adekoya M (2011). Molecular characterization of twenty-nine okra accessions using the Random Amplified Polymorphic DNA (RAPD) Molecular Marker. Acta SATECH 4(1):1-14.
Oppong-Sekyere D, Akromah R, Nyamah EY, Brenya E, Yeboah S (2012). Evaluation of some okra (*Abelmoschus spp* L.) germplasm in Ghana. Afr. J. Plant Sci. 6(5):166-178.
Saifullah M, Rabbani MG (2009). Evaluation and characterization of okra (*Abelmoschus esculentus* L. Moench.) genotypes. SAARC J. Agric. 7:92-99.

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Full Length Research Paper

Re-evaluating $TiCl_4$ and UV assays for detection of vicine and convicine in high-throughput screening of immature and mature seeds of faba bean

Yurdagul Ferhatoglu* and Albert Vandenberg

Department of Plant Sciences, University of Saskatchewan, 51 Campus Drive, Saskatoon SK, S7N 5A8, Canada.

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Genomic resources have just started to focus on the faba bean; the genes for synthesis of vicine and convicine (V-C) have not been determined, and recently developed genetic markers for these anti-nutritionals have not been used to examine these traits in very large scale in faba bean. Simple, rapid and cost-effective technologies are crucial in crop breeding programs, especially in the developing world, and in some cases, traditional methodologies are used in combination with genetic markers to assess agronomic traits and the value of gene markers. Here, two methodologies (TiCl4 assay and 274 nm absorption) are re-evaluated for their application in detection of V-C in faba beans. In comparison with TiCl4 assay, the method of 274 nm UV absorption without an HPLC analysis offers more reliable analysis for detection of V-C in immature and mature seeds of faba bean. Its application in high throughput screening by 60 min agitation of immature seeds or mature seed flour in 2% trichloroacetic acid (TCA) allows quick screening of low V-C faba beans. The level of V-C was maximum when seed moisture was 80% and V-C level was measured as 0.92% in CDC Fatima flour. Though V-C from 2% TCA extract of mature and immature seeds of CDC Fatima was detected by 274 wavelength in the TiCl4 assay reaction, a Ti-aglycone complex was not clearly detectible at 480 nm as previously suggested.

Key words: Vicine, convicine, TiCl4 assay, UV assay, high throughput, seed, faba bean.

INTRODUCTION

Faba bean (*Vicia faba* L.) is the third most important feed grain legume after soybean and pea. The largest producer is China, followed by Australia, France, the Americas, Egypt, Sudan, Morocco and Ethiopia (FAO, 2013). In North America, faba bean has been grown on a limited basis on the Canadian prairies since the 1970s (Gade, 1994). Production has expanded since 1997 and the annual growth rate was 5.82% between 2003 and 2013 in Canada (FAO, 2013).

Faba bean is rich in protein (Burstin et al., 2011) and essential amino acids (Alghamdi, 2009; Mortuza et al., 2009; Sosulski and Hold, 1980). It has potential as a meat substitute in many parts of the world where there is demand for non-animal protein sources. Faba bean produces the highest percentage of protein (24–38%) per seed (Burridge, 1999; Burstin et al., 2011) and per unit

*Corresponding author. E-mail: ygungor27@gmail.com. Tel: 306-2414668.

land area as compared to all other temperate and tropical pulse crops, including lentil (*Lens culinaris*), common bean (*Phaseolus vulgaris*), chickpea (*Cicer arietinum*) and pea (*Pisum sativum*) (Broad beans world yield, FAOSTAT 2013). Mature faba beans provide high-quality carbohydrates (58%), fiber (25%) (USDA Nutritional Database, http://www.nal.usda.gov/fnic/foodcomp/search), antioxidants, and nutrients, with a comparatively low glycemic index (GI) (40 and 48 GI per 150 g serving for baked bean and dark durum wheat pasta, respectively [GI database, University of Sydney]). They could be an alternative to wheat in the food industry.

Faba bean flour alone and mixed with wheat flour at 100 and 70% provides a gluten-free option for the bread and pasta industries (Sozer, personal communication, VTT Technical Research Centre of Finland, 2014). Immature faba beans used as vegetable are a significant source of vitamin A (350 iU per 100 g) and vitamin C (0.033%) (http://www.nal.usda.gov/fnic/foodcomp/search, Kmiecik et al., 1990) and polyphenols with antioxidant activity (Baginsky et al., 2013). Green pods of faba bean are a significant source of L-DOPA (Burbano et al., 1995) which is used for treating Parkinson disease (Seeberger and Hauser, 2009) and for overcoming low libido in human (Hulse, 1994). Faba beans contain significant amounts of flavanoid glycosides which inhibit xanthine oxidase (XO) (Spanou et al., 2012), XO inhibitors are beneficial in vascular disorders (George and Struthers, 2008).

Presence of some anti-nutritional factors limits faba bean use in the food and feed industry**.** In humans, aglycones of vicine (2,6-diamino-4,5-dihydroxypyrimidine-5-[*β*-D-glucopyranoside]) and convicine (2,4,5-trihydroxy-6-aminopyrimidine-5-[*β*-D-glucopyranoside]), divicine and isouramil, respectively, are responsible for the favism in individuals who carry specific alleles that cause deficient, non-functional or low functional G6P dehydrogenase (G6PD) enzymes (Mason et al., 2007). Favism is a potential life-threatening oxidative damage that results from the ingestion of faba beans. These individuals are unable to regenerate reduced glutathione (GSH) which results in deposition of toxic substances in the membranes of red blood cells, causing cell apoptosis or destruction (Arese et al., 2012). This X-linked gene is known to be the most common human enzyme defect (> 400 million people) worldwide (Mason et al., 2007; Cappellini and Fiorelli, 2008) and natural variants are expected to be generally found in both animal and human populations. The negative effects of toxic glycosides were also reported in animal models such as poultry (Muduuli et al., 1982).

Earlier studies of faba bean tissue analysis for vicine and convicine (V-C) showed it could be detected in seeds from 13-15 cm long pods but not from younger pods. The substrate [6-14C], orotate incorporation into the pyrimidine ring of V-C in pod and seed showed V-C synthesis synthesis in both tissues, but with accumulation in seeds and not in pod tissue (Brown and Robbins, 1972). A contradicting study that analyzed V-C content during seed development showed that vicine content was highest when seed contained 80% moisture but was present even in seeds of new developing pods (Burbano et al., 1995). Another study showed the highest amount of V-C in mature resting seed, and lesser amounts in roots during vegetative growth. The V-C was present in root tissues until seed maturation and also in stem before the start of seed development (Ramsay and Griffiths, 1996). Among faba bean flour extracts that have commercial value, the amount of V-C was higher in protein concentrate than in starch and flour of faba bean (Kim et al., 1982). Reduction of V-C in faba bean is of ongoing interest for its breeders (Abd Allah et al., 1988; Jamalian et al., 2006), processes are ineffective and costly for the food and feed industry. The V-C compounds are thermally stable [melting point: 242-244°C (Olaboro et al., 1981; Marquardt et al., 1983) and the cooking and boiling processes do not remove V-C by more than 50%, although natural genetic variation in V-C reduction by these processes does exist (Martinez et al., 2012). Ultimately V-C has to be removed by crop development programs.

The lack of genomic data was limited until now by the enormous nuclear genome of *V. faba* (predicted as ~13 000 mb, which is 30 times greater than the sequenced *Medicago truncatula* genome (Bennett et al., 1982; Johnston et al., 1999), and the small size of sequence database (579 assembled genome nucleotide, 75870 transcript, 64079 un-annotated nucleotide from various resources) at public domain (Vicia faba nucleotides, NCBI 2015) have eliminated gene based marker development for V-C content screening in faba bean. The low V-C gene locus has originally been discovered from Polish collection by Duc et al. (1989), by discovery of low V-C mutant (with 0.046% V+C of seed dry matter) which led to development of genetic markers (Gutierrez et al., 2006 and Khazaei et al., 2015) for V-C trait or concentration analysis but neither genes for their synthesis nor the wide use of genetic marker application has been determined. Genetic markers, generally speaking, are valuable when tightly linked to agronomic traits in breeding programs, ideally they are very valuable when whole genome is known.

Acid, alkaline and alcohol extractions have been used for V-C estimation (Higazi and Read, 1974; Jamalian, 1978; Sosulski and Pitz, 1979; Hegazy and Marquardt, 1983). Three different spectrophotometric methods have been used for measuring V-C in faba bean samples. One is based on UV (273.5 nm) spectrophotometry of protein free (Collier, 1976) while the others three are colorimetric methods. One is based on the formation of a Ti-complex with V-C, detected at 480 nm (Kim et al., 1982), the second is based on the reduction of Folin-Ciocalteau phenol reagent reads at 650 nm (Higazi and Read, 1974), and in

Table 1. Faba bean cultivars and characteristics.

Genotypes and definitions	Flower color	Vicine – convicine	Hilum color	Seed coat color-tannin-total phenolic of mature seed
Disco* (D)	White	Low	light brown	Light-low- low
Snowbird* (SN)	White	Normal	light brown	Light-high-high
CDC Fatima* (F)	Normal type (white with black wing spots)	Normal	Black	Dark-high-moderate
CDC Snowdrop (SD)	White	Normal	light brown	Light-unknown-unknown
CEB04928 (CEB)	White	Normal	light brown	Light-unknown-high

Source: *Oomah et al. (2011) Phenolics, phytic acid, and phytase in Canadian-grown low-tannin faba bean (*Vicia faba* L.) genotypes, J. Agric Food Chem. 2011 Apr 27; 59(8):3763-71. doi: 10.1021/jf200338b. Epub 2011 Mar 10.

the third method, the reduction of o-ferriphenanthroline to o-ferrophenanthroline by V-C aglycones is read at 610 nm (Chevion and Novak, 1983). The visual color forming Folin-Ciocalteau phenol and $TiCl_4$ assays are also used for analysis of phenolic compounds [example: sinapic acid (Ismail and Eskin, 1979). Additionally, TiCl4 is used for analyzing H_2O_2 (Gupta and Eskin, 1977). More accurately, V-C content measuring analytical methods include C18 reverse phase HPLC connected to a uv-vis detector (Marquardt and Frohlich, 1981; Zhang et al., 2003) and GC-MS (Sosulski and Pitz, 1979) which are quite costly and the TLC (Jamalian and Bassiri, 1978), which is cost effective but very slow technique. Low-cost, reliable, simple, less time-consuming tests are needed for routine analysis of V-C in faba bean breeding programs. Here, we aimed to re-evaluate TiCl4 and UV spectrophotometric assays of immature and mature seeds.

Improving crops using genomic tools is still in its infancy, especially in legumes (Varshney et al., 2015). There is little evidence on the types of cases in which they work, and their application on very large scales is still limited (Varshney et al., 2015). Utilization of the faba bean genome by its breeders or for any other application, especially in developing countries, takes a long time. Costly and time-consuming V-C screening strategies can be overcome with quick, cheap, simple, practical phenotypic markers and analytical tools. Here, TiCl4 and UV assays were compared to clarify whether any of these methods could be a better option in large-scale low V-C faba bean analysis.

MATERIALS AND METHODS

Plant material

Mature and immature seeds of the faba bean cultivars listed in Table 1 were obtained from the Crop Development Centre (CDC) of the University of Saskatchewan. Dry seeds were from the 2014 harvest of field research plots located at Preston, Saskatoon, whereas immature seeds were collected from plants at the CDC greenhouses within the same year. Dry seeds were kept at room temperature and fresh seeds in a –20°C freezer until analysis. V-C was determined in mature and immature seeds and immature seed tissues, including cotyledon, radical, seed coat and pod.

Mature seeds were manually de-hulled and then ground in a coffee mill at the day of analysis. An unknown cultivar protein concentrate from dry milling process was obtained from Parrheim Foods (PF) (Saskatoon, SK, CA) and was used in purification and analysis of V-C.

Other materials

All reagents were of analytical grade. Titanium tetrachloride was obtained from Sigma-Aldrich Canada Co. (Oakville, Ontario, CA). V-C standards were not commercially available; therefore they were purified from faba bean protein concentrate of PF using the method of Marquardt et al. (1983). Purity of the extracted V-C was determined by reversed phase analytical HPLC (RP-HPLC) and mass spectrophotometer (HP1100 series of Agilent, Germany) equipped with UV detection coupled to a Quattro LC (Waters, UK) triple quadrupole mass spectrometer (MS) equipped with an electrospray ionization (ESI) interface. Identifications of V-C were initially made by specific absorbance spectra (Merck Index, 2006) and then by MS.

All HPLC analyses were performed using a C18 column (CAPCELL C18 AG120, 25 × 4.6, 5 micron, Phenomenex, Torrance, CA, USA) connected to a UV detector that was adjusted to 274 nm. Extracts were diluted ten times in 2% TCA (trichloroacetic acid), then were applied to the column equilibrated and run with water (Quemener, 1988) at 0.8 µl/min.

HPLC mass spectrophotometer conditions were a short RP-HPLC column (Genesis C18 2.1 × 100 mm, 3.5 µm, Germany) with the 97% A (5% formic acid) and 3% B (95:5 methanol : water with 5% formic acid) as a mobile phase at 0.6 ml/min, for 20 min.

Extraction and UV spectrophotometer analysis of V-C

Percentage V-C analysis in 2% TCA extracts of mature and immature seed

Ground, mature, dehulled seed samples (100 mg) were extracted in duplicate with 2% TCA (0.6 ml), and the aqueous sample solution was suspended, then sonicated (5 min, two times). For immature seeds, a single seed from each of three pods was extracted with extraction solvent

Table 2. V-C content of faba bean with 2% TCA extract.

Sample type	Fresh seed weight (%)	Total absorbance at 274 nm (%)		
Immature seed- CDC Fatima (weight 0.5±0.07 g, mortar pestle extract)		**Convicine (C)**	**Vicine (V)**	**V+C**
Seed coat	31	17±2	69±3	86±4
Cotyledon	67	17±2	73±3	86±4
Seed radicle	2.5	4±0.5	5±0.5	9±0.5
Green pod	357	none	none	none
Mature seed flour, sonicated	**Total absorbance at 274 nm, relative to CDC Fatima**			
CDC Fatima	1	17±3	67±4	84±5
Snowbird	1	15±3	71±3	86±4
Disco	0.33	18±4	47±2	65±3
Protein concentrate	2.36	12±3	59±3	71±3
Immature green seed (weight (gram), mortar pestle extract)	**Total absorbance at 274 nm, relative to CDC Fatima**			
CDC Fatima (0.45±0.12)	1	26±3	56±3	83±3
Snowbird (0.70±0.26)	1	16±3	67±2	86±6
Disco (0.47±0.1)	0.5	-	-	-

(0.4 ml, 2% TCA/100 mg), as described in the dehulled mature seed sample analysis, by mortar and pestle disruption. Immature seed sections including cotyledon, seed coat, radical and pod were also extracted as in the whole immature seeds in the triplicate sample set. All sample extracts were suspended and then centrifuged (12,000 g for 15 min). The V-C percentage (% V+C) in all samples was determined by comparing the V and C peak areas to the whole run area in chromatograms obtained from the HPLC analysis described above; data are presented in Table 2. Identification of V and C peaks on chromatograms was determined by an HPLC run of analytical standards. A typical HPLC chromatogram of V and C in immature seed tissue is shown in Figure 1.

CDC Fatima V-C quantification

CDC Fatima was used as a control sample and its V-C level was determined in dehulled ground mature seeds by duplicate sample analysis. The 100 mg samples were consecutively extracted three times with 2% TCA in a sonicator and centrifuged; consecutive extracts were combined. Another set of duplicate samples was extracted from the same source of CDC Fatima by soaking two seed cotyledons in 30 ml of hot water for 3.5 h, then adding 100 ul HCl followed by centrifugation (12,000 g for 15 min) (Gutierrez et al., 2006). Analytical standards (V and C) were also prepared in the same buffer (2% TCA) and read by UV assay. Extinction coefficients (ε) of both V and C were taken as 16.400 at 274 nm (Merck Index, 2006) and Beer's law (Absorbance = ε L c) was applied to determine the concentration of standard solutions. The amount of standard solutions injected on HPLC corresponding peak areas was used in quantification of V+C in HPLC runs and extracts.

CDC Fatima buffer tests to maximize V-C extraction

CDC Fatima immature seeds (~0.45 g) were extracted in triplicate with mortar and pestle as mentioned above, with the aqueous solutions including acetone : water (70:30 ratio), 2% TCA, water, 0.01 N NaOH, or ethanol : water (70:30; 0.6 ml). Supernatants were used immediately for the 220-300 nm (UV spectrum) data collection by UV plate reader (FLUOStar Omega). Mean data were used in plotting wavelength versus absorbance (OD) (Figure 2A).

Assessing developing seed for V-C accumulation

The V+C level of CDC Fatima developing seed (Figure 6A) was determined by UV assay (274 nm absorbance), and samples were extracted as in immature seeds as earlier mentioned. Mean OD data ± standard deviation (SD) versus seed moisture levels as seed stage determinant was plotted (Figure 2B). Moisture level versus wet weight of Fatima immature seed was assayed, and the related mean was plotted (Figure 2C). The V+C level of Snowbird, Disco and CDC Fatima genotype seeds was determined similarly by UV assay during development (assayed by seed wet weight), and mean ± SD of samples is presented in Figure 3.

Quick extraction of V-C from immature seeds

Single seeds from three pods split into two cotyledons were sonicated for 5 min in microcentrifuge tubes, each containing a single seed, or were shaken (200 rpm) at room temperature for 60 min in 2% TCA in 96 well plate samples. Samples were then centrifuged at 4000 g. All quick extractions were processed by UV plate reader, which was set to 220-300 nm (FLUOStar Omega). Sample means of 274 nm absorbance were used for plotting minutes versus absorbance (OD274; Figure 4).

Titanium tetrachloride assay

Greenhouse-grown developing immature seeds (~82% moisture

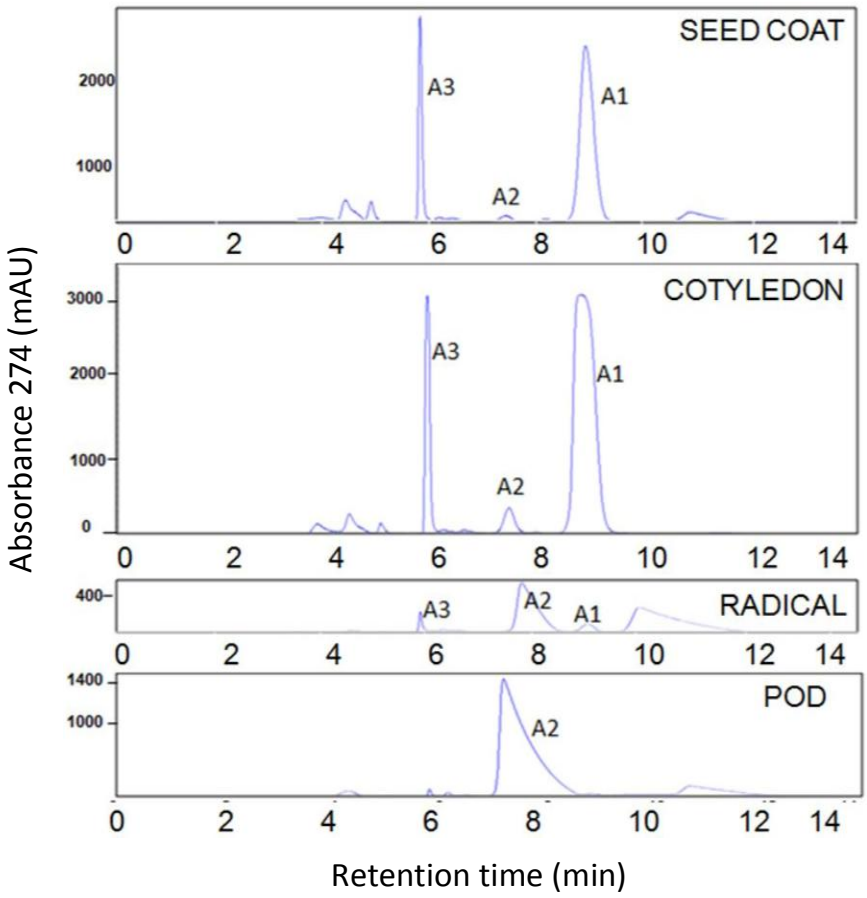

Figure 1. Typical HPLC chromatograms of vicine and convicine from 100 mg of single seed coat, cotyledon, radicle and pod tissue of CDC Fatima (0.5±0.07 g seed fresh weight) extract with 400 µl of 2% TCA. Eluents: Water. Flow: 0.8 ul/min. Injection: 80 ul/min of 1/10 dilution of extract. Absorbance 274 nm versus retention time for vicine (A1), unknown (A2), convicine (A3).

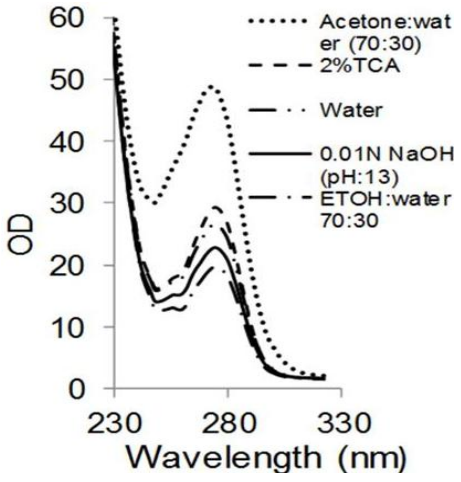

Figure 2A. Optical density (OD) in relation to 220-300 nm wavelength of CDC Fatima immature seed extracted with five solvents.

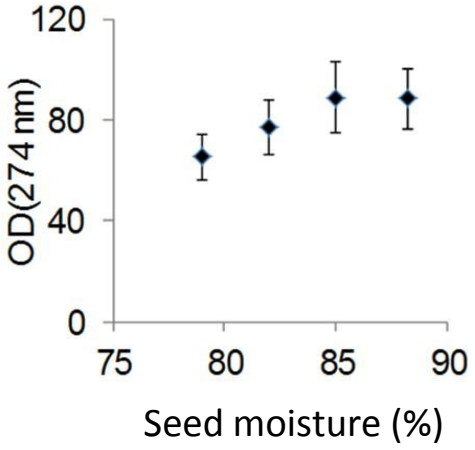

Figure 2B. Wavelength 274 nm in relation to % seed moisture of CDC Fatima immature seed extracted with 2% trichloroacetic acid (TCA).

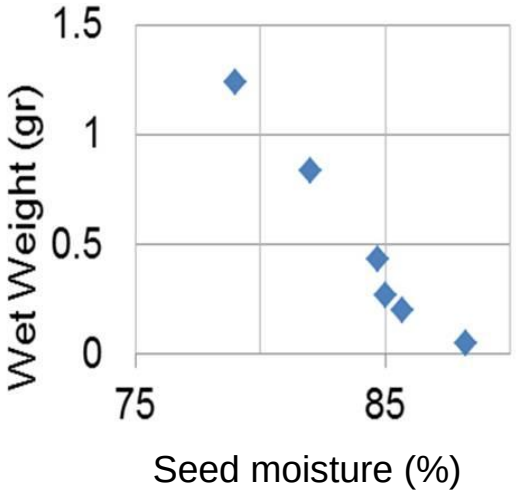

Figure 2C. Wet weight in relation to % seed moisture of CDC Fatima immature seed extracted with 2% trichloroacetic acid (TCA).

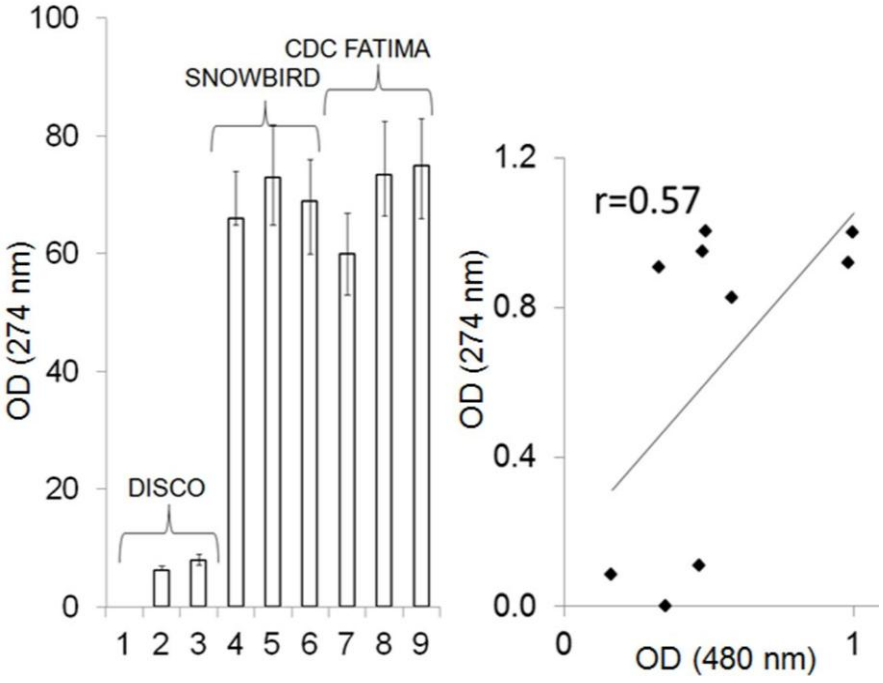

Figure 3. UV and $TICl_4$ assay in V-C detection from Disco, Snowbird, and CDC CDC Fatima green seeds. A: Disco (1-3), Snowbird (4-6), CDC Fatima (7-9) immature seed development and corresponding UV assay (274 nm) profile. Weight of seeds: 1, 4, 7=0.1±0.03; 2, 5, 8=0.2±0.05; 3, 6, 9=0.4±0.07. 6. B). B. shows correlation of UV (274 nm) and $TiCl_4$ assay (480 nm) in green seed samples from faba bean varieties Disco, Snowbird and CDC Fatima. Expressions of ODs are relative to CDC Fatima seed (0.5 g).

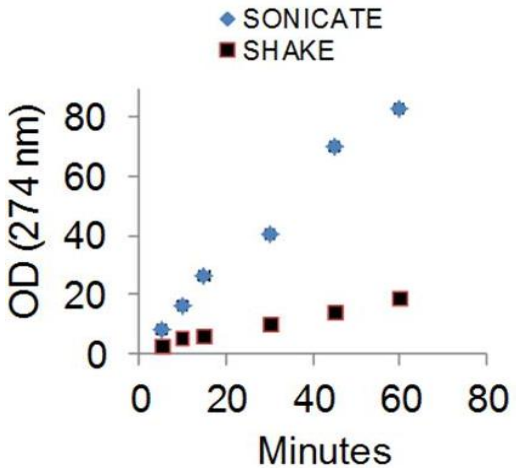

Figure 4. Wavelength 274 nm in relation to extraction time of CDC Fatima immature seed extracted with 2% trichloroacetic acid (TCA). Seed shaking and sonication extraction time in quick extraction process.

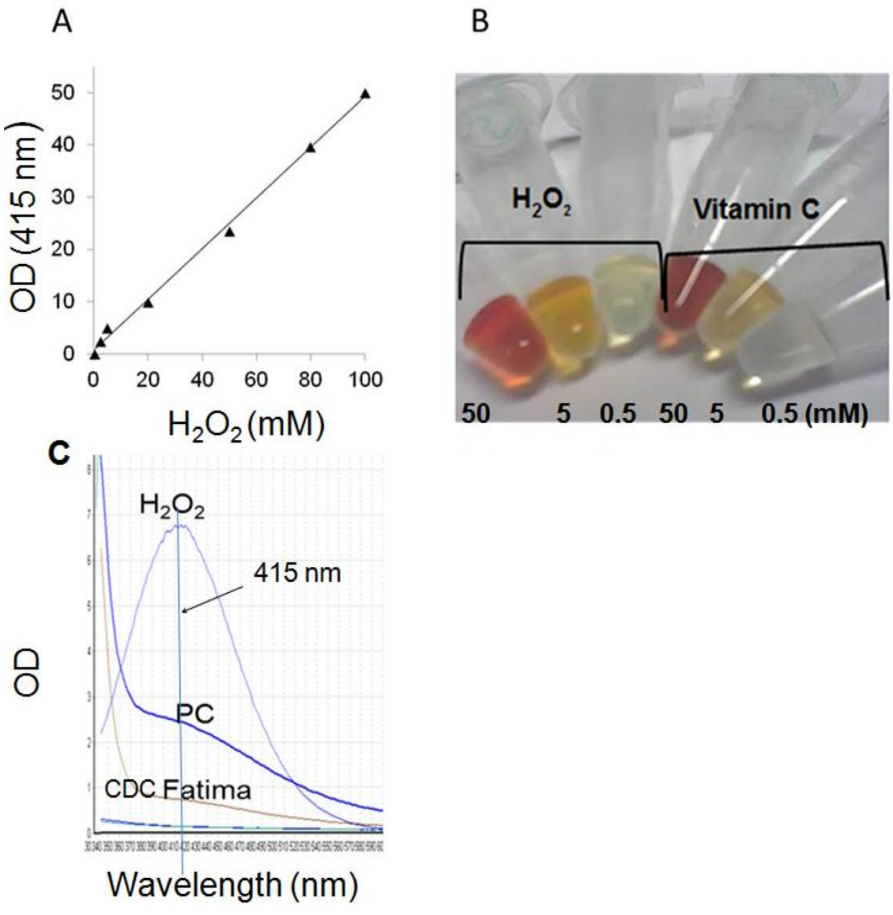

Figure 5. A) Standard curve (absorption plot) for Ti-peroxy complex ($TiCl_4$+ H_2O_2). Absorbance (415 nm) versus H_2O_2 concentration. B) Ti-peroxy and ascorbate color scale. H_2O_2 and vitamin C concentrations (left to right): 50, 5 and 0.5 mM. C) PC, CDC Fatima, H_2O_2 indicate faba seed protein concentrate, CDC Fatima flour and Ti-peroxy complex λ_{max}: 415 nm, respectively.

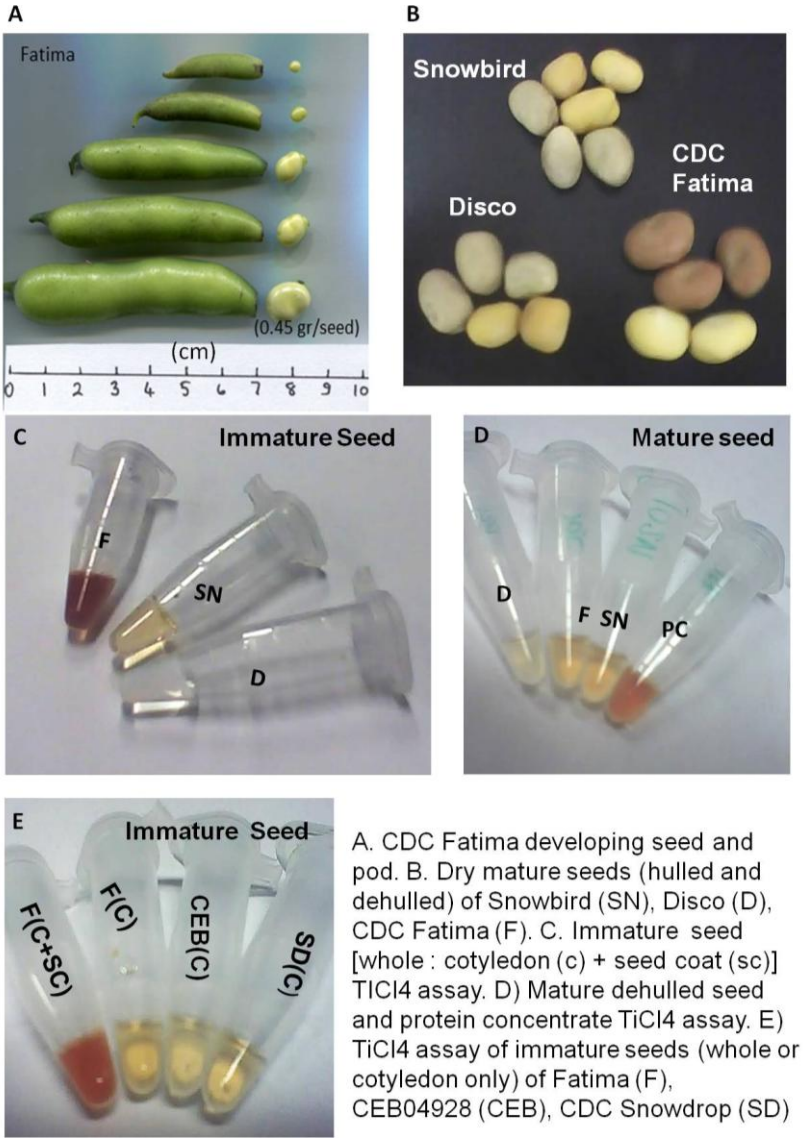

Figure 6. Faba bean seeds and $TiCl_4$ assay color formation.

and 0.58 ± 0.13 g) that reached maximum levels of V+C according to the earlier assay (Figures 2B, C and 3), and ground, mature, dehulled seed samples (100 mg) were extracted in duplicate in 2% TCA, as mentioned in the percentage V-C analysis of mature and immature seeds, then TiCl4 assay was applied to precentrifuged aqueous solutions. Data are presented in Figures 5C, 6C, D and E. All color assays were done twice using the extracts. A 15 % titanium tetrachloride (TiCl4) in concentrated HCl (15 ml of $TiCl_4$ in 85 ml of 20% HCl) was prepared in an ice bath in a fume hood and by adding $TiCl_4$ (99%) into 20% HCl slowly, drop by drop. $TiCl_4$ reaction was measured with minor modifications of the rapid colorimetric method (Kim et al., 1982) by adding 50 µl $TiCl_4$ (15%) and 50 µl of extract from mature or green seeds and then heating the reaction at 80°C for 5 min since application of Kim's method had not formed any color of the indicator product [Ti-aglycone (λmax_{480}) (Kim et al., 1982), Ti-peroxy (λmax_{415}) (Gupta and Eskin 1977), or any major peak that is in the visible spectrum. Initial modifications of the assay (Kim et al., 1982), included directly heating 2% TCA V-C extract (0.6 ml /100 mg and 0.4 ml/100 mg tissue for mature and immature seed, respectively) in HCl (4 N) for 5 min at 80°C and then adding $TiCl_4$ (15%) for color formation. V-C hydrolysis was monitored by spectrophotometer and disappearance of V-C peak was evident in acid hydrolysed samples as similar event was evident at earlier studies. For comparative purpose, theTiCl4 assay was applied to H202 and ascorbic acid dissolved in 2% TCA (Figure 5B).

British research lines V+C from HPLC analysis

Areas of v-c (V+C) obtained from HPLC runs of 159 recombinant inbred lines (RILs) including the single plant progeny parents Melodie (LVC), normal VC parent and the LVC parent Betty (Gutierrez et al., 2006) were used in the calculation of correlation coefficient (**r**=Pearson correlation) with the data obtained from the same sample set (mature faba bean seed ground flour provided by Donal O'Sullivan University of Reading, Berkshire, UK) but extracted and analyzed differently. Our ground mature seed

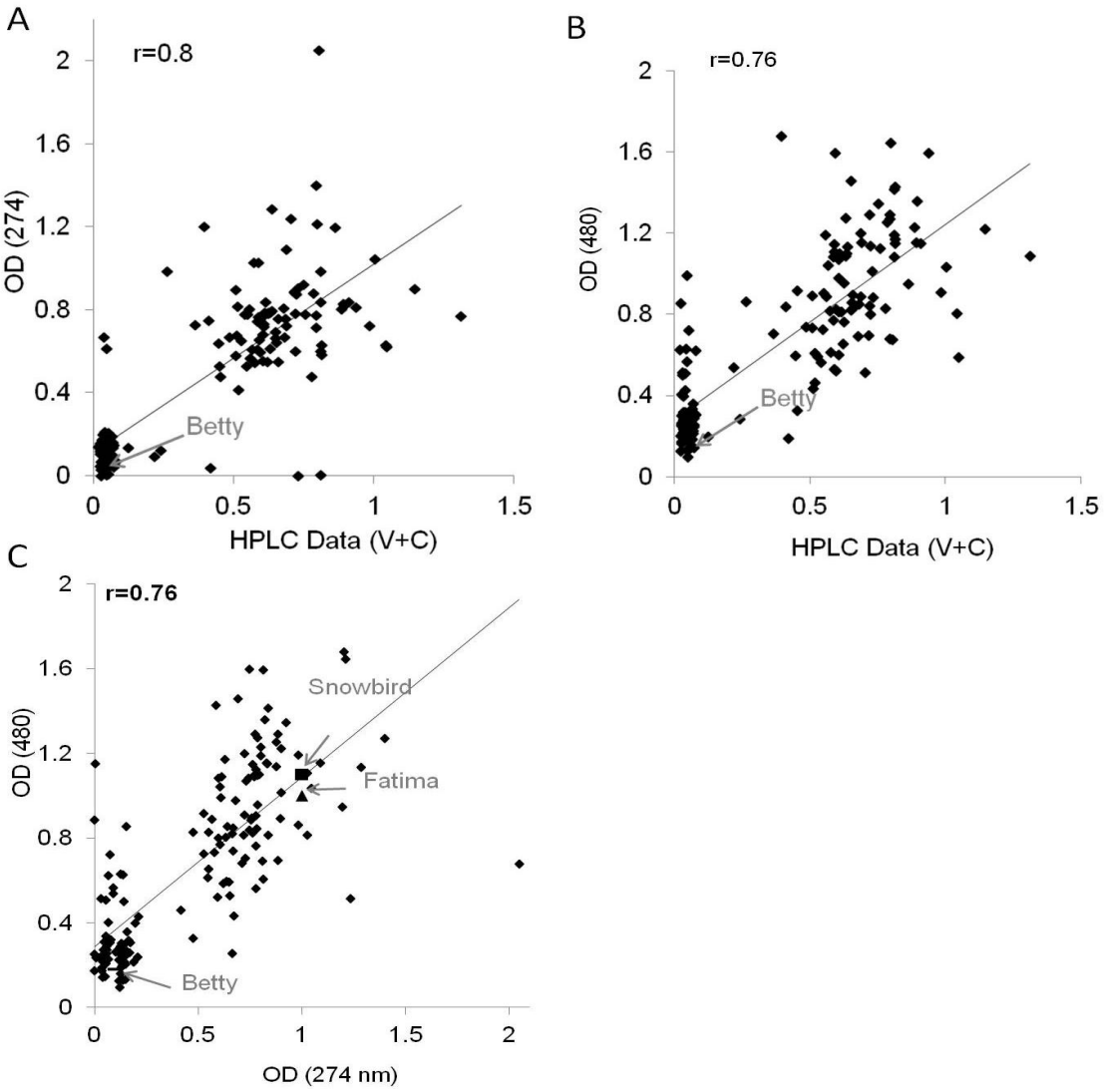

Figure 7. Mature seed flour from 159 Faba bean RILs and V-C negative (Betty, zero V-C) and positive control varieties (Snowbird and CDC Fatima, normal v-c). A) UV assay (OD 274 nm) versus HPLC data (V-C), B) $TICl_4$ assay (OD 480 nm) versus HPLC data (V-C), C) $TiCl_4$ assay versus UV assay.

analysis was done as described in the method section of this study by 2 × 5 min sonication in 2% TCA (100 mg flour/600 µl 2% TCA) of duplicate samples and centrifugation at room temperature. Data were collected at 274 nm wavelength at UV plate reader (FLUOStar Omega) and duplicate sample means were used plotting of HPLC data (V+C) versus absorbance (274 nm) . The r was determined using the Microsoft Excel function Pearson tool. The same extract was used for the TiCl4 assay and data were used in r calculation with the both HPLC and OD (274 nm) data of Britain Research lines (Figure 7).

RESULTS AND DISCUSSION

UV assay in V-C detection and analysis

V-C was detectible in seed coat (maternal tissue), seed radicle and cotyledons of immature seed but not in maternal seedpod tissue of test variety CDC Fatima (Table 2). In earlier studies, trace amounts of V-C were de-tected in shells of young (4-cm pod) and mature pod of low V-C and normal V-C genotypes in addition to seeds (Duc et al., 1989). It has not been quantified, but a significant amount (86% of 274 nm absorbed extract) of V-C was detected in seed coats of immature seeds (Figure 1 and Table 2), though trace amounts of V-C existed in dry mature seed coats (data not shown), which is similar to earlier studies (Burbano et al., 1995). Mature seeds of the low V-C variety Disco (not single-plant progeny) contained about 25% V-C (expressed as the percentage of CDC Fatima total absorbance and of V+C of total area absorbed at 274 nm) of the normal V-C va-rieties CDC Fatima and Snowbird (Table 2), and the V-C

content of protein concentrate of an unknown variety was about twice that of the flour V-C of CDC Fatima and Snowbird.

The V-C content of dry mature seed flour of the CDC Fatima variety was predicted to be 0.92% according to the two extraction methods (2% TCA and hot-water soaking HCl). Similar results were observed by Collier (1976), Hussein et al. (1986) and Khamassi et al. (2013). Khamassi et al. (2013) reported V-C from 0.02 to 1.32% (by weight) of dry seeds in normal commercial varieties and UK research lines, whereas Hussein et al. (1986) reported ~0.83% V-C in Egyptian-grown varieties.

Varieties of aqueous extraction buffers were studied in immature seed analysis. Using alcohol : water (70:30) at high pH in extraction enhanced the UV (274 nm) absorption (Figure 2A) but also reduced the specificity of the TiCl4 assay (data not shown); therefore, 2% TCA was the better option. In earlier studies, acetone: water enhanced the isolation of V-C from mature faba bean protein concentrate (Marquardt et al., 1983). The result from water was not better than the results from 2% TCA. Zhang et al. (2003) extracted vicine with water from bitter melon seed and leaves, and the HPLC run of these extracts (methanol: 0.025 mol/L phosphate [10:90 v/v, pH 3.0]) contained a clear vicine chromatogram in seed tissue but minor impurities in leaf.

V-C was detected in immature seeds as small as 2 mm (0.1 g seed) (Figure 2C) and their presence was at its maximum (85% of total absorbance at 274 nm) when seed moisture was ~85% and wet seed weight was 0.4 g (Figure 2B and C), which is in agreement with Burbano's studies using different genotypes (1995). In variety Snowbird, as compared to CDC Fatima, accumulation of V-C seems to start earlier (Figure 3), and seeds as small as 0.1 g have showed V-C differences between low and normal V-C varieties, as was the case for the Disco, Snowbird and Fatima genotypes (Figure 3).

Seed shaking and sonication for 60 and 5 min, respectively, provided enough signal for UV analysis in immature green seeds (Figure 4) of CDC Fatima and Snowbird (80-85% moisture), and mature seeds contained ~85% V-C of total extract in both types of extraction samples, according to HPLC analysis (data not shown).

$TICl_4$ assay re-evaluation, modification

The standard curve for H_2O_2 levels by $TiCl_4$ is shown in Figure 5A. A linear relationship was evident for the Ti-peroxy complex, which was detectable at 415 nm and by the naked eye at 0.5 mM concentration (Figure 5A and B). The same visible color was observed in 0.35 and 0.5 mM concentrations of vicine and ascorbic acid, respectively. Darker colors were formed in 5 and 50 mM concentrations of both H_2O_2 and vitamin C (Figure 5B), and the corresponding lambda max was observed at 415 (Figure 5C) and 410 nm (not shown), respectively. In contrast to the results of Kim et al. (1982), divicine and isomural at 480 nm were not detectible in purified vicine and protein concentrate (Figure 5C). Pedersen et al. (1988) stated that the chemical cleavage of vicine is not divicine, but actually the deamino divicine later formed the H_2O_2 in a non-air-sealed environment. There is no previous study on color assay (Ti-complex), if the suggested air-sealed environment is necessary, it is not easily doable on a large scale. On a small scale, this was mimicked by creating a medium containing extracted V-C, then adding $TiCl_4$ directly before or after heating for divicine and isomural formation. N_2 bubbled (air-free) V-C extract was tested in our modified $TiCl_4$ assay, but in none of these attempts were we able to detect the proposed Ti complex (Kim et al., 1982).

Our attempt to use $TiCl_4$ and UV assays comparatively in V-C analysis had poor to medium correlation (Pearson r = 0.57) of both analysis methods for immature seeds of CDC Fatima and Snowbird (Figure 3). In another set of immature seeds (weight: 0.43 g) of faba bean research lines, the correlation was very poor (-0.25; data not shown).

$TiCl_4$ analysis of immature seeds showed that CDC Fatima (normal-type flower) had the darkest color seed, which contained a high-tannin seed coat (Figure 6B). Though CDC Fatima and Snowbird contain similar percentages of V-C in immature seeds (Table 2), the color formed in the $TiCl_4$ reaction was much richer and darker for CDC Fatima than for Snowbird (Figure 6C). The white-flowered cultivars, Snowbird, CDC Snowdrop (SD), CEB04928 (CEB) and CDC Fatima, had identical colors by $TiCl_4$ assay when the seed coat of immature seeds of CDC Fatima was removed (Figure 6E). This shows $TiCl_4$ interacts with phenolic compounds and possibly also with tannins in the seed coats of fresh cotyledons of CDC Fatima. Baginsky et al. (2013) reported significant amounts of phenolic compounds (anthocyanins, flavonoids and other compounds structurally similar to V-C) in immature faba bean seeds. Our analytical studies (not published) done later showed significant amounts of phenolic compounds (for example, cathechin) in normal-type flowers of *V. faba* (Ferhatoglu et al., unpublished).

The $TiCl_4$ assay has been suggested for analyzing V-C in mature seeds (Kim et al., 1982). In mature seed analysis, we observed a color difference between the low V-C, low phenolic, zero-tannin variety Disco (zt-2 white flower genotype) (Khamassi et al., 2013; Pedersen et al., 1988) and the normal V-C, normal phenolic, tannin-containing seed varieties Snowbird (zt-1 white flower gene) and CDC Fatima (normal flower color) (Figure 6D) (Pedersen et al., 1988). The protein concentrate had the most intense color using the $TiCl_4$ assays, as expected by the corresponding high V-C values (Table 2) and possible high tannins and phenolic compounds (Figure 6D).

A single 2% TCA extract from a mature CDC Fatima seed contains enough V-C for visual determination of the

Ti-aglycone complex, which should carry a color lighter than the 5 mM Ti-peroxy and Ti-Vit C complex (Figures 5B and 6D) because the Ti-alyocone complex concentration was predicted to be about 1.6 mM in colorimetric assays from corresponding UV quantitation (data not shown).

In earlier studies, $TiCl_4$ assay had not been applied on a large scale to genotypically different faba bean lines or immature seeds. In this study, mature seed flour of faba bean Melodie × Betty RIL lines was analyzed for V-C using the modified $TiCl_4$ assay and two extraction methods (solvent and method wise) and HPLC chromatography. When both types of data were plotted (Figure 7), fair levels of correlation (r ~ 0.76) existed between $TiCl_4$ data (OD480) and HPLC data (Figure 7B). This means that the $TiCl_4$ assay without significant prepurification may be applied in detecting V+C in mature seeds, but outliers do exist, as shown in Figure 7B. A slightly better correlation between UV assay and HPLC data was evident (Figure 7A).

This study, especially given a choice of Disco (white-flowered, low V-C, low phenolic, zero tannin), CDC Fatima (white with black wing spots, normal V-C, high phenolic, tannin), or Snowbird (white-flowered, normal V-C, high phenolic, tannin), appears to provide enough information on the interaction between unspecific compounds and $TiCl_4$ in addition to V-C in immature seeds. The $TiCl_4$ assay is simple and can be evaluated by the naked eye (colorimetrical) at ~1% w/w V-C seed content with 600 µl 2% TCA/0.1 g extract, but specificity is low, possibly because of the complexity of the plant metabolic system in immature seeds. The $TiCl_4$ assay can and should be used together with the UV assay for selecting low V-C but high phenolic/tannin (nutritionally valuable, rich in antioxidants) genotypes in cotyledons and/or whole seeds that are immature or mature. Oomah et al. (2011) determined a very high correlation between tannins and phenolic compounds in screened faba bean varieties. They measured higher levels of phenolic compounds and tannin in Snowbird relative to CDC Fatima, similar to the results observed in our $TiCl_4$ assay (480 nm absorbance; Figure 7). Here, we were able to identify normal and white-colored flower varieties by simple visualization of $TiCl_4$ assay and phenolic differences of Snowbird and CDC Fatima by $TiCl_4$ assay at 480 nm absorbance.

Removal of the unwanted metabolites with neutral alumina prior to $TiCl_4$ assay, as previously suggested (Kim et al., 1982), will be incomplete, as this was observed in the analysis of sinapic acid samples (Fenwick, 1981) and in our attempts in V-C analysis in protein concentrate, even though the extract was passed through a neutral alumina column (Supelclean LC-Alumina, Supelco, PA), according to Kim et al. (1982). The extraction of faba bean flour V+C with simple 2% TCA and its analysis by UV assay (274 nm absorbance) and data correlation (by Pearson correlation) with the HPLC quantified V-C (extracted differently) from the same sample set (Figure 7) has shown that UV assay (r = 0.80 for OD 274 nm versus V+C area from HPLC) can be applied directly to the high-throughput platform for the selection of low V-C seeds (Figure 7). Nucleosides, nucleotides and peptides absorbed by UV must be at a very low level in the freshly prepared 2% TCA extract, which was prepared at room temperature because the correlation is very high between both types of UV-based V-C analysis with or without HPLC. The solvent/extract ratio is low; therefore, 2% TCA should precipitate most of the interference compounds. The $TiCl_4$ assay with the dry flour extract had poorer correlation (r = 0.76) relative to the UV assay (Figure 7). The 2% TCA extraction makes V-C extraction and detection simple for low V-C faba bean. There is no report on V-C degradation by 2% TCA (0.125 N). HCl, whose acid strength is about the same as that of TCA at 0.7 N, is known to hydrolyze V-C (Marquardt et al., 1983).

Supporting this study, our most recent low-cost low–V-C screening studies in faba bean breeding programs during seed development suggest that both $TiCl_4$ and UV assay on 2% TCA extracts can be applied successfully to large-scale sample sets in selecting low V-C and low to high tannin/phenolic seed genotypes (Ferhatoglu et al., unpublished).

Appendices

Supplementary data on UV (280 nm) and mass analysis of vicine and convicine by HPLC mass spectrophotometer are shown in supplementary Figure 1. Vicine (ppt#2) is pure and convicine (Xtal yellow ppt#1) contains some minor impurities.

Conflict of interests

The authors have not declared any conflict of interest.

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REFERENCES

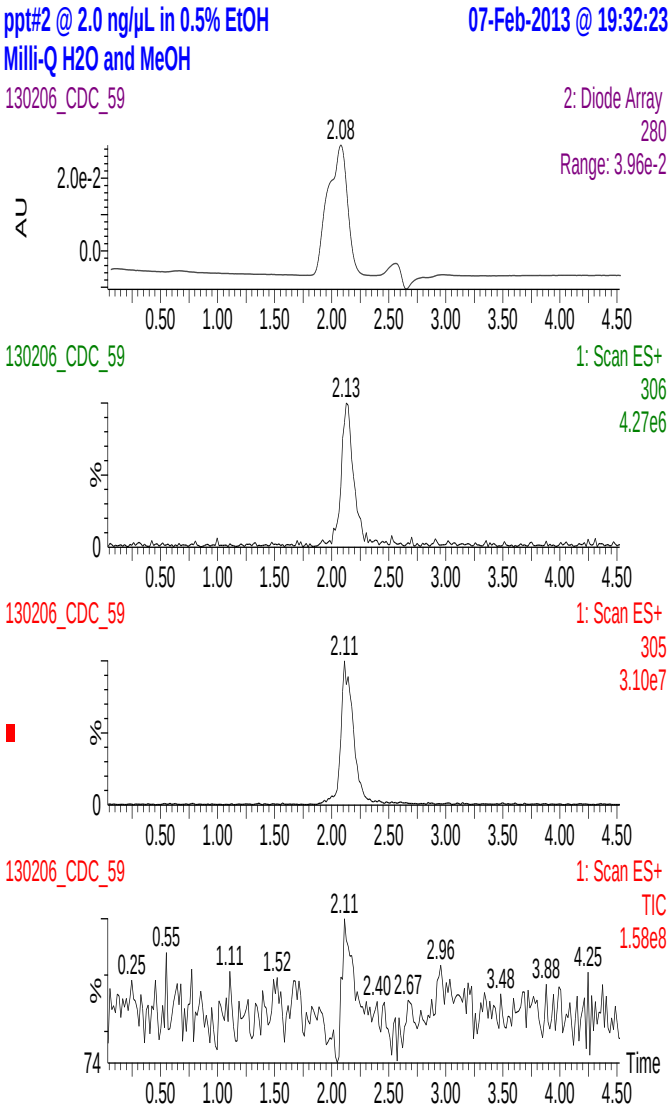
Abd Allah MA, Foda YH, Abu Salem FMA, Abd Allah ZS (1988). Treatments for reducing total vicine in Egyptian faba bean (Giza 2 variety). Plant Foods Hum. Nutr. 38:201-202.

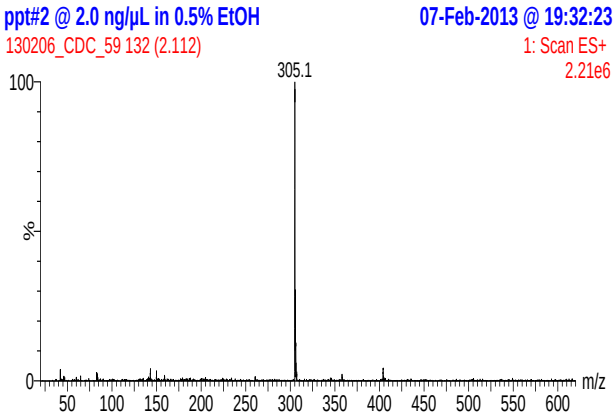
Alghamdi SS (2009). Chemical composition of faba bean (Vicia faba L.) genotypes under various water regimes. Pak. J. Nutr. 8(4):477-482.

Arese P, Gallo V, Pantaleo A, Turrini F (2012). Life and death of glucose-6-phosphate dehydrogenase (G6PD) deficient erythrocytes - role of redox stress and band 3 modifications. Transfus. Med. Hemother. 39(5):328-34.

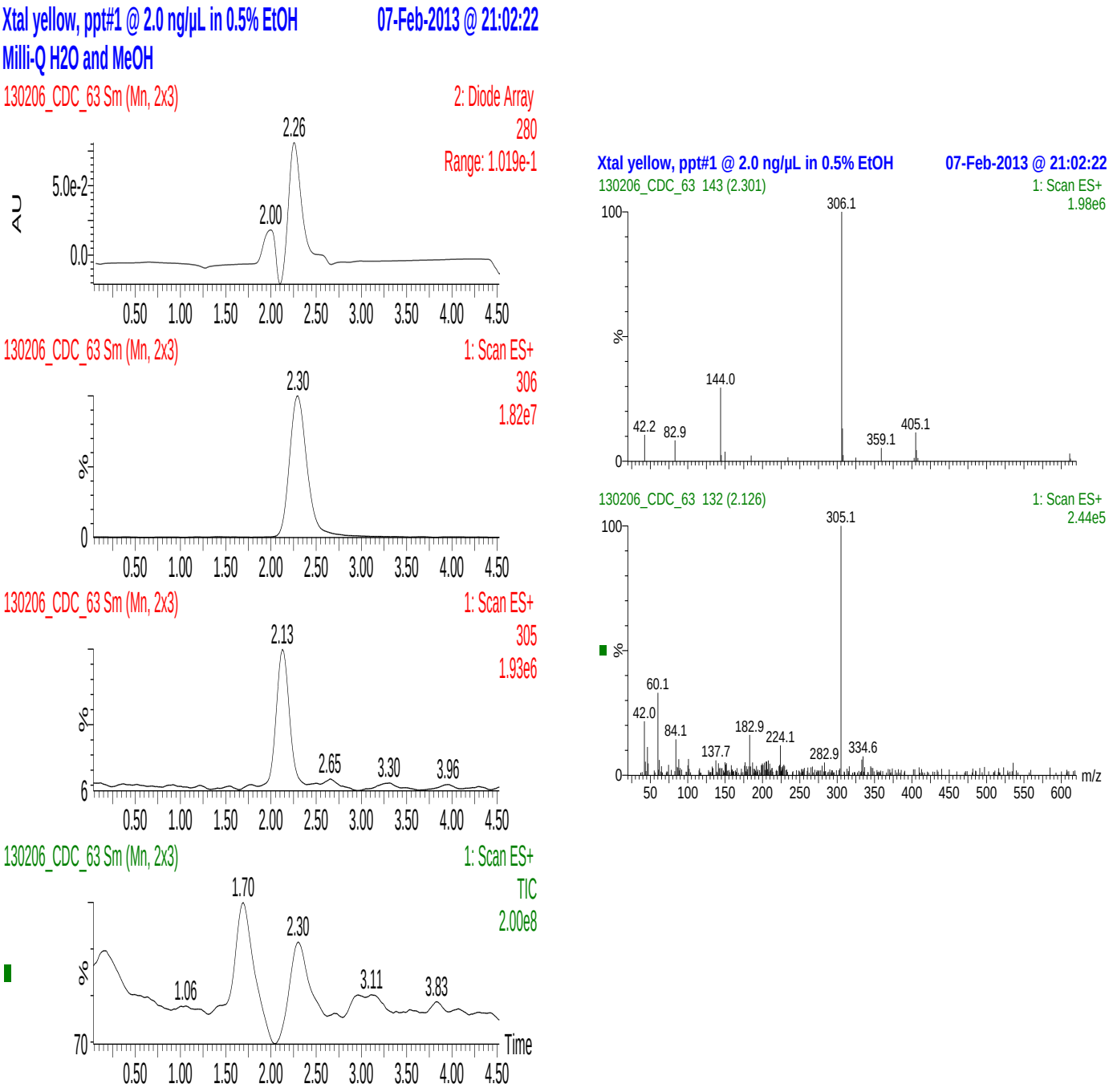
Baginsky C, Caceres A, Pena-Neira A, Hernandez T, Estrella I, Morales H, Pertuze R (2013). Phenolic compound composition in immature

seeds of faba bean (*Vicia faba* L.) varieties cultivated in Chile. J. Food Compost. Anal. 31(1):1-6.
Bennett MD, Smith JB, Heslop-Harrison JS (1982). Nuclear DNA amounts in angiosperms. Proc. R. Soc. Lond. B Biol. Sci. 216:179-199.
Brown EG, Roberts F M (1972). Formation of vicine and convicine by *Vicia faba*. Phytochemistry 11(11):3203-3206.
Burridge P (1999). GRDC Project DAW440B, Australia.
Burbano C, Cuadrado C, Muzquiz M, Cubero J (1995). Variation of favism-inducing factors (vicine, convicine, and L-DOPA) during pod development in *Vicia faba* L. Plant Foods Hum. Nutr. 47:265-274.
Burstin J, Gallardo K, Mir RR, Varshney RK, Duc G (2011). Chapter 20: Improving protein content and nutrition quality. In: A. Pratap, Kumar, J. (Eds.), Biology and Breeding of Food Legumes. CPI Group Ltd; Croydon, UK. pp. 314-318.
Cappellini MD, Fiorelli G (2008). Glucose-6-phosphate dehydrogenase deficiency. Lancet 371:64-74.
Chevion M, Navok T (1983). A novel method for quantitation of favism-inducing agents in legumes. Anal. Biochem. 128:152-158.
Collier HB (1976). The Estimation of Vicine in Fababeans by an Ultraviolet Spectrophotometric Method. Can. Inst. Food Sci. Technol. J. 9:155-159.
Duc G, Sixdenier ML, Furtoss V (1989). Search of genetic variability for vicine and convicine in *Vicia faba* L. A first report of a gene codes for nearly zero-vicine and zero-convicine contents. In: J. Huismen, A.F.B. Van der Poel, E.E. Liener (Eds.). Recent Advances in Antinutritional Factors in Legume Seeds. Wageningen, Netherland, Wageningen Academic Publishers pp. 305-313.
FAOSTAT (2013). Broad beans world yield. http://faostat.fao.org
Fenwick GR (1981). The quantitative determination of sinapine using titanium tetrachloride. Comments relating to the specificity of the method. Food 25:795-798
Gade DW (1994). Growth in the world Fabas. In: el DW Gade, "Environment, Culture and Diffusion: The Broad Bean in Quebec". Cahiers de geographie du Quebec 38:137-150.
George J, Struthers AD (2008). The role of urate and xanthine oxidase inhibitors in cardiovascular disease. Cardiovasc. Ther. 26(1):59-64.
Gupta CB, Eskin NAM (1977). An improved method for the determination of residual hydrogen peroxide in milk. J. Food Sci. 42:537-538.
Gutierrez N, Avila CM, Duc G (2006). CAPs markers to assist selection for low vicine and convicine contents in faba bean (*Vicia faba* L.). Theor. Appl. Genet. 114:59-66.
Hegazy MI, Marquardt RR (1983). Development of a simple procedure for the complete extraction of vicine and convicine from faba beans (*Vicia faba* L.). J. Sci. Food Agric. 34:100-108.
Higazi MI, Read WC (1974). A method for determination of vicine in plant material and in blood. J. Agric. Food Chem. 22(4):570-571.
Hussein L, Motawel H, Nassib A, Khalil S, Marquardt RR (1986). The complete elimination of vicine and convicine from the faba beans by combination of genetic selection and processing techniques. Plant Food Hum. Nutr. 36:231-242.
Hulse JH (1994). Nature, composition and utilization of food legumes. In: FJ Muehlbauer and WJ Kaiser (eds.), Expanding the Production and Use of Cool Season Food Legumes. Dordrecht, Netherland, Kluwer Academic pp. 77-97.
Ismail F, Eskin NAM (1979). A new quantitative procedure for determination of sinapine. J. Agric. Food Chem. 27: 917-918.
Jamalian J (1978). Favism-inducing toxins in broad beans (Vicia faba). Determination of vicine content and investigation of other non-protein nitrogenous compounds in different broad bean cultivars. J. Sci. Food Agric. 29: 136–140.
Jamalian J, Bassiri A (1978). Variation in vicine concentration during pod development in broad bean (*Vicia faba* L.). J. Agric. Food Chem. 26: 1454-1456.
Jamalian J, Ghorbani M (2006). Extraction of favism-inducing agents from whole seeds of faba bean (*Vicia faba* L var. major). J. Sci. Food Agric. 86:1055-1060.
Johnston JS, Bennett MD, Rayburn AL, Galbraith DW, Price HJ (1999). Reference standards for determination of DNA content of plant nuclei. Am. J. Bot. 86:609-613.
Khamassi K., Jeddi FB, Hobbs D, Irigoyen J, Stoddard F, O'Sullivan DM, Jones H (2013). A baseline study of vicine-convcine levels in faba bean (Vicia faba L.) germplasm. Plant Genet. Resour. 11:1-8.
Khazaei H, O'Sulllivan DM, Jones H, Pitts N, Sillanpaa MJ, Parssinen P, Manninen O, Stoddard FL (2015). Flanking SNP markers for vicine–convicine concentration in faba bean (*Vicia faba* L.). Mol. Breed. 35:38.
Kim S, Hoehn E, Michael E, Ismail F (1982). A simple and rapid colorimetric method for determination of vicine and convicine. J. Agric. Food Chem. 30:144-147.
Kmiecik W, Lisiewska Z, Jaworska G (1990). Vitamin C levels in fresh, frozen and canned broad beans in relation to their varieties and degree of maturity. Rocz Panstw Zakl Hig 41:17-24.
Marquardt RR, Frohlich AA (1981). Rapid reversed-phase high-performance liquid chromatographic method for the quantitation of vicine, convicine and related compounds. J. Chromatogr. 208:373-379.
Marquardt RR, Muduuli DS, Frohlich AA (1983). Purification and some properties of vicine and convicine isolated from faba bean (*Vicia faba* L.) protein concentrate. J. Agric. Food Chem. 31(4):839-844
Martínez AC, Ocaña KM, Moreno AO, Cabrera HEB, Ortiz DG, Múzquiz M, Pedrosa MM, Burbano C, Cuadrado C, Martínez JC (2012). Effect of Roasting and Boiling on the Content of Vicine, Convicine and L-3,4-dihydroxyphenylalanine in *Vicia faba* L. J. Food Qual. 35:419-428.
Mason PJ, Bautista JM, Gilsanz F (2007). G6PD deficiency: the genotype-henotype association. Blood Rev. 21:267-283.
Mortuza G, Hannan A, Tzen JTC (2009). Chemical composition and functional properties of *Vicia faba* L. from Bangladesh. Bangladesh J. Bot. 38:93-97.
Muduuli DS, Marquardt RR, Guenter W (1982). Effect of dietary vicine and vitamin E supplementation on the productive performance of growing and laying chickens. Br. J. Nutr. 47:53-60.
NCBI (2015). *Vicia faba* nucleoides, NCBI sequence database. http://www.ncbi.nlm.nih.gov
Olaboro G, Marquardt RR, Campbell LD, Fröhlich AA (1981). Purification, identification and quantification of an egg-weight-depressing factor (vicine) in fababeans (*Vicia faba* L.). J. Sci. Food Agric. 32:1163-1171.
Oomah BD, Luc G, Leprelle C, Drover JC, Harrison JE, Olson M (2011). Phenolics, phytic acid, and phytase in Canadian-grown low-tannin faba bean (Vicia faba L.) genotypes. J. Agric. Food Chem. 59:3763-3771.
Pedersen JZ, Musci G, Rotilio G (1988). Electron Spin Resonance Characterization of the Radicals Produced by Enzymatic or Chemical Cleavage of Vicine. Biochemistry 27:8534-8536.
Quemener B (1988). Improvements in the High Pressure Liquid Chromatography Determination of Amino Sugars and alpha-galactosides in faba Bean, lupine, and pea. J. Agric. Food Chem. 36:754-759.
Ramsay G, Griffiths W (1996). Accumulation of vicine and convicine in *Vicia faba* and *V. narbonensis*. Phytochemistry 42:63-67.
Seeberger LC, Hauser RA (2009). Levodopa/carbidopa/entacapone in Parkinson's disease. Expert. Rev. Neurother. 9:929-940.
Sosulski FW, Pitz WJ (1979). Determination of vicine and convicine in faba bean cultivars by gas-liquid chromatography. Can. Inst. Food Sci. Technol. J. 12:93-97.
Sosulski FW, Holt NW (1980). Amino acid composition and nitrogen to protein factors for grain legumes. Can. J. Plant Sci. 60:137-1331.
Spanou C, Veskoukis AS, KerasiotiT, Kontou TM, Angelis A, Aligiannis N, Skaltsounis AL, Kouretas D (2012). Flavonoid glycosides isolated from unique legume plant extracts as novel inhibitors of xanthine oxidase. PLoS One 7(3):e32214.
Varshney RK , Kudapa H , Pazhamala L, Chitikineni A, Thudi M, Bohra A, Gaur PM, Janila P, Fikre A, Kimurto P, Ellis N (2015). Translational Genomics in Agriculture: Some Examples in Grain Legumes. Crit. Rev. Plant Sci. 34:169–194.
The Merck Index (2006). 14th edition, Merck & Co., Inc. Whitehouse Station, NJ, USA.
Zhang H, Wang Y, Zhang X, Liu M, Hu Z (2003). Analysis of Vicine in Bitter Melon with High Performance Liquid Chromatography. Anal. Lett. 36:1597-1605.





Supplementary Figure 1A. UV (280 nm) and mass analysis of vicine by HPLC mass spectrophotometer.



Supplementary Figure 1B. UV (280 nm) and mass analysis of convicine by HPLC mass spectrophotometer.

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Cytotoxicity study on *Maerua pseudopetalosa* (Glig and Bened.) De Wolf tuber fractions

Manal A. Ibrahim[1]* and El Bushra E. El Nur[2]

[1]Department of Botany, Faculty of Science and Technology, Omdurman Islamic University, Sudan.
[2]Department of Botany, Faculty of Science, University of Khartoum, Sudan.

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Ethyl acetate and ethanol extracts of the tuber parts of *Maerua pseudopetalosa* were subjected to further separation by column chromatography technique and eight fractions were obtained for the former and twelve for the latter one. The brine shrimp lethality assay was used for assessment of the toxicity. Remarkable cytotoxicity against brine shrimp larvae was shown, for the first time, by the ethanol extract. The fractions F_8, F_9, F_{11} and F_{12}, with high cytotoxic values (1.25, 7.98, 0.185, 0.041 µg/ml, respectively), were subjected to gas chromatography/mass spectrometry analysis. Thirty three compounds were detected; which were not recorded in any previous work in the available literature. Fractions 8 and 9 were found to be cytotoxic due to the presence of oleate and linoleate compounds; with more cytotoxicity in fraction 8 as a result of the additional presence of decenoic acid. Also, fraction 12 was more cytotoxic than fraction 11 and this was attributed to the presence of a proline derivative (proline-N-methyl-butyl ester). This compound might be considered as the cause of the high toxicity of the fraction; since free proline was used as an inhibitor of breast cancer development. Surprisingly, *M. pseudopetalosa* tubers were used in the folkloric medicine by the natives of the South Blue Nile State for the treatment of breast cancer growth without any knowledge of its chemical constituents.

Key words: Capparaceae, brine shrimp larvae, bioactive compounds, column chromatography, GC/MS analysis, proline derivative.

INTRODUCTION

The use of plants in medicine is not limited or restricted to any region of the world. It is an old practice in various parts of the globe for both preventive and curative purposes. Dependence on herbs as medicine in the treatment of diseases is an adopted practice by a large proportion of the rural population; because of their availability and affordability (Sani et al., 2009).

The foliage parts of *M. pseudopetalosa* provide much relished browsing for goats in Somalia, while in parts of the Republic of South Sudan, the plant is eaten, but only

*Corresponding author. E-mail: manalabdalla071@gmail.com.

as a famine-food, after careful preparations to remove any toxic component (Henry, 1948). The fruit is eaten in Sudan under the belief that it provides physical strength and the roots are used to make sweet drinks (Doka, 2001). However, the same parts are used in Nigeria in topical application to the chest for cure of cough (Rajesh et al., 2009). The roots may also be used to purify stored water in rural areas (Burkill, 1985). And its use to cure tumors is practiced in the southern part of the Blue Nile State-Sudan. The toxic principle caused by tetra-methyl ammonium iodide (known as tetra-amine for short) is reported for the tuberous roots, roots and leaves of *Maerua pseudoheptalosa* (Henry, 1948).

Pisutthanan et al. (2004) had shown that brine shrimp lethality test is a general and excellent bioassay for toxicity screening of medicinal plants popularly used for several purposes. The bioassay is also used for monitoring the isolation of biologically active compounds. The brine shrimp assay has been established as a safe, practical and economic method for the determination of the bioactivity of synthetic compounds (Almeida et al., 2002) as well as plant products (Meyer et al., 1982; McLaughlin et al., 1991). The brine shrimp assay had been successively employed for bioassay-guided fractionation of active cytotoxic and antitumor agents as reported by Parra et al. (2001); who also referred to a positive correlation between the lethality to brine shrimp and the corresponding lethal oral dose in mice. The plant kingdom represents an enormous reservoir of biologically active molecules and so far, only small fractions of plants with medicinal activity have been assayed. Nearly 50% of drugs used in medicine are of plant origin. There is therefore much current research devoted to the phytochemical investigation of higher plants that have ethanobotanical information associated with them. The phytochemicals (secondary metabolites) isolated are then screened for different types of biological activity (Harborne, 1998).

In addition, there will be need for the permanent search and development of new natural drugs. This need also arise from the advantage of certain natural products in controlling some diseases that chemicals fail to do. For example, the annonaceous acetogenins, extracted from pawpaw tree (*Asimina triloba Dunal*) were found to be the best effective against selected tumor type, e.g., squamtacin is selective against the human prostate carcinoma cell line (PC-3) and a series of 9-carbonyl compounds work best against the human pancreatic tumor cell line (PaCa-2) (Ahammadsahib et al., 1993).

In this regard, a simple bioassay was used for screening purposes (Hostettmann, 1991). Thus *Artemia salina* larvae (brine shrimps nauplii) has been used as a target organism to detect bioactive compounds in plant extracts and toxicity to this crustacean has a good correlation with anti-tumor activities in man (McLaughlin, 1991) since the brine shrimp responds similarly to the corresponding mammalian system (Solis et al., 1993).

Cytotoxicity via the brine shrimp test is studied in order to reveal new anticancer compounds (Harborne, 1998). Therefore, this study aimed to evaluate the cytotoxicity of tuber fractions of *M. pseudopetalosa* against brine shrimp larvae as a new potential source of natural anti-tumor agent and the bioactive fractions were subjected to GC/MS analysis.

MATERIALS AND METHODS

Plant collection and extraction

The investigated plant (*M. pseudopetalosa*) was collected from Upper Nile State (Republic of South Sudan). Its geographical coordinates are: 9° 32' 13" North, 31° 39' 22" East. The plant was authenticated at the Department of Botany by Prof. Hatil H. Alkamali, Omdurman Islamic University. The dried ground tubers (1 kg) of *M. pseudopetalosa* were soaked for 3 days in 1500 ml ethyl acetate and ethanol, consecutively. They were subjected to silica gel (230 -400 mesh) column chromatography separation; using stepwise gradient elution of n-hexane to chloroform, and chloroform to ethyl acetate and finally washing with pure methanol. Using suitable solvent systems, portions of 100 ml were collected, concentrated and combined according to their similarity in spectrometric and TLC separation behaviors. Ethyl acetate gave eight fractions while ethanol gave twelve fractions (Harborne, 1998).

Brine shrimp lethality test

Brine shrimp lethality bio-assay was carried out to investigate the cytotoxicity of the plant extracts. *A. salina* (Leach) eggs (50 mg) were added to a hatching chamber containing sea water (45 ml). The hatching chamber was kept under an inflorescent bulb for 48 h for the eggs to hatch into shrimp larvae. Test fractions (20 mg) were separately dissolved in 2 ml of methanol; then 5, 50 and 500 µl of each solution were transferred into vials corresponding to 10, 100 and 1000 µg/ml, respectively. Each dosage was tested in triplicates. The vials (9 for each test) and one control containing 500 µl of the solvent were allowed to evaporate to dryness in 48 h at room temperature (Meyer et al., 1982).

Ten larvae of *A. salina* Leach (taken 48-72h after the initiation of hatching) were added to each vial and the final volume of the solution in each vial was adjusted to 5 ml with sea water; immediately after adding the shrimps. One drop of dimethyl sulphoxide (DMSO) was added to the test and control vials before the addition of the shrimps to enhance the solubility of the plant extract (Meyer et al., 1982). The LC_{50} values of the brine shrimps obtained for the tested plant extracts were recorded. The reference cytotoxic drug (Etoposide) was used as a positive control with LC_{50} (7.465 µg/ml) (Ahmad et al., 2009).

Gas chromatography/mass spectrometry technique (GC/MS)

The GC/MS analysis was done on a thermo-gas chromatograph /mass spectrometer (model Shimadzu 2010) equipped with DB-5 capillary column (30 m long, 0.25 mm in diameter, film thickness 0.25 µm). The carrier gas was helium and the maximum usable temperature was 325°C. The separated compounds were identified by computer searches in commercial libraries of NIST and WILEY

Table 1. Brine shrimps lethality of plant fractions.

S/N*	LC_{50} (µg/ml)	
	Ethanol fraction	Ethyl acetate fraction
1	>1000	37.80 (0.00-298.16)
2	>1000	883 (186.5-12552)
3	>1000	>1000
4	102 (50.43-206.05)	375.4 (205.2-840.8)
5	807 (7.85-142.1)	>1000
6	>1000	744.3 (236.1-16738.7)
7	89.9 (22.5-326.7)	299.7 (72.7-14875.1)
8	1.25 (18.71429988)	520.5 (199.8-3591.6)
9	7.98 (.9082-20.12)	
10	30.69 (2.79-95.82)	
11	0.1853 (7.6-445180	
12	0.0413 (2.95-9.32)	

*****Number of fractions.

(Ronald, 1997).

Statistical analyses

LC_{50} values were determined at 95% confidence intervals by analyzing the data on a computer loaded with a Finney Program (Mclaughin et al., 1991).

RESULT AND DISCUSSION

Brine shrimp toxicity assay

Two solvent fractions, namely ethanol and ethyl acetate of *M. pseudopetalosa* were used to test their cytotoxic effects against the brine shrimp *A. salina.* The mortality end point (LC_{50}) was calculated after 24 h; according to the method described by Meyer et al. (1982). The results of this study are classified as: LC_{50} less than 20 µg/ml were considered as highly toxic, LC_{50} from 20 to 100 µg/ml as toxic, LC_{50} from 100 to 500 µg/ml as moderately toxic and from 500 to 1000 µg/ml was weakly toxic according to Padmaja et al. (2002). However, Meyer et al. (1982) considered the LC_{50} values >1000 µg/ml as non-toxic or safe.

As a matter of fact, the results in Table 1 revealed that the ethanol extract is a very promising one with remarkable toxicity against brine shrimp larvae. The four fractions f_8, f_9, f_{11} and f_{12} have the highest cytotoxic effects with values equal to 1.25, 7.98, 0.185 and 0.041 µg/ml, respectively.

This is a clear indication of a first time achievement which was not preceded by any other reports in the available literature. The importance of the cytotoxicity stems from the fact that it is linked with the discovery of anticancer compounds (Moshi et al., 2004, 2006).

From a pharmacological point of view, a good relationship has been found with the brine shrimp lethality test to detect antitumoral compounds in terrestrial plant extracts (Mackeen et al., 2000; Zani et al., 1995). The significant correlation between the brine shrimp assay and *in vitro* growth inhibition of human solid tumor cell lines were demonstrated by the National Cancer Institute (NCI, USA). It is significant because it shows the value of this bioassay as a pre-screening tool for antitumor drug research (Anderson et al., 1991). Not only that there is positive correlation between brine shrimp toxicity and 9KB (human nasopharyngeal car-cinoma) cytotoxicity ($p = 0.036$ and kappa = 0.56). The brine shrimp test was being used as a prescreen for a panel of six human solid tumor cell lines at the Cell Culture Laboratory of the Purdue Cancer Center (*McLaughin* and Rogers, 1998). This is an internationally accepted bioassay for screening of antitumor compounds (Meyer et al., 1982).

Some of the other fractions including F_7 with a value equal to 89.9 µg/ml and F_{10} (LC_{50} 30.6 µg/ml) are considered to be toxic, whereas F_5 (LC_{50} 807 µg/ml) was weakly toxic. However, the other fractions showed non-toxic effects on brine shrimp larvae. Similar results were obtained by Adoum (2009) after application of an aqueous and ethyl acetate extracts of roots of *Cochlospermum tinctorium* and the chloroform fraction of stem bark of *Entada sudanica* which had exhibited very high lethality on brine shrimps at LC_{50} values of 8, 10 and 6 µg/ml.

Moderate toxicity for fractions 7 and 4 was shown by ethyl acetate fractions at LC_{50} (299.7 and 375.4 µg/ml), whereas F_2, F_6 and F_8 were weakly toxic. Moreover, F_3 and F_5 (LC_{50}>1000) gave non-toxic effects and were considered to be inactive or safe. F_1 is the only fraction which exhibited high toxic effect. However, the other fractions showed non-toxic effects on brine shrimp larvae. Bastos et al. (2009) examined four fractions of *Zeyheria tuberculosa* and reported that two of them were not toxic, while the other two were weakly toxic. Bose et al. (2011) studied the cytotoxicity of a member of the Capperaceae family (*Cleome viscose*) on the brine shrimp *A. salina* and had shown that its crude extract produced the most prominent cytotoxicity (LC_{50} 28.18 µg/ml).

Cytotoxicity may be linked to anticancer activity; since compounds with high toxicity (7.46 µg/ml LC_{50} and less), are considered as anticancer agents. The United States National Cancer Institute had a fixed standard level for cytotoxicity which is set according to the type of drug used (Mojica and Jose, 2007).

Gas chromatography/mass spectrometric (GC/MS) analysis

The GC/MS chromatographic separation technique was

Table 2. Molecular weights, retention times, formulae and peak areas of compounds present in F_8 as revealed by GC/MS analysis.

S/N	Compound	Mw.	Rt. time	Formula	Peak area (%)
1	Octadecanoic acid /stearic acid	284	37.425	$C_{18}H_{36}O_2$	0.95
2	Hexadecanoic acid, ethyl ester (ethyl palmitate)	284	38.083	$C_{18}H_{36}O_2$	7.08
3	7-Tetradecyne	194	40.067	$C_{14}H_{26}$	1.20
4	9-Octadecenoicacid,(Z)-methyl oleate	296	40.175	$C_{19}H_{36}O_2$	0.55
5	9,12-Octadecadienoic acid	280	40.83	$C_{18}H_{32}O_2$	3.07
6	Cis-9-Hexadecenal	238	40.925	$C_{16}H_{30}O$	2.94
7	Ethyl linoleate	308	41.325	$C_{20}H_{38}O_2$	45.53
8	Ethyl oleate	310	41.417	$C_{20}H_{38}O_2$	37.06
9	Ethyl-9-hexadecenoate	282	41.525	$C_{18}H_{34}O_2$	0.85
10	Heptadecanoic, ethyl ester/ ethyl -n-heptadecanaote	298	41.875	$C_{19}H_{38}O_2$	0.78

Mw. = Molecular weight; Rt. Time = retention time.

Table 3. Molecular weights, retention times, formulae and peak areas of compounds present in F_9 as revealed by GC/MS analysis.

S/N	Compound	Mw.	Rt.time	Formula	Peak area (%)
1	Furan-3-carboxaldehyde	96	6.667	$C_5H_4O_2$	2.12
2	Propanol,2,3-dihydroxy/ Glycerose	96	8.492	$C_3H_6O_3$	5.06
3	3-Pyridinecarboxylic acid 1,2,5,6-tetrahydro-1-methyl-methyl ester	155	14.808	$C_8H_{13}NO_2$	8.01
4	2-Furancarboxaldehyde,5-(hydroxyl- methyl)	126	19.633	$C_6H_6O_3$	58.54
5	Octanoic acid, 2-methyl,methyl ester/methyl2-methyl octanoate	172	38.075	$C_{10}H_{20}O_2$	0.80
6	(Z,Z)-heptadeca-8,11-dien-1-yl bromide	314	40.067	$C_{17}H_{31}Br$	0.61
7	Ethyl linoleate	308	41.317	$C_{20}H_{36}O_2$	12.27
8	Ethyl oleate	310	41.408	$C_{20}H_{38}O_2$	9.60

Mw. = Molecular weight; Rt. Time = retention time.

used for identification of the four ethanol fractions (8, 9, 11 and 12) which were selected according to their cytotoxic effects against brine shrimp larvae.

The detection of 33 different compounds found in the four fractions in this study is considered as a pioneer achievement which has not been reported before. F_8 and F_9 are composed of ten and eight compounds, respectively (Tables 2 and 3, Figures 1 and 2). Out of these compounds, two were found to be common in the two fractions; these are ethyl linolate and ethyl oleate derivatives of the corresponding unsaturated fatty acids. These two fatty acids are possible causes of the cytotoxicity observed for the two fractions (F_8 and F_9); since Ortsater (2011) reported that the ethyl derivatives of unsaturated fatty acids oleate and linoleate are known to display some toxicity. However, he also referred to the potent toxicity of the palmitate derivatives which may provide another explanation for the increased cytotoxicity of fraction 8; since their presence is restricted to this fraction. A further possible explanation for the increased cytotoxicity of fraction 8 might be as a result of the presence of the ethyl derivatives of the additional fatty acids (other than oleate and linoleate ethyl derivatives) present in this fraction which are replaced by the methyl derivatives in fraction 9.

Furthermore, the presence of decenoic derivative in fraction 8 might also be taken as another proof for the increased cytotoxicity of the fraction as compared to fraction 9 which lacks this derivative. Also, hexadecenoic acid, which was also reported in the royal jelly, of the nurse bees is known to have bactericide, anti- inflamematory and anticancer activities (Isidrov et al., 2011).

As for F_{11} and F_{12} and despite the fact that they have five compounds in common (Tables 4 and 5, Figures 3 and 4), it was found that F_{11} has more detectable compounds (15 compounds) and less cytotoxicity than F_{12} which has got only 9 compounds.

The only compound which bears some kind of resemblance (not exactly similar) is the compound proline, N-methyl- butyl ester, in fraction 12, as compared to proline betaine ethyl ester identified from the same *Maerua sp*. by William et al. (1996).

One of the compounds present in fraction 12 is a derivative of proline (proline-N-methyl- butyl ester). This

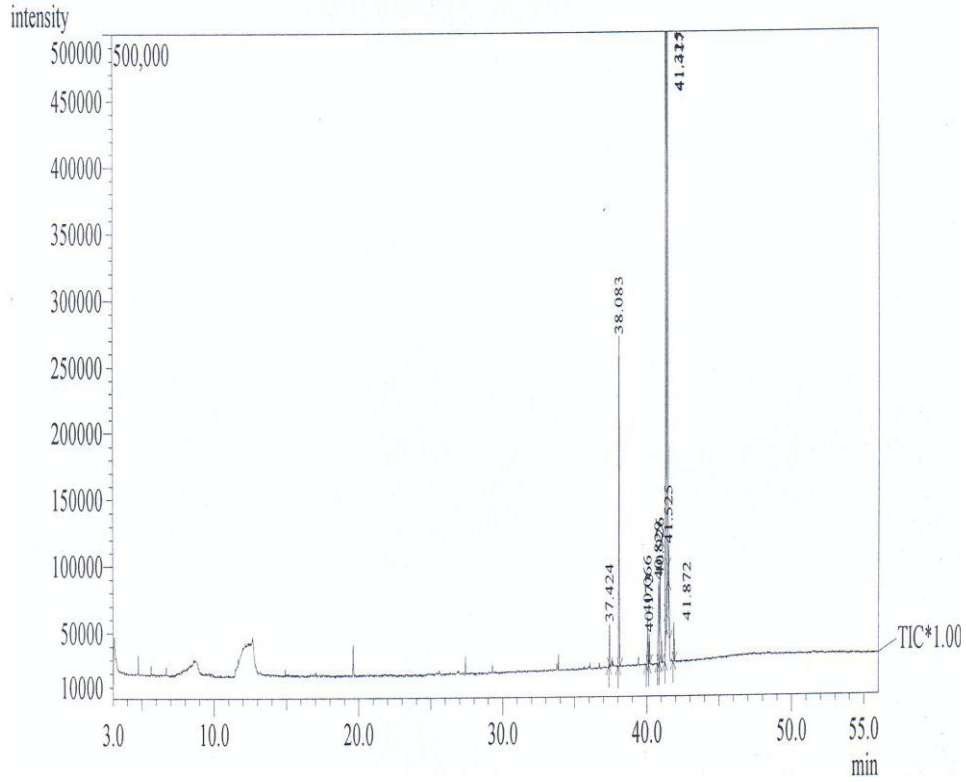

Figure 1. GC/MS total chromatogram of compounds detected in fraction (8).

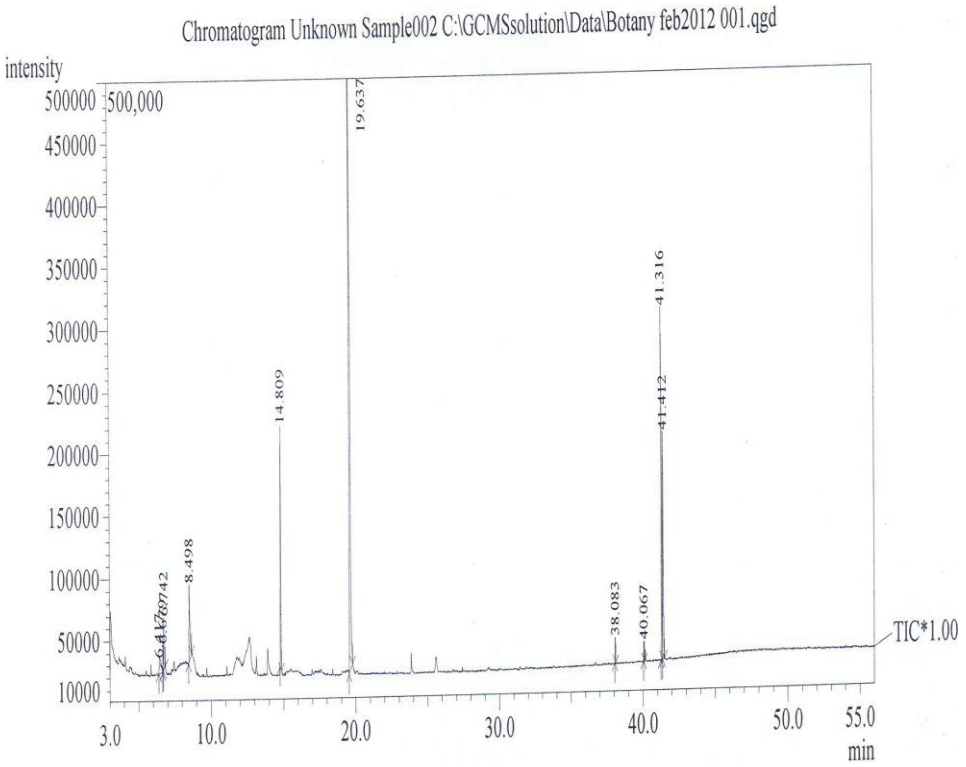

Figure 2. GC/MS total chromatogram of compounds detected in fraction (9).

Table 4. Molecular weights, retention times, formulae and peak areas of compounds present in F_{11} as revealed by GC/MS analysis.

S/N	Compound	Mw.	Rt. time	Formula	Peak area (%)
1	Methanamine,N-(3-methyl-2-butenylidene	97	6.725	$C_6H_{11}N$	15.26
2	N,N-Dimethyl-1,3-butadien-1-amine	97	7.417	$C_6H_{11}N$	4.34
3	Cyclobutanone,2,3,3,4-tetramethyl	126	10.692	$C_8H_{14}O$	27.40
4	Hygrine/2-prpanone,1-(methyl-2-pyrrolidinyl)-,	141	13.783	$C_8H_{15}NO$	0.46
5	1-(3-amino propyl)piperidine	142	14.342	$C_8H_{18}N2$	3.39
6	3-Pyridinecarboxylic acid,1,2,5,6-tetrahydro-1-methyl,methyl ester	155	14.817	$C_8H_{13}NO_2$	0.76
7	Morpholine,4-(2-methyl-1propenyl	141	15.308	$C_8H_{15}NO$	22.55
8	6-methyl-6-azabicyclo(3,2,1)octane	125	17.717	$C_8H_{15}N$	2.86
9	1-Methyl-pyrrolidine-2-caroxylic acid	129	18.075	$C_6H_{11}NO_2$	1.12
10	5-hydroxy piperidine carboxylic acid	145	18.217	$C_6H_{11}N_3O$	9.65
11	1,4-cis-Cyclohexanedicarboxylic acid,dimethyl ester	200	18.392	$C_{10}H_{16}O_4$	2.21
12	Boranamine,N-ethyl-1,1-dipropyl	141	18.625	$C_8H_{20}BN$	0.92
13	2-(E)-Hexenoic acid,(4S)-amino-5-methyl	143	18.742	$C_7H_{13}NO_2$	6.14
14	1-Piperidinepropanenitrile	138	31.667	$C_8H_{14}N_2$	1.39
15	2-Pyrolidine methanol,1-methyl(1-methyl-2pyrrolidinyl)-methanol	115	31.217	$C_6H_{13}NO$	1.57

Mw. = Molecular weight; Rt. Time = retention time.

Table 5. Molecular weights, retention times, formulae and peak areas of compounds present in F_{12} as revealed by GC/MS analysis.

S/N	Compound	Mw.	Rt. time	Formula	Peak area (%)
1	(trans/cis)-3-(Dimethylamino)-4,5-dicyanocyclohex-1-ene	175	6.742	$C_6H_{13}N_3$	2.19
2	Methanamine,N-(3-methyl-2-butenyliden	97	7.300	$C_6H_{11}N$	19.13
3	N,N-Dimethyl-1,3-butadien-1-amine	97	7.433	$C_6H_{11}N$	28.05
4	Cyclobutanone,2,3,3,4-tetramethyl	126	10.700	$C_8H_{14}O$	1.71
5	Hygrine/2-propanone,1-(methyl-2-pyrrolidinyl)-,(R)	141	13.808	$C_8H_{15}NO_2$	11.31
6	N-(6-chloro-2-pyrazinyl)-2-(1-piperidinyl)acetamide	245	14.367	$C_{11}H_{15}CIN_4O$	2.49
7	Morpholine,4-(2-methyl-1-propenyl)	141	15.317	$C_8H_{15}NO$	7.35
8	Proline, N-methyl-butyl ester	185	17.925	$C_{10}H_{19}NO_2$	2.01
9	Cyclohexanol,2-amino-1-methyl-4-(1-methylethyl)	155	18.625	$C_{10}H_{21}N$	2.28

Mw. = Molecular weight; Rt. Time = retention time.

compound might be considered as the cause of the high toxicity of the fraction; since free proline was found to inhibit the growth of mammary tumors induced by N-methyl-N-Nitrosourea in rats as reported by Kalinovsky et al. (2005). They suggested its use as an inhibitor of breast cancer development.

Surprisingly, *M. pseudopetalosa* tubers were used in the folkloric medicine by the natives of the South Blue Nile State for the treatment of breast cancer growth without any knowledge of its chemical constituents. Furthermore, acetamide which is present in F_{12} was found to be cytotoxic at high concentrations in maternal animals (Kennedy, 1986) and may consequently add to the increased cytotoxicity of the fraction at the appropriate concentration.

Conclusion

The overall results indicated that the ethanol fractions

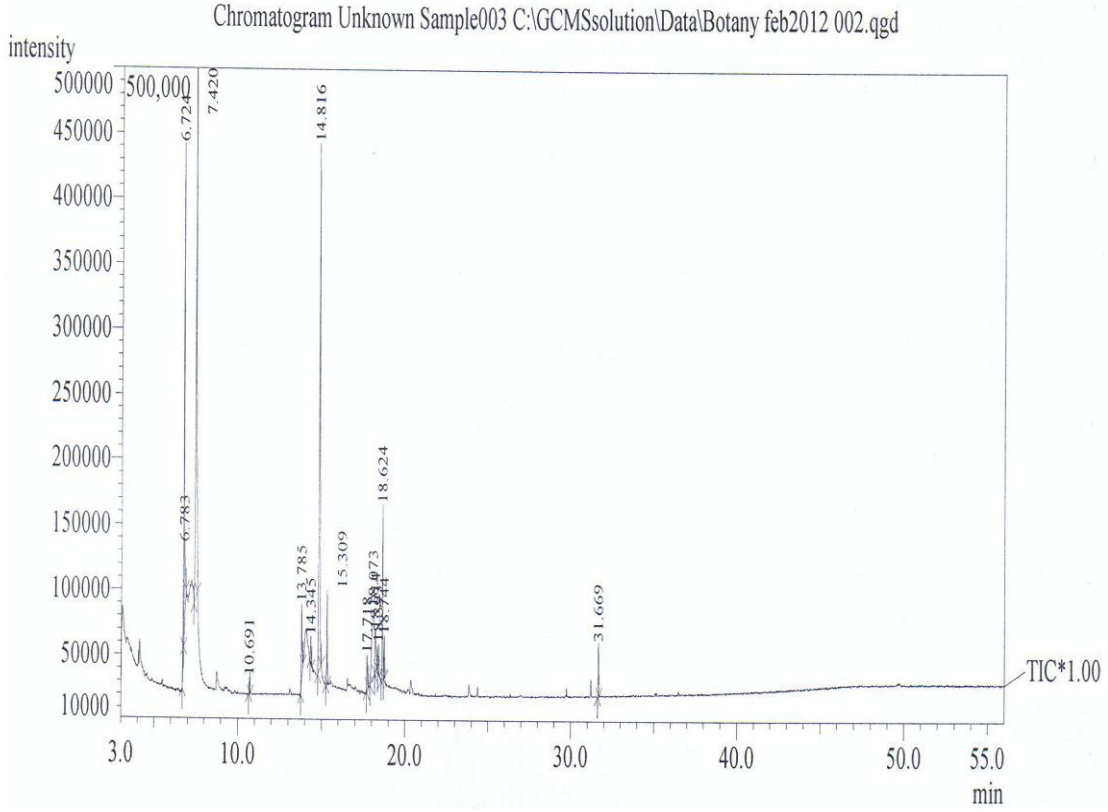

Figure 3. GC/MS total chromatogram of copounds detected in fraction (11).

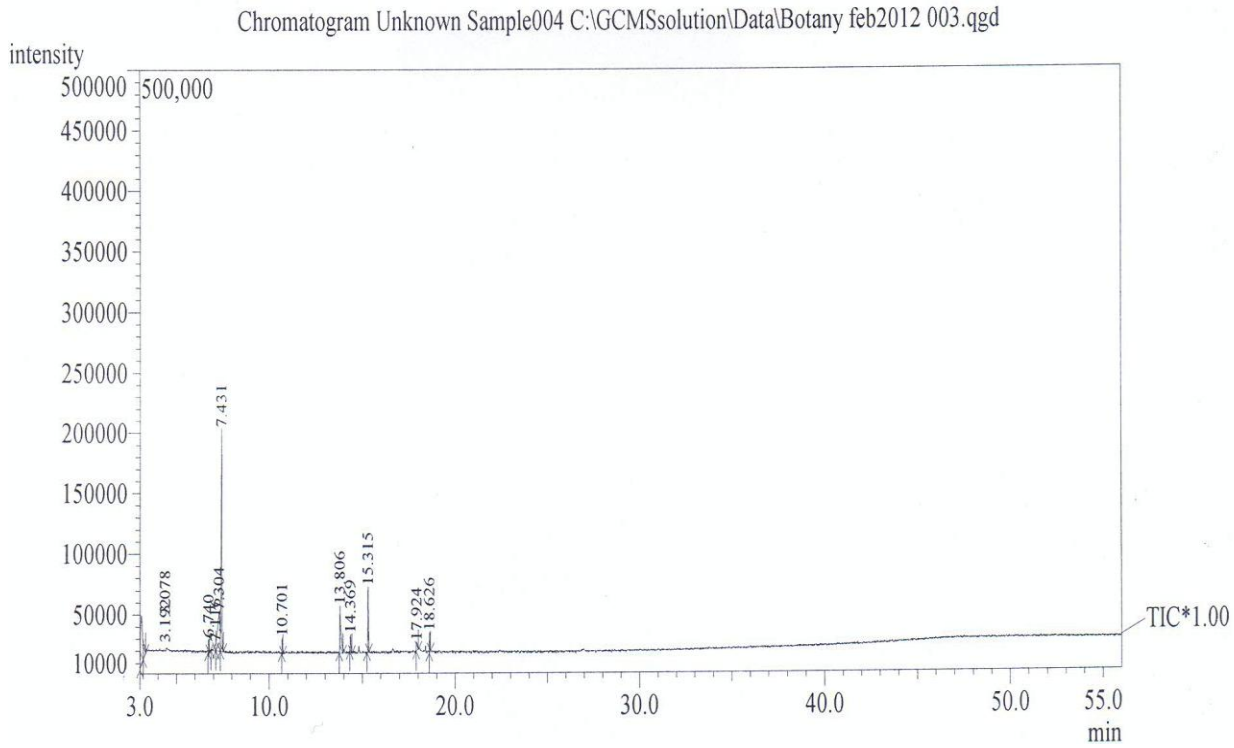

Figure 4. GC/MS total chromatogram of compounds detected in fraction (12).

exerted more cytotoxic effects than those obtained through ethyl acetate fractions.

The four ethanol fractions (F_8, F_9, F_{11} and F_{12}) with highest cytotoxicity are composed mainly of derivatives of fatty acids, derivatives of amino acids, amines, acetamide, ester cyclobutanone, methane amines and carboxylic acid derivatives.

The importance of cytotoxicity, in this study is brought about by its highly acclaimed characteristics that are closely linked to antitumor activity.

Conflict of Interest

The authors have not declared any conflict of interest.

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REFERENCES

Adoum OA (2009). Determination of toxicity levels of some savannah plants using brine shrimp tests (BTS). Bayero J. Pure Appl. Sci. 2(1):135-138.

Ahammadsahib KI, Hollingworth RM, McGovern PJ, Hui YH, McLaughlin JL (1993). Inhibition of NADH: ubiquinone reductase (mitochondrial complex I) by bullatacin, a potent antitumor and pesticidal Annonaceous acetogenin. Life Sci. 53:1113-1120.

Ahmad B, Ali N, Shumaila B, Choudhary MI (2009). Biological activities of aerial parts of *Tylophora hirsuta Wall.* Afr. J. Biotechnol. 8(18):4627-4631.

Almeida PA, Silva TMS, Echevarria A (2002). Mesoionic 5-alkyl-1,3-dithiolium-4-thiolates: Synthesis and brine shrimp toxicity. Heterocycl. Comm. 8:593-600.

Anderson JE, Goetz CM, McLaughlin JL, Suffness M (1991). A blind comparison of simple bench-top bioassay and human tumour cell cytotoxicities as antitumor prescreens. J. Phytochem. Anal. 2:107-111.

Bastos ML, Lima MR, Conserva LM, AndradeVS, Rocha EM, Lemos RP (2009). Studies on the antimicrobial activity and brine shrimp toxicity of *Zeyheria tuberculosa* (vell.) Bur. (Bignoniaceae) extracts and their main constituents. Ann. Clin. Microbiol. Antimicrob. 8:16.

Bose V, Bala V, Ghosh NT, Gunasekaran K, Rahman A (2011).Antinociceptive, cytotoxic and antibacterial activities of *Cleome viscose* leaves. Rev. Bras. Farmacogn. 21(1):45-51.

Burkill HM (1985).The useful plants of West Tropical Africa. Royal Botanical Garden.1(1):18-26.

Doka IGM (2001). Some medicinal plants of Western Kordofan folklore and phytochemical screening. MSc. Thesis. Faculty of Science Unv. Of Khartoum, Sudan. pp. 18.

Harborne JB (1998). Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. 3rd Edn., Chapman and Hall, London. pp. 302.

Henry AJ (1948). The toxic principle of *Courbonia virgata*: Its isolation and identification as a tetramethylammonium salt. Birth. J. Pharmacol. 3:187.

Hostettmann K (1991). Methods in Plant Biochemistry: Assays for Bioactivity. Academic Press, London. UK. 6:360.

Isidrov VA, Czyzewska V, Jankowsk M , Bakier S (2011). Determination of royal jelly acid in honey. J. Food Chem. 124:387-391.

Kalinovsky T, Waheed M, Nusrath W, Ivanov V, Niedzwiecki A, Rath M (2005). Modulation of N-methyl-N- nitrosourea induced mammary tumors in Sprague-Dawley rats by combination of lysine,proline, arginine, ascorbic acid and green tea extract. J. Breast Cancer Res. 7:291-295

Kennedy GL (1986).Biological effects of acetamides and formamides and their monomethyl and dimethyl derivatives. J. Crit. Rev. Taxicol. 7(2):129-182.

Mackeen MM, Ali AM, Lajis NH, Kawazu K, Hassan Z, Amran M, Habsah M, Mooi LY, Mohamed SM (2000). Antimicrobial, antioxidant, antitumour-promoting and cytotoxic activities of different plant part extracts of *Garcinia atroviridis* Griff. Ex T. Anders. J Ethnopharmacol. 72:395-402.

McLaughin JL, Rogers LL (1998). The use of biological assays to evaluate botanicals. J. Drug Inform. 32:513-524.

Mclaughin JL, Chang CJ, Smith DL (1991). "Bench – Top" Bioassays for discovery of bioactive natural products: an update. Stud. Nat. Prod. Chem. 9:383-409.

Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL (1982). Brine shrimp: A convenient general bioassay for active plant constituents. Planta Med. 45:31-34.

Mojica E, Jose R (2007). Bioactivity study of *Barringtonia asiatica* (L.) Kurz. seed aqueous extract in *Artemia salina*. Int. J. Bot. 3(3):325-328.

Moshi MJ, Cosam JC, Mbwam BOH, Kapingu M, Nkunsya MHH (2004). Testing beyond ethnomedical claims: Brine shrimp lethality of some Tanzanian plants. J. Pharmaceut. Biol. 42:547-551.

Moshi MJ, Mbwambo ZH, Nodo RS, Masmba PJ, Kamuhabwa A, Kapingn MC, Thomas P, Richard M (2006). Evaluantion of ethnomedical claims and brine shrimp toxicity of some plants used in Tanzania as traditional medicines. Afr. J. Tradit. Complement. Altern. Med. 3:48-58.

Ortsater H (2011). Arachidonic acid fights palmitate :New insights into fatty acid toxicity in β-cells. J. Clin. Sci. 120:179-181.

Padmaja R, Arun PC, Prashanth D, Deepak M, Amit A, Anjana M (2002). Brine shrimp lethality bioassay of selected Indian medicinal plants. J. Fitoterapia. 73(6): 508-510.

Parra AL, Yhebra RS, Sardinas IG, Buela LI (2001). Comparative study of the assay of *Artemia salina* L. and the estimate of the medium lethal dose (LD_{50}avalue) in mice to determine oral acute toxicity of plant extracts. J. Phytomed. 8(5):395-400.

Pisutthanan S, Plianbangchang P, Pisutthanan N, Rvanruay S, Muanrit O (2004). Brine shrimp Lethality. Activity of Thia medicinal plants in the family Meliaceae. Naresuan Univ. J. 12:13-18.

Rajesh P, Selvamani P, Lath S, Saraswathy A, Rajesh VK, (2009). A review on chemical and medicobiological applications of capparidaceae family. J. Pharmacogn. 31(6):378-387.

Ronald HA (1997). Gas Chromatography Mass Spectroscopy: Handbook of Instrumental Techniques for Analytical Chemistry. pp. 609-611.

Sani D, Sanni S, Ngulde SI (2009). Phytochemical and antimicrobial screening of the stem aqueous extract of *Anisopus mannii.* J. Med. Plant Res. 3:112-115.

Solis PN, Wright CW, Anderson MM, Gupta MP, Phillipson JD (1993). A microwell cytotoxicity assay using *Artemia salina*. Plant. Med. 59:250-252.

William F H, Mclean G, Blunden G, Jeweres K (1996). Quaternary ammonium compounds in the Capparaceae. Biochem. Syst. Ecol. 24(5):427-434.

Zani CL, Chaves PPG, Queiroz R, Mendes NM, Olivii AB (1995). Brine shrimp lethality assay as a prescreening system for anti-*Trypanosoma crusi*. J. Phytomed. 2:47-50.

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