CHARACTERIZATION OF DESERT DATE (*Balanites aegyptiaca*) SAPONINS AND THEIR BIOLOGICAL ACTIVITIES

Thesis submitted in partial fulfillment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

by

Bishnu P. Chapagain

Submitted to the Senate of
Ben-Gurion University of the Negev

February 2006

Beer-Sheva

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Approved by the advisor:_____

Approved by the Dean of the Kreitman School of Advanced Graduate Studies:

_____

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Abstract

The desert date (*Balanites aegyptiaca* L.) is an evergreen tree belonging to the Zygophyllaceae family. It is mainly grown in the arid regions of Africa, the Middle East, and South Asia. Israel is considered the Northern-most hemisphere where Balanites trees grow naturally. In Israel, Balanites is found growing naturally in the Ein-Gedi Oasis, the Arava rift Valley, and Bet-Shean valley. Balanites has multi-use potential—from ethno-medicine to fire wood. Various parts of this plant have been used for many folk medicines in Africa and Asia. Early studies have shown that the saponin compounds are the main attributer behind its ethno-medicinal uses. Balanites oil is considered to be a good source for cosmetics and it was found to be used by ancient Egyptian royalty. Furthermore, Balanites is highly adapted to the arid regions where no other trees have adapted to the harsh arid conditions. In spite of the multi-use potential and ecological significance, Balanites is the most neglected tree species in the arid regions and the plant has not yet been domesticated.

Saponins belong to a complex and chemically diverse group of compounds which derive their name from their ability to form stable, soap-like foam in aqueous solutions. In plants, saponins play a role as secondary metabolites and assigned for defense mechanism. In chemical terms, saponins contain a carbohydrate moiety attached to a triterpenoid or steroid aglycone. Saponins are currently attracting considerable interest as a result of their diverse beneficial properties. Recent studies have suggested that saponins affect the immune system in ways that help to protect the human body against cancers, and also lower cholesterol levels. Saponins decrease blood lipids, lower cancer risks, and lower blood glucose response. With increasing demand for pharmacological and nutritive values for the saponins and sapogenin, alternate sources of saponins are sought. Until now, diosgenin and related steroidal saponins have been obtained commercially from the tubers of various Dioscorea species, however it is crucial to discover new and alternative sources of these compounds because of decreasing plant resources as well as increasing demand.

So far, studies have reported on saponin content in Balanites only from African and Indian samples. So the prime objective of this study was to characterize the saponins in

the various tissues of the *B. aegyptiaca* grown in Israel. For this purpose, liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-mass spectrometry /mass spectrometry (LC-MS^n) techniques were employed for the identification of the saponins in the fruit mesocarp, seed kernel, and root extracts of Balanites, and high field nuclear magnetic resonance (NMR) was used for structural elucidation of the major saponins in support of the LC-MS. The second aim of this study was to characterize the biological activities of the saponins from Balanites tissues. Various saponins and mostly saponin-rich extracts (SREs) were evaluated for the antifungal and larvicidal activity using some of the most prevalent phytofungi, and mosquito larvae. To increase the understanding of the interaction of saponin molecules with the plant membrane, another part of the study focused on the use of SREs as delivery adjuvants across isolated leaf cuticle membranes (CMs). A comparative study of saponin content in seed kernels and its correlation among five Israeli, as well as five international, provenances and the possibility of production of diosgenin (sapogenin) in callus culture in Balanites was also carried out.

The LC-ESI-MS^n characterization of the methanol extracts of fruit mesocarp of *B. aegyptiaca* grown in Israel found five major saponins with main one as1064 Da (*ca.* 42% of the total saponins). Other saponins found in the fruit mesocarp were: 1078, 1196, 1210, and 1046 Da. The structures elucidated by 800 MHz NMR reveled that the main saponin (1064 Da) of fruit mesocarp was 26-(O-β-D-glucopyranosyl)-3β,22,26-trihydroxyfurost-5-ene 3-O-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**1**). Subsequently, the second (1210 Da) and third (1078 Da) major saponins in the fruit mesocarp were 26-(O-β-D-glucopyranosyl)-22-O-methylfurost-5ene.3β,26-diol 3-O-β-D-xylopyranosyl-(1→3)- β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**2**) and 26-(O-β-D-glucopyranosyl)-22-O-methylfurost-5ene.3β,26-diol 3-O-β-D- glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**3**)**,** respectively**.** The LC-ESI-MS^n analysis of the methanol extract of the kernel detected six saponins with main one being 1210 Da (*ca.* 36% of total saponin). Similarly, nine saponins, with main one 1196 Da (*ca.* 52.6% of the total saponins), were found in root extracts. In all these tissues, diosgenin was found only the sole aglycone for saponins. The chemical characterization experiment

of various Balanites tissues suggested that LC-ESI-MS^n can effectively be used for rapid identification of saponins in Balanites using minimum manipulations and chromatographic separation.

All SREs of *B. aegyptiaca* including fruit mesocarp (ME), kernel (KE), and root extracts (RE) were found highly larvicidal against the vector of Denuge (*Aëdes aegypti*) and West Nile Virus (*Culex pipiens*). Among the SREs, the highest larvicidal effects were found in KE followed by RE and ME. The larvicidal experiments with pure isolated saponins (**1**), (**2**), and (**3**) revealed that saponins with a methyl group in the C-22 position was found to be highly larvicidal against mosquito larvae, compared with the saponin lacking the methyl group at this position. The presence of a xylose sugar unit at the sugar chain was found to be less active compared to saponons lacking this molecule.

Regarding the fungicidal activity, fungal and/or dose specific inhibition of SREs was achieved. Moderate and high inhibition of SRE of fruit mesocparp (ME) was found against the growth of *Alternaria solani* and *Pithium ultimum*, respectively, whereas no inhibition was found against the growth of *Fusarium oxysporum*, *Verticillium dahliae*, and *Colletrotrichum coccodes*.

SREs of fruit mesocarp, kernel, and root of Balanites were also found to accelerate the delivery of 2,4-D (^{14}C) through isolated CMs. Among the three SREs, mesocarp extract showed better performance. The transmission electron microscope (TEM) and dynamic light scattering (DLS) characterization showed that Balanites saponins produce nano size vesicles and these nano vesicles could have played a vital role in 2,4-D delivery together with the association of saponin molecule with the phytosterol present in the CMs.

Among the tested Balanites provenances, the highest sapogenin (2.74 % DW) level in kernels was found in Bet-Shean provenance (Israel) whereas the lowest sapogenin level (1.41% DW) was found in Rajasthan provenance (India). A strong positive correlation (R^2 = 0.849) between sapogenin level and oil content in seed kernels was also observed. *In vitro* study of callus cultures and subsequent determination of the total sapogenin accumulation, revealed that shoot-derived callus produced the highest level of total sapogenin *in vitro* when raised on MS basal media supplemented with 1.0 mg l^{-1} 2,4-D alone or in combination with 0.5 mg l^{-1} BAP, compared to the root, hypocotyl, and epicotyl derived callus. However, the total sapogenin was found to be significantly affected by the

growth regulators and somatic embryogenesis together with the explants.

In summary, it is hoped that the outcome of this study would certainly help to further increase the understanding of saponins and to help to expand the use of *B. aegyptiaca* through exploitation of new uses concerning its saponin content. That would ultimately help to domesticate this highly potential but most neglected plant in arid regions of Africa and Asia, which could certainly help to improve the ecological and financial situation of the world's most difficult area in long run.

Key words: Balanites, provenances, saponins, diosgenin, biological activities, LC-ESI-MS^n, NMR, larvicidal, antifungal, *Aëdes*, *Culex*, *Pithium*, *Alternaria*, *Fusarium*, *Verticillium*, *Colletrotrichum*, leaf cuticles, nano-vesicles, callus culture, PGR, embryogenesis.

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Glossary

CID	collision induced dissociation
CMs	leaf cuticular membrane
COSY	two-dimensional ^{1}H correlation spectrometry
DEPT	distortionless enhancement by polarization transfer
DDW	double distilled water
DW	dry weight
ELS	evaporative light scattering
Gal	β-D-Galactose
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
Glu	β-D-Glucose
HMBC	^{1}H-detected heteronuclear one bond spectroscopy
HPLC	high-performance liquid chromatography
HPPL	high performance particle size
HSQC	heteronuclear single quantum coherence
IR	infra-red
LC-MS	liquid chromatography-mass spectrometry
m/z	mass-to-charge ratio
Me	methyl
NMR	nuclear magnetic resonance
PPM	parts per million
Rha	α-L-rhamnose (6-deoxymannose)
RI	refractive index
SEM	scanning electron microscope
SOFU	simulation of foliar uptake
TEM	transmission electron microscope
TLC	thin layer chromatography
UV	ultra-violet
Xyl	β-D-Xylose

Articles written from this thesis

1. Wiesman Z, **Chapagain BP** (2003). Laboratory evaluation of natural saponin as bioactive agent against *Aedes aegypti* and *Culex pipiens*. Dengue Bull 27: 168-173.
2. **Chapagain BP**, Wiesman Z (2005). Variation in diosgenin level in seed kernel among different provenances of *Balanites aegyptiaca* Del (Zygophyllaceae) and its correlation with oil content. Afric J Biotechnol 4: 1209-1213.
3. **Chapagain BP**, Wiesman Z (2005). Larvicidal effects of aqueous extracts of *Balanites aegyptiaca* (desert date) against the larvae of *Culex pipiens* mosquitoes. Afric J Biotechnol 4: 1351-1354.
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6. **Chapagain BP**, Wiesman Z (2006). Phyto-saponins as a natural adjuvant for delivery of agro-materials through plant cuticular membranes. J Agric Food Chem 54: 6277-6285.
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8. Stærk D, **Chapagain BP**, Lindin T, Wiesman Z, Jaroszewski JW (2006). Structural analysis of complex saponins of *Balanites aegyptiaca* by 800 MHz ^{1}H NMR. Magn Reson Chem 44: 923-928.
9. **Chapagain BP**, Wiesman Z. Structural characterization of saponins in *Balanites aegyptiaca* Del. kernel and root extracts by liquid chromatography – electrospray ionization-mass spectrometry (LC-ESI-MS^{n}). Phytochem Anal, *Submitted.*
10. **Chapagain BP**, Wiesman Z, Tzor L. *In-vitro* study of antifungal activity of saponin-rich extracts against most prevalent phytopathogenic fungi. Ind Crops Prod, *Submitted.*
11. **Chapagain BP**, Saharan V, Wiesman Z. Larvicidal activity of saponin-rich extract of Balanies aegyptiaca callus against Aedes aegypti mosquito. Bioresource Technol, *Submitted*.
12. **Chapagain BP,** Wiesman Z. Desert date (Balanites aegyptiaca L.): a potential source for control of dengue vector in isolated rural dry land areas (Review), Dengue Bull, *Submitted*.

Thesis organization

This thesis is divided into seven chapters: **Chapter 1** is a general introduction; **Chapters 2** and **3** report the chemical characterization of the saponins from the various tissues of *Balanites aegyptiaca* grown in Israel; **Chapter 4** mentions larvicidal properties of Balanites saponin-rich extracts and/or pure saponins; **Chapter 5** describes antifungal activities of saponin rich extracts; **Chapter 6** deals with the interaction of saponin with isolated leaf cuticular membranes (CMs), and **Chapter 7** presents the general conclusions. Each chapter reporting the research (**Chapters 2–6**) is designed to stand by itself, with materials and methods, results and discussion, and conclusions sections. Since the general introduction in **Chapter 1** covers most of the introductions for later chapters, the introductions in **Chapters 2–6** have been shortened to reduce redundancy.

The variation in sapogenin (diosgenin) level in kernels from different provenances of Balanites and their correlation with oil content and the production of sapogenin (diosgenin*) in vitro* in callus cultures of Balanites are reported only as published articles (**Appendices A1** and **A2**) in order to address the production potential of the *B. aegyptiaca* saponins. Similarly, larvicidal effects of the aqueous extracts and saponin fraction of Balanites tissues are also presented only in published articles (**Appendices A3** and **A4)** to reduce redundancy. **Appendices B1–B3** presents the NMR spectral data of the major Balanites saponins.

CHAPTER 1

General Introduction

Balanites aegyptiaca

Description of the plant

Desert date (*Balanites aegyptiaca* L. Delile) is a semi-evergreen, usually spiny, extremely variable shrub or small tree of the Zygophyllaceae family that grows up to 12 m high. The bole is usually straight with a 60 cm diameter, often fluted, the branches are generally spread irregularly or pendulous, and sometimes form a round crown. The tree produces yellow date-like fruit. The trees bear heavy yields—as many as 10,000 fruits annually on a mature tree in good condition (**Fig 1.1**). Each fruit, weighing 5–8 g, consists of an epicarp (5–9%), a mesocarp or pulp (28–33%), an endocarp (49–54%), and a kernel (8–12%) (**Fig 1.2**). The oil content of *B. aegyptiaca* seed kernels approaches 50% (Chapagain and Wiesman, 2005).

History of the plant

The Balanites tree has a long history of use as a resource, especially in the African continent where it is the most wide-spread woody plant. According to Sprague (1913), *B. aegyptiaca* has been planted in Egypt for over four thousand years. Stones of the fruits were placed as votive offerings in the tombs of the Twelfth Dynasty. The tree also has biblical connections. It is believed that Balanites was the source of one of the ingredients of the perfume 'spikenard' as used by the Egyptian royalty (Hall and Walker, 1991). Today this plant plays a diverse cultural and traditional role in different societies. The Huasa community of West Africa uses the fruit of *B. aegyptiaca* to ensure immunity and protection against defeat in boxing (Irvine, 1961). Chips of the wood placed in elephant dung are used to prevent elephants from attacking people or property in East Africa. The mistletoe grown in *B. aegyptiaca* is taken as concoctions to enhance scholastic ability. The fruit is used to hang around the neck of a potential victim to ward off blood-sucking sorcerers in Saharan Morocco (Burkhill, 1985). If, as is generally believed, humanity began in Africa, then the bittersweet Balanites fruit is likely among the oldest of all foods. Certainly this resilient evergreen tree has been helping mankind for thousands of years. Its

fruits have been found in Pharaohs' tombs dating back to at least the 12th dynasty in ancient Egypt; thus even royalty has appreciated it for 4,000 years (Ladipo, 1989).

Distribution

Balanites aegyptiaca is perhaps one of the most wide-spread woody plants of the African continent. It is distributed through much of Africa from costal Mauritania and Senegal to Somalia and Egypt, southwards to Zambia and Zimbabwe, as well as in the Middle East from Yemen to Jordan and Israel (Sands, 2001). Benin, Burkina Faso, Cameroon, Chad, Djibouti, Ethiopia, Gambia, Ghana, Guinea, Bissau, Guinea, Ivory Coast, Kenya, Mali, Mauritania, Nigeria, Niger, Senegal, Sudan, Somalia, Tanzania, Togo, Uganda, Zaire, and Zambia are the primary African countries where Balanites are grown (Booth and Wickens, 1988). Algeria, Angola, Burundi, Central African Republic, Libya, Morocco, and Rwanda are the other African countries where Balanites are found (Hall and Walker, 1991).

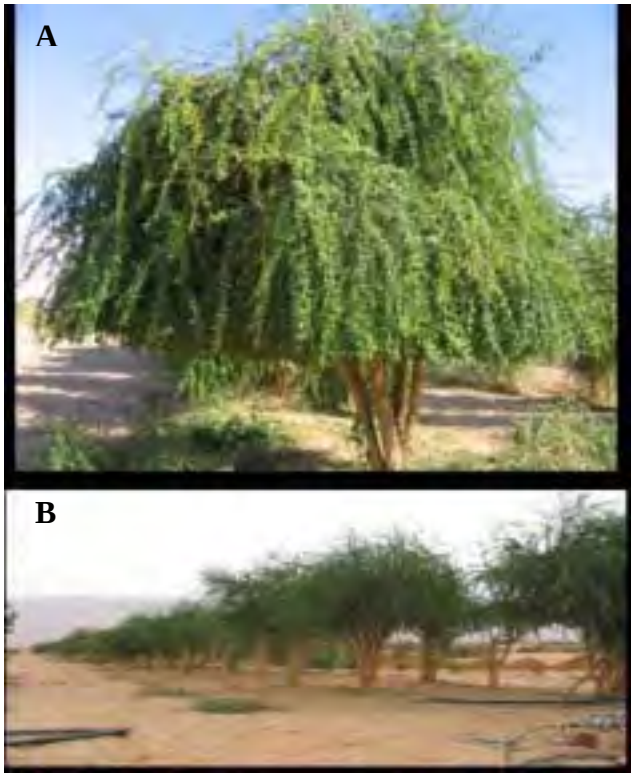


Fig 1.1: A – mature tree of *B. aegyptiaca* grown in the Arava Valley, Israel. B – a landscape view of a Balanites plantation site at Kibbutz Samar, Israel.

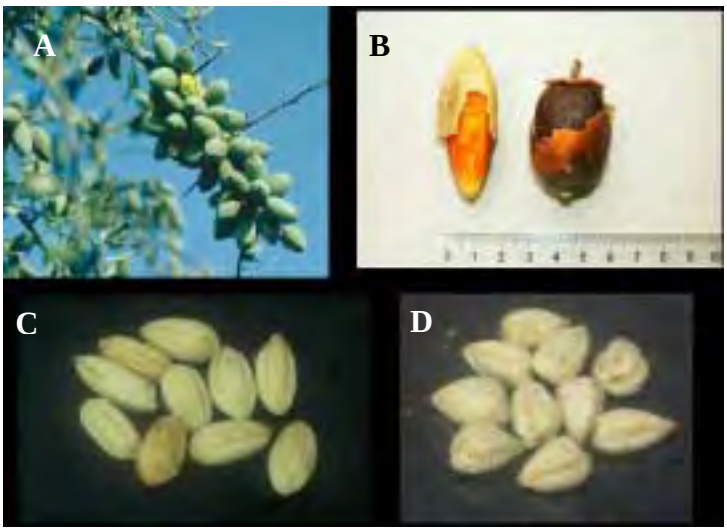


Fig 1.2: Immature fruiting branch (A), mature fruits (B), nuts (C), and kernels of Balanites.

Balanites are not only grown throughout the African continent but also in the Middle East, the Arabian Peninsula, and Southern Asia. Israel, Jordan, Saudi Arabia, North and South Yemen, India, and Myanmar are the countries beyond Africa where Balanites naturally grow. In Israel, Balanites are found in the Arava Valley (near the Jordanian border), Eilat (near Red Sea coast), Ein-Gedi oasis (near the Dead Sea), and Bet-Shean Valley (near the Sea of Galilee) (Chapagain and Wiesman, 2005). The growth range of Balanites was found to extend across more than 50º of latitude: from 35º N (Bet-Shean Valley, Israel) to about 19º S (Budi district, Zimbawe) (Zohary, 1973). However, it is mainly distributed in semiarid and arid zones in tropical Africa **(Fig 1.3)**. The tree is called as *Lalob* in Arabic, *Aduwa* in Hausa, *Hingota* in Hindi, and *Zaqum mitzri* in Hebrew.

In the Indian subcontinent, so far *B. aegyptiaca* and its subspecies are reported to be found only in India and neighboring Burma (Union of Myanmar) (**Fig 1.4**). In India, Balanites are widely grown in Rajasthan and neighboring states, whereas in Burma this plant is recorded so far only from the Irrawady Valley and one of two adjacent areas between Yeu (*ca.* 22º 50' N) in the north and Prome (*ca.* 18º 15' N) in the south. The Burmese species is better known as *B. triflora*. The Indian species of Balanites is known *Balanites roxburghii).* Although some taxonomists have indicated the differences between these two species of Balanities, until they are precisely examined, the relationships between *triflora* and *roxburghii* remain uncertain (Sands, 2001).

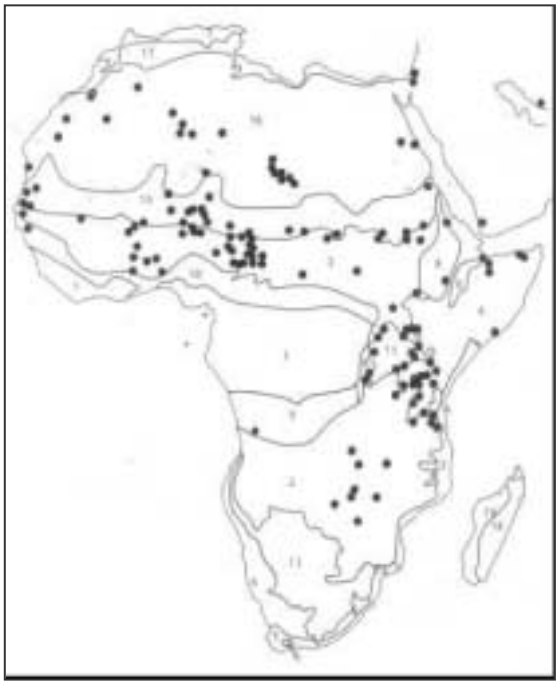


Fig 1.3. Map showing the *Balanites aegyptiaca* population distribution in Africa (according to Hall and Walker, 1991)

A tree adapted for arid and semiarid regions

B. aegyptiaca has developed an armory of weapons against the conditions it must endure in its native semiarid and arid regions. In the Sahel and sub-Saharan Africa, the tree rarely exceeds 10 m in height, but the thick and tough leaves with a glossy coating provide protection from the dry air (Hall and Walker, 1991). The double root system runs vertically and horizontally, finding water up to 7 m below the surface and within a radius of up to 20 m from the trunk. This root system also helps the tree endure sandstorms which are common in this region and which uproot and severely damage other trees. Under such harsh conditions, the coating of sand around each root provides an insulating layer of air, which helps moderate temperature fluctuations and reduce evaporation. The long green spines and branches continue to photosynthesize even long after the leaves have fallen off, and thus ensure the trees' survival. Thus, from the crown of the tree down to its roots, *B. aegyptiaca* is well adapted to surviving under the extreme conditions of the desert.

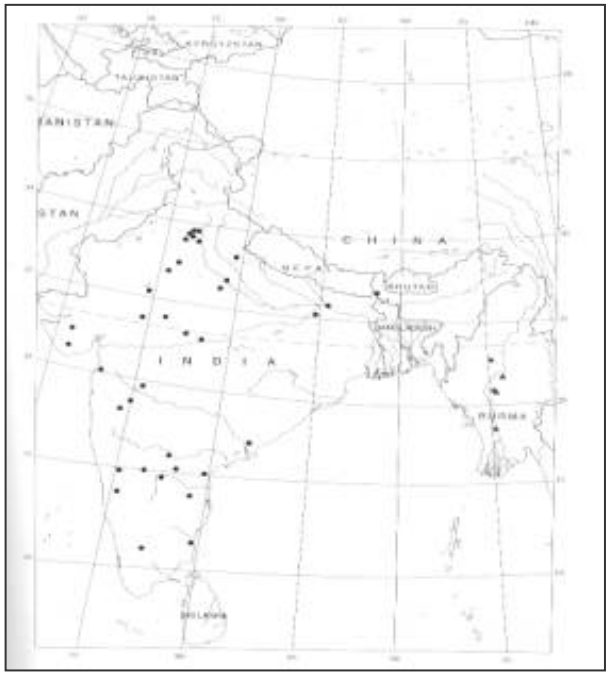


Fig 1.4. Map showing the distribution of *Balanites aegyptiaca* and associated species in Indian sub-continent (according to Sands, 2001).

The uses

The fruit, which contains many valuable nutrients, is used in a variety of ways in different parts of Africa. The mesocarp is sweet and is eaten fresh by some local populations. More commonly, it is used for preparing beverages, cooked foods, and medicines (Newinger, 1996; Mohamed et al., 2002). In Ethiopia, where Balanites is known as *kute*, the fruit is eaten after it has been boiled and skinned. The kernel may also be eaten, but only after removing the skin, washing the inner part with ash, cooking it, and mixing it with salt to make it somewhat more palatable (Guinad and Lemess, 2001). Balanites kernels contain a high percentage of oil, sometimes known as *zachun oil* or *betu oil*, which is highly familiar and prized in most parts of Africa. Its culinary quality is comparable to that of good-quality vegetable oil.

Most parts of the *B. aegyptiaca* tree can be utilized—not only the fruit. In times of famine, the flowers, leaves, and even the bark are used as food. The seeds and foliage serve as fodder for livestock, especially in arid areas and during drought periods. The wood is highly prized for cooking because it burns without smoke. In Chad, it is used in the construction of houses and to make "slates" for schools, mortars for grinding millet and corn, and handles for agricultural equipment. Twigs are used to clean teeth. The trees

themselves provide shade and shelter, and the older plants, with their razor-sharp spines, may be planted as protective hedges around farms (personal communication with Dr. Idriss, Chad).

Various parts of the tree have been used in many regions of Africa and Asia to prepare traditional medicines (Hall and Walker, 1991; Iwu, 1993; Mohamed et al., 1999; Newinger, 1996). A literature survey has revealed antifeedant, molluscidal, antidiabetic, anthelmintic, and contraceptive activities in various *B. aegyptiaca* extracts (Jain and Tripathi, 1991; Kamel et al., 1991). Most of these studies reported the active compounds to be saponins.

Though *Balanites aegyptiaca* has multi-potential uses from ethnomedicinal to cosmetics, from food to fodder, this plant is considered one of the most neglected plant species in the arid regions and has not yet been domesticated. For all its age-old history as a resource, this is still a "lost" crop. Balanites is seldom, if ever, included in text books. It is unknown to horticultural science. And a concerted effort to develop its true potential using modern capabilities has yet to be attempted.

Saponins

Structure and basic properties

Saponins are naturally occurring high-molecular-weight amphiphilic glycosidic compounds. Saponin compounds are naturally occurring in a variety of higher plants and a few marine species (Price et al., 1987; Hostettmann and Marston, 1995). These amphiphilic compounds consist of a hydrophobic aglycone linked to a hydrophilic sugar moiety. Saponins are basically classified as triterpenoids, steroids, or steroid alkaloids (**Fig. 1.5A, B, and C**) according to the structure of the aglycone (non-sugar part) and monodesmosidic, bidesmosidic, or tridesmosidic (Greek *desmos*=chain) according to the number of the sugar moieties attached to the aglycone (Hostettmann and Marston, 1995). The steroid saponins comprise two categories, based on the structure of the aglycone ring. They are said to be furostane when the E ring of the aglycone is opened and spirostan when it is closed (**Fig. 1.5**). All classes of aglycones may have a number of functional groups (–OH, –COOH, –CH_3) causing big natural diversity. This diversity can be further expanded by the composition of sugar chains, sugar numbers, branching patterns, and type of substitution. Hexoses (glucose, galactose), 6-dehydroxyhexoses (rhamnose, furanose), pentoses (xylose,

arabinose), and uronic acids (glucuronic, galacturonic) are the most common sugar residues in the saponin molecules that are attached at the C-3 position (monodesmosidic), and C-26 or C-28 (in bidesmosidic). Tri-desmosidic saponins (saponins having three sugar chains) are seldom found in nature (Hostettmann and Marston, 1995). The sugar moiety is linked to the aglycone through an ether or ester glycosidic linkage at one or two sites (Liu et al., 2003). The configuration of inter-glycosidic linkage is either α or β and the monosaccharide can be in the pyranose or furanose forms.

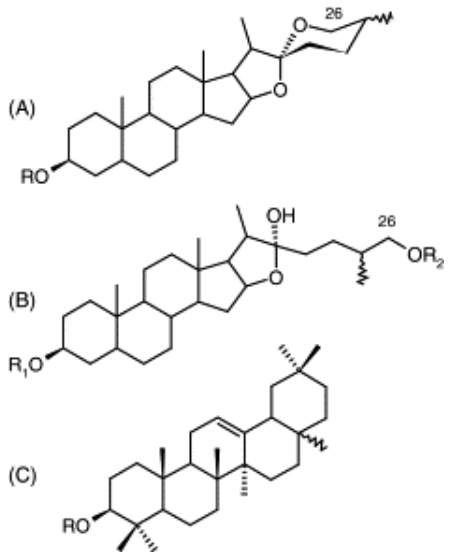


Fig. 1.5. Aglycone skeletons of (A) steroidal spirostane, (B) steroidal furostane, and (C) triterpenoid saponins. R = sugar moiety (according to Sparg et al., 2004)

Since the aglycone is very hydrophobic and the sugar chains are very hydrophilic, these characteristics provide saponin molecules with excellent foaming and emulsifying properties. The presence of both polar (sugar) and non-polar (steroid or tritepene) groups provides saponins with strong surface-active properties (Liener, 1994) that possess a strong foaming capacity in aqueous solutions. In aqueous solutions they form small micelles individually, and these hydrophobic micelles of triterpene or steroid groups stack together like small piles of coins (Kersten and Crommelin, 2003). Saponin molecules also form micelles with sterols, such as cholesterol and bile acids. The hydrophobic portion of the saponin (the aglycone or sapogenin) associates (lipophilic bonding) with the hydrophobic sterol molecules, in stacked micelle aggregation (Oakenfull and Sidhu, 1989). Indeed, it

should be no surprise that the name 'saponin' comes from the Latin word *sapo* meaning soap.

Biological properties

In plants, saponins are secondary metabolites. The term "secondary metabolites" indicates compounds that are not required for plant growth and development but presumed to function in communication or defense (Luckner, 1990). Although little is known about the influence of the biological activities of saponins on the growth of the plant itself, both inhibitory and stimulatory effects (allelopathic activities) of saponins have been reported in the literature (Hoagland et al., 1996). One of the earliest works on steroidal saponins reported that an optimal concentration of saponins doubled the growth of wheat embryo (Grunwald, 1974). It has also been reported that the saponins exhibit phytohormone-like activities (Mahmoud, 1996), and that pea saponin exerts a stimulatory effect on lettuce root growth but not on seed germination (Tsurumi and Tsujino, 1995).

It is reported that saponin molecules that occur constitutively in healthy plants act as a barrier to infection and protect the plants against attack by a wide range of potential pathogens (Mansfield, 1983; Osbourn, 1996). For example, the well-known saponins avenacin A-1 from oat roots, α-tomatine from tomato, and α-chalonine from potato, are known to exhibit antifungal activities (**Fig. 1.6**) (Osbourn 1996; Morrissey and Osbourn 1999; Papadopoulou et al., 1999; Bouarab et al., 2002). The nature of the particular aglycone moieties and sugar constituents of a saponin dictates the way in which it will react with different living organisms, such as microbes, plants, and animals (Oleszek et al., 1999). Saponins are, for example, very toxic to cold-blooded organisms, but apparently not to mammals (Hall and Walker, 1991; Harborne and Baxter, 1993).

Earlier studies have revealed that many saponins or saponin-rich extracts (SRE) from various plants showed antifungal activities (Oleszek et al., 1990; Hostettmann and Marston, 1995; Osbourn et al., 1996). Sindambiwe et al. (1998) have reported growth inhibition of *Epidermaphyton flooccosum*, *Microides interdigitalis*, and *Trichophyton rubrum* fungi by a saponin-rich extract of *Maesa lanceolata*. Many saponins have also been found to have an inhibitory effect against *Candida albicans*, *Crytococus neoformans*, and *Aspergillus fumigatus* (Li et al., 1999). Similarly, saponins from *Chenopodium quinoa*, *Capsicum*

annum, *Phytolacca tetramera*, and *Panax notoginseng* were also reported to have antifungal activities (Woldemichael and Wink, 2001; Escalante et al., 2002; Iorizzi et al., 2002).

A study of the literature also revealed that saponins reduce larval growth and cause mortality in the flower beetle, *Tenebrio molitor* (Adel et al., 2000), European grape moth, *Lobesia botrana* (Tiva et al., 1992), and European corn borer, *Ostrimia nubialis* (Nozzolillo et al., 1997). Studies have also reported that the high saponin content cultivar of alfalfa (*Medicago sativa* L.) is correlated with resistance to the pea aphid (*Acyrthosiphon pisum*) (Pedersen et al., 1976).

Nutritive properties

In the past, saponins were recognized as antinutritional constituents due to their adverse effects, such as toxicity to fish and cold blooded animals and haemolytic effects, however, recent studies have shown that saponins are beneficial to humans. In high concentrations, saponins impart a bitter taste and astringency; however, recent studies have shown that saponins possess hypocholesterolemic, immunostimulatory, and anticarcinogenic properties (Carrol and Kurowska, 1995; Kenarova et al., 1990; Kikuchi et al., 1991). Saponins are also considered natural antioxidants since they bind to cholesterol and prevent cholesterol oxidation in the colon (Shi et al., 2004).

Extracts of the saponin-containing plants like *Yucca schidigera* and *Quillaja saponaria* have been reported being used for various food applications since time immemorial (Price et al., 1987). The beneficial effects on human and animal health of supplementation with saponin-rich extracts have also been documented (Anthony et al., 1994). The US Federal Drug Administration has also designated Yucca and Quillaja saponin-rich extracts safe for human consumption (Sen et al., 1998). It has been suggested that the prime reasons for the beneficial effects of saponin-rich extracts was the influence of antimicrobial activities of the saponin compound present in the extracts. It has also been reported that growth inhibition of different bacteria occurred when used against *Y. schidigera* in the gut (Van Nevel and Demeyer, 1990). Sen et al. (1998) found growth inhibition of *E. coli* by extracts of *Q. saponaria* and *Y. schidigera*.

Association with membranes

Most biological properties of saponins are attributed to their action on membranes – especially pore formation in membranes. The ability to rupture erythrocytes, hemolytic activity, is generally attributed to the interaction between the saponins and the sterols of the erythrocyte membrane, which bursts as a result (Baumann et al., 2000). This haemolytic action of saponins is believed to be the result of the affinity of the aglycone moiety for membrane sterols, particularly cholesterol (Glauert et al., 1962) with which they form an insoluble complex (Bangham and Horne, 1962). However, the interaction between saponins and membranes seems to be complicated, with the composition of the target membrane, the type of side chain, and the nature of the aglycone to which these are attached all having an effect (Gee et al., 1996). The precise details of the interaction between saponins and membranes need further elucidation (Francis et al., 2002).

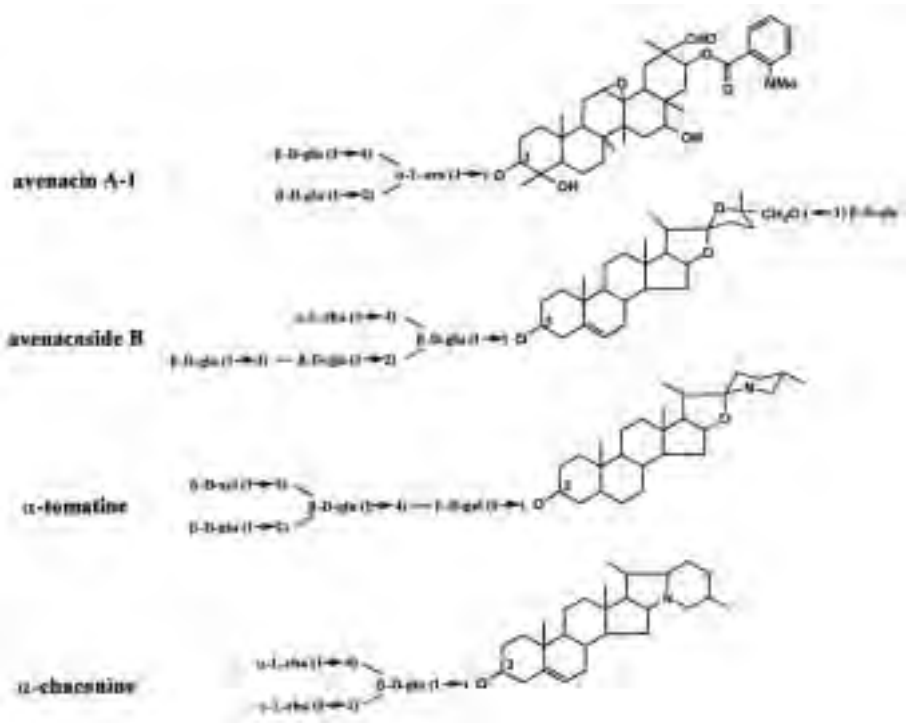


Fig 1.6. Examples of antifungal saponins from oats and solanaceous plants. The major oat root saponin avenacin (A-1 and avenacoside B) and tomato and potato saponins (a tomatine and a chaconine) (according to Morrissey and Osbourn, 1999).

Although the precise mechanism of the interaction of saponins with the cell membrane is not fully understood (Morrissey and Osbourn, 1999), it is generally accepted that the antifungal activity of saponins is related to their ability to complex with sterols in the fungal membranes and thereby cause a loss of membrane integrity (Keukens et al. 1995; Morrissey and Osbourn 1999). It has also been suggested that the saponin-sterol membrane complexes ultimately form aggregates that lead to the formation of membrane pores (Armah et al., 1999).

B. aegyptiaca and saponins

A literature survey shows that *B. aegyptiaca* contains both spirostanol and furostanol saponins (Liu and Nakanishi, 1982; Pettit et al., 1991; Hosney et al., 1992; Kamel, 1998) together with flavonoid glycosides (Kamel et al. 1991), sapogenol, and 6-methyldisgenin (Hosney et al., 1992). Liu and Nakanishi (1982) isolated three spirostanol saponins, balanitin-1, -2, and -3 from 60% aqueous methanol extracts of the bark and root of *Balanites aegyptiaca*. Pettit et al. (1991) isolated four new cytostatic steroidal saponins, called balanitin-4, -5, -6, and -7, from the CH_2Cl_2/MeOH (1:1) extract of the kernels. Hosney et al. (1992) reported balanitin-3 from fruit mesocarp using methanol extracts. They also isolated a new furostanol saponin, balanitoside, and 6-methyldiosgen. Kamel et al. (1991) have isolated two new steroidal saponins and two previously known saponins from the water-extract of the mesocarp. The known saponins were previously isolated from the rhizomes of *Dioscorea gracillima* (Kawasaki et al., 1974). These compounds can be used as the starting materials in the synthesis of oral contraceptives, sex hormones, and other steroids, such as corticoids and anabolic compounds for which there has been an excellent market since the 1960s. Up to the present, diosgenin and related steroidal saponins have been obtained commercially from the tubers of various Dioscorea species. However, the need to discover alternative sources for these compounds has become crucial because of decreasing supply and increasing demand for the plant resources currently in use.

A significant number of works on isolation and structure determination of natural products from the *B. aegyptiaca* have been carried out. However, almost all are from either

the African or Indian samples. No work so far has been reported on samples from the Israeli provenances; so one of the aims of this study was to characterize these saponins.

Determination of saponins

The early determination procedures for saponins in plant material were predominantly based on gravimetric and physical (foam forming ability) or biological properties (haemolytic ability) (Oleszek, 2002). However, because of the variability in these characteristics, the determination procedures have largely been replaced by colorimetric and, more recently, spectrometric-like high performance liquid chromatography (Marston et al., 2002).

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is the most commonly used method for saponin detection. Separation of components in HPLC is based on mass-transfer between stationary and liquid mobile phases in a column. Choosing suitable solvent(s) for the sample and mobile and stationary phases may have an effect on the separation process. An important advantage in HPLC analysis of saponins is that entire glycosides and aglycones can be analyzed without any derivatization. However, a problem can arise because of the weak absorption and low sensitivity of the saponins in the short UV wavelengths (200–215 nm) because of the lack of strong chromophores. To overcome this problem, the refractive index detector (RI) is used in common practice. HPLC with RI detector gives better chromophores; however, when gradient elution is used, RI measurement is not practiced (Kostova and Dinchev, 2005). Alternatively, the saponins can be derivatized and chromophores can be detected at a higher wave length (254 nm); however, all these methods need authentic samples and the identities of peak can be confirmed only by their retention times (Slacanin et al., 1988). Since the introduction of the first HPLC methods for saponin separation and quantification (Bushway et al. 1986), continuous developments and improvements of solvent systems, sample preparations, and column chemistry have been made (Bushway et al. 1986; Friedman et al. 1994; Edwards and Cobb 1996).

Mass spectrometry

Mass spectrometry (MS) provides information about the molecular weight and chemical structure of the compound analyzed. The mass spectrometer creates charged ions from molecules. These ions are extracted into the analyzer region of the mass spectrometer and separated according to their mass-to-charge ratios (m/z). The separated ions are detected and the signals are sent to the data system where the m/z ratios are stored, together with their relative abundance, for presentation in the format of an m/z spectrum. Because of its specific "finger-print" property, MS is very useful in qualitative analyses of unknown compounds. MS is generally used as a detector in GC or HPLC apparatuses.

MS has played an important role in structural analysis of saponins. In early studies, derivatization was required for using electron impact mass spectra (Tomova et al., 1974). Later, field desorption and fast atom bombardment spectra were employed to analyze derivatized and underivatized saponins, providing evidence about molecular mass and sugar sequence by cleavage of glycosidic bonds (Wu et al., 1996; Wang et al., 1997). More recently, electrospray ionization (ESI) in the positive and negative ionization mode has become one of the most effective analytical tools for structural characterization of native saponins (Cai et al., 2001). Multi-stage tandem mass spectroscopy (ESI-MS^n) of molecular ions is used for detailed structural analysis of underivatized saponins (Fang et al., 1999). The fragment ions from glycoside cleavage provide information about the mass of aglycone, the type of the monosaccharide, and the sequence and branching of the oligosaccharides. The fragment ions from cross-ring cleavages of sugar residues give information about the linkages between units.

LC-MS and LC-MS/MS have shown easy and rapid methods for the identification of saponin and sapogenin composition compared to the traditional approaches which required extensive purification and, in the case of GC-MS, conversion of the saponins to their corresponding sapogenins and volatile derivatives. Both the extensive purification and the derivatization steps are time-consuming and are known to create artifacts (Muir et al., 2000).

Objective and Hypothesis

Objectives

The main aim of this study is to characterize saponins extracted from *B. aegyptiaca* of Israeli origin and to improve the understanding of the biological activities of these compounds and their interaction with biological membranes. It is hoped that the findings of this study on *B. aegyptiaca* will provide support for efforts currently under way to draw attention to the economic potential of the natural compounds produced by this highly adapted but mostly neglected arid species for the benefit of the people, with emphasis on some of the world's most poverty stricken populations.

The following are the specific objectives of the study:

- To characterize the main saponins in extracts of various Balanites plant tissues;
- To determine the "production potential" of these saponins in various Balanites provenances;
- To study the larvicidal and fungicidal activities of Balanites saponins;
- To deepen the understanding of the interaction of Balanites saponins with biological cuticle membranes.

Hypothesis

Since saponin content in the plant depends on factors such as the cultivar (provenances), the physiological state, and the geographical location of plant, there is a possibility of finding variation in saponin content among different tissues and/or provenances of *B. aegyptiaca*.

Callus culture has been found to be quite successful for the *in vitro* mass production of some secondary metabolites including triterpenoid saponin, so there is the possibility of producing Balanites saponin using this technique with proper adjustment of media, etc.

As the amphiphilic saponin compounds increase their association with biological membranes – with a high affinity to sterol compounds in these membranes – the saponins extracted from *B. aegyptiaca* tissues may also have an affinity to the sterol compounds (mainly cholesterol) present in the mosquito larva cuticle membrane and the cell

membranes of some fungi. Therefore, there is a good chance that these saponins will cause a loss of cuticle integrity and, as a result, exhibit significant larvicidal or fungicidal activity.

B. aegyptiaca saponins may also exhibit considerable affinity for the phytosterols of the plant cuticle membrane that may lead to membranes with reversible pores or with a certain degree of disorder, which would enhance the delivery of agro-materials through this cuticle membrane. That could ultimately make Balanites saponins a good candidate for the delivery of biomaterials with some sort of antifungal properties through the plant cuticle membrane. Such a delivery system could ultimately be exploited in the foliar application of pesticides, fertilizers, or herbicides, and this ultimately helps the domestication process of the most neglected plant species. This may also help in ecological conservation by growing Balanites plants in a harsh arid region like the Israeli Negev desert, where other plant species barely grow.

CHAPTER 2

Characterization of the fruit mesocarp saponins of *Balanites aegyptiaca*

Introduction

Saponins are the most widely distributed group of natural secondary metabolite compounds found in plants and have numerous uses, traditional as well as modern. They are high molecular weight glycosides consisting of either a triterpenoid or a steroid type aglycone; and one or more sugar chains (Hostettmann and Marston, 1995). The structural diversity and resulting wide range of polarities, including numerous sugar moieties, makes saponin a complex compound and presents a considerable challenge for structural elucidation (Oleszek, 2002). With the recognition of additional beneficial uses for saponins, their identification in different plants has taken on greater significance in phytochemistry. Traditional methods for identifying saponins in plants are complicated and time-consuming because saponins are highly polar, thermally labile, and structurally complex molecules found at low levels in most plant species (Huhman and Sumner, 2002). Therefore, there is increasing demand for better methods of identifying and structurally characterizing saponins.

To date, mass spectrometry (MS) has been very popular for identification of certain unknown biomolecules. MS determines the mass to charge ratio (*m/z*) of gas phase ions required to vaporize the sample molecule. For this, a nano-scale sample, and sometimes even a pico-scale sample, is sufficient (Chatman et al., 1999). Until recently, when electron impact (EI) or chemical ionization (CI) methods were used in MS, samples were needed for derivatization. However, thermally labile samples, like saponins, were difficult to detect. After the development of soft-ionization methods, e.g., electrospray (ES), detection of mass by direct analysis of polar and thermally labile molecules without prior derivatization became a possibility (Fenn et al., 1989). Furthermore, since the development of tandem mass spectrometry (MS/MS), a technique in which a parent ion is mass-selected and induced to fragment into structurally significant products (De Hoffmann, 1996), MS has become more reliable in identification of the mass of various biomolecules. Liquid chromatography mass spectrometry (LC-MS/MS) has already been used to detect and

identify some saponins from plant extracts (Liu et al., 2003; Liang et al., 2002; Cui et al., 2000).

Balanites aegyptiaca is a desert plant species that is primarily grown in most of the arid regions of the world. Although it is considered to be the most adapted plant species of all dry arid and semiarid region plants, it is highly neglected. Literature study has revealed that various parts of this plant contain saponins, and fruit mesocarp contains the highest quantity. Studies show that most of the ethnobotanical uses of this plant are attributed to its saponin content. Saponin content in *B. aegyptiaca* has been reported in the literature. However, to the best of our knowledge, neither the saponin in *B. aegyptiaca* provenance grown in Israel nor the technique of using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) have been reported. This study describes the separation of methanol-extracted *B. aegyptiaca* mesocarp saponins by high-performance liquid chromatography-refractive index (HPLC-RI) to detect the whole spectrum of its major saponins, followed by electrospray ionization-mass spectrometry (ESI-MS) combined with multistage mass spectrometry (MS^n) to identify the major steroidal saponins of *B. aegyptiaca* grown in Israel. The structural determination of selected major saponins was also carried out using high-field NMR techniques.

Material and Methods

Plant material

Fully ripened fruits of *B. aegyptiaca* were collected from *B. aegyptiaca* plants grown in Kibbutz Samar located in the Arava rift valley of southern Israel between the Dead Sea and Red Sea, close to the Jordanian border, 40 km north from the Rea Sea's Gulf of Eilat. The plant was botanically authenticated by Prof. Uzi Plitman from the herbarium in the Hebrew University of Jerusalem. Voucher specimen (76816) was deposited in the herbarium of the Hebrew University of Jerusalem. The epicarp (outer cover) was gently removed by hand and the mesocarp (pulp) was manually extracted with a knife. The mesocarps were first freeze-dried with a lyophilizer (Christ Alpha 1-4, Germany), then stored in an electric desiccator (Sanplatec Corp., Israel) for further use.

Extraction and separation

For the extraction**,** the freeze-dried mesocarp was pulverized with a mortar and pestle, then combined with methanol (1:10) and shaken continuously overnight in a high-speed electric shaker (Tuttnauer, Jerusalem, Israel), followed by centrifugation at 3500 rpm for 18 min at 20ºC (Hermble, Germany) and the supernatant collected. Two subsequent extractions of the residue were carried out in methanol using only vortexes and centrifugation. After three successive extractions, the supernatant was clear. The supernatants were combined and the methanol was evaporated off in a rotary evaporator (Heta-Holten A/S, Denmark) under reduced temperature (below 40ºC). After dissolving the residue in H_2O (1:2 w/v), the extract was defatted three times using a 1:4 ratio of n-hexane. The water was then removed from the defatted extract with a lyophilizer. The semi-crystalline dried saponin extract (55% by weight of mesocarp) was designated ME (methanol extract) and kept refrigerated (4ºC) for further use.

Liquid chromatography

A Waters (Milford, MA, USA) 2690 HPLC system equipped with an auto sampler, a Luna 5u 100A C18 column (250 × 2 mm, 5 μm), an HPLC pump, and an Agilent (Palo Alto, CA, USA) G134A RI detector, was operated at room temperature. The mobile phase was 70:30 MeOH/H_2O at a flow rate of 0.2 mL/min. The injection volume was 10 μL. In some experiments, the ME was eluted in methanol in solid phase extraction (SPE) cartridges (C18, 35 mL, 10 g), after discarding the first elution in water and then 35% methanol before injection. The methanol was again evaporated under reduced temperature and the extract was dissolved in methanol-water (70:30) and filtered (0.45 μm) before injection. Twenty-two percent by weight was obtained from the SPE elution.

The evaporative light scattering (ELS) detector, a Sedex 75 (SEDERE, France), Luna C18 column (250 × 2.0 mm, 5 μm particle size; Phenomenex), equipped with a guard, was used for the separations, with the same isocratic mobile phase of 70:30 MeOH/H_2O. A 100 μL sample was injected at a flow rate adjusted to 0.3 mL/min. The ELS detector separation was performed at ambient temperature. The ELS detector probe temperature was set at 50°C, the gain was set at 9, and the nitrogen nebulizer gas was adjusted to 2.0 bar. This

experiment gave a drastically reduced free sugar peak. This experiment was undertaken to collect the individual peak saponins.

Mass spectrometry

All the experiments were performed with a single-ion-trap mass spectrometer (Esquire 3000 Plus, Bruker Daltonik, Germany) equipped with an ESI interface as the ion source for MS analyses. The electrospray voltage was set at 4.5 kV. The temperature of the ion source capillary was 300ºC. Negative ion mode was used for all experiments. Mass spectrometer conditions were optimized to achieve maximum sensitivity. Full scan spectra from *m/z* 25 to 2000 in the negative ion mode were obtained (scan time 1 s). The ion trap was set for acquisition in automatic gain control mode with an accumulation time of 159 μs. For MS^n analyses, the isolation width for $[M-H]^-$ molecular ions was 3 *m/z* units; for MS^n experiments, fragmentation was induced using activation amplitudes of 0.95 to 1.12 V.

NMR spectrometry

^1H, ^{13}C, and DEPT 135 NMR spectra were recorded at 25°C with a Varian Unity Inova 800 spectrometer (proton frequency 799.809 MHz) in CD_3OD with TMS as internal reference. NOESY spectra were obtained with mixing times of 700 ms. HMBC experiments were optimized for $^nJ_{C,H}$ = 5 Hz. Mass spectra were obtained on a JEOL JMS-AX505W double-focusing mass spectrometer operating in EI mode. The NMR study was carried out in the Danish University of Pharmaceutical Sciences, Copenhagen, Denmark.

Results

Analysis of B. aegyptiaca mesocarp extracts by LC-MS

Figure 2.1 shows the HPLC (RI) and MS total-ion-current (TIC) chromatograms of the methanol extracts of *B. aegyptiaca* mesocarp (ME). There are eight separation peaks at 4.2, 4.8, 6.0, 7.2, 8.2, 8.6, 11.2, and 12.4 min retention time in HPLC-RI. There is a large void volume at the beginning of the chromatogram, perhaps due to the free sugar compounds in the *B. aegyptiaca* mesocarp (**Figure 2.1a**). However, this large void volume was drastically reduced when the extract was further eluted by water, using an SPE Sep-Pak C18 cartridge

and analysed with an ELS detector (**Figure 2.1b**). A clear correlation between the peaks obtained from the RI (HPLC chromatogram) and the MS (TIC chromatogram) are also observed. A comparison of the ELS and RI chromatograms revealed a very similar pattern of peaks; however, the retention times were different. Since the ELS detector was not coupled to the MS, further analysis of each MS peak was carried out with an RI detector.

The ESI mass spectra $[M-H]^-$ of each of the eight RI peaks are presented in **Figure 2.2**, with their corresponding retention times. A summary of the peaks with retention time, *m/z* value $[M-H]^-$, and area is also listed in **Table 2.1.** Among the eight peaks there are actually only five masses, *m/z* 1195, 1063, 1077, 1209, and 1045, in negative ion mode (**Table 2.1**). The ion at *m/z* 1077 repeated three times: at 6.0, 8.2, and 11.2 min; the ion at *m/z* 1209 repeated twice: at 7.2 and 9.6 min. An estimated quantification shows that the *m/z* 1063 peak at 4.8 min retention time is the largest, corresponding to 18.75%, followed by peaks at *m/z* 1209 (11.8%), 1077 (10.89%), 1045 (1.55%), and 1195 (1.35%) of the injected sample.

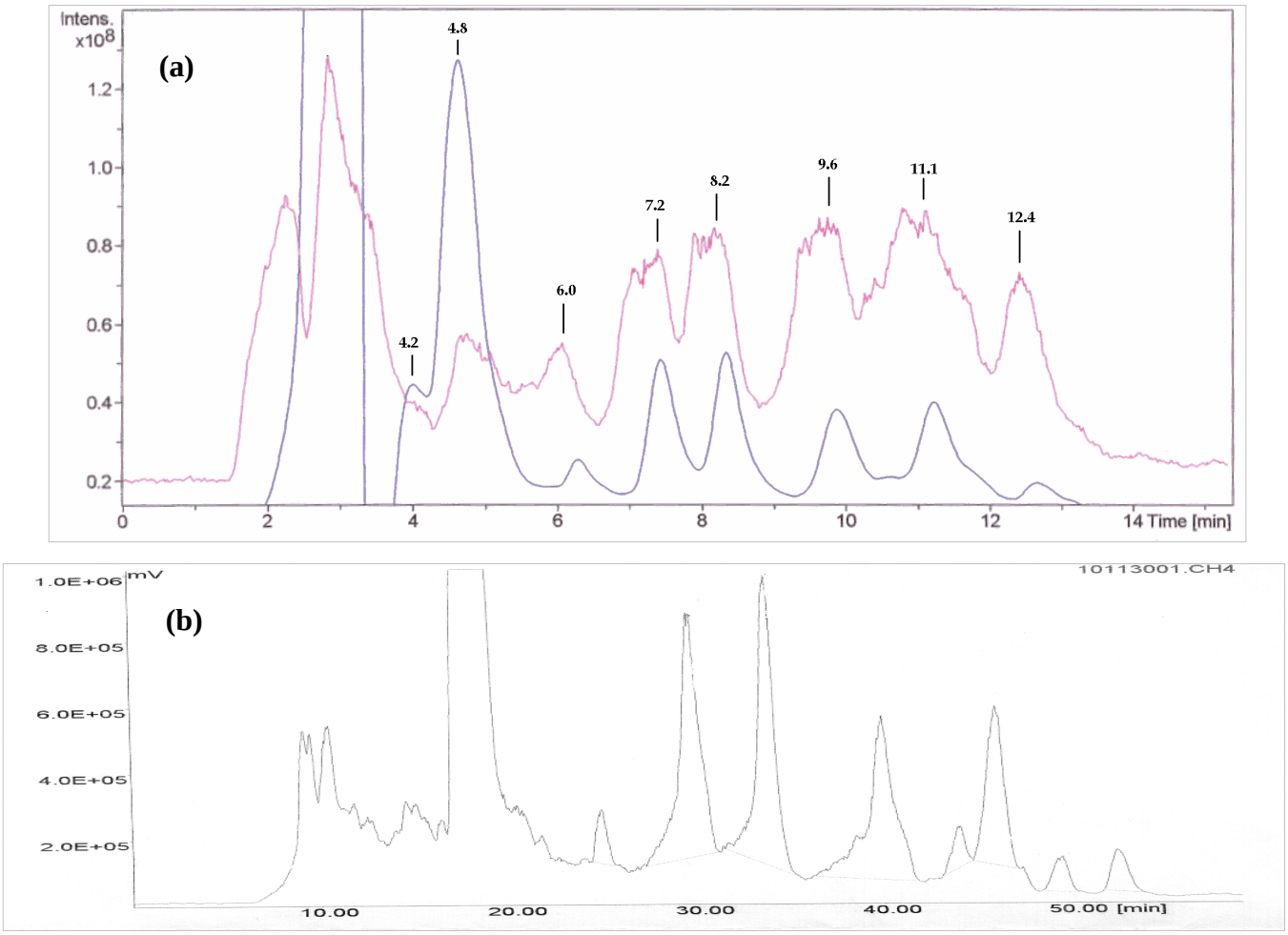


Figure 2.1. LC-MS chromatogram of methanol-extracted *B. aegyptiaca* mesocarp in negative ion mode. (a) TIC chromatogram superimposed on RI chromatogram. (b) HPLC chromatogram of further eluted extract of *B. aegyptiaca* mesocarp by SPE Sep-Pak C18 cartridge with ELS detection**.**

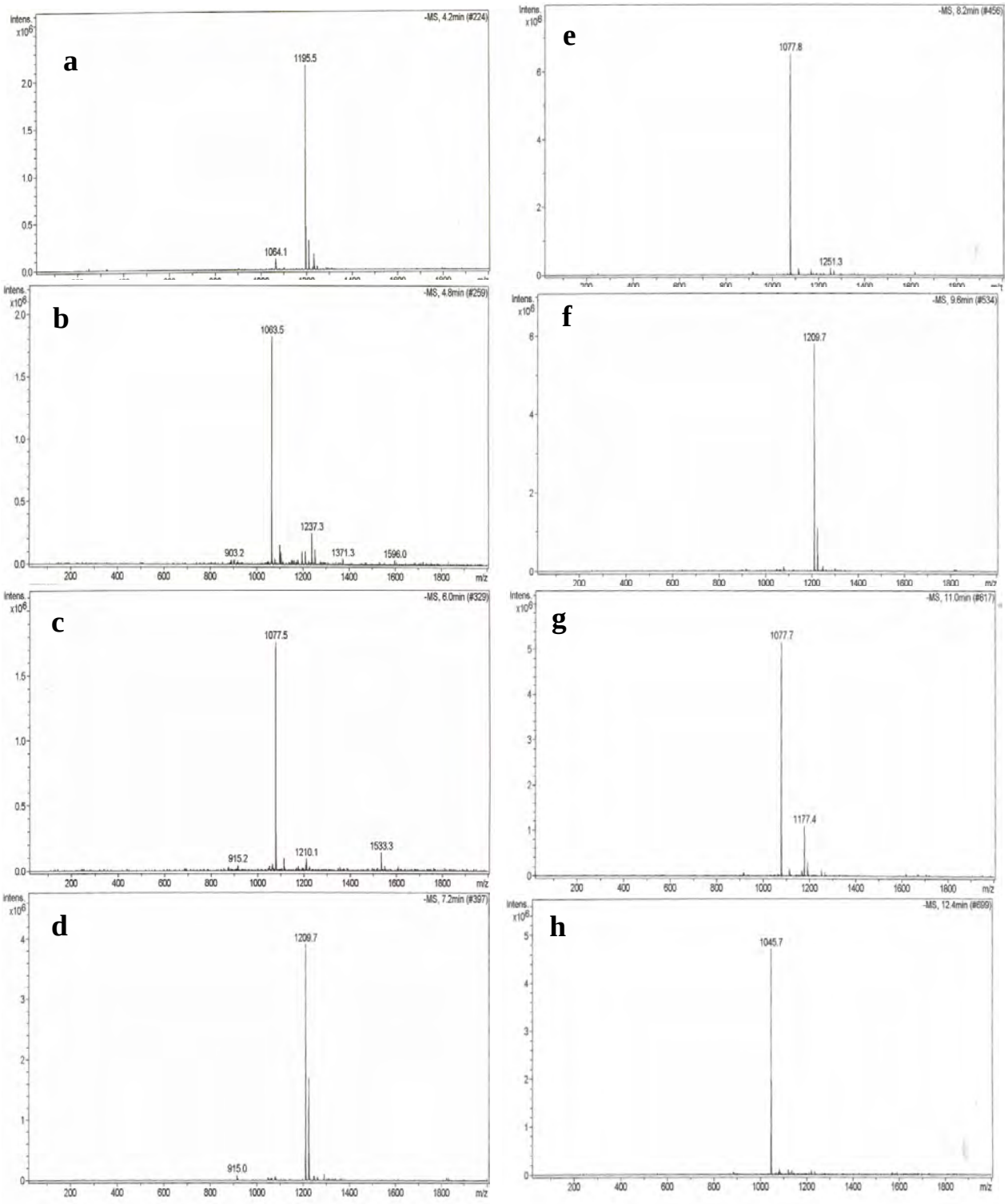


Figure 2.2. Electrospray mass spectra $[M-H]^-$ of methanol-extracted *B. aegyptiaca* mesocarp. (a) through (h) are mass spectra of peaks 1 to 8 obtained at the respective retention times.

Table 2.1. Major HPLC-RI peaks of methanol-extracted *B. aegyptiaca* mesocarp, with *m/z* values obtained from ESI-MS.

Peaks	Retention Time (min)	*m/z* [a] $[M-H]^-$	Amount (%)[b]
1	4.2	1195	1.35
2	4.8	1063	18.75
3	6.0	1077	0.95
4	7.2	1209	7.15
5	8.2	1077	5.62
6	9.6	1209	4.65
7	11.1	1077	4.32
8	12.4	1045	1.55

[a] The *m/z* values cited are integers obtained by truncating (not rounding off) measured values.
[b] Amount is calculated based on the chromatographic peak area from the injected sample.

Characterization of the* B. aegyptiaca *mesocarp saponins by LC-ESI-MS^n

Characterization of the saponin peaks obtained from the LC-MS was carried out by MS^n experiment in order to determine the structural pattern of each of the saponin peaks. The ESI-MS/MS in negative ion mode of saponin peaks of *B. aegyptiaca* fruit mesocarp obtained from the LC-RI and LC-ELS (*m/z* 1195, 1063, 1077, 1209, and 1045) is presented in **Figure 2.3**. The ESI-MS/MS spectra of the largest peak (Peak 2, *m/z* 1063) shows the six main fragment ions at *m/z* 917, 901, 755, 593, 431, and 413 (**Figure 2.3b)**. The differences in mass between the parent ion (*m/z* 1063) and the fragment ions (*m/z* 917 and 901) are 146 and 162 Da, indicating the loss of a deoxyhexose sugar and a hexose sugar unit, respectively. The competing lost sugar units suggest that there are two different terminal residues in the oligosaccharide chains, one a hexose unit (most probably glucose), the other a deoxyhexose unit (most probably rhamnose) which fits with the earlier study of saponins (Cui et al., 2000). The fragment ion at m/z 755 corresponds to loss of a disaccharide consisting of a deoxyhexose and a hexose. The ion at *m/z* 593 shows the loss of a hexose unit; the ion at *m/z* 431 shows the loss of two hexose units from the ion at *m/z* 755. The smallest fragment ion (*m/z* 413) corresponds with the further loss of one H_2O unit. This, the smallest of the fragments, fits the molecular mass of dehydrated diosgenin ($-H_2O$), indicating that this is the type of aglycone that comprises *B. aegyptiaca* saponin. This

corresponds with the claims of earlier studies that diosgenin is the sapogenin of *B. aegyptiaca* (Liu and Nakanishi, 1982; Kamel, 1998). In addition, in the CID spectrum of the main fragment ions, the elimination of 18 Da, corresponding to cleavage of the H_2O, was also observed. However, the loss of 18 Da was always at low intensity (**Figure 2.3b**). For further characterization, an MS^n experiment was carried out on the *m/z* 1063 parent ion**.** The cleavages of the main peak (MS^2) are at m/z 917, 901, 883, 755, 737, 593, 431, and 413. The intensity of the peak at m/z 901 is greater than that at m/z 917, demonstrating that the $[M-H]^-$ ion preferentially expels glucoside from the C-26 position (Liu et al., 2004). The MS^3 of the m/z 901 produces m/z 755, m/z 739, and m/z 593. Further, MS (MS^4) of the m/z 755 produces fragment ions at m/z 737, m/z 593, and m/z 575 **(Table 2.2**).

Figure 2.3a shows the fragment ions of the *m/z* 1195 parent ion (Peak 1). There are seven fragment ions: at *m/z* 1063, 1049, 917, 901, 755, 593, and 431. The mass differences between the parent ion and the fragment ions at *m/z* 1063 and 1049 are 132 and 146 Da, respectively. These indicate losses of a pentose sugar and a deoxyhexose sugar, respectively. The competing losses of a pentose and a deoxyhexose sugar suggest that there are two different terminal residues in the oligosaccharide chains, most probably a xylose and a rhamnose. The presence of the ESI-MS pattern of the other succeeding fragment ions (917, 901, 977, and 413) shows the similarity of the behavioral pathway of this saponin with the saponin (m/z 1063) described earlier. This suggests that this saponin peak has one more pentose (most probably xylose) unit than the main saponin of *B. aegyptiaca* fruit mesocarp (MW 1064 Da) described previously with the same aglycone, diogenin.

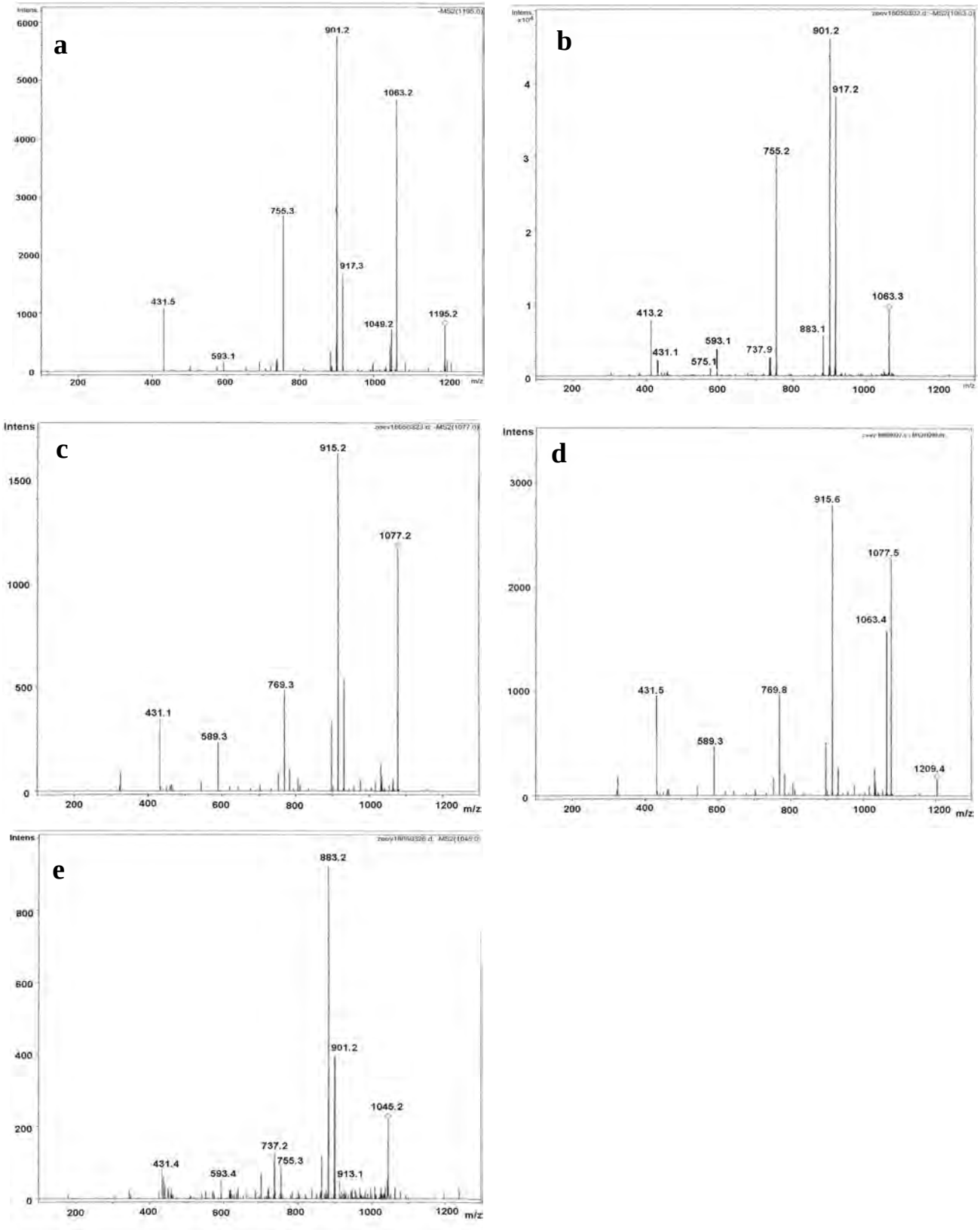


Figure 2.3. MS/MS spectra $[M-H]^-$ showing fragment ions of the major saponins of methanol-extracted *B. aegyptiaca* mesocarp (**a–e**), at *m/z* 1195, 1063, 1077, 1209, 1045, respectively.

Table 2.2. MS^n Electrospray mass spectral data of the major compound of the *Balanites aegyptiaca* fruit mesocarp.*

MS	MS^2	MS^3	MS^4
1063 $(M-H)^-$	917 $[M-H-Rha]^-$		
	901 $[M-H-Glu]^-$	755 $[M-H-Glu-Rha)]^-$	737 $[M-H-Glu-Rha-H_2O]^-$
			593 $[M-H-Glu-Rha-Glu]^-$
			575 $[M-H-Glu-Rha-Glu-H_2O]^-$
		739 $[M-H-Glu-Glu]^-$	
		593 $[M-H-Glu-Rha-Glu]^-$	
	883 $[M-H-Glu-H_2O]^-$		
	755 $[M-H-Glu-Rha]^-$		
	737 $[M-H-Glu-Rha-H_2O]^-$		
	593 $[M-H-Glu-Rha-Glu]^-$		
	575 $[M-H-Glu-Rha-Glu-H_2O]^-$		
	431 $[M-H-Glu-Rha-Glu-Glu]^-$		
	413 $[M-H-Glu-Rha-Glu-Glu-H_2O]^-$		

*Values are the mass/charge ratio (m/z) of the ion. Values in brackets show cleavage of the compound in successive MS^n negative ion modes. The m/z values quoted are integers obtained by truncating (not rounding off) measured values

Figure 2.3c shows the ESI-MS^2 spectrum of the $[M-H]^-$ ion peak at *m/z* 1077. There are four major fragment ions at: *m/z* 915, 769, 589, and 431. These fragment ions show the respective losses of: 162; 308 (162 plus 146); 488 (2×162 plus 146 plus 18); and 646 (3×162 plus 146 and 14) Da masses from the parent ion. The smallest fragment at *m/z* 413, which could also be obtained by the combined losses of 664 Da masses (3×162 plus 146 plus 14 plus 18) from the parent ion, is the dehydrated diosgenin ($-H_2O$). This also suggests that the peak at *m/z* 1077 $[M-H]^-$ belongs to a different saponin, with the same aglycone (diosgenin), but with an additional 14 Da mass attached to the main *B. aegyptiaca* saponin (MW 1064). This syggests the addition of a $–OCH_3$ molecule in place of a –OH molecule in the *B. aegyptiaca* MW 1064 Da saponin. The formation of a methoxy derivative at C-22 during extraction with methanol has also been reported in earlier studies of furostanol saponins (Agrawal et al., 1985).

Figure 2.3d shows the negative-mode MS^2 fragment ions of the parent ion at *m/z* 1209. The major fragment ion peaks observed in this precursor ion are at *m/z* 1077, 1063, 915, 769, 589, and 431. The mass differences between the parent ion at *m/z* 1209 and the fragment ions at *m/z* 1077 and *m/z* 1063 are 132 Da and 146 Da. This shows the loss of a pentose (xylose) and a deoxyhexose (rhamnose). This also suggests that there are two such sugars in the terminal residue of the saccharidic chain. However, the high intensity of the 1077 ion indicates that first expulsion of xylose unit from the parent ion at m/z 1209. The fragment ions from *m/z* 1077 to 431, inclusive, give the same pattern of sugar-unit loss as the previous ion 1077 (**Fig 2.3c**). This suggests that this saponin peak is a very similar saponin to that of the main MW 1064, with an additional xylose and methyl unit.

The $[M-H]^-$ ion MS^2 spectra of the peak ion at *m/z* 1045 is presented in **Figure 2.3e**. These spectra show that there are two main cleavages from the parent ion, at *m/z* 901 and at *m/z* 883, with a deletion of 144 and 162 Da from the parent ion, respectively. The intensity of the peak at *m/z* 883 is much greater than that at *m/z* 901, which demonstrates that the $[M-H]^-$ parent ion at *m/z* 1045 preferentially jettisons a 162-unit glucoside (glucose). The next fragment ion, at *m/z* 737, shows a loss of 162 Da (glucose) plus a loss of 146 Da (rhamnose) from the parent ion. The fragment ion at *m/z* 593 shows a further loss of 144 Da. This is 2 Da smaller than a rhamnose molecule and thus is not rhamnose. The elimination of 144 Da, corresponding to cleavage of the E-ring, is generally observed in furostanol saponins. Such a cleavage phenomenon has also already been reported by Liang et al. (2002) and Liu et al. (2004) in a furostanol saponin of *Asparagus cochinchinensis*. Thus, this fragmentation pathway suggests that this peak is a genuine furostanol saponin. The smallest fragment ion, at *m/z* 431, shows further elimination of a glucose unit (162 Da) from the fragment ion at *m/z* 593. As with most of the other parent ions, this ion (*m/z* 1045) could not expel a sequential glucoside to produce the $[Aglycone-H_2O]^-$ ion (*m/z* 413).

Actually, LC-ESI-MS analysis of the methanol extract of *B. aegyptiaca* mesocarp produced eight peaks (**Figure 2.1a**). Among these eight peaks, we have discussed the MS/MS analysis of the five major peaks—in terms of area—at 4.2 (*m/z* 1195), 4.8 (*m/z* 1063), 7.2 (*m/z* 1209), 8.2 (*m/z* 1077), and 12.4 (*m/z* 1045) min retention time. The other three peaks, at 6.0, 9.6, and 11.1 min retention time, repeat the values of *m/z* 1077 and

1209, and could be isomers of these respective saponins. The availability of isomeric saponins in plant extracts has also been reported in earlier studies (Cui et al., 2000).

Structural analysis of the major saponins of* B. aegyptiaca *mesocarp by NMR

For structural identification, peaks of the biggest and most distinguished fraction of the major saponin peaks obtained from the HPLC-RI and ELS (Peak 2, tR 4.8 min; Peak 4, tR 7.2 min; and Peak 5, tR 8.2 min) were chosen and the fractions were collected separately using semi-preparative HPLC. The methanol was evaporated and the aqueous solutions were freeze dried. After this, the residues were dissolved in D_2O and freeze dried again. D_2O exchanged the hydroxylic protons to deuterium, which diminishes the HOD signal in the ^1H NMR spectra. The residues were dissolved in CD_3OD and the NMR spectra were recorded.

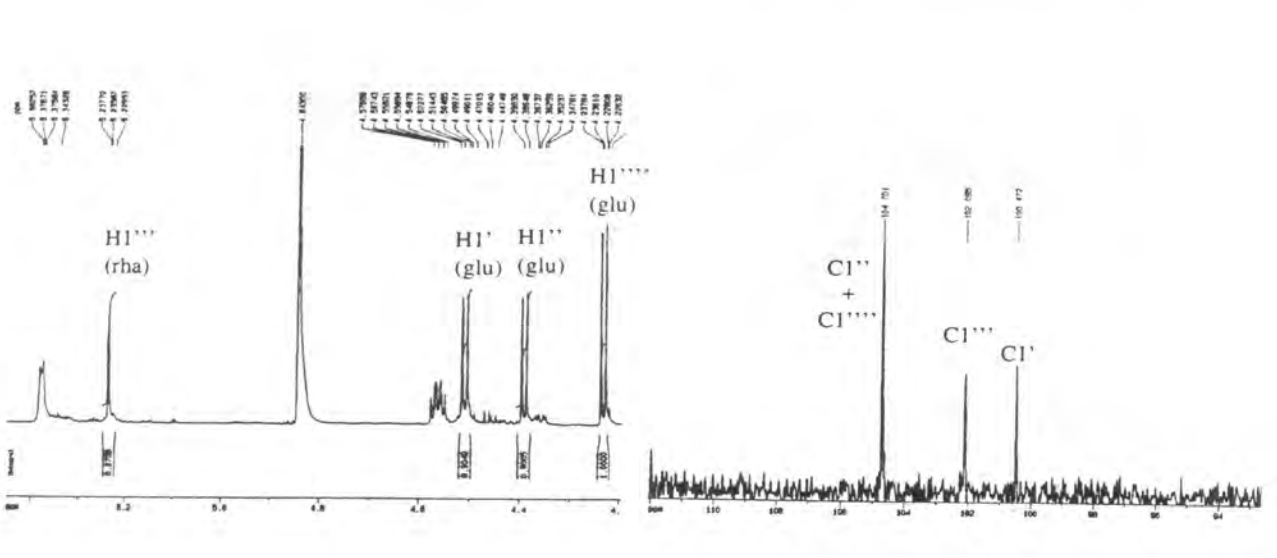


Figure 2.4. Peaks from the anomeric carbons and protons in the sugar residues of Peak 2, tR 4.8 min.

Peak 2, tR 4.8 min

In the ^1H NMR spectra of the peak 2, resonances for four anomeric protons appeared at δ 5.24 *d,* J = 1.5 Hz (H1’’’), 4.51, *d,* J = 7.9 Hz (H1’), 4.39, *d,* J = 7.9 (H1’’), and 4.23, *d,* J = 7.8 (H1’’’’), indicating the presence of four pyranoses. The four peaks of the anomeric

carbons were seen at δ 104.7 (× 2) (C1'' and C1''''), 102.1 (C1''') and 100.5 (C1') (**Figure 2.4**).

Furthermore, ^{13}C NMR data clearly reveal the presence of three glucose units at δ 62.9 (C-6''), 62.5 (C-6''''), and 62.0 (C-6'), and one rhamnose unit at δ 18.0 (C-6'''), as well as characteristic furostanol carbon signals (**Appendix B1**), previously reported by Hosny et al. (1992). The positions of the various sugar protons were determined by HSQC spectra and the cross peaks in the COSY spectrum (**Figure 2.5**). Thus, C-3 (δ 79.4) showed HMBC correlation to H1', which indicates that C3 and C1' are attached through an ether linkage. C2' (δ 78.7) shows HMBC correlation to H1''', which indicates that C2' and C1''' are attached through an ether linkage. C4' (δ 81.0) shows HMBC correlation to H1'', which indicates that C4' and C1'' are attached through an ether linkage (**Figure 2.6**). C26 (δ 76.1) shows HMBC correlation to H1'''', which indicates that C26 and C1'''' are attached through an ether linkage. There were also NOEs from H-1' to H-4_{eq}, H-4_{ax} and H-3, from H-1'' to H-4', H-6_a' and H-6_b', from H-1''' to H-2', and from H-2' to H-5'''.

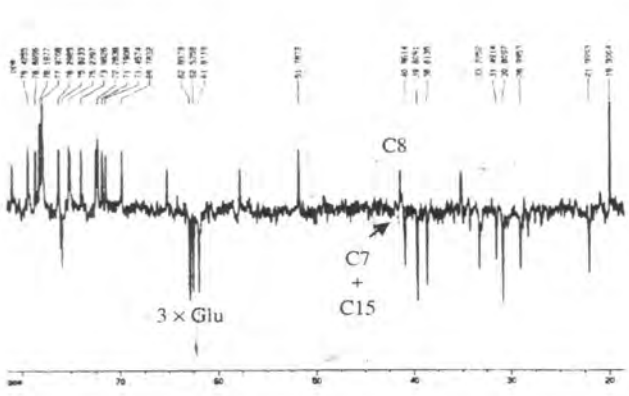


Figure 2.5. The DEPT 135 spectra show the methylene groups with negative peaks.

The anomeric configurations of all glucose residues were deduced as β from the large vicinal coupling constants (7.8–7.9 Hz). The rhamnose orientation was determined as α from the small coupling (1.5 Hz). This leads to the determination of the structure of the

compound as 26-(O-β-D-glucopyranosyl)-3β, 22, 26-trihydroxyfurost-5-ene 3-O-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**Figure 2.7**)

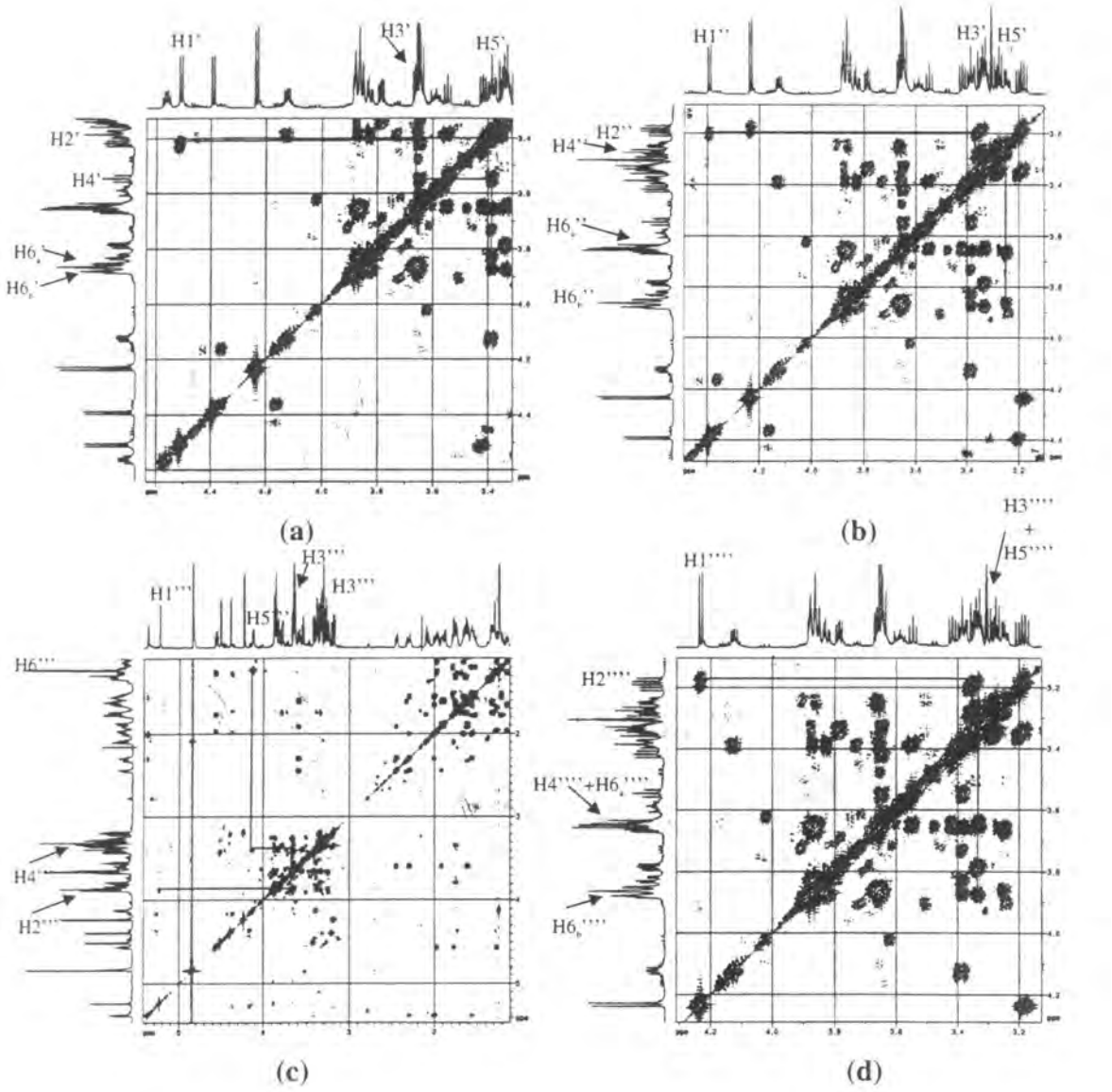


Figure 2.6. COSY spectra showing the cross-peaks from Glu' (**a**), Glu'' (**b**), Rha (**c**), and Glu'''' (**d**).

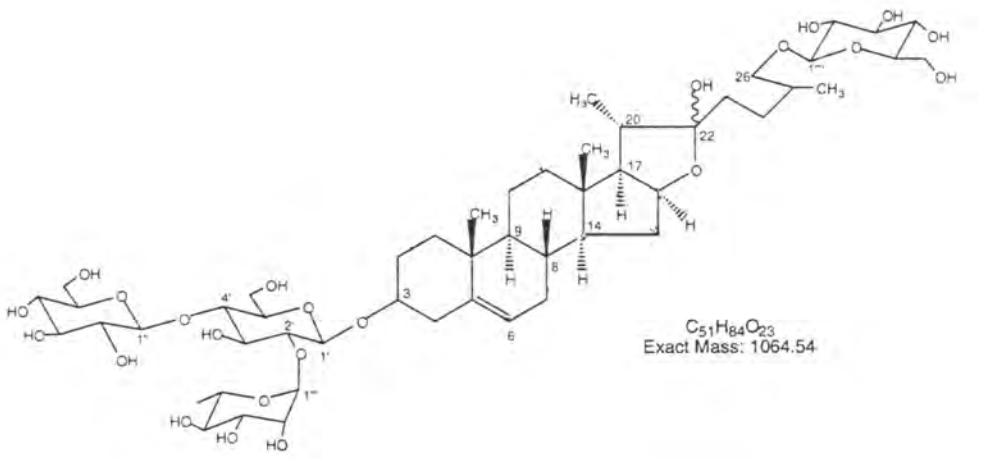


Figure 2.7. Structure of the main saponin in *Balanites aegyptiaca* fruit mesocarp, isolated from peak 2 (tR, 4.2 min) of the methanol extract.

Peak 4 (tR 7.2 min)

The NMR data of the saponin of this fraction (peak 4) clearly reveal resonances of five anomeric carbon and proton atoms, instead of four in the previous fraction. This indicates the presence of five pyranoses.

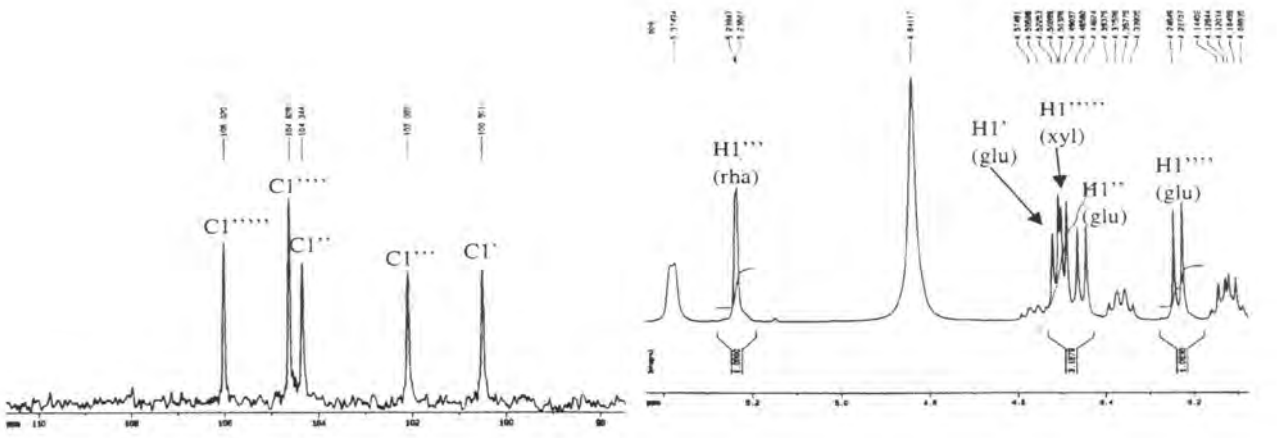


Figure 2.8: Peaks from the anomeric carbon and proton atoms in the sugar residues of the compound of peak 4 (tR, 7.2 min)

In the ^{1}H NMR spectrum, resonances for the anomeric protons are observed at δ 5.24, *d*, **J** =1.0 Hz (H1 ’’’), δ 4.51, *d*, **J** = 7.9 Hz (H1 ’), δ 4.50, *d*, **J** = 7.8 (H1’’’’), δ 4.46, *d*, **J** = 7.9 (H1’’), and δ 4.24, *d*, **J** = 7.8 (H1’’’’). Resonances of the corresponding anomeric carbons can be seen in the ^{13}C NMR spectrum at δ 106.0 (C1’’’’’), δ 104.6 (C1’’’’), δ 104.3 (C1’’), δ 102.1 (C1’’’), and δ 100.5 (C1’) (**Appendix B2**). In fact, there are five more carbons in the compounds in this fraction than in the fraction of peak 5, tR 8.2 min and one of them belongs to a methylene group (δ 67.2 C5’’’’), revealing that the last sugar residue is xylose (**Figure 2.8**). The anomeric configuration of xylose was deduced as β from the large J $^{1}H^{1}H$ coupling (J=7.8 Hz). The ^{1}H NMR spectrum shows a singlet at δ 3.14. This is the signal from the methoxy group at C22 (**Figure 2.9**).

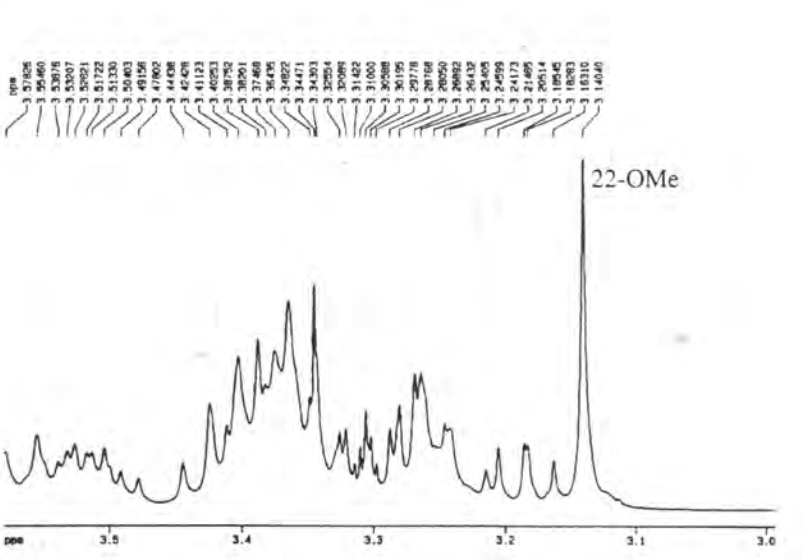


Figure 2.9. ^{1}H NMR spectrum showing the methoxy group at C-22 in the compound in fraction peak 4 (tR, 7.2 min).

The structure of the saponin of peak 4 (tR, 7.2 min) is thus determined as 26-(O-β-D-glucopyranosyl)-22-O-methylfuros-5-ene-3β, 26-diol 3- O-β-D-xylopyranosyl-(1→3) -β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**Figure 2.10**). Spectral data are presented in **Appendix B2**.

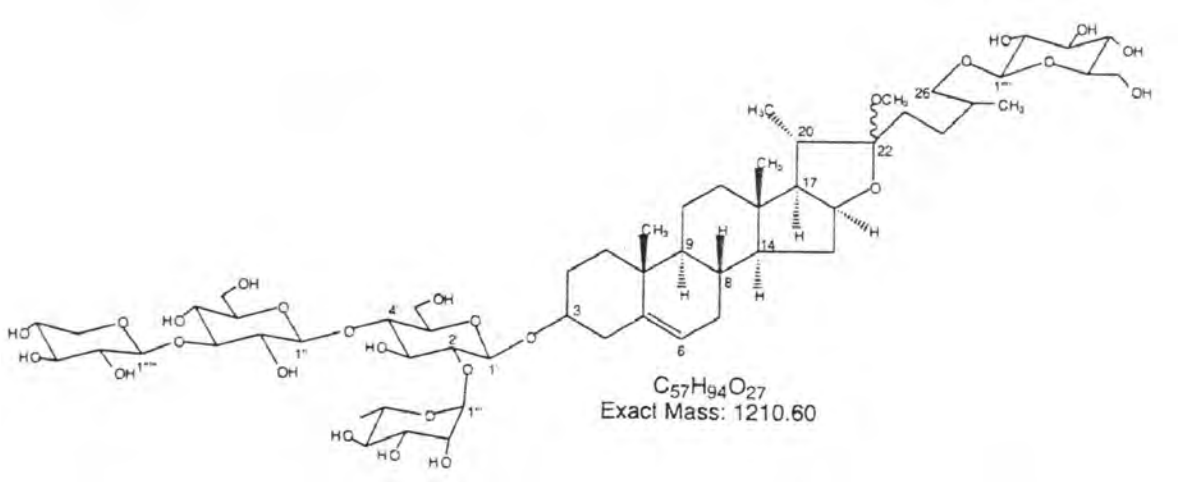


Figure 2.10. Structure of the saponin isolated from Peak 4 (tR, 7.2 min) of the *Balanites aegyptiaca* fruit mesocarp.

Peak 5, tR 8.2 min

The NMR data of the saponin of peak 5, tR 8.2, are quite similar to those of peak 2, but there is one major difference. As in peak 4, this compound gives a large singlet at δ 3.14. Some of the atoms in positions close to C-22 (positions 16, 17, 20, and 24) show epimer character, as seen in NMR spectra from the compound of peaks 2 and 4. These factors indicate that the structure of this compound is 26-(O-β-D-glucopyranosyl)-22-O-methylfurost-5-ene-3β,26-diol 3- O-β-D-glucopyranosyl-(1→4) -[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**Fig 2.11**). See **Appendix B3** for spectral data.

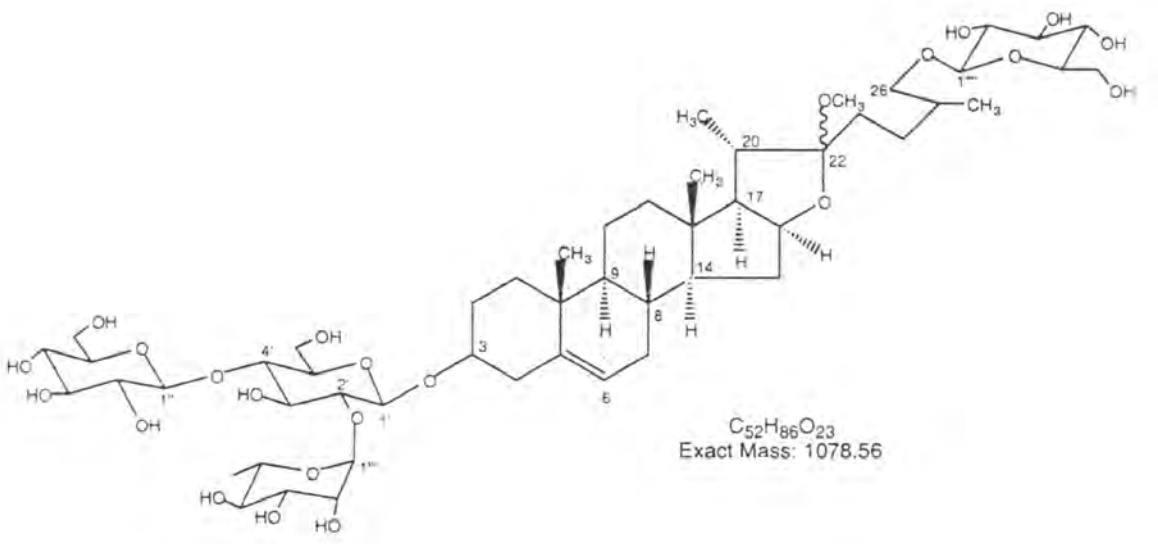


Figure 2.11. Structure of the saponin isolated from peak 5 (tR, 8.2 min) of the *Balanites aegyptiaca* fruit mesocarp.

NMR analyses have shown that, as in agreement with previous work described in the literature, the aglycone of all the major saponin fractions was found to be of the fursotanol type. For compounds of the spirostanol type, the signal for C-22 should appear at δ 109.5 ± 0.1, but its position can vary (by 108.7–110.0 ppm) depending on the solvent and structural environment, but for compounds of the furostanol type, the signal for C-22 is at approximately δ 110.8 for C-22 hydroxy furostane derivatives and approximately δ 113.5 for methoxy furostane derivatives (Agrawal et al., 1985).

Discussion

Two main purposes were behind this study: the first is the characterization of fruit mesocarp saponins of the *B. aegyptiaca* grown in Israel and the second is to develop a rapid and efficient method of isolation of *B. aegyptiaca* saponins. As mentioned in the Introduction (Chapter 1), although quite a few studies regarding the isolation and structural characterization of saponins from *B. aegyptiaca* have been carried out, so far no study has used samples of Israeli provenance. Since Israel is also one of the natural habitats of Balanites (northern-most latitude), it will be worthwhile to know about the saponin profile of *B. aegyptiaca* grown in Israel itself.

The results of this study clearly show that HPLC with RI detector could easily separate the major saponin peaks of the *B. aegyptiaca* with an isocratic gradient of methanol-water (70:30). The hyphenated MS detector had been found to be a very effective procedure to detect the saponin compound in the extract. The very similar results of the three most dominant saponin peaks of methanol extract of *B. aegyptiaca* fruit mesocarp by NMR suggests that the ESI-MS^n experiment was equally effective for the determination of the saponins of the *B. aegyptiaca* fruit mesocarp. This led us to further analysis of the other saponins from other tissues using only LC-ESI-MS^n, because this technique is much more convenient than the former one.

None of the saponins isolated in this study were previously reported from *B. aegyptiaca*. The most dominant saponin of the fruit mesocarp of *B. aegyptiaca* isolated in this study, 26-(O-β-D-glucopyranosyl)-3β, 22, 26-trihydroxyfurost-5-ene 3-O-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (MW 1064 Da), was very similar to the earlier isolated balanitoside by Hosney et al. (1992), but there are also differences in the point of attachment of rhamnopyranosyl. However, literature study revealed that this saponin compound has already been isolated by Watanabe et al. (1983) and Ori et al. (1992) from the methanolic extract of the subterranean part of *Ophiopogan planiscapus* and from the hot methanolic extract of fresh bulbs of *Lilium hansonii*, respectively. The second largest saponin compound isolated in this study, 26-(O-β-D-glucopyranosyl)-22-O-methylfuros-5-ene-3β,26-diol 3- O-β-D-xylopyranosyl-(1→3) -β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (MW 1210 Da), has never been reported previously and is presumably a new furostanol saponin. The third largest saponin compound isolated was in fact very similar to the first one. The difference was just one additional $–CH_3$ molecule in C-22. The tendency to form 22-methoxy derivatives during the extraction with methanol is common and it is widely reported in literature (Agrawal et al., 1985). To obtain the genuine 22-hydroxy furostanol, solvent extraction is recommended using a solvent like pyridine; however, from the applied point of view, methanol is the most convenient solvent for extraction.

Conclusions

From this study we can suggest that the methanol extract of the fruit mesocarp of *B. aegyptiaca* grown in Israel contains at least five major steroidal saponins with diosgenin as aglycone, and glucose, rhamnose, and xylose are the attached sugar units. Among them, the main constituent (42.3% of the total saponin) is a bi-desmosidic furostanol saponin with molecular mass 1064 Da containing three units of glucose and one unit of rhamnose attached to the aglycone. The other saponins are also found to be structurally very similar to this saponin molecule, with the addition of one xylose (molecular mass 1196 Da), one rhamnose (molecular mass 1210 Da), or additional methyl units (molecular mass 1078 Da). The saponin molecule, with molecular mass 1046 Da, that was found to have different cleavage patterns, was also structurally close to the main saponin.

The LC-ESI-MS^n procedure applied in this study enabled rapid determination of *B. aegyptiaca* saponins using a minimum number of manipulations and chromatographic separation steps. When the MeOH extract is injected directly into the HPLC column, it shows a large proportion of sugars, which were eluted with the front band; however, further elution by Sep-Pak (C18) was found to be very effective in removing free sugar bands. In terms of dry weight basis, a 55% methanol extract (ME) was obtained from mesocarp. When ME was eluted further by methanol after discarding the first water elution, using Sep-Pak a 22% extract was obtained. Finally, 44.3% of the total saponin was obtained from this Sep-Pak eluted extract. In totality, 5.36% total saponin content was obtained from the fruit mesocarp of *B. aegyptiaca*. Hence, it is concluded that HPLC-RI combined with ESI-MS^n is a proper method for isolation and characterization of the saponin compound in *B. aegyptiaca* mesocarp extract. Although electrospray ionization mass spectrometry coupled with MS^n can only verify the structure of the compound, it was found to be an easy and rapid method for identifying the saponins with their sugar chains and aglycone status in the *B. aegyptiaca* fruit mesocarp extract.

CHAPTER 3

Detection of saponins in *B. aegyptiaca* kernel cake and root extracts by LC-ESI-MS^n

Introduction

Being one of the widely grown tree species of the arid and semiarid regions, tons of fruits of *B. aegyptiaca* are produced every year in this region. One estimate shows that every year more than 400,000 tons of *B. aegyptiaca* fruits are produced in Sudan alone and a significant part of this goes for oil extraction (Mohamed et al., 2002). Local people use the pulp as a sweet fruit and the seed kernel, which is rich in oil, is used as a source of edible oil (Newinger, 1996); however, the oil cake (the meal produced after oil extraction) is regarded as unsuitable as food, unlike many other oil crops, because of the presence of many toxic substances. Literature study has revealed that *B. aegyptiaca* fruit is used against stomach pain, as an anthelminthic, and as an oral antidiabetic, while the kernel extract is used against bilharzias (Kamel et al., 1991; Iwu, 1993; Mohamed et al., 1999). It has also been reported that *B. aegyptiaca* roots have been used in many folk medicines, *viz*: for the treatment of abdominal pains and asthma, as a purgative, and as an anthelmintic (Farid et al., 2002). The molluscicidal properties of the fruit and whole plant have also been recognized and subsequently recommended as a means for the control of the fresh water snail which acts as intermediary host of Bilharzia (Schistosomiasis) (Kloos and McCullough, 1987). The molluscicidal and other properties of the *B. aegyptiaca* are attributed to their saponin constituents. Earlier studies have shown that *B. aegyptiaca* kernel and root extract contain steroidal saponins (Pettit el al., 1991; Farid et al., 2002).

Although *B. aegyptiaca* has been used for various purposes, from ethno-medicinal to fire wood, this plant is considered one of the most neglected tree species in the arid regions and has not yet been domesticated (Hall and Walker, 1991). Even though Israel is one of the native homelands of *B. aegyptiaca* and it is considered to be the northern-most latitude where *B. aegyptiaca* grows naturally (Zohary, 1973), to the best of our knowledge no study has so far reported on the saponin content in *B. aegyptiaca* seed kernel and root extract from Israeli provenances. Though saponins are extremely widely distributed in the plant kingdom, saponin content depends on factors such as cultivar, age, physiological state, and geographical location of the plant (Hostettman and Marston, 1995). Hence, it is surmised

that a study of the saponin profile in *B. aegyptiaca* seed kernels and roots from Israeli samples will help in one way to increase the understanding of Israeli provenance *B. aegyptiaca* and in another way to help to expand the use of mostly waste product (kernel cake) and to promote new plantations that will ultimately help in the domestication process. Traditional analytical protocol for detection and identification of saponins in plants are complicated and time consuming procedures because saponins are of high polarity, are thermally labile, and are present at low levels (Cui et al., 2000), so in this study we used liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) for the identification and characterization of the seed kernel and root saponins of Israeli provenance *B. aegyptiaca*.

Materials and methods

Sample collection

Well-ripened fresh fruits and tender roots of *B. aegyptiaca* were collected from the *B. aegyptiaca* plants grown at the Kibbutz Samar in southern Israel. Kibbutz Samar is located in the Arava Rift Valley of Israel, 40 km north from the Rea Sea Gulf of Eilat, and very close to the Jordanian boarder.

Saponin extraction

The epicarp (outer cover) and mesocarp (pulp) of the fruits were removed by hand and the nuts were washed with tap water. After washing, the nuts were oven-dried at 70°C for 72 h. Decortications of the nuts were carried out manually, releasing the kernel (approximately 10% of the total fresh weight). The kernels were coarsely ground using a mortar and pestle. After grinding, the ground kernel was defatted three times using n-hexane. In brief, 3 g of kernel powder were put in a plastic tube (50 ml capacity, centrifuge grade) and 30 ml of n-hexane was added. The tubes were put in electric shaker (Z. Tuttnauer Ltd. Jerusalem) overnight at high speed, followed by centrifugation (3500 rpm, 18 min, 20°C), and supernatant was collected. Two subsequent extractions were carried out on the residue using only vortexes and centrifuging. After three successive extractions, the supernatant was clear; all the supernatants were collected, the n-hexane evaporated using a

rotary evaporator at a reduced temperature, and the oil was collected. A range of 45–50% oil by weight was obtained.

The cake (kernels that remained after oil extraction) was kept under the hood overnight to dry all the hexane. The next day, 30 ml MeOH was added to each tube and kept on the shaker overnight followed by centrifugation. The second and third extractions by methanol were also carried out as with the n-hexane. At the end, all methanol extract supernatants were pooled and the methanol was evaporated using a rotary evaporator. Finally, a yellowish white crystal powder of crude saponins was obtained (approximately 12.9% dry weight of kernel weight); this product is named KE (kernel extract).

The fresh roots were chopped into small pieces and oven dried below 50ºC. The dried pieces were powdered in a coffee grinder. The powder was first defatted using n-hexane and then extracted with MeOH, similar to the procedure used with the kernels. Finally, a yellowish crystal powder was obtained (approximately 14.6% of root by dry weight); this product is named RE (root extract).

ESI-mass spectrometry

All the experiments were performed using a single ion-trap mass spectrometer (Esquire 3000 Plus, Bruker Daltonik) equipped with an electrospray interface (ESI) as the ion source for MS analyses. Instrument control and data analysis were by means of Bruker Daltonics Data Analysis version 3. The electrospray voltage was set to 4.5 kv. Temperature of the ion source capillary was 300ºC. A negative ion mode was used in all cases. Mass spectrometer conditions were optimized to achieve maximum sensitivity. Full scan spectra from *m/z* 25 to 2000 in the negative ion mode were obtained (scan time 1s). Ion trap condition: acquisition in automatic gain control with an accumulation time of 159 μs. For the MS^n analyses, the $[M-H]^-$ molecular ions were isolated with an isolation width of 3 *m/z* units and fragmented using activation amplitude of 0.95 to 1.12 for MS^2 experiments.

Liquid chromatography

A Waters (Miliford, MA, US) 2690 HPLC system with an auto sampler was operated at room temperature with a Luna 5 U 100 A C18 column (250 × 2 mm, 5 μm) included an

HPLC pump and a refractive index (RI) detector G1314 A (Agilent). The mobile phase was MeOH/H_2O (70:30) at a flow rate of 0.2 ml/min. The injection volume was 10 µl. Both the KE and RE were eluted by methanol in Solid Phase Extraction (SPE) cartridges (C 18) after discarding the first water elution. The methanol was again evaporated under reduced temperature and each extract was dissolved in methanol: water (70:30) and filtered using a 0.45 µm filter before injection. From the SPE elution, 40% and 35% by weight of KE and RE, respectively, were obtained.

Results and discussion

Kernel extract

HPLC-RI analysis

Developing analytical methods for saponins is considered a challenge because most saponins have no or weak chromospheres in most common detectors such as UV, and an evaporating light scattering detector is recommended for saponin detection (Ganzera et al., 2001). However, the chromatogram developed by the RI detector with isocratic solvent of methanol:water (70:30) in our experiment was clear. The full scan HPLC-RI chromatogram of the methanol extract of the *B. aegyptiaca* seed kernel is presented in **Figure 3a**. There are nine major peaks at 5.4, 6.1, 7.5, 9.5, 10.9, 12.6, 14.6, 16.4, and 23.3 min retention time (tR). An estimation based on the peak area shows that peak 6 is the largest, followed by peak 4, peak 7, peak 5, peak 8, peak 2, peak 7, peak 1, and peak 3 (**Table 3.1**).

LC-ESI-MS analysis

The mass spectrometric analysis of each of the nine peaks in negative ion mode gave deprotonated molecular mass from which molecular weights were calculated (**Table 3.1**). Full scan negative ion ESI mass spectrum of KE is presented in **Figure 3.1**. In the figure, the RI (LC) chromatogram is presented in **Figure 3.1a** and the TIC (MS) chromatogram is presented in **Figure 3.1b**; they are quite fitted to each other. The negative ion ESI mass spectra of all major peaks are presented **Figure 3.2**. The mass spectra of all the saponin peaks are shown as $[M-H]^-$. The $[M-H]^-$ at *m/z* of the peaks are 1195, 1063, 1209, 1223, 1077, 1209, 1077, 1045, and 1045 with tR 5.4, 6.1, 7.5, 9.5, 10.9, 12.6, 14.6, 16.4, and 23.3 minutes, respectively. The m/z values quoted are integers obtained by truncating (not

rounding off) the measured values. Further MS/MS analysis of each peak in negative ion mode by collision-induced dissociation (CID) was carried out in order to know the structural profile of each peak. The compounds related to peaks in **Figure 3.1** exhibited intense deprotonated molecular ions $[M-H]^-$ in negative ion mass spectra. The retention times, values of the mass-to-charge ratios (*m/z*) for the $[M-H]^-$ ions, and the CID fragments of the nine peaks are listed in **Table 3.2**. The molecular mass of peaks 1–9 were confirmed to be 1196, 1064, 1210, 1224, 1078, 1210, 1078, 1046, and 1046 Da by LC-ESI-MS, respectively. Peaks 3 and 6 had the same $[M-H]^-$ ion at *m/z* 1209, which gave the MW of 1210 Da for both of these compounds; similarly, peaks 5 and 7, as well as peaks 8 and 9, had the same MW of 1078 Da and 1046 Da, respectively.

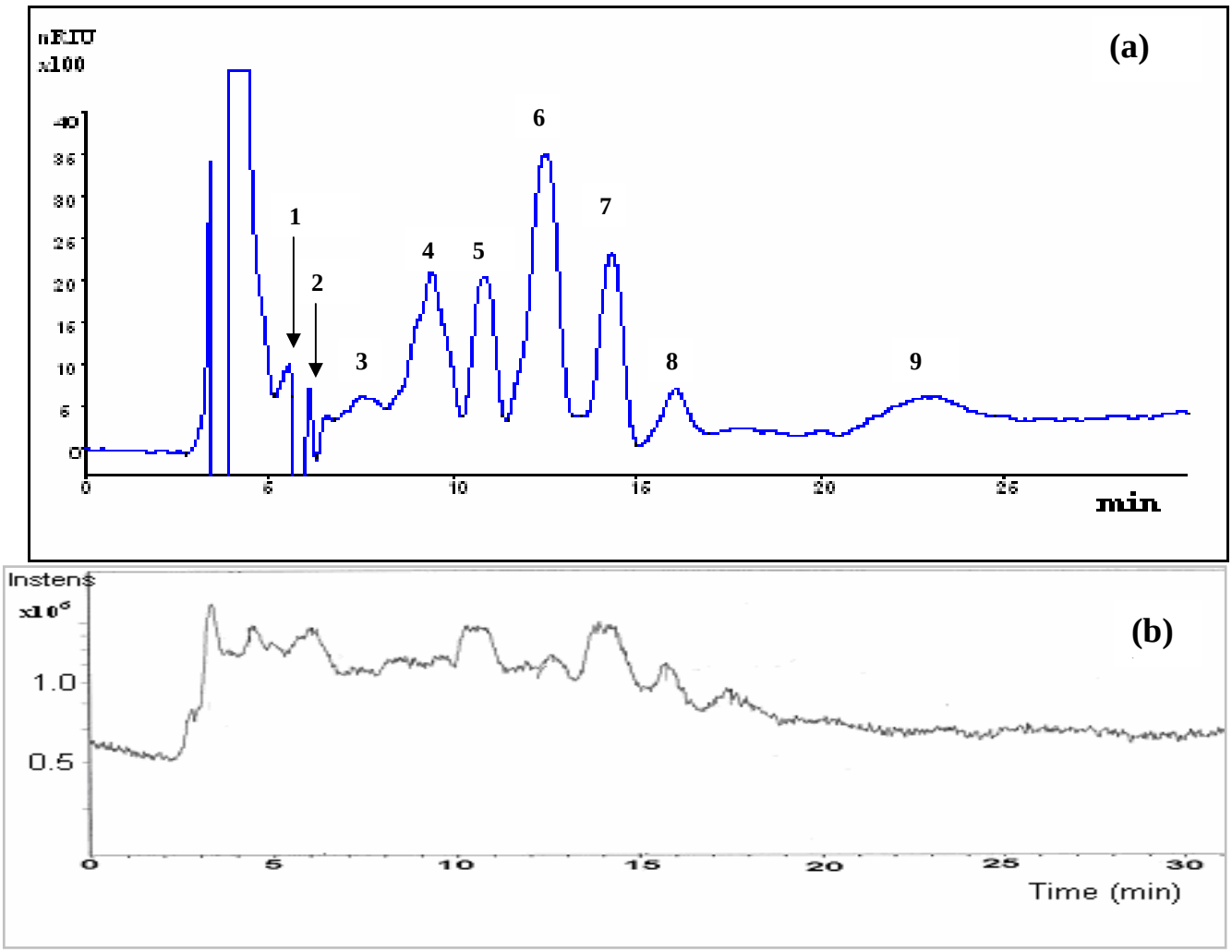


Figure 3.1. Major saponin peaks of methanolic extract of *B. aegyptiaca* seed kernel. (a) HPLC-RI chromatogram. (b) MS-TIC chromatogram.

Table 3.1 Peaks, retention time, amount, and corresponding mass of the methanolic extracts of *B. aegyptiaca* seed kernel saponins.

Peaks	Retention time (min)	m/z [a](M-H)$^-$	Amount (%)[b]
1	5.4	1195	0.4
2	6.1	1063	0.6
3	7.5	1209	0.4
4	9.5	1223	6.7
5	10.9	1077	4.5
6	12.6	1209	11.2
7	14.6	1077	5.9
8	16.4	1045	2.0
9	23.3	1045	0.5

[a]Values quoted are integers obtained by truncating (not rounding off) measured values.
[b]The percent is calculated based on the chromatographic peak area from the injected sample.

The CID spectrum of LC-ESI-MS/MS of peak 1 at *m/z* 1195 shows 7 fragmented ions at *m/z* 1063, 1049, 901, 755, 593, 431, and 413 (**Fig. 3.3a** and **Table 3.2**). This fragmented pathway shows the subsequent loss of monosaccharide units, suggesting a pattern of the characteristic cleavage of glycosidic bonds (Liu et al., 2004) and this provides information about the monosaccharidic sequences of the saponin compound. The subsequent fragmentation pattern shows that this saponin is the one of the saponins present in the fruit mesocarp of *B. aegyptiaca* i.e., a saponin with a five monosaccharide unit glucose, rhamnose, and xylose (3:1:1) as sugar moieties and diosgenin as aglycone moiety as described in Chapter 2.

The LC-ESI-MS of peak 2 (**Fig. 3.2b**, **Table 3.2**) gave a pattern of fragmentation similar to that of peak 1 starting with the first fragment ion at *m/z* 901. Other fragment ions at *m/z* 755, 593, 431, and 413 are same as in peak 1. This indicates that this peak with *m/z* 1063 is also a very similar saponin compound as that seen in peak 1 with one fewer pentose sugar (xylose). This also suggests that this is the same saponin as described in Chapter 2 as the main saponin of *B. aegyptiaca* fruit mesocarp i.e., MW 1064 Da.

The LC-ESI-MS/MS of peak 3 observed five fragment ions at *m/z* 1077, 897, 589, 431, and 413 from the parent ion at 1209 (**Fig. 3.3c**, **Table 3.2**). The fragment ion at *m/z* 1077 corresponded to the loss of a xylose unit from the parent ion at *m/z* 1209. This shows the cleavage of a pentose sugar (xylose) first from the parent ion; it is unlikely that there two

simultaneous sugar units in former peaks. This fragmentation pattern and ultimate aglycone molecule also show that this was one of the main saponins of ***B. aegyptiaca*** as mentioned in Chapter 2.

The CID spectrum of LC-ESI-MS/MS of peak 4 at *m/z* 1223 shows 7 fragmented ions at *m/z* 1077, 1061, 915, 769, 589, 431, and 413 (Fig. 3.3d, Table 3.2). The ion fragments at *m/z* 1077 and 1061 show a corresponding loss of a deoxyhexose unit (rhamnose) and a hexose sugar unit (glucose) from the parent ion. Similarly, the fragment ion at *m/z* 915 corresponds with the loss of both a hexose sugar unit (glucose) and a deoxyhexose sugar unit (rhamnose) from the parent ion. The subsequent fragment ions at *m/z* 769 and 589 correspond to loss of a glucose unit (hexose sugar) and two units of rhamnose, and two units of glucose and one rhamnose unit with a water unit from the parent ion. The subsequent fragment ion at *m/z* 431 corresponded with loss of all sugar units (3 glucose and 2 rhamnose) as well as one methyl unit from the parent ion (an aglycone, a diosgenin). The last fragment ion at *m/z* 413 indicates the further loss of a water unit from the previous fragment. This cleavage pattern suggests that the peak 4 is a different type of saponin with 3 hexose sugar units (glucose), and two deoxyhexose sugars (rhamnose) are attached in the similar aglycone.

The CID fragmentation of peak 5 gave four fragmented ions at *m/z* at 915, 769, 589, and 431 from the parent ion at *m/z* 1077 (**Fig. 3.3e**, **Table 3.2**). The fragment ion at *m/z* 915 corresponds to loss of a hexose sugar unit (glucose) from the parent ion. The subsequent fragment ions at *m/z* 769 and 589 show the cleavage of a hexose sugar unit (glucose) and a deoxyhexose sugar unit (rhamnose), and two hexose sugar units (glucose), one deoxyhexose sugar unit (rhamnose), and a water unit from the parent ion, respectively. Similarly, the fragment ion at *m/z* 431 corresponds with the loss of all sugar units (3 glucose and 1 rhamnose) and a methyl unit from the parent ion. This indicates that this is also the one of the saponins present in the fruit mesocarp as described earlier in Chapter 2.

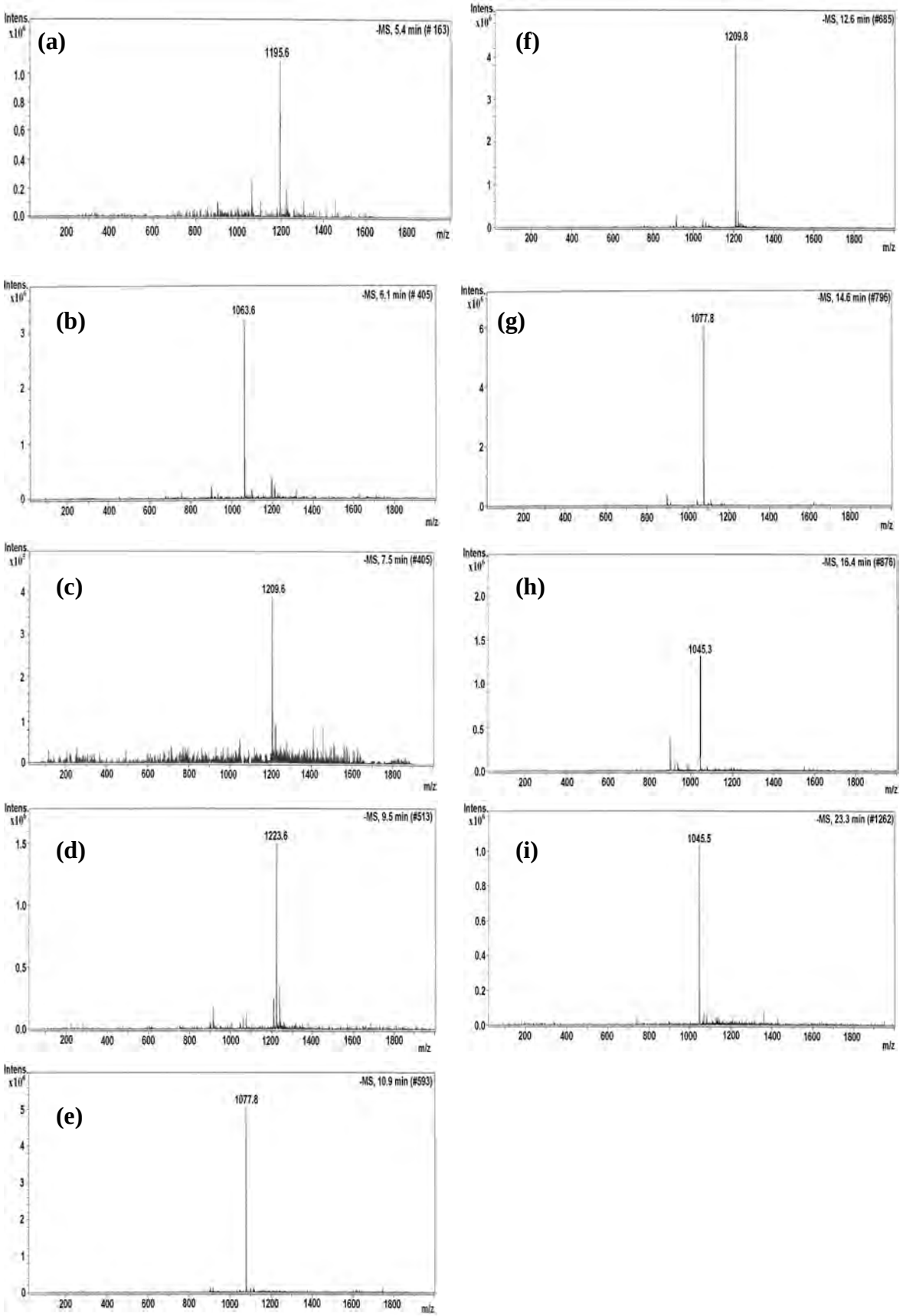


Figure 3.2 ESI-MS spectra of *B. aegyptiaca* fruit kernel extract; (**a**) to (**i**) show different retention times.

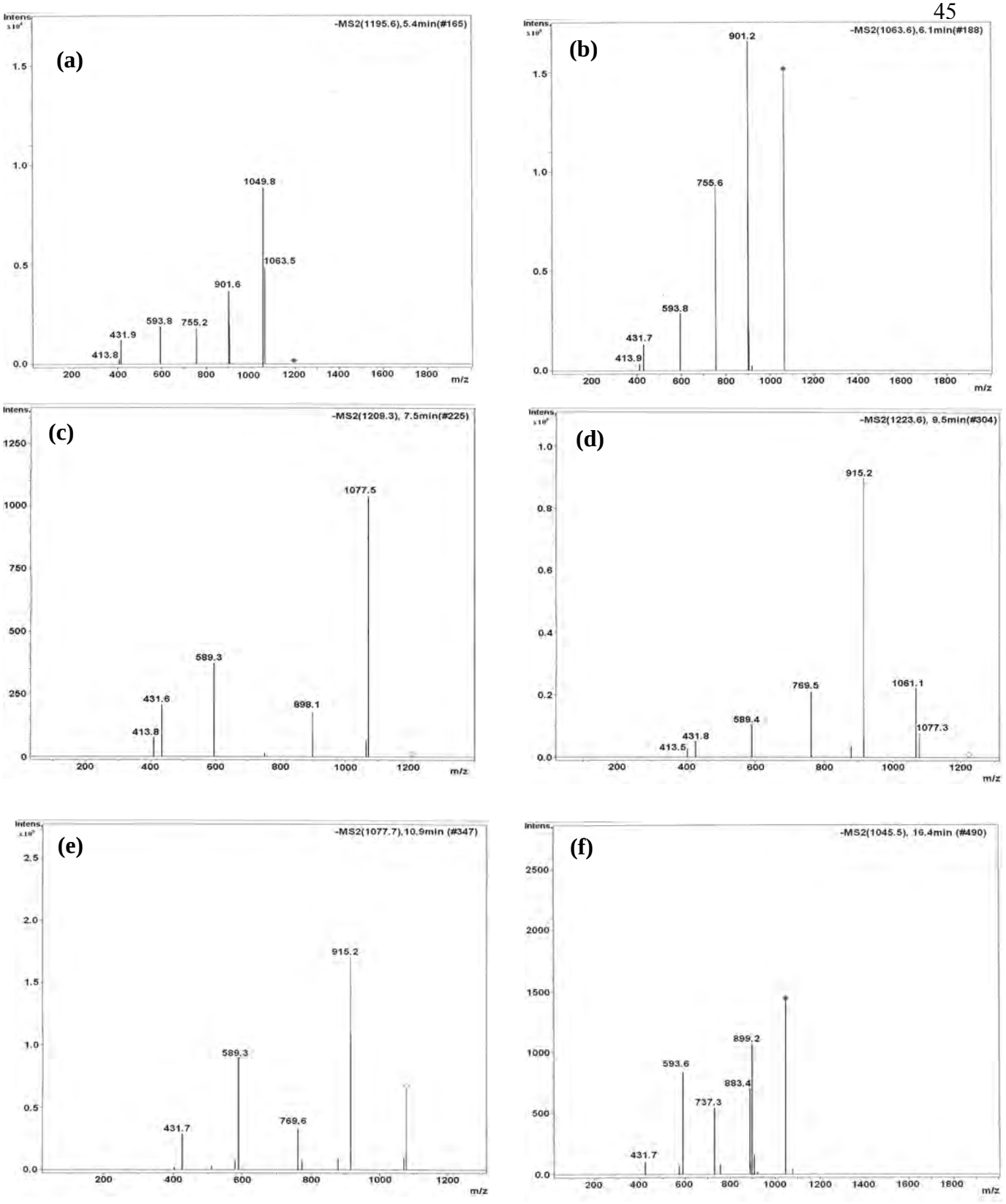


Figure 3.3 MS/MS spectra of *B. aegyptiaca* kernel cake (**a**) peak 1, (**b**) peak 2, (**c**) peak 3, (**d**) peak 4, (**e**) peak 5, (**f**) peak 8.

Table 3.2 The LC-ESI-MS and corresponding CID data in negative ion mode (*m/z* values) of peaks 1–9 in LC profile of *B. aegyptiaca* kernel cake.

Peak	tR (min)[a]	(M-H)[-b]	CID[c]
1	5.4	1195	1063 [M-H-Xyl], 1049 [M-H-Rha], 901[M-H-Glu-Xyl], 755 [M-H-Glu-Rha-Xyl], 593 [M-H-2Glu-Rha-Xyl], 431[M-H-3Glu-Rha-Xyl], 413[M-H-3Glu-Rha-Xyl-H_2O]
2	6.1	1063	901 [M-H-Glu], 755 [M-H-Glu-Rha], 593 [M-H-2Glu-Rha], 431[M-H-3Glu-Rha], 413[M-H-3Glu-Rha-H_2O]
3	7.5	1209	1077 [M-H-Xyl], 897[Glu-Xyl-H_2O], 589[M-H-2Glu-Rha-Xyl-H_2O], 431[M-H-3Glu-Rha-Xyl-14], 413[M-H-3Glu-Rha-Xyl-14-18]
4	9.5	1223	1077 [M-H-Rha], 1061[M-H-Glu], 915[M-H-Glu-Rha],769[M-H-Glu-2Rha], 589[M-H-2Glu-2Rha-H2O], 431[M-H-3Glu-2Rha-14], 413[M-H-3Glu-2Rha-14-H2O]
5	10.9	1077	915 [M-H-Glu], 769[M-H-Glu-Rha], 589[M-H-2Glu-Rha-H2O], 431[M-H-3Glu-Rha-CH3]
6	12.6	1209	1195 [M-H-14], 1077[M-H-Xyl], 915[M-H- Glu-Xyl], 769[M-H-Glu-Rha-Xyl], 589[M-H-2Glu-Rha-Xyl-H2O], 431[M-H-3Glu-Rha-Xyl-H2O]
7	14.6	1077	931[M-H-Rha], 915[M-H-Glu], 897[M-H-Glu-H2O], 769[M-H-Glu-Rha], 589[M-H-2Glu-Rha-H2O], 431[M-H-3Glu-Rha-H2O]
8	16.4	1045	899[M-H-Rha], 883 [M-H-Glu], 737[M-H-Glu-Rha], 593[M-H-Glu-Rha-144], 431[M-H-2Glu-Rha-144]
9	23.3	1045	[M-H-Rha], 883[M-H-Glu], 737[M-H-Glu-Rha], 593[M-H-Glu-Rha-144], 431[M-H-2Glu-Rha-144]

[a] Retention time, [b] Deprotonated molecular ion, [c] Fragmented ions
The *m/z* values quoted are integers obtained by truncating (not rounding off) measured values.

The LC-ESI-MS/MS of peak 8 shows five fragmentations at *m/z* 899, 883, 737, 593, and 431 from the parent ion at *m/z* 1045 (**Fig. 3.3f**, **Table 3.2**). This also suggests that this is also one of the saponins present in the fruit mesocarp of the *B. aegyptiaca* described earlier in Chapter 2.

Peak 6 had the same MW of 1210 Da as peak 3. This peak gave a slightly different fragmentation pattern than did peak 3. In this case, the first fragment ion was at *m/z* 1195, which corresponds to a loss of one methyl unit from the parent ion. The fragment ion at *m/z* 1077 shows a cleavage of one xylose unit from the parent ion peak. The subsequent ions at *m/z* 915 and 769 show the loss of a glucose and a xylose sugar unit and a glucose, a rhamnose, and a xylose sugar unit from the parent ion. The other two fragments at *m/z* 589

and 431 had the same cleavage pattern as peak 3. This suggested that this peak is actually the same saponin as peak 3. The appearance of different retention time could have been connected with the different fragment patterns. As with peaks 3 and 6, peaks 7 and 5 had the same MW of 1078 Da. In peak 5, the only cleavage of a glucose unit was found as an independent fragment in the beginning, whereas in peak 7, the first peak was loss of rhamnose unit; only then did the fragment with loss of glucose unit from the parent ion appear. In peak 5, the fragment ion at *m/z* 769 appeared before the fragment ion at 589; whereas in peak 7, two fragment ions at *m/z* 897 and 769 appeared before the fragment ion at *m/z* 589. The same molecular mass peak appearing at different retention times could again be assumed from their fragmentation behaviors.

Peak 9 actually shows the same fragmentation pattern as peak 8. It can be suggested that these two peaks are an isomeric saponin; however, the conformity can not be verified only from the ESI-MS/MS experiment.

Root extract

HPLC-RI analysis

The n-hexane defatted and MeOH extracted root of *B. aegyptiaca* yielded 11 chromatographic peaks. The full scan of the peaks of the HPLC-RI is presented in **Figures 3.4** and **3.5**. An quantitative estimation based on the peak area shows that among 11 peaks, peak 2 was the biggest followed by peak 5 and peak 9. Peaks 1, 4, 6, 7, 8, 10, and 11 are less than 1% (**Table 3.3**).

LC-ESI-MS analysis

The preliminary information about the major compounds with their mass in the methanol extract of *B. aegyptiaca* root was obtained from direct injection of the extract into the ESI-MS (**Fig. 3.4**). However, for the comprehensive study, the spectrometric analysis of each of the eleven peaks obtained from the RI was carried out. The negative ion ESI mass spectra of all 11 peaks are presented **Figure 3.5**. The LC-ESI-MS analysis in negative ion mode gave the deprotonated molecular mass as was shown for the kernel extract from which molecular weights were calculated (**Table 3.3**). The $[M-H]^-$ at *m/z* of the peaks are 1339, 1195, 1063, 1209, 1223, 1529, 1585, 1571, 1223, 1515, and 1529 with tR at 4.2, 5.4, 6.1, 7.4, 9.3, 9.9, 10.7, 11.6, 12.5, 13.6, and 14.5 minutes, respectively. The *m/z* values

quoted here also are integers obtained by truncating (not rounding off) the measured values. Further MS/MS analysis of each peak in negative ion mode by CID exhibited intense deprotonated molecular ions [M-H (**Fig. 3.6**). The retention times, values of the mass-to-charge ratios (*m/z*) for the $[M-H]^-$ ions, and the CID fragments of the nine peaks are listed in **Table 3.4**. Peaks 5 and 9 had the same $[M-H]^-$ ion at *m/z* 1223, which gave the molecular mass of 1224 Da for both of these compounds; similarly, peaks 6 and 11 had same molecular mass of 1530 Da.

The CID spectrum of LC-ESI-MS/MS of peak 1 at *m/z* 1339 shows 6 fragmented ions at m/z 1195, 1063, 901, 755, 593, and 431 (**Fig. 3.7a** and **Table 3.4**). The first fragmented ion, at *m/z* 1195, shows a loss of 144 Da from the parent ion at *m/z* 1339. Actually this is not a rhamnose unit, but is 2 Da less than a rhamnose molecule. The elimination of 144 Da, corresponding to cleavage of the E-ring, is generally observed in furostanol saponins. Such type of cleavage phenomenon has already been reported by Liang et al. (2002) and Liu et al. (2004) in a furostanol saponin of *Asparagus cochinchinesis*. So this fragmentation pathway suggests to us that this peak is a furostanol saponin. The corresponding loss of a pentose sugar unit (xylose) in ions at *m/z* 1063 and onward showed a similar pattern as described earlier. Based on the fragmentation pattern of the ESI-MS/MS spectrum, the compound corresponding to peak 1 was suggested to be a saponin with six monosaccharide glucose units, rhamnose, and xylose (3:2:1) as sugar moieties and diosgenin as the aglycone moiety.

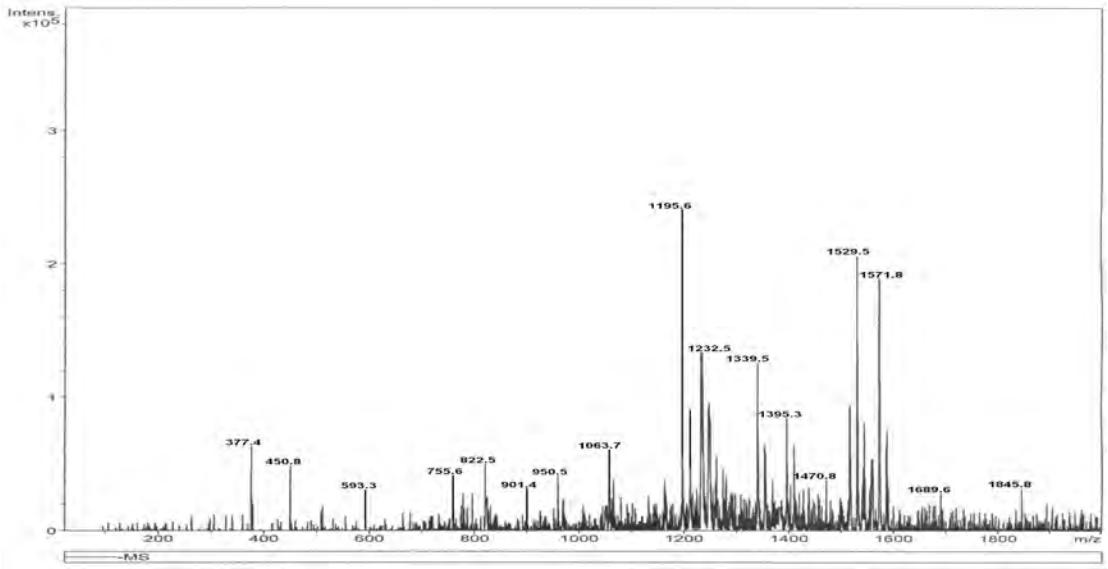


Figure 3.4 Full scan ESI-MS spectra of the methanol extract of *Balanites aegyptiaca* root.

Table 3.3 Major saponin peaks of the methanolic extract of the *Balanites aegyptiaca* root with corresponding retention time (tR), area, and mass charge ratio in negative ion mode.

Peak	Retention time	m/z[a] (M-H)$^-$	Amount (%)[b]
1	4.2	1339	0.8
2	5.4	1195	20.6
3	6.1	1063	1.7
4	7.4	1209	0.8
5	9.3	1223	7.4
6	9.9	1529	0.6
7	10.7	1585	0.7
8	11.6	1571	0.4
9	12.5	1223	5.1
10	13.6	1515	0.6
11	14.5	1529	0.4

[a]Values quoted are integers obtained by truncating (not rounding off) measured values.
[b]The percent is calculated based on the chromatographic peak area of the injected sample**.**

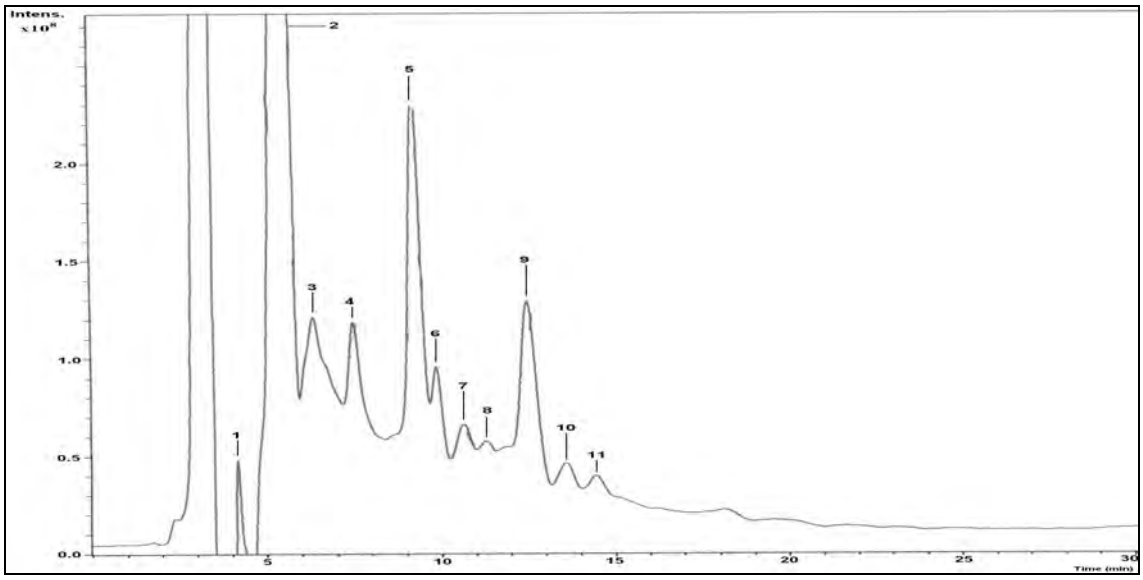


Figure 3.5 Separation of the methanol extract of *Balanites aegyptiaca* root by reserved phase HPLC-RI. The numbers show the major saponin peaks.

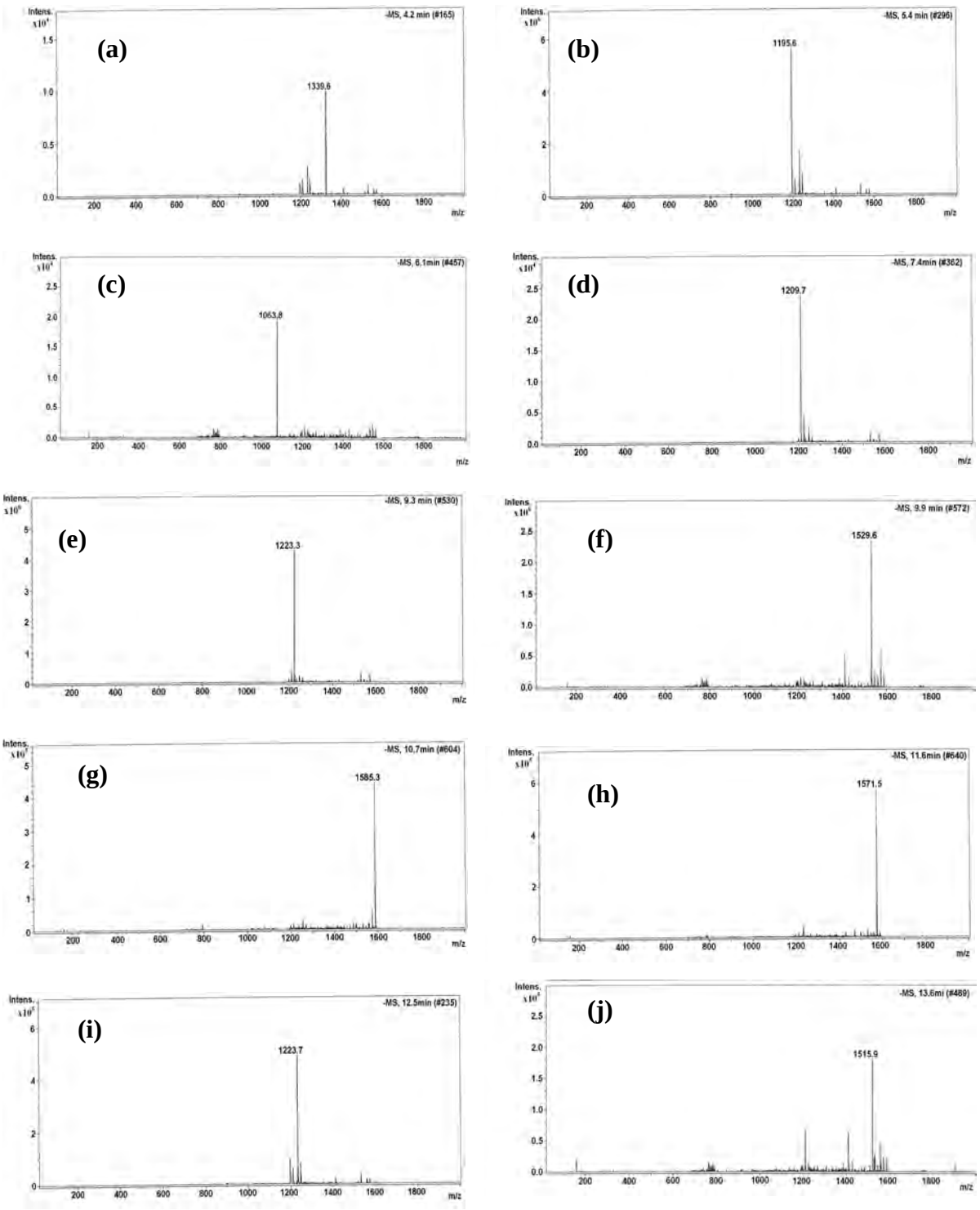


Figure 3.6 Electrospray mass spectra $[M-H]^-$ of methanol extract of *Balanites aegyptiaca* root. (**a**)–(**k**) are the mass spectra of peaks 1 to 11 of the HPLC-RI chromatogram of Figure 3.5, respectively.

The LC-ESI-MS of peak 2 at *m/z* 1195 (**Fig. 3.7b**, **Table 3.4**) gave a similar fragmentation pattern to peak 1 from the kernel extract described earlier. This suggests that this peak 2 is the already isolated saponin of fruit mesocarp (Chapter 1) and kernel extract with five monosaccharide units glucose, rhamnose, and xylose (3:1:1) as sugar moieties and diosgenin as aglycone.

The fragmented ions of peak 3 at *m/z* 1063 gave a very similar cleavage pattern to that of peak 2 of the kernel extract as described earlier (**Fig. 3.7c**, **Table 3.4**). These data suggested that peak 3 is the same saponin of peak 2 of kernel extract and major saponin of mesocarp described in Chapter 2.

The LC-ESI-MS/MS of peak 4 in negative ion mode showed the same fragment ions as peak 3 of the kernel extracts (**Fig. 3.7d**, **Table 3.4**). This suggested that this is also the same saponin found earlier in kernel and fruit mesocarp with MW 1210 Da described in Chapter 2.

The CID spectrum of LC-ESI-MS/MS of peak 5 at *m/z* 1223 shows similar fragmented ions to those of peak 4 of the kernel extracts (**Fig. 3.7d**, **Table 3.4**). This fragmentation pattern suggested that this is the same saponin molecule that was separated in a kernel extract with MW 1224 Da.

The MS/MS of peak 6 gave eight fragmented ions at *m/z* at 1385, 1223, 1077, 1045, 901, 755, 593, and 413 from the parent ion at *m/z* 1529 (**Fig. 3.7e**, **Table 3.4**). The difference between the first fragmented ion (*m/z* 1385) and the parent ion is 144 Da, which shows a similar pattern of cleavage as peak 1. The subsequent fragments are very similar to peak 5 except for the last fragmentation at *m/z* 413. The last daughter fragment at *m/z* 413 corresponds to the loss of a water unit from the aglycone. The MS and corresponding MS/MS of peak 6 suggest that this peak is similar to peak 5 with the addition of 144 Da mass.

The ESI-MS/MS of peak 7 gave nine fragmented ions at 1571, 1427, 1385, 1223, 1077, 915, 901, 755, and 593 (**Fig. 3.7f**, **Table 3.4**) from the parent ion at *m/z* 1585. The fragment ions at *m/z* 1385 to *m/z* 593 appear similar to peak 6. The first fragment ion at *m/z* 1571 corresponds to the loss of a methyl unit from the parent ion, whereas subsequent fragments ion at *m/z* 1427 and onwards show the loss of 144 Da and a unit of 42 Da thereafter, suggesting a sugar molecule. Unlike previous peaks, this ion did not demonstrate cleavage until the aglycone; however, the observed fragmentation behavior could predict that this peak also has the same aglycone as the former one. Though the loss of 42 Da could not be

considered cleavage behavior, from these data it can be suggested that this peak is also very similar to the saponin in peak 6 with 144 Da cleavage behaviors.

The fragmented ion shows that peak 8 is a similar saponin to that in peak 7 without a methyl unit (**Table 3.4**). The fragmented ions of peaks 9 and 11 show that they are similar to peaks 5 and 6, respectively, with a molecular mass of 1224 and 1530 Da each.

The ESI-MS/MS of peak 10 produced seven fragment ions from the parent ion at m/z 1515 (**Table 3.4**). The first fragment ion at *m/z* at 1371 corresponds with a loss of 144 Da from the parent ion. This also indicates a furostanol saponin as discussed earlier. The subsequent ions at *m/z* 1209, 1063, 901, 755, and 593 follow the cleavage pattern described as seen in earlier peaks with loss of a glucose; a glucose and a rhamnose; two glucose and a rhamnose; two glucose and two rhamnose; three glucose and two rhamnose units, respectively, less than the first fragmented ion. The last fragmented ion at *m/z* 431 indicates a diosgenin unit of an aglycone moiety. This suggests that peak 10 is a different saponin with MW 1516 Da.

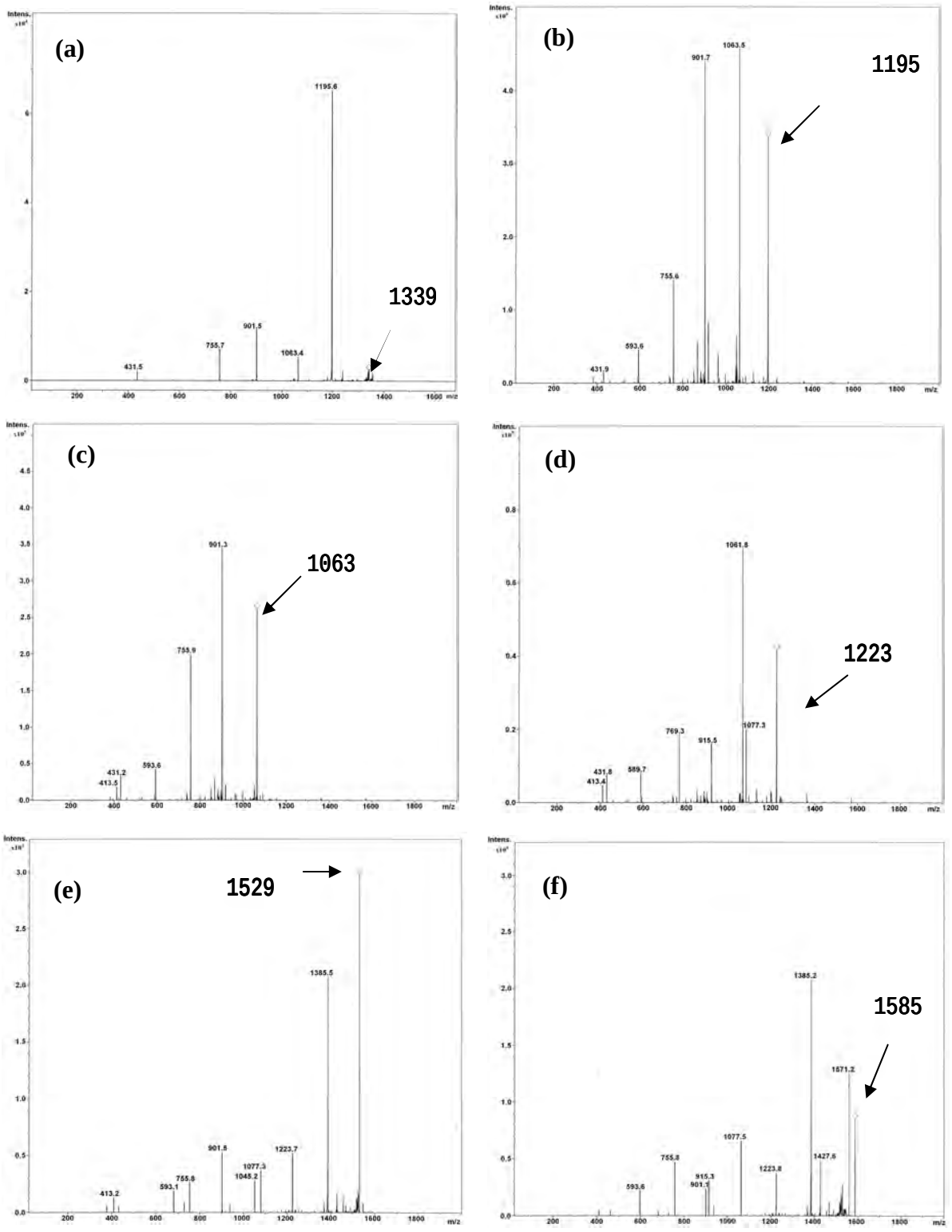


Figure 3.7 ESI-MS/MS spectra in negative ion mode $[M-H]^-$ of the selected saponin peaks of *Balanites aegyptiaca* root extract. Figures (**a**) to (**f**) are the MS/MS of the corresponding *m/z* at 1339, 1195, 1063, 1223, 1529, and 1585, respectively.

Table 3.4 The LC-ESI-MS and corresponding CID data in the negative ion mode (*m/z* values) of peaks 1–11 in LC profile of methanolic extracts of *Balanites aegyptiaca* root.

Peak	tR (min)[a]	(M-H)[-b]	CID[c]
1	4.2	1339	1195 [M-H-144], 1063 [M-H-144-Xyl], 901 [M-H-144-Xyl-Glu], 755 [M-H-144-Xyl-Glu-Rha], 431 [M-H-144-Xyl-Rha-3Glu],
2	5.4	1195	1063 [M-H-Xyl], 901[M-H-Glu-Xyl], 755 [M-H-Glu-Rha-Xyl], 593 [M-H-2Glu-Rha-Xyl], 431[M-H-3Glu-Rha-Xyl],
3	6.1	1063	901 [M-H-Glu], 755 [M-H-Glu-Rha], 593 [M-H-2Glu-Rha], 431[M-H-3Glu-Rha], 413[M-H-3Glu-Rha-H_2O]
4	7.4	1209	1077 [M-H-Xyl], 1063 [M-H-Rha], 1045 [M-H-Xyl-H2O-CH3], 915 [M-H-Xyl-Glu], 901[M-H-Rha-Glu], 883[M-H-Glu-Rha-H_2O], 769[M-H-Glu-Rha-Xyl], 755[M-H-Glu-Rha-Xyl-14], 593[M-H-2Glu-Rha-Xyl-14], 431[M-H-3Glu-Rha-Xyl-14]
5	9.3	1223	1077 [M-H-Rha], 1061[M-H-Glu], 915[M-H-Glu-Rha],769[M-H-Glu-2Rha], 589[M-H-2Glu-2Rha-H2O], 431[M-H-3Glu-2Rha-14], 413[M-H-3Glu-2Rha-14-H2O]
6	9.9	1529	1385 [M-H-144], 1223[M-H-144-Glu], 1077[M-H-144-Glu-Rha], 1045[M-H-144-Glu-Rha-H2O-14], 901[M-H-144-2Glu-Rha-14], 755[M-H- 144-2Glu-2Rha-14], 593[M-H- 144-3Glu-2Rha-14], 4131[M-H-144-4Glu-2Rha-14-H2O]
7	10.7	1585	1571[M-H-14], 1427[M-H-14-144], 1385[M-H-14-144-42], 1223[M-H-14-144-42-Glu], 1077[M-H-14-144-42-Glu-Rha], 915[M-H-14-144-42-2Glu-Rha], 901[M-H-2x14-144-42-2Glu-Rha], 755[M-H-2x14-144-42-3Glu-Rha], 593 [M-H-2x14-144-42-4Glu-Rha]
8	11.6	1571	1427[M-H-144], 1385[M-H-144-42], 1223[M-H-144-42-Glu], 1077[M-H-144-42-Glu-Rha], 915[M-H-144-42-2Glu-Rha], 901[M-H-14-144-42-2Glu-Rha], 755[M-H-14-144-42-3Glu-Rha], 593 [M-H-14-144-42-4Glu-Rha]
9	12.5	1223	1077 [M-H-Rha], 1061[M-H-Glu], 915[M-H-Glu-Rha], 901[M-H-Glu-Rha-14], 769[M-H-Glu-2Rha], 589[M-H-2Glu-2Rha-H2O], 431[M-H-3Glu-2Rha-14], 413[M-H-3Glu-2Rha-14-H2O]
10	13.6	1515	1371[M-H-144], 1209[M-H-144-Glu], 1063[M-H-144-Glu-Rha], 901[M-H-144-2Glu-Rha], 755[M-H-144-2Glu-2Rha], 593[M-H-144-3Glu-2Rha], 431[M-H-2Glu-Rha-144]
11	14.5	1529	1385 [M-H-144], 1223[M-H-144-Glu], 1077[M-H-144-Glu-Rha], 1045[M-H-144-Glu-Rha-H2O-14], 901[M-H-144-2Glu-Rha-14], 755[M-H- 144-2Glu-2Rha-14], 593[M-H- 144-3Glu-2Rha-14], 4131[M-H-144-4Glu-2Rha-14-H2O]

The *m/z* values quoted are the integers obtained by truncating (not rounding off) measured values. [a] Retention time, [b] Deprotonated molecular ion, [c] Fragmented ions

Conclusions

The result of this study shows that five major furostanol saponins with diosgenin as aglycone are found in the methanol extracts of the kernel of the *B. aegyptiaca* grown in Israel with MW 1196, 1064, 1210, 1224, 1078, and 1046 Da; whereas, nine saponins with MW 1340, 1196, 1064, 1224, 1530, 1586, and 1582 Da are found in methanol extracts of the root. Among them, 1196, 1064, 1210, and 1078 Da MW saponins are the same saponins as those already isolated in the methanol extracts of fruit mesocarp of the *B. aegyptiaca* described in Chapter 2. The remaining saponins are new and have never been previously reported.

This study also provides the quantitative information of the saponin compounds in kernel and root extracts of *B. aegyptiaca*. The major saponin in the kernel was the MW 1210 that amounted to 11.6% of the injected extract, which corresponds to 36% of the total saponins; whereas the major saponin of the root extract was MW 1196 Da that amounted 20.6% of the injected extracts and corresponds to 52.6% of the total saponins. Each kernel and root had 12.9% and 14.6% methanol extracts, respectively. When these extracts were further eluted by methanol after discarding the first elution by water, using Sep-Pak (C18), 40% and 35% of the KE and RE extracts, respectively, were obtained. When these extracts were injected to the HPLC-RI, 32.2% and 39.1% of the total saponins were obtained. In this way, 1.66% and 1.99% total saponin on a dry weight basis in kernel and root, respectively, were found in *B. aegyptiaca* of Israeli provenance.

These findings are different than the earlier findings of *B. aegyptiaca* kernel cake saponins by Pettit et al. (1991) where they reported monodesmosidic spirostanol saponins corresponding to molecular mass of 1065, 1049, 903, and 604 Da, which they named Balanitin 4, 5, 6, and 7, respectively. However, Pettit et al. (1991) had isolated saponins from the kernel using dichloromethane and methanol (1:1) extraction and the sample was collected from Africa. Except for the saponin with MW 1196 Da, other saponins are new since the earlier report of root saponins by Farid et al. (2002). They had also extracted using methanol but defatted by petroleum ether and further eluted by Sephadex LH-20 from the sample from Sudan. The variation could be the differences in provenances and extraction methods. This also indicates that variation in the saponin content may prevail in different provenances as well as extraction procedures.

The results of this study also show that the LC-ESI-MS/MS technique is very helpful for the identification of the saponins in plant extracts and this could give considerable structural information on saponins. The use of LC-ESI-MS helps to avoid the traditional protocol of saponin detection which is a long and tedious process. Although considerable information regarding the aglycone and sugar chain can be obtained from this technique, confirmation of the alignment of the saccharidic chain along the molecule of saponin can not be verified using only this technique.

CHAPTER 4

Larvicidal properties of *Balanites aegyptiaca* saponins

Introduction

Mosquitoes constitute a major public health menace as vectors of several serious human diseases (El-Hag et al., 1999). Among mosquito-borne diseases, Dengue (which occurs as dengue fever or dengue hemorrhagic fever (DF/DHF) is severe and caused by a virus transmitted by the *Aedes aegypti* mosquito. This globally prevalent disease has recently spread drastically and is now found to be endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia, and the Western Pacific. Every year, more than 100 million people residing in these areas are infected by DF/DHF (Halstead, 2000). The West Nile Virus (WNV), that causes encephalitis or meningitis, is another serious mosquito-borne disease which is transmitted by *Culex pipiens* (the northern house) mosquito. This disease affects the brain tissue and the most serious cases can result in permanent neurological damage and be fatal (Hubalek and Halouzka, 1999). WNV is distributed throughout Africa, the Middle East, and southern temperate and tropical Eurasia, and was recently introduced into North America as well (Campbell et al., 2002). There is no vaccine to prevent these infections, nor are there drugs to combat the disease in infected persons, so vector control is the most prevalent solution so far available for reducing morbidity.

Most of the widely used vector interruption methods are synthetic insecticide-based, which not only affect the non-target population, but also generate constantly increasing resistance by the vector (Wattal et al., 1981). The search for natural insecticides which do not have any ill effects on the non-target population and are easily biodegradable remains a top priority (Redwane et al., 2002). A considerable number of plant derivatives have been shown to be effective against mosquitoes; however, due to the dramatic increase in resistance of mosquitoes to familiar chemicals, better alternative means of control are sought (El-Hag et al., 1999).

During the course of last two decades, many plant extracts have been evaluated for their larvicidal activities (Jang et al., 2002) in order to find methods for biological control of mosquitoes. Literature study has revealed that, among other biological activities, saponin-

rich extracts of *Agave sisalana* (Pizarro et al., 1999) and *Quillaja saponaria* (Pelah et al., 2002) have been shown to have larvicidal properties against mosquito larvae. Saponin-rich extracts such as the extract of the sea cucumber body wall have also been reported to have larvicidal properties against the *Culex pipens* fatigans mosquito (Thakur et al., 2004). Taking into consideration the naturalistic and environmentally safe position of saponin compounds and the presence of these compounds in *B. aegyptiaca* plant tissues, and their uses in many folk remedies including against the mollusks, and the severity of Dengue and WNV and the socioeconomic situation in Balanites growing areas, this research has been carried out to identify the larvicidal activities of various saponin-rich extracts of *B. aegyptiaca* plant tissues against the larvae of *A. aegypti* and *C. pipens* mosquitoes. A side experiment with pure saponins isolated from the fruit mesocarp to gain more understanding about the structure-function of saponins and their larvicidal effects is also discussed.

Materials and Methods

Plant material

Well-ripened fresh Balanites fruits and tender roots were collected from the Balanites plantation site located at Kibbutz Samar in southern Israel near the Red Sea. The plant species was kindly identified taxonomically by Prof. Uzi Plitman from the herbarium in The Hebrew University of Jerusalem.

Preparation of saponin-rich extracts (SREs)

Upon being brought to the lab, the epicarp (outer cover) of the fruits was removed by hand and the mesocarp (pulp) of the fruits scrapped by a hand knife; the nuts were washed with tap water. The mesocarp of the fruit was first lyophilized using Christ Alpha 1-4 lyophilizer (Germany). After lyophilization, the mesocarp was ground to powder. The ground mesocarp was first defatted with n-hexane (3 × 1:10 ratio). The defatted mesocarp was then extracted by methanol (3 × 1:10 ratio). The methanol was evaporated using a rotary evaporator (Heta-Holten A/S, Denmark) under reduced temperature and the extract was called ME (mesocarp extract). The washed nuts were oven dried at 70ºC for 96 h. Decortications of the nuts were carried out manually and released the kernel (approximately 10% of the total fresh fruit weight). The kernels were coarsely ground using a mortar and

pestle and defatted (oil extraction) by n-hexane as in the mesocarp. The n-hexane defatted kernels (kernel cake) were extracted by methanol, the methanol was evaporated, and the extract named KE (kernel extract). The roots were first rinsed thoroughly in running tap water, then chopped into small pieces, dried in the oven at 70ºC for 96 h, and ground using a coffee mill. The ground root powder was also defatted using n-hexane, as with the mesocarp. The defatted powder was extracted by methanol; the methanol was evaporated as described earlier, the extract was named as RE (root extract).

Saponin determination

Diosgenin (sapogenin) quantity in each extract was determined by measuring absorbance at 430 nm on color reaction of anisaldehyde, sulfuric acid, and ethyl acetate as described by Baccou et al. (1977) and Uematsu et al. (2000) with some modification by Chapagain and Wiesman (2005). Total saponins were calculated based on the diosgenin equivalent and major saponin present in the extracts as described earlier in Chapters 2 and 3.

Larvicidal test

Eggs of the *A. aegypti* and *C. pipens* mosquitoes were obtained from the Entomology Laboratory of the Israel Ministry of Health, Jerusalem, and necessary larvae were prepared in the laboratory of the Institutes for Applied Research, Ben-Gurion University, as described in the WHO standard protocol (1973). Each SREs (ME, KE, and RE) was dissolved in stabilized tap water to a standard series of concentrations (0, 25, 50, 100, 200, 500, 1000, 2000, and 4000 ppm) and twenty young (first–second instars) and old (third–fourth instars) larvae were placed separately into 250 ml disposable plastic cups containing 100 ml of treatment solutions as described by Pelah et al. (2002). After adding the larvae, the plastic cups were kept in the growth room maintained at 27±2°C with a 14 h day (light period) and 40±5% relative humidity.

The effects of the SREs on larval mortality were monitored by counting the number of dead larvae each day. The concentration at which 50% of the test larvae showed mortality (LC_{50}) and the concentration at which 90% of the test larvae showed mortality (LC_{90}) were calculated after two, four, and six days of exposure. During the course of the experiment, a

food preparation based on baby food was provided to the larvae. The percent mortality was corrected using Abbott's formula as suggested by Finney (1971).

In another series of experiments, observations on adult emergence at sub-lethal doses of all three extracts were made and the emergence of fifty percent of the test larvae (EC_{50} values) was determined using the Probit program. Each set of experiments was replicated thrice and mean and ANOVA were calculated by using JMP soft ware (SAS, 2000).

Table 4.1 The percentage of saponin in the saponin-rich extracts (SREs) of *B. aegyptiaca* fruit mesocarp (ME), kernel cake (KE), and root (RE) used in this study.

SREs	**Saponins (% DW)**
ME	12.01 ± 0.98
KE	14.91 ± 1.04
RE	13.26 ± 1.00

Values are the mean of 10 samples ± SE. The percent of saponins was calculated based on the sapogenin (diosgenin) equivalent.

Results

Table 4.2 shows the LC_{50} and LC_{90} values at two, four, and six days of assay time of the three SREs of *B. aegyptiaca* in young (first–second instars) and old (third–fourth instars) larvae of *A. aegypti* mosquito. The results show that both LC_{50} and LC_{90} values are highest with ME compared to KE and RE in both young and old larvae experiments. A concentration of 560, 98, and 133 ppm, and 1605, 185, and 260 ppm of ME, KE, and RE, respectively, were found to kill 50% (LC_{50}) and 90% (LC_{90}) of the tested young larval population two days after exposure, whereas 482, 75, and 102 and 1330, 125, and 185ppm concentrations of ME, KE, and RE, respectively, were needed to kill 50 and 90% of tested larvae after four days of exposure. The LC_{90} values were found to be almost half after six days of exposure in all three SREs, whereas LC_{50} values were around two-thirds in the same assay time. The mortality pattern was similar with all SREs for all assay times in old larvae experiments; however, both LC_{50} and LC_{90} values were found to be higher in old larvae experiments compared to the young larvae experiments.

Table 4.2 LC_{50} and LC_{90} (ppm) values of the saponin-rich extracts (SREs) of the *B. aegyptiaca* fruit mesocarp (ME), kernel cake (KE), and root extract (RE) against *Aedes aegypti* mosquito larvae at different assay times.

SREs	2 days		4 days		6 days	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}
First–second instars larvae						
ME	560 a	1605 a	482 a	1330 a	430 a	995 a
KE	98 c	185 c	75 c	125 c	55 c	85 c
RE	133 b	260 b	102 b	185 b	75 b	120 b
Third–fourth instars larvae						
ME	770 a	1995 a	595 a	1580 a	488 a	1175 a
KE	125 c	200 c	94 c	150 c	60 c	95 c
RE	165 b	290 b	130 b	195 b	85 b	140 b

Each value is the mean of 3 ± SE (n=3). Different letters in each column represent the significant difference at 1% level of significance (Tukey-Kramer HSD).
LC_{50} = Lethal concentration at which 50% of the larvae showed mortality.
LC_{90} = Lethal concentration at which 90% of the larvae showed mortality.

Table 4.3 shows the LC_{50} and LC_{90} values at two, four, and six days of assay time of *B. aegyptiaca* ME, KE, and RE in young and old larvae of *C. pipens*. A very similar mortality pattern was observed in this experiment as in the former one with *A. aegypti*. The KE also showed the significantly highest larval mortality (lowest LC values) compared to the ME and RE, and young larvae were shown to be more susceptible than the old larvae for all exposure times. However, comparing the two experiments, the *C. pipens* larvae were shown to be more susceptible than the *A. aegypti* larvae against all SREs. In the *A. aegypti* experiment, a concentration of 55, 75, and 430 ppm (in young larvae) and 60, 85, and 488 ppm (in old larvae) of KE, RE, and ME, respectively, killed 50% of tested larvae after six days, whereas only 42, 68, and 405 ppm (in young larvae) and 48, 76, and 433 ppm (in old larvae) of KE, RE, and ME, respectively, were needed to kill 50% larvae after six days in *C. pipens*.

Table 4.3 LC_{50} and LC_{90} (ppm) of saponin-rich extracts (SREs) of the *B. aegyptiaca* fruit mesocarp (ME), kernel cake (KE), and root (RE) against *Culex pipens* mosquito larvae at different assay times.

SREs	2 days		4 days		6 days	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}
First–second instars larvae						
ME	524 a	1510 a	485 a	1035 a	405 a	790 a
KE	86 c	170 c	80 c	110 c	42 c	70 c
RE	114 b	245 b	72 b	170 b	68 b	110 b
Third–fourth instars larvae						
ME	630 a	1735 a	565 a	1370 a	433 a	970 a
KE	115 c	185 c	85 c	125 c	48 c	83 c
RE	140 b	270 b	120 b	185 b	76 b	131 b

Each value is the Mean of 3±SE (n=3). Different letters in each column represent the significant difference at 1% level of significance (Tukey-Kramer HSD).
LC_{50} = Lethal concentration at which 50% of the larvae showed mortality.
LC_{90} = Lethal concentration at which 90% of the larvae showed mortality.

All SREs of *B. aegyptiaca* were also found to be very active with regard to its efficacy in inhibiting the adult emergence. A concentration of 340, 40, and 50 ppm (in young larvae) and 395, 50, and 70 ppm (in old larvae) in *A. aegypti,* and 305, 25, and 40 ppm (in young larvae) and 355, 35, and 55 ppm (in old larvae) in *C. pipens* mosquito, were found to prevent adult emergence of 50% of the tested larvae populations (**Table 4.4**).

Table 4.4 EC_{50} (ppm) value of the saponin-rich extracts (SREs) of *B. aegyptiaca* fruit mesocarp (ME), kernel cake (KE), and root (RE) against *Culex pipens* mosquito larvae.

SREs	EC_{50}	
	A. aegypti	*C. pipens*
First–second instars larvae		
ME	340 a	305 a
KE	40 c	25 c
RE	50 b	40 b
Third–fourth instars larvae		
ME	395 a	355 a
KE	50 c	35 c
RE	70 b	55 b

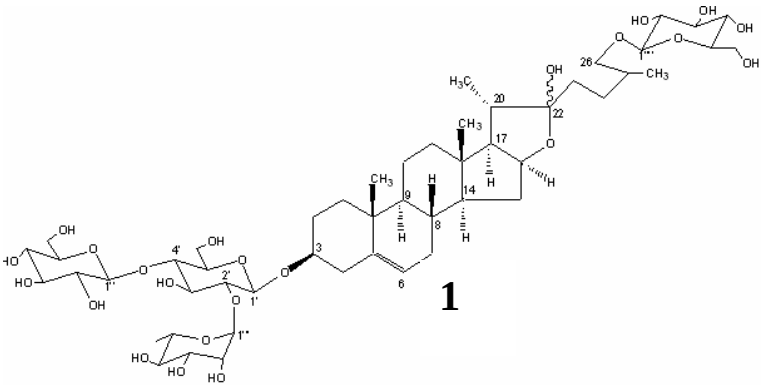
Each value is the Mean of 3 (n=3). Different letters in each column represent the significant difference at 1% level of significance (Tukey-Kramer HSD).
EC_{50} = Concentration at which 50% of the total test larvae emerge to adult

Discussion and conclusions

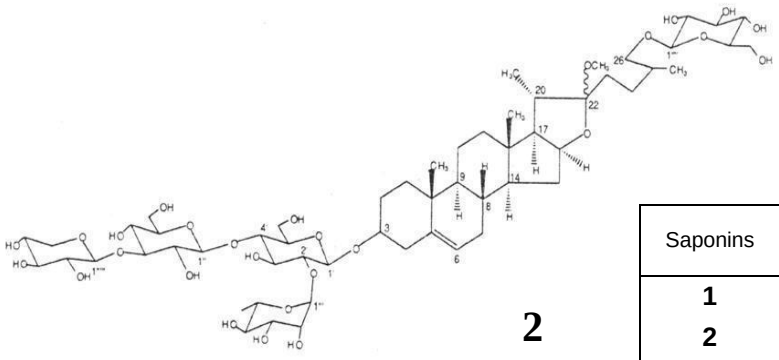
The results of this study clearly show that all saponin-rich extracts of *B. aegyptiaca* i.e., fruit mesocarp, root, and kernel cake, produce high larval mortality. Among them, higher larval mortality was achieved with kernel cake compared to the root and mesocarp extracts. However, the larvicidal effect of kernel and root extracts was very similar compared to the mesocarp extract. Since less than 50 ppm of KE and RE are shown to be sufficient to inhibit the emergence of 50% of the larvae population, this can certainly help to drastically reduce the mosquito population. The older larvae were found to be less susceptible than the younger and *C. pipens* was found to be more susceptible than *A. aegypti*.

When all extracts were tested for their total saponin content by spectrophotometric methods, the total saponin was found to be very similar in all of them (**Table 4.1**). A very similar quantity of total saponins in the three extracts was also achieved in the spectrometric study reported earlier (Chapters 2 and 3). These earlier studies (Chapters 2 and 3), revealed that the major saponin in the three extracts of Balanites was not the same. The major saponin of ME was MW 1064 Da (*ca.* 42% of total saponin) whereas the major saponin of KE was MW 1210 Da (*ca.* 36% of total saponin), and MW 1196 Da was the main saponin of RE (*ca.* 52%). This indicates that the structure of the saponin could play a more vital role in larval mortality than the quantity of saponin.

To increase further understanding about the structure-function relation of saponins and their larvicidal effects, a brief separate study was also conducted. In this study, three pure saponins isolated from ME as described in Chapter 2, namely 26-(O-β-D-glucopyranosyl)-3β,22,26-trihydroxyfurost-5-ene 3-O-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**1**), 26-(O-β-D-glucopyranosyl)-22-O-methylfurost-5ene.3β,26-diol 3-O-β-D-xylopyranosyl-(1→3)- β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**2**), and 26-(O-β-D-glucopyranosyl)-22-O-methylfurost-5ene.3β,26-diol 3-O-β-D- glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**3**) (**Fig. 4.1**), were used to test larvicidal activities against the larvae of *A. aegypti*. In this experiment, 50 ml of test solution were prepared in triplicate (0, 25, 50, 100, 200, 500, and 100 ppm) and 20 larvae of 3–4 instars were used with same procedure as described earlier. Mortality was calculated each day and corresponding LC_{50} and LC_{90} were drawn from the data after two days of exposure (**Table 4.4**).

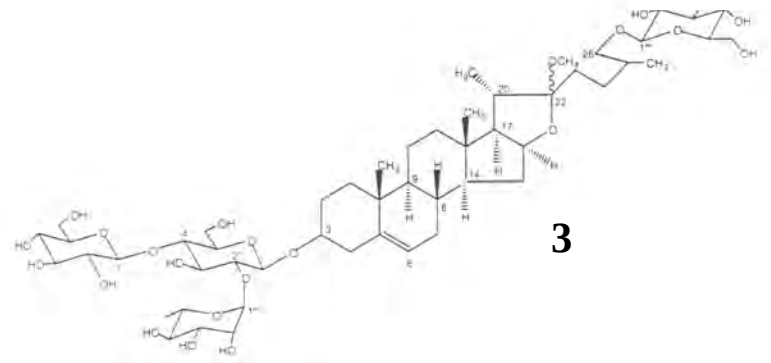


1



2

Saponins	MW	Sugar		
		C-26	C-3	C-22
1	1064	Glu	Glu, Glu, Rha	-OH
2	1210	Glu	Glu, Glu, Rha, Xyl	$-CH_3$
3	1078	Glu	Glu, Glu, Rha	$-CH_3$



3

Figure 4.1 Structure of the three (**1, 2, 3)** major saponins of methanol extract of *Balanites aegyptiaca* fruit mesocarp (ME) used in the experiment.

Table 4.5 LC_{50} and LC_{90} (ppm) values of the major saponins of methanol extract of *Balanites aegyptiaca* fruit mesocarp (ME) two days after exposure against *A. aegypti* mosquito larvae.

Saponins	**LC_{50}**	**LC_{90}**
Saponin (**1**)	>1000	>1000
Saponin (**2**)	120	525
Saponin (**3**)	70	275

Each value is the Mean of 3 (n=3).
LC_{50} = Lethal concentration at which 50% of the larvae showed mortality.
LC_{90} = Lethal concentration at which 90% of the larvae showed mortality

From the results of this side experiment, it is apparent that the larvicidal effect is higher in the saponins that contained a methyl group at C-22 (saponins **2** and **3**) compared to the saponin lacking a methyl group at C-22 (saponin **1**). This shows that the attachment of a methyl group at C-22 is essential for larval mortality. The lack of a methyl group or attachments of OH in saponin **1** (MW 1064) showed significantly less larval mortality than the saponins with having a methyl group at C-22. When comparing the larval mortality in saponins **2** and **3,** both of them have a methyl group attached, the difference in effect might be the difference in the present of sugar chain. In saponin **2** there is a xylose molecule but there is no such molecule in saponin **3**. This shows that the presence of xylose sugar in the sugar chain also has a strong influence in the larvicidal properties, and this significantly reduced larval mortality. The presence of a methyl group and xylose in association in the biological activities in the furostanol saponins has also been reported by Iorizzi et al. (2002). In that study, high fungal growth inhibition was found to be heavily affected by the presence and absence of a xylose unit in the oligosaccharide chain. The high mortality in the saponin-rich extract of the fruit mesocarp (ME) (**Table 4.2**), compared to saponin **1**, might be due to the presence of saponin **2** and saponin **3** in ME (Chapter 1). The synergetic effect of the presence of other compounds in the ME might also have had an influence in the mortality of the larvae; however, the presence of a methyl group and the xylose sugar seems to strongly influence mortality.

When the images of dead larvae were taken in scanning electron microscope (SEM), a rupture of the body surface of the larva was clearly seen in the micrograph (**Fig. 4.2**). The body of insects, including mosquito larvae, is covered by the cuticle membrane which is comprised largely of cholesterol (Lasser and Clayton, 1966). The results indicate that the cause of rupturing of the body wall of the mosquito might be the disarrangement of this membrane by the association of the saponin molecule with the cholesterol molecules present in the cuticle membranes of the larvae as discussed in earlier report by Morrissey and Osbourn (1999) in fungus; this could be the most probable reason for the larval death. However, further study would be useful in this regard.

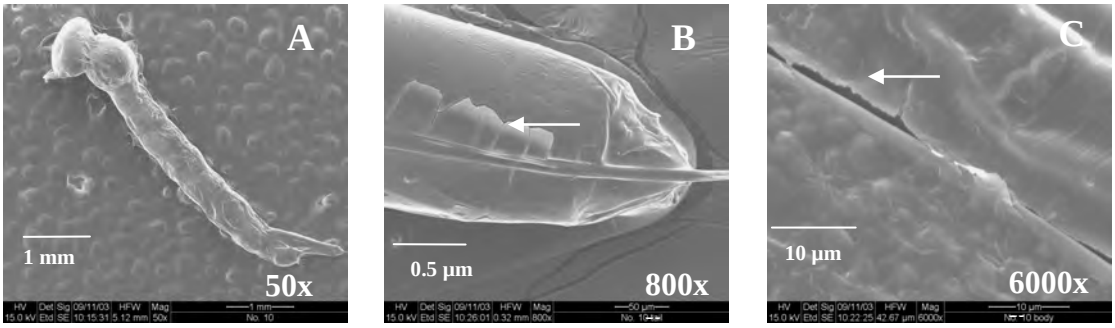


Figure 4.2 Scanning electron microscope (SEM) characterization of dead larvae from the use of saponin. **A**. a dead larva, **B**. larva tail showing rupture, **C**. the ruptured place in close view at high magnification. Arrow showing the place of rupture.

Most of the literature reports showed that bidesmosidic furostanol saponins have no or very weak biological activity (Hostettmann and Marston, 1995). However, the results of the present study clearly show that bidesmosidic furostanol saponins can have strong biological activities, but the activities depend on the structure of the saponins. This study clearly points out that generalization of the saponins in terms of functionality is very difficult as seen here that all three saponins used in the experiment are structurally very close, but the results are very different**.** A further study in this regard would certainly be important to develop our understanding about the structure–function relation of saponins.

From the application point of view, the use of pure saponins from any particular plant for larvicidal purposes is very difficult due to high costs of purification; however, the cost of SREs is much lower and could be utilized for their high larvicidal effects. Earlier studies also indicated the possible use of saponin-rich extract as a natural larvicidal agent against

the mosquito (Pelah et al., 2002; Pizarro et al., 1999); however no study has so far been done regarding inhibition of the emergence of the adult mosquito. The result of low EC_{50} values of a saponin-rich extract of kernel cake extract and root extract of *B. aegyptiaca* in this study provides more reliable evidence for using such extract as a natural larvicidal agent. Since a large proportion of the populations living in areas where Dengue and WNV are serious problems suffer from varying degrees of poverty, the discovery of plant-derived compounds that could help with the control or eradication of these diseases would be of great value, particularly when the concerned plants were readily available to those who most need to use them. In this context, the highly bioactive compounds of *B. aegyptiaca*, which are widely grown in most of these disease-infected areas, offer an opportunity for developing alternatives to rather expensive and environmentally hazardous organic insecticides. The use of specific and ecologically friendly control measures, therefore, would give considerable advantages over conventional insecticides. Consequently, since Balanites is widely grown in rural and remote areas, their commercial exploitation would contribute toward rural economic development. Furthermore, the kernel cake of Balanites is the waste product after extraction of oil, so in this way the community gains considerable benefit from the marginal product.

CHAPTER 5

Antifungal activity of saponin-rich extracts of *B. aegyptiaca* against prevalent phytopathogenic fungi

Introduction

One-third of all global agricultural production is lost each year due to various pests and diseases (Bajwa et al., 2003). Among the losses, nearly 20% is the result of pathogenic fungal diseases (Agrios, 2000). Chemical compounds are the major control measure to avoid loss of yield or quality due to these fungi. No doubt the use of chemicals has been found to be effective in controlling these diseases, but some major problems threaten to limit their continued use. One problem is the tendency of fungi to develop resistance to chemicals, necessitating a higher dose or the development of new chemicals to replace those to which fungi are resistant (Bajwa, et al., 2003). Another is the creation of a hazardous environment both for human beings and other flora and fauna by these chemical fungicides because of their non-biodegradable nature (Hayes and Laws, 1991). These problems highlighted the need to develop alternative methods for controlling plant diseases; this in turn has stimulated research on the occurrence of natural botanical pesticides and the potential for commercialization of these materials (Arnason et al., 1989). Furthermore, some synthetic pesticides are currently prohibited in several countries, and the trend is continuing. In this context, the search for natural, biologically active, and renewable plant products that could replace hazardous pesticides is relevant.

Plant secondary metabolites are rapidly degraded in soil, they generally have no mammalian toxicity, and they can have an effective role in sustainable agriculture (Saxena, 1983). Studies show that most antifungal studies of saponins have been carried out on yeast and other fungi, and a meager study has been done on pathogenic plant fungi. This encouraged us to test the effects of Balanites saponins on the growth of some common important plant pathogenic fungi. Introduction of pure saponins from any particular plant into the specific pathogen is very difficult due to the high cost of purification, so this study was designed to perform *in vitro* studies using only SREs, at a much lower cost.

In this study, together with the SREs of fruit mesocarp of *B. aegyptiaca,* antifungal activity of bark extract of *Quillaja saponaria,* and plant extract of *Yucca schidigera* against

the prevalent phytopathogenic fungi were tested *in vitro. Q. saponaria* is an evergreen tree of the Rosaceae family, found in arid regions of Chile, Peru, and Bolivia, and contains triterpenoid saponins mainly in its bark (Cheeke, 2000). *Y. schidigera* is a plant of the Agavaceae family which is native to the desert southwest United States, Baja California, and Mexico, whose extract contains steroidal saponins (Wang et al., 2000). Recently, *Y. schidigera* has been recognized as a superior natural wetting agent and surfactant via flood or drip irrigation, and as a foliar spray to increase water and fertilizer penetration, reduce surface tension of agricultural sprays, and soften hard compacted soils (Karnan and Marx, 2000). *Q. saponaria* and *Y. schidigera* are the two most exploited saponin containing plants to date. The comparative effect of three SREs is discussed. The objective of this study was to search for more environmentally and toxicologically safe and more selective and effective fungicides.

Materials and methods

Preparation of the saponin-rich extracts (SREs)

SRE of *Balanites* was made by dilution of the laboratory-prepared fruit mesocarp extract of the *B. aegyptiaca* (ME). The preparation of ME was the same as described earlier in Chapter 4. The SRE of Quillaja (QE) was prepared by dilution of a commercial saponin extract of *Q. saponaria* bark purchased from Sigma (St. Louis, MO, US). *Y. schidigera* SRE (YE) was obtained from Cellu-con Inc. (Strathmore, CA, US). The total saponin content in ME, QE, and YE was determined by measuring absorbance at 430 nm on color reaction of anisaldehyde, sulfuric acid, and ethyl acetate as described by Baccou et al. (1977) and Uematsu et al. (2000) with some modification by Chapagain and Wiesman (2005).

Fungal Isolates

Cultures of *Pythium ultimum*, *Alternaria solani, Colletotrichum coccodes*, and *Verticillium dahliae* were isolated from diseased specimens of potato, and *Fusarium oxysporum* from jojoba, grown in the experimental field of Gilat Station, Negev, Israel. Isolates were placed on potato dextrose agar (PDA, Difco, Detroit, MI, US) supplemented with 100 ppm streptomycin and incubated in the dark at 27ºC. Pathogens were identified

visually and microscopically, and sub-cultured on PDA without antibiotics as described by Tsror et al. (2001).

Growth Inhibition Measurements

Five concentrations (0.1, 0.5, 1.0, 2.0, and 4.0% w/v) of ME, QE, and YE were tested. A negative control (0%) and positive controls of registered fungicides were added to separate Erlenmeyer flasks containing sterilized (121ºC, 1.2 atm, 20 min) PDA and mixed properly. Samples of PDA containing ME, QE, or YE, and controls (15 ml) were poured separately into sterilized Petri dishes and allowed to solidify. For the positive controls, flustriafol 100 ppm, metalaxyl 5 ppm, benomyl 5 ppm, and prochloraz 100 ppm and 200 ppm were used for *A. solani*, *P. ultimum, F. oxysporum, V. dahliae, and C. coccodes*, respectively.

A 2 mm diameter plug of the actively growing mycelium of the fungal isolate was placed in the center of the each plate. The plates were then incubated at 27ºC, in the dark (10 plates per treatment). Measurement of the colony diameter of the radial mycelium growth was carried out according to the growth rate of each fungus. The growth of *A. solani* and *F. oxysporum* was measured on the 2nd, 4th, and 7th days. Growth measurement of *V. dahliae* and *C. coccodes* was carried out on the 3rd, 7th, and 10th days. Because of its fast growing nature, the colony diameter of *P. ultimum* was measured daily for two days.

For growth inhibition analysis, colony diameter data taken after 2 days (*P. ultimum*), 7 days (*A. solani* and *F. oxysporum*), and 10 days (*V. dahlia* and *C. coccodes*) were used. The inhibitory activity of each treatment was expressed as the percent growth inhibition compared to the negative control (0%) using the following formula, where DC = diameter of control, and DT = diameter of fungal colony with treatment (Pandey et al., 1982):

$$\text{Growth inhibition (\%)} = [(DC-DT)/DC] \times 100$$

Growth inhibition is defined as negative for the case of no inhibition, no inhibition for 0–10% growth inhibition, low inhibition for 10–33% growth inhibition, moderate for 33–66% growth inhibition, and high inhibition for >66% growth inhibition. The concentration of each of the SREs that inhibits 50% of the growth of each fungus (LC_{50}) was determined using the Probit program. The experiments were repeated twice and the data presented here

are the average of two experiments. All the experiments were done in the Gilat Agricultural Station, Gilat, Israel.

Statistical analysis of the data was performed with JMP software (SAS, 2000) using the Tukey-Kramer HSD test for determining significant difference among treatments at p = 0.05 level of significance.

Results

Total saponin content in the SREs

The saponin content in the three SREs used in this study, BE, QE, and YE, was found to be very similar (**Table 5.1**). The total saponins in BE, QE, and YE were 11.2%, 12.03%, and 13.5% of DW, respectively.

Table 5.1 Saponin amounts in the saponin-rich extracts (SREs): Fruit mesocarp extract of *Balanites aegyptiaca* (ME), bark extract of *Quillaja saponaria* (QE), and plant extract of *Yucca schidigera* (YE).

SRE	Saponins (% DW)
ME	11.7 ± 0.88
QE	12.03 ± 0.94
YE	13.50 ± 1.00

Values are the mean of 10 samples ± SE as determined by Baccou et al. (1977) and Uematsu et al. (2000), with some modification by Chapagain and Wiesman (2005).

Effects of the SREs on in vitro *growth of* A. solani

The radial growth of *A. solani* was reduced by all SREs at all concentrations (**Table 5.2**), and a dose dependent effect was observed in the three extracts. The highest growth inhibition was observed with 4% YE (76.0%), followed by QE (47.5%), and ME (34.7%) at the same concentration. Two and four percent YE were significantly more effective in growth inhibition than 100 ppm flustriafol, which was used as a positive reference. In the case of ME and QE, none of the concentrations inhibited 50% fungal growth; the LC_{50} of YE was 0.3% (Table 5.3).

Effects of the SREs on in vitro *growth of* P. ultimum

With *P. ultimum*, all SREs also showed a dose dependent effect (**Table 5.2**). The highest concentration (4%) of ME inhibited fungal growth by 81%, whereas 4% of YE and QE caused 63.4 and 59.1% growth inhibition, respectively. The inhibition by 4% ME was significantly higher than the fungicide metalaxyl at 5ppm. The LC_{50} of ME, QE, and YE was 2, 1.6, and 1.2 %, respectively (**Table 5.3**).

Effects of the SREs on in vitro *growth of* F. oxysporum

Among the three SREs, ME showed no inhibitory effect against *F. oxysporum* (**Table 5.2**). QE showed fairly stable inhibition with regard to concentration. The lowest concentration of QE (0.1%) showed 40.8% inhibition, whereas all other concentrations (from 0.5 to 4.0%) inhibited growth by 52.0 to 56.9%, which was not significantly different from the fungicide benomyl (5 ppm) used as a reference. The inhibition pattern in YE was found to be different than that of QE, where 0.1% showed only 13.8% inhibition and 4.0% showed a 57.3% inhibition. However, the inhibition by 2 and 4% YE was not significantly different than benomyl 5 ppm. None of the tested concentrations of ME inhibited 50% of the growth, but 0.6 and 2% of each QE and YE, respectively, caused 50% growth inhibition (LC_{50}) (**Table 5.3**).

Effects of the SREs on in vitro *growth of* V. dahliae

The effect of ME, QE, and YE on the growth of *V. dahliae* and *F. oxysporum* was similar (**Table 5.2**). ME showed a very weak inhibition; 4% ME caused only 9.7% growth inhibition. QE showed a moderate growth inhibition, with 35.9% inhibition at 4%. YE at 4% inhibited 59.2% of the growth. Although there was a difference in growth inhibition effect with the three SREs, a dose dependent pattern was observed in all extracts. The results showed that none of the treatments equaled prochloraz 100 ppm which was used as the positive control. None of the concentrations of ME and QE were able to suppress 50% growth, but 50% inhibition was obtained with 2.0% YE (**Table 5.3**).

Effects of the SREs on in vitro *growth of C*. coccodes

YE showed the strongest growth inhibitory effects; 0.1% YE inhibited 55.8% growth, whereas 4.0% inhibited 100%. In contrast, negative inhibitory effects of ME and QE against the growth of this pathogen were observed (**Table 5.2**). Prochloraz (at 200 ppm), which was used as positive control, inhibited 95% of the growth and even 0.1% of YE was able to inhibit 50% growth (**Table 5.3**).

Table 5.2 Effect of saponin rich extracts (SREs) on *in vitro* mycelial colony growth of several fungi.[a]

Treatments	% Growth inhibition[b]				
	Alternaria solani	*Pythium ultimum*	*Fusarium oxysporum*	*Verticillium dahliae*	*Colletotrichum coccodes*
ME					
0.1	1.03 f	0.50 f	0.76 b	3.28 c	-10.21 b
0.5	8.60 e	7.85 e	0.46 b	7.54 b	-9.14 b
1.0	17.49 d	28.00 d	1.25 b	8.84 b	-8.58 b
2.0	29.16 c	70.91 c	3.28 b	9.41 b	-10.51 b
4.0	34.70 b	81.09 a	2.15 b	9.74 b	-12.69 b
Positive control[c]	65.01 a	75.05 b	56.85 a	68.11 a	95.25 a
QE					
0.1	19.70 f	26.69 f	40.81 b	17.72 c	-1.44 b
0.5	25.81 e	37.51 e	52.01 a	20.54 c	-2.44 b
1.0	34.32 d	46.32 d	53.68 a	29.35 b	-1.33 b
2.0	39.10 c	53.21 c	55.66 a	33.48 b	-0.36 b
4.0	47.50 b	59.12 b	56.11 a	35.92 b	-3.85 b
Positive control[c]	65.01 a	75.05 a	56.85 a	68.11 a	95.25 a
YE					
0.1	28.19 e	7.87 f	13.76 d	35.10 e	55.79 e
0.5	56.13 d	48.31 e	32.63 c	46.12 d	81.95 d
1.0	65.20 c	52.36 d	44.32 b	48.95 d	87.01 c
2.0	71.84 b	57.59 c	52.16 a	52.12 c	93.21 b
4.0	75.98 a	63.37 b	57.25 a	59.17 b	100.00 a
Positive control[c]	65.01 c	75.05 a	56.85 a	68.11 a	95.25 b

[a] Mesocarp extract of *Balanites aegyptiaca* (ME), bark extract of *Quillja saponaria* (QE), and plant extract of *Yucca schidigera* (YE).
[b] Each value is the mean of the 20 from two experiments (n=20). Means followed by same letter in each column are not significant different at p=0.05 by Tukey-Kramer HSD. % growth inhibition was calculated compared to the growth of the control (0%).
[c] For the positive control, flustriafol (100 ppm), metalaxyl (5 ppm), benomyl (5 ppm), and prochloraz (100 ppm and 200 ppm) were used for *A. solani, P. ultimum, F. oxysporum, V. dahliae, and C. coccodes*, respectively.

Table 5.3 LC_{50} values (% w/v) of saponin rich extracts (SREs)[a]

SRE	LC_{50}[b]				
	Alternaria solani	*Pythium ultimum*	*Fusarium oxysporum*	*Verticillium dahliae*	*Colletotrichum coccodes*
ME	> 4.0	2.0	>4.0	>4.0	–
QE	>4.0	1.6	0.6	>4.0	–
YE	0.3	1.2	2.0	1.5	< 0.1

[a] Mesocarp extracts of *Balanites aegyptiaca* (BE), bark extract of *Quillja saponaria* (QE), and plant extract of *Yucca schidigera* (YE) on *in vitro* growth inhibition of *Alternaria solani*, *Pythium ultimum, Fusarium oxysporum, Verticillium dahliae*, and *Colletotrichum coccodes*.
[b]LC_{50}, concentration (% w/v) of the SREs at which fifty percent of the radial growth of the mycelium of indicated fungi suppressed compared to the growth of the control. The LC_{50} value was determined using the Probit program.

Discussion and conclusions

Saponins are generally considered to have antifungal properties (Oleszek et al., 1990; Hostettmann and Marston, 1995; Osbourn et al., 1996; Sindambiwe et al., 1998). The results of the present study agree with these earlier findings. The results show a dose-dependent antifungal and/or saponin-specific response of the saponin-rich extracts against the mycelial growth of commercially important phytofungi. The dose-dependent antifungal, as well as saponin-specific, behavior of the anti-microbial activity has also been reported (Sparg et al., 2004).

The total saponin content in the three saponin-rich extracts used in this study was similar (**Table 5.1**). The ME was moderately active (>33 and <66%) against *A. solani*, but highly active (>66%) against *P. ultimum*, showing significantly higher growth inhibition compared with the fungicide metalaxyl 5 ppm. However, for the other fungi (*F. oxysporum, V. dahliae, and C. coccodes*), ME showed either a very weak or a negative effect. QE was moderately active (>33 and <66%) against *A. solani, P. ultimum, F. oxysporum, and V. dahliae*, but negative for *C. coccodes*. However, the growth inhibition by 0.1 to 4.0% QE was not significantly different than the growth inhibition obtained with the fungicide benomyl 5 ppm. YE, on the other hand, was found to be very active against all fungi tested in this study. The YE had either moderate or high growth inhibitory effect against all fungi. Less than 0.1 to 2.0% of YE was found to reach 50% growth inhibition of the tested fungi

(**Table 5.3**). In the case of *C. coccodes*, 100% growth inhibition occurred with the highest concentration of YE (**Table 5.2**), and even the lowest concentration (0.1%) inhibited growth by 55.8%. Two and 4% of YE significantly suppressed growth of *A. solani* compared with the fungicide used as positive control. This suggests that YE is very active against the growth of *C. coccodes.*

The antifungal and/or saponin-specific results achieved in this study indicate differences in the mode of action of SREs as well as the nature of the fungi. Fruit mesocarp of the *Balanites aegyptiaca* extract (ME) contains mostly steroidal saponins mainly (*ca.* 42%) bi-desmosidic furostanol steroidal saponins (MW 1064 Da) with diosgenin as aglycone with sugar units attached in C-3 and C-26 positions (Chapter 2). The main saponin constituents of the QE are triterpenoid saponins with quallic acid as aglycone (Hostettmann and Marston, 1995). YE contains both spirostanol and furostanol glycosides, but the predominant one is the spirostanol, primarily sarsapogenin (66%) as an aglycone (Oleszek et al., 2001). This shows that the differences in composition of the saponins in the extract may play different roles in antifungal activities, not only that the same extract has shown different activities with different fungi. For instance, while ME was most active against *P. ultimum*, it had a negative impact on *C. coccodes*. This might have been related to involvement of the saponins. It is generally believed that the antifungal activities of the saponin are related to the association of the saponin molecule with the sterol (mainly cholesterol) present in the fungal cell (Morrissey and Osbourn, 1999). The different antifungal activities of the individual saponins and/or extracts with different fungi might be related to the differences in the sterol composition in the different fungus cells that were tested.

Extracts of *Quillja saponaria* (QE) have been used for decades in diverse industries, such as food and beverages and cosmetics. The Yucca extract (YE) has also been used in various agricultural practices as an additive in flood irrigation to soil or in spray over foliage. It has also been reported that YE has the ability to interact with cells in the plant roots to increase water and nutrient absorption and also create a more favorable rhizosphere for the plants; this is the reason *Yucca schidigera* products have been used in agriculture for years as soil improvers, foliar sprays, wetting agents, stress control agents, and plant growth promoters, but no clear reason is available. The result of the present study may

provide the answer to this question.

Although the fruit of *Balanites aegyptiaca* is eaten and quite popular in traditional folk remedies, particularly in the native areas where this plant grows, such as the Sudano-sahelian area of Africa and the Rajasthan state of India, most people do not like to eat the fresh fruit because of its bitter taste. In this context, the use of Balanites fruit mesocarp extract can play vital role to combat some fungi like Pythium. Pythium is a soil-borne pathogen and causes many economically important diseases.

Although the results obtained in this study were only from *in vitro* experiments, it has been observed that in almost all cases substances which are found to be fungicidal *in vitro*, also kill the fungus *in vivo* (Kuhn and Hargreaves, 1987). The results of this study may ultimately help in the search for novel, environmental approaches to plant disease control. Saponin-rich extracts may be useful in their own right as an attractive alternative for control of fungi that attack crops, avoiding chemical fungicide applications.

CHAPTER 6

Effect of saponins on delivery of 2,4-D [^{14}C] through plant cuticle membranes (CMs)

Introduction

Plant cuticle is a thin (<0.1–10μm) continuous layer or membrane of predominantly lipid material that covers the entire external surface of plants (Kerstiens, 1996). The main function of the plant cuticle is to minimize water loss when stomata are closed and to protect the plant against physical, chemical, and biological attack; however, cuticle remains the main barrier to the penetration of foliar and ground-applied compounds (Kirkwood, 1999). Along with lipids, various phytosterols such as pentacylic triterpenols and Δ^5-sterols are also present in the cuticular membrane in leaves (Killops and Frewin, 1994).

To enhance the delivery of foliar-applied agro-materials to the inner tissue of the plant through the cuticular layer, the use of agricultural adjuvants and/or surfactants has become common practice (Chapagain and Wiesman, 2003). Among the surfactants, silicone-based nonionic surfactants are the most commonly recommended and used adjuvants these days. Although these surfactant-type adjuvants increase the diffusive mobility of agro-materials across the cuticle, thereby increasing the penetration potential, since these adjuvants were originally designed for herbicides, severe necrotic damage to the treated leaves is commonly encountered when using these surfactants with foliar nutrients (Wiesman et al., 2002).

Adjuvants are generally considered “inert” or essentially non-hazardous; however, environmental questions have always been raised about adjuvants because of their synthetic nature. Traditional amine ethoxylate surfactants are now less preferred due to unfavorable toxicity profiles of aquatic fauna, while nonylphenol ethoxylates have recently been under public scrutiny for alleged oestrogenic side-effects. The trend within the oil-type adjuvants is likely to continue to move towards vegetable oils and their derivatives due to poor biodegradation of mineral oils (Cornish et al., 1993). However, biodegradable and environmentally safe adjuvants are always being sought.

Saponins have been used as an adjuvant in veterinary medicines to promote penetration of drugs through the stratum corneum (SC), the major barrier layer in the skin; interest in using the saponin-based adjuvants in humans is also constantly increasing (Magnusson et

al., 2004). Their immunological role and divergent biological activities, with the presence of both hydrophilic and lipophilic moieties, has made glycosidic saponins the best adjuvant for drug delivery (Ferreira and Llodra, 2000). Although various saponin-rich extracts have commonly been used in agriculture for their various activities, there has been no report about the use of these extracts as an agricultural adjuvant. Considering the use of saponins in vaccine delivery and the protective activities of saponins, we surmised that saponins may also be used as a non-ionic, environmentally safe bio-adjuvant for foliar application of agrochemicals.

In this study, the delivery of 2,4-D (^{14}C) using three saponin-rich extracts from *B. aegyptiaca* fruit mesocarp, kernels, and roots, and the most common adjuvant used in veterinary medicines, the *Quillaja saponaria* extracts, were carried out and their efficiency for penetration was compared. The objective of this study was to investigate an environmentally friendly bioadjuvant for the delivery of agro-materials through the plant cuticular barrier.

Materials and Methods

Preparation of the saponin-rich extracts (SREs)

Saponin-rich extracts (SREs) of *Q. saponaria* (QE), and fruit mesocarp (ME), kernel (KE), and root (RE) of *B. aegyptiaca* were made following the same protocol as described earlier in Chapters 4 and 5. To enhance the saponin concentration in each SRE, further elution was done by methanol using Solid Phase Extraction (C18) after discarding the first water elution for removal of excess of free sugars, giving the total saponins in all four SREs ≥ 25% of dry matter.

Leaf cuticle isolation

Full-grown matured leaves of the *Citrus grandis* L. having astomatous cuticles were collected and washed in de-ionized distilled water (DDW). After punching 20 mm diameter discs (using a cork-borer) out of the leaves, the cuticles were isolated enzymatically by incubating the leaf discs in a mixture (1:1) of cellulose 203-13L (Biocatalysts, UK) and Pectinase 62L (Biocatalysts, UK) as described by Schonherr and Riederer (1986) in a 1% concentration (w/w) of citric acid buffer (0.1M) at 40^{o}C and pH 4. After a few days,

astomatous cuticles from the upper leaf epidermis were collected, rinsed extensively, desorbed in DDW, air-dried on Teflon discs, and stored in a refrigerator until used. These isolated astomatous adaxial cuticles are referred to hereafter as cuticular membrane (CMs).

Delivery Experiments

In the first phase of the study, four donor solutions (1% of each QE, ME, KE, and RE) were prepared by adding ^{14}C-labeled 2,4-D (specific activity 19.2 mCi/mmol, Sigma) as a tracer (30,000–40,000 cpm/µl) and compared with both negative (deionized distilled water, DDW) and positive (1% Triton X-100, Sigma) controls. Rates of cuticular penetration were measured at 30% RH and 30ºC using SOFU procedure as described by Schonherr (2000) and modified by Wiesman et al. (2002). A special delivery system with a thermostat desorption chamber and controlled environment was designed for the experiment. CMs were mounted between the lid and bottom of the desorption chambers using silicon grease (Bayer, Germany). Each CM was tested for leaks. DDW was added to the desorption chamber for 24 h, after which the DDW was withdrawn and a 10 µl droplet of donor solution (1% solution of QE, ME, KE, RE, and DDW with 2,4-D [^{14}C]) was placed in the center of the CM. After the water evaporated from the donor solution, the chambers were filled again with DDW which served as the receiving solution.

Receiver solution (DDW) was quantitatively withdrawn after 1, 4, 24, 48, 72, 96, 120, 144, and 168 h for scintillation counting and was replaced with fresh solution. At the end of the experiment, the CM was removed from the chamber and, after adding the scintillation cocktail, counted to determine the amount of radioactive material left on its surface. A Beckman LS 1701 scintillation counter (Beckman Coulter, US) was used to determine the radioactivity of samples. The amount applied (M_0) was calculated by summing the amounts penetrated (M_t) plus the amount left on the CMs at the end of the experiment. Thus, M_t / M_0 is the fraction that penetrated and 1 - (M_t/M_0) is the fraction remaining on the surface of the CM. Data were plotted as –ln (1 - M_t/M_0) vs. time as described by Schonherr (2000). The experiment was repeated three times.

In the second phase of the study, six concentrations of the ME (0.1, 0.25, 0.5, 1.0, 2.0, and 5% w/v) were tested as donor solutions by adding ^{14}C labeled 2,4-D and compared with the negative control (DDW) following the same protocol as in the first phase of study. In

the third phase of the study, the effect of the penetration of 1% ME was compared at different humidity levels (30, 60, and 90% RH) and temperatures (30, 45, and 60ºC). While comparing humidity, the temperature was set at 30ºC; during the temperature study humidity was set at 30% RH. In all experiments, at least 20 CMs were used in each treatment.

Microscopic study

Transmission electron microscopy (TEM) characterization of the different saponin-rich solutions was carried out on a JEOL-JEM-1230 Electron microscope (Japan) using negative staining technique, employing saturated uranyl acetate solution (after centrifuging), using Ultra-pure water (Biological industries, Israel). The grid (300 mesh copper Formvar/carbon) was immersed in a 1.0% solution of each ME, RE, KE, and QE for 1.5 minutes and then stained in the uranyl acetate solution for 1.5 min. The grid was then dried at room temperature on Whatmann filter paper (Ottaviani et al., 2000). The dried grids were examined at 8000 KV accelerating voltage and 25 K magnification.

Particle sizes of the extract solutions were measured using light scattering measurement techniques on an ALV-NIBS High Performance Particle Sizer (Germany). Each 1% of ME, KE, RE, and QE was prepared using Ultra-pure water in dust-free conditions. Measurements were performed at a 173^{o} angle at 632nm λ at 25^{o}C. The light source was an argon ion laser and the photoelectron count-time autocorrelation function was calculated with a BI2030AT (Brookhaven Instruments, Germany) digital correlator and analyzer, using the method of cumulants or the constrained regularization algorithm CONTIN applying the Stokes-Einstein relationship to the translational diffusion coefficients, providing an intensity weighted distribution of hydrodynamic sizes (Finsy, 1994).

Statistical analysis of the data was performed with JMP software (Version 4; SAS Institute, Inc., Cary, NC, US) using the Tukey-Kramer HSD test for determining significant difference among treatments at $p=0.05$ level of significance.

Results

Effect of the saponin source on penetration of 2,4-D across the CMs

The effect of the different saponin sources on the penetration of the 2,4-D (^{14}C) is presented in **Figure 6.1**. The first order plot (natural logarithms) of the fraction of 2,4-D that had not yet penetrated was plotted vs. time, thus 2,4-D penetration can be completely described by a single constant, the rate constant (*k*) of penetration, which is equivalent to the slope of the straight line (Schonherr, 2000). When no adjuvant was added to the 2,4-D (control – DDW), the rate penetration was 0.595×10^{-5} h^{-1}. The penetration rate was 8.9-times higher (5.83×10^{-5} h^{-1}) when RE was used. Similarly, 9.5-times (6.18×10^{-5} h^{-1}) and 13.7-times (8.75×10^{-5} h^{-1}) higher penetration was achieved in KE and QE treatment, respectively, whereas 16.3-times higher (10.20×10^{-5} h^{-1}) penetration was observed with ME treatment, and 17.6-times higher (11.000×10^{-5} h^{-1}) with Triton treatment. Among all the SREs, the highest penetration was achieved with ME and this rate penetration was not significantly different than that of Triton (a positive control) (**Table 6.1**).

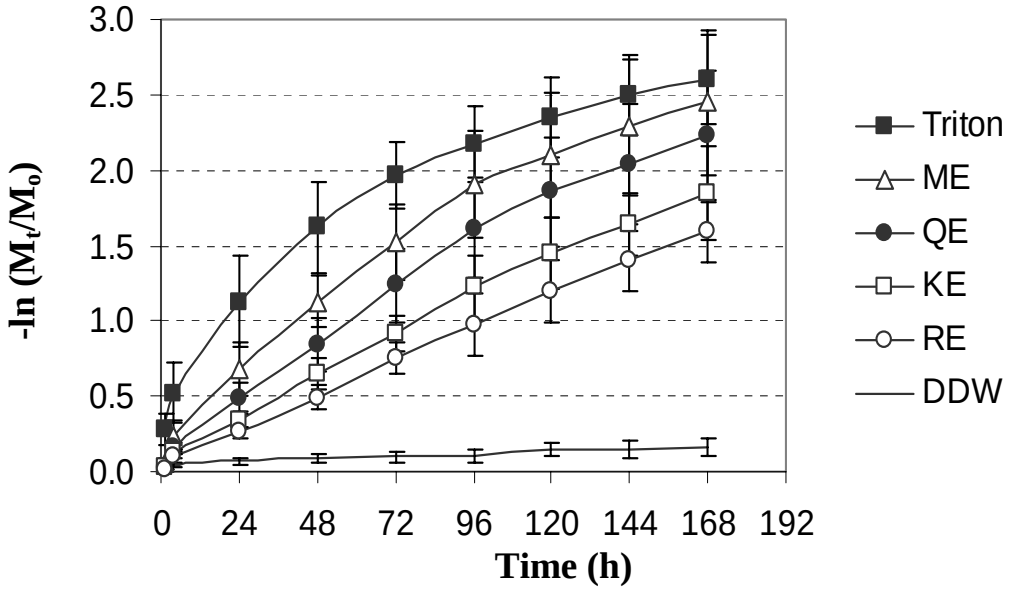


Figure 6.1 Time-course effect of the different SREs on penetration of 2,4-D (^{14}C) across the *Citrus grandis* leaf cuticular membrane (CMs) at 30ºC and 30% RH. Together with 2,4-D, a 1% (w/v) solution each of Triton, ME, RE, KE, or QE was added in each treatment as an adjuvant. QE, ME, KE, RE, and DDW refer to *Quillaja saponaria* extract saponin, *Balanites aegyptiaca* fruit mesocarp extract saponin, *B. aegyptiaca* kernel extract saponin, *B. aegyptiaca* root extract saponin, and deionized distilled water, respectively. The DDW contained only 2,4-D solution as a control. Each value is the mean of 60 CMs ± SE.

Table 6.1 Effect of SREs as an adjuvant on rate penetration of 2,4-D (^{14}C) across the *Citrus grandis* leaf cuticular membrane (CMs) at 30ºC and 30% RH. Together with 2,4-D, a 1% (w/v) solution of Triton, ME, RE, KE, or QE was added in each treatment as an adjuvant.

Treatment	Rate penetration (h^{-1})	Factor increase (times)
DDW	0.59×10^{-5} d	0.0
RE	5.83×10^{-5} c	8.9
KE	6.18×10^{-5} c	9.5
QE	8.75×10^{-5} b	13.7
ME	10.20×10^{-5} a	16.3
Triton	11.00×10^{-5}a	17.6

Each value is the mean from the pool data of 60 CMs. Means sharing common postscripts are not significantly different ($p < 0.05$). Triton, QE, ME, KE, RE, and DDW refer to Triton X-100, *Quillaja saponaria* extract saponin, *Balanites aegyptiaca* fruit mesocarp extract saponin, *B. aegyptiaca* kernel extract saponin, *B. aegyptiaca* root extract saponin, and deionized distilled water, respectively. The DDW contained only 2,4-D solution as a control.

Effect of ME concentration on penetration of 2,4-D across CMs

The first phase of the study showed that the commercial foliar adjuvant Triton and ME significantly enhanced the penetration of 2,4-D across the CMs in comparison to the control and other saponin sources. Since there was no significant difference in rate penetration between ME (1%) and Triton, in the second phase of the study a series of concentrations of ME were tested. In this study, ME concentrations were used in a range from 0.1 to 5.0%. The rate penetration of the control treatment (without addition of an adjuvant, only 2,4-D called here DDW) was just 0.59×10^{-5} h^{-1}, which was almost 19 times lower than the value of 2% ME, which had the highest penetration rate among all treatments. However, the rate penetration of 1% ME was not significantly different than that of 2% ME (**Table 6.2**). Rate penetrations increased with an increase in ME concentration from 0.1% to 2.0%; however, the rate was drastically decreased when 5% ME was used (5.65×10^{-5} h^{-1}) in comparison to 2% ME (11.90×10^{-5} h^{-1}) (**Fig. 6.2**).

Effect of humidity and temperature on penetration of 2,4-D across CMs

In the third phase of the study, the effects of humidity and temperature on the rate of penetration of 1% ME across the CMs were tested. When humidity was increased from 30% to 60% and then to 90%, the rate of penetration of 2,4-D was found to increase from 10.46×10^{-5} to 13.32×10^{-5} and 16.35×10^{-5} h^{-1}, respectively. At all three tested humidity levels, the rate of 2,4-D penetration was dramatically increased in comparison to the control (**Table 6.3**). As with humidity, the rate of 2,4-D penetration also increased with increasing temperature from 30°C through 60°C up to 90°C, 10.35×10^{-5}, 12.32×10^{-5} and 16.15×10^{-5} h^{-1}, respectively.

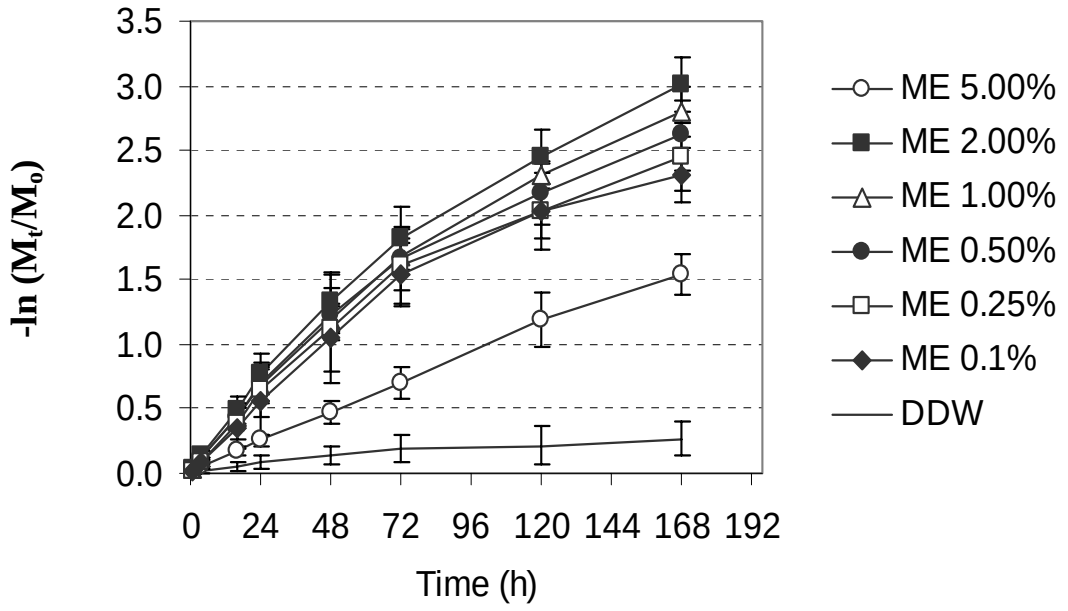


Figure 6.2 Time-course effect of the concentration of the *Balanites aegyptiaca* fruit mesocarp extract saponin (ME) on penetration of 2,4-D (^{14}C) across the *Citrus grandis* leaf cuticular membrane (CMs) at 30ºC and 30% RH. Each treatment contained the same concentration of 2,4-D together with the amount of ME as an adjuvant as listed on the graph. The DDW (deionized distilled water) contained only 2,4-D solution as a control. Each value is the mean of 60 CMs ± SE.

Light scattering characterization of particle size distribution of saponin solution

Using dynamic light scattering measurement, the average diameter of the mean mass particle population of the ME, KE, RE, and QE solutions (1.0%) was obtained (**Fig. 6.3**). The mean diameter of the ME and QE solutions were 167nm and 177nm, respectively, whereas the average diameters of the particle of the mass population of KE and RE were 2.0- and 2.3-times higher than the two saponin rich extracts i.e., 502 and 587nm, respectively.

TEM characterization of the particles contained in saponin solution

When all four saponin solutions (QE, ME, KE, and RE) that were used earlier in different 2,4-D delivery experiments were characterized in TEM, small nano-sized vesicles were clearly observed in all four solutions (**Fig. 6.4**). The size and structure of the vesicles in both QE and ME were similar, whereas, in agreement with the light scattering analysis, both the size and structure of RE and KE were slightly different than QE and ME. In QE and ME, the nano-sized vesicles were small and tightly spherical, whereas large and loose spherically shaped structures were observed in both KE and RE.

Table 6.2 Effect of *Balanites aegyptiaca* fruit mesocarp saponin extract (ME) concentration as an adjuvant on rate penetration of 2,4-D (^{14}C) across the *Citrus grandis* leaf cuticular membrane (CMs) at 30ºC and 30% RH. Each treatment contained the same concentration of 2,4-D together with the reported amount of ME as listed.

Treatment	Rate penetration (h^{-1})	Factor increase (times)
DDW	0.59×10^{-5} f	0.0
ME 0.10%	9.46×10^{-5} c	15.0
ME 0.25%	9.88×10^{-5} bc	15.7
ME 0.5%	10.53×10^{-5} b	16.8
ME 1.0%	11.07×10^{-5} a	17.7
ME 2.0%	11.90×10^{-5} a	19.1
ME 5.0%	5.65×10^{-5} e	8.5

Each value is the mean from a data pool of 60 CMs. Means sharing common postscripts are not significantly different ($p < 0.05$). DDW refers to deionized distilled water as a control.

Table 6.3 Effect of humidity and temperature on rate penetration of the 2,4-D (^{14}C) across the astomatous adaxial *Citrus grandis* leaf CM with 1% *Balanites aegyptiaca* fruit mesocarp extract saponin (ME) as an adjuvant.

Treatments	Rate penetration (h^{-1})
[a]***Humidity (%)***	
30	10.46×10^{-5} (0.71×10^{-5})
60	13.32×10^{-5} (2.45×10^{-5})
90	16.35×10^{-5} (4.36×10^{-5})
[b]***Temperature (ºC)***	
30	10.35×10^{-5} (0.59×10^{-5})
60	12.32×10^{-5} (2.44×10^{-5})
90	16.15×10^{-5} (4.76×10^{-5})

Value in brackets is the rate penetration of the control in treatment at respective conditions.
[a] Humidity experiment was conducted at 30ºC.
[b] Temperature experiment was conducted in 30% humidity.

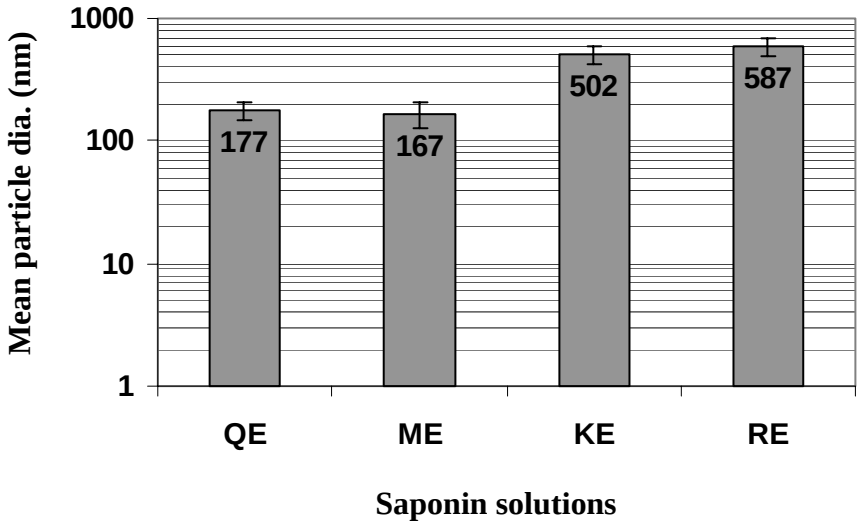


Figure 6.3. Mean particle size of the different saponin extract solutions (1.0%) determined by light scattering measurements (ALV-NIBS High Performance Particle Sizer) at a 173° angle at 632nm λ at 25°C. QE, ME, KE, RE, and DDW refer to *Quillaja saponaria* extract saponin, *Balanites aegyptiaca* fruit mesocarp extract saponin, *B. aegyptiaca* kernel extract saponin, *B. aegyptiaca* root extract saponin, and deionized distilled water, respectively. Each value is the mean of 6 samples ± SE.

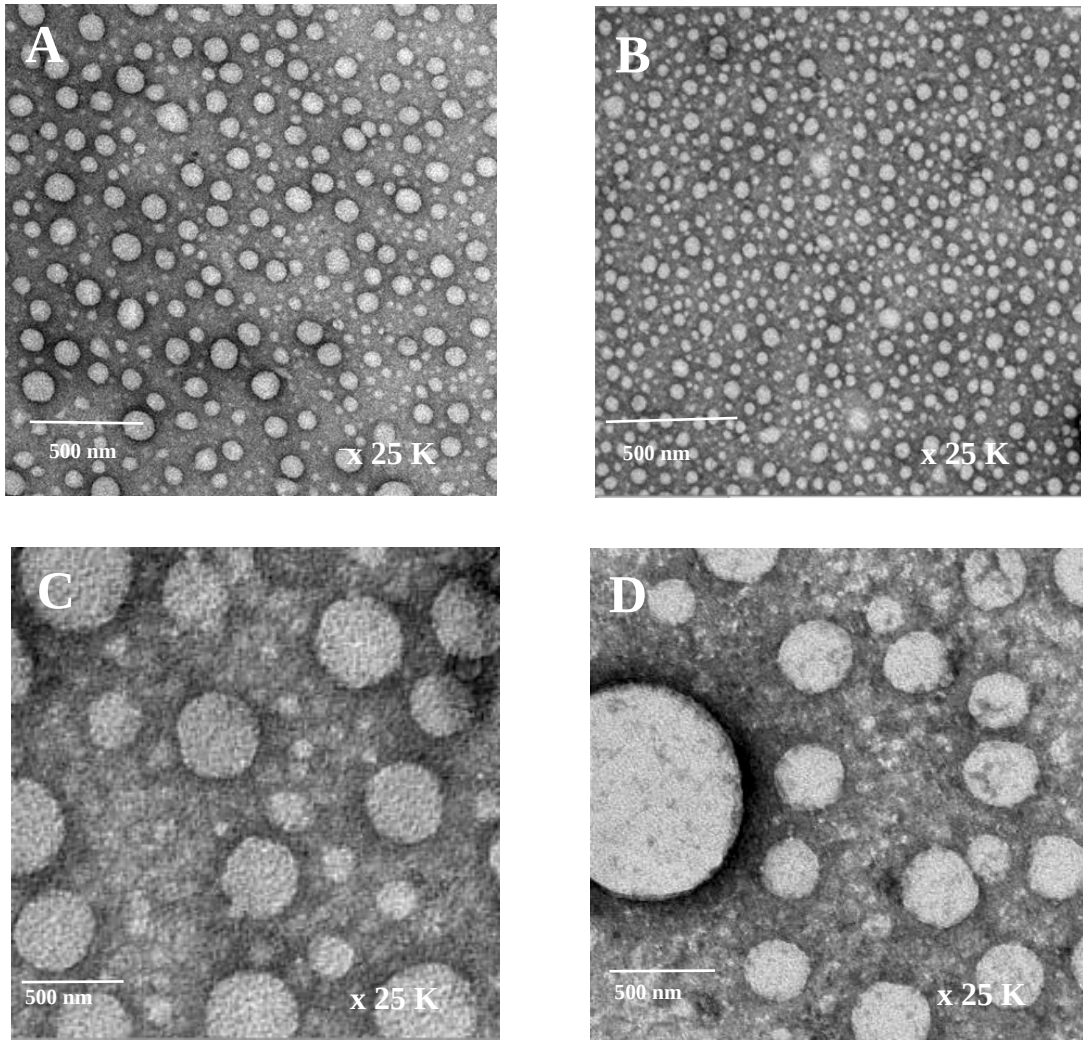


Figure 6.4 Transmission electron microscope (TEM) characterization of the nano-vesicles present in the different saponin extract solutions (1.0%). **A** – *Quillaja saponaria* extract saponin (QE); **B** – *Balanites aegyptiaca* fruit mesocarp extract saponin (ME); **C** – *B. aegyptiaca* kernel extract saponin (KE); and **D** – *B. aegyptiaca* root extract saponin (RE) with uranyl acetate background as negative staining.

Discussion and conclusions

Agro-material delivery is highly important in modern agriculture. Many variables, both external and internal, regulate the rates of absorption of applied materials by plant foliage. Humidity, temperature, and the chemical constituents of the agro-materials are the main factors that influence foliar penetration of active materials into a plant (Baur, 1999). Since the cuticular membrane is composed of a lipophilic layer, the nature of applied materials (hydrophilic or lipophilic) affects penetration. That is why lipophilic surfactants are commonly used to support penetration by lowering the surface tension and consequently increasing permeability and absorbance of applied materials. High humidity and moisture on external surfaces, and low moisture tension within the plant, favor rapid foliar uptake (Crafts, 1962).

This study was an attempt to develop a natural delivery adjuvant system for agro-materials through the plant cuticle membrane. Significant ability of a natural saponin preparation to deliver 2,4-D through isolated leaf CMs was observed.

The first phase of the delivery experiment showed saponin extracts from *Q. saponaria* bark and various parts of *B. aegyptiaca* accelerated the penetration of 2,4-D through the astomatous adaxial *C. grandis* leaf CMs. The most accepted, and one of the leading synthetic surfactants used in foliar application, Titron × 100, showed the highest rate of 2,4-D penetration; however, this was not significantly different than the fruit mesocarp saponin extract of *B. aegyptiaca* (ME).

In the second phase of the study, when a series of concentrations of ME was used, 1% and 2% ME optimized the rate of penetration of the 2,4-D. Raising the concentration to 5% inhibited the penetration, which might be explained by crystallization of saponin molecules in the over-saturated solution (Wiesman et al., 2002; Luber, 2001). This suggestion is supported by scanning electron microscope (SEM) characterization of saponin concentration interaction with CMs (data not shown).

Increasing the humidity to 90% markedly accelerated the rate of 2,4-D penetration through isolated CMs. Similarly, increasing the temperature from 30°C to 60°C markedly increased the rate penetration from 10.345×10^{-5} h^{-1} to 15.357×10^{-5} h^{-1} (**Table 6.3**). These results are in good agreement with literature reports with regard to surfactant behavior (Wiesman et al., 2002; Schonherr, 2000).

Various possible reasons might be suggested for acceleration of the penetration rate of 2,4-D with the help of saponin as a delivery adjuvant. One of the possibilities is the high affinity of the saponin molecules for phytosterol present in leaf CMs. Although the precise mechanism of how saponins interact with the membrane is not fully understood, it is generally accepted that the mechanism of antifungal activity of saponins is apparently due to their ability to complex with sterol in fungal membranes and cause loss of membrane integrity (Keukens et al., 1995). It has also been reported that saponins complex with sterols in membranes and ultimately form aggregates which then lead to the formation of membrane pores (Armah et al., 1999). Hence, one of the possibilities behind the penetration could be the affinity of glycosidic saponin compounds from saponin-rich extracts for increased association with cuticle membranes (CMs), and their affinity with the sterol compounds of these membranes. Therefore, there is a good chance for these saponins to cause significant disorders in the cuticles. The data obtained in the 2,4-D delivery system clearly demonstrated the interaction between saponin extract and cuticle membrane. These data extend and support the previous data on interaction of saponins with biological membranes (Hostettmann and Marston, 1995). This may also explain the interference of saponins with a wide range of biological systems.

The second possibility is the effect of the saponin molecule on the cuticle layer to increase the diffusion process. It is common to use surface-active agents or surfactants as spray adjuvants in formulations of agrochemicals to improve their effectiveness following application to foliage (Abert et al., 2002). Amphiphilic saponin molecules function as a surface-active agent because of the lipophilic and hydrophilic moieties present in the molecule. Hence, these saponins are known as natural surfactants (Hostettmann and Marston, 1995). Saponins, which are steroidal and triterpenoid glycosides, have the ability to lower the surface tension of aqueous solutions (Samuelsson, 1992). The glycolipid saponins, as natural surfactants, keep the delivered biomaterial in a solubilized or partially solubilized form, depending mainly on the environmental humidity and temperature (Schonherr, 2000), and they increase the plasticity and permeability of biological membranes (Wiesman et al., 2002).

The third possible reason behind the penetration enhancement is the formation of small nano-vesicles or micelles that help the penetration. Microscopic study of the saponin

solutions used in our studies has shown the formation of natural nano-sized micelle type vesicles (**Figs. 6.3, 6.4**). Vesicles, especially liposomes, have been suggested as vehicles for the delivery of different compounds (mainly encapsulated drugs) (Gregoriadis et al., 1998).

The reason for different rate penetrations of 2,4-D across the leaf cuticle membrane with ME, KE, and RE, could be differences in the saponin constituents in these extracts. Although ME, KE, and RE all are saponin-rich extracts obtained from the same plant, *B. aegyptiaca*, characterization of the saponins in different tissues of this plant show that there are compositional differences in the major saponins in Balanites plant tissues (Chapters 2 and 3). The major saponin in fruit mesocarp contains one pentose sugar unit (xylose) fewer than the root and kernel saponins (Chapter 3). Furthermore, there is also a difference in the presence of a $–CH_3$ or –OH group in the C-22 position in the different saponins of the Balanites plant tissue. It was found that SREs from the kernel and root of Balanites containing high saponin levels with a xylose unit and $–CH_3$ group in position C-22 of their aglycone moiety, showed higher larval mortality than the fruit mesocarp extract saponin which contains mostly saponins without a xylose unit in the sugar chain (Chapter 4). The differences in the presence of a xylose sugar unit in the sugar chain with $–CH_3$ and/or –OH groups in the aglycone moiety of the KE, RE, and ME might have worked differently to lead to different results for the delivery of the 2,4-D across the CMs.

From the results achieved in this study, it is clearly shown that SREs of both *Q. saponaria* and *B. aegyptiaca* accelerate the delivery of 2,4-D through isolated leaf cuticle membranes. These results suggest that amphiphilic saponins could be used as foliar penetrants for the delivery of agrochemicals. They also show the potential of these natural saponins for use in other delivery mechanisms. The use of *Q. saponaria* has already been exploited commercially; however, the use of Balanites is very negligible. Since Balanites is highly adapted to most of the arid land where other crops are extremely difficult to grow (Hall and Walker, 1991), the products of *B. aegyptiaca* could be very inexpensive. Furthermore, *B. aegyptiaca* contains a large quantity of easily extractable saponins in its fruit mesocarp (Chapter 2), which is not particularly popular for fresh fruit consumption. Thus, the results of this study also open the door for the commercialization of the neglected desert plant species, Balanites, for its valuable products. Since saponins are environmentally friendly, natural substances, the plant-originated natural saponin-based

delivery system could play a vital role in saving the environment from pollution. The antifungal and antimicrobial properties of these saponins would further help with judicious placement of agrochemicals in the framework of integrated pest management, as well.

CHAPTER 7

General conclusions and suggestion for future

Although saponin-containing plants have been used in various traditional folk medicines, especially in the Orient, for hundreds of years, saponins are not as attractive for researchers compared to the many other secondary metabolites, for example phenolics. One of the reasons for this is the problem of separation and quantification of saponins which is still a challenge. In these circumstances, the method employed in this study for identification, separation, and structural elucidation of complex saponins (Chapters 2 and 3) using the direct preparative-scale HPLC-RI of crude extracts followed by ESI-MS^n and supported by high filed NMR investigation represents a new and easy approach. The present work, in addition to elucidation of the structures of the major saponins of *B. aegyptiaca*, describes a useful, compact protocol for rapid isolation of saponins, and demonstrates the power of ESI-MS^n and high-field NMR spectroscopy for unambiguous de novo structure determination of complex saponins without derivatization or degradation. Since the traditional protocol for saponin characterization is long and tedious and needs a series of chromatographic steps, this protocol would certainly be useful for identification and analysis of the complex steroidal saponins. The method employed in this study had no problems for lacking chromospheres and hindrances to detection. This work provides a saponin profile of various tissues of *B. aegyptiaca* grown in Israel. This study also shows that saponins in various tissues of *B. aegyptiaca* of Israeli provenances are quantitatively, as well as qualitatively, different than the earlier reports of African provenances. This work also identifies some new saponins which have not been previously reported in the literature. This study also concludes that major saponins in the fruit mesocarp, kernel, and roots of the Balanites are furostanol as the steroid with diosgenin as the aglycone.

The results of this study (Chapter 4) suggest a possible use of various saponin-rich extracts of *B. aegyptiaca* as natural larvicidal agents for controlling the mosquito population. Killing one hundred percent of the mosquito larvae would be a difficult task using extracts from various parts of the Balanites plant, as with most synthetic chemicals; however, the substantial reduction in emerging adult mosquitoes by a considerably very small amount would certainly help to control mosquito population drastically. In fact, as adult mosquitos transmit diseases, the critical concentrations that inhibit 50% of the larvae from emerging as adults (EC_{50}) are

more meaningful. The use of Balanites saponin extracts not only helps to reduce the mosquito population in the area where the *Balanites* has already been available, but it could also open an export front.

The kernel oil is the most prominent economic products from Balanites so far and the cake (left over after oil extraction) is considered to be a waste product. No study has so far reported about the relationship between oil content and saponin level in the seed kernel of Balanites. The result of this study which shows a high positive correlation between sapogenin (diosgenin) and the oil content in Balanites seed kernel (Appendix A1) provides clear information regarding selection for the desirable germplasm for Balanites in the domestication process. The high larvicidal activities of the kernel extract (Chapter 4) provide a new use of kernel cake from the so-called waste product. In this circumstance, the Bet-Shean provenance (Israel) which showed high oil content and sapogenin level in seed kernel (Appendix A1) would be a desirable germplasm source at least for beginning the domestication. However, more investigations, using greater number of provenances are needed, which may provide additional and even more useful characters.

One of the most encouraging results obtained from this study was the effective production of micelle-like nano-vesicles from the Balanites saponin (Chapter 6). Although formation of micelles prefers a stacked structure with the hydrophobic nuclei stacking together like a pile of coins, hydrophilic carbohydrate side chains of the saponin molecule extending out from the interior core have been reported in the literature from the triterpenoid saponins (Oakenfull and Sidhu, 1989); however, no report has yet been found about the production of vesicles from steroidal saponins. The microscopic characterization study of this work has clearly shown the presence of vesicles in the solution of the SREs of steroidal saponins of ***B. aegyptiaca***. Considerable research has been focused in recent years about the use of vesicles (liposomes) for delivery of drugs to the target tissues, especially in the pharmacy and medical fields as a carrier for active substances. The outcome of the poor efficiency of conventional liposomes for efficient delivery of drugs in certain important diseases like cancer, pharmacists have been looking new liposomes that can efficiently enhance delivery and magnitude to immunological response. Enhanced delivery of 2,4-D across the CMs by SREs could also be attributed to the presence of nanovesicles in the SREs together with the association of saponin molecules with the sterol integrated in the CMs. This shows the possibility of using Balanites saponin vesicles

as a delivery adjuvant, not only in agro-materials like nutrients, pesticides, and herbicides, but also in delivery of the nucleic acids, proteins, toxins, and other materials through the cuticle membranes and also for drug delivery as well. Furthermore, such a delivery system could ultimately be exploited in the foliar application of pesticides, fertilizers, or herbicides and this ultimately help in the domestication process of most neglected plant species. This may also help ecological conservation by growing Balanites plants in harsh arid regions such as the Israeli Negev desert where other plant species hardly grow. Further research in this regard is strongly suggested; this would be an excellent endeavor for the use of Balanites plant for the future.

The *in vitro* study of diosgenin accumulation in various callus cultures (Appendix A2) shows the possibility of mass production of diosgenin, an important sapogenin used in the pharmacy and cosmetics industries, in callus cultures of Balanites; this can be an alternative means of production of diosgenin. However, the significant differences in diosgenin content among explants, various plant growth regulators, and embryogenesis indicate that further study with more possible alternative parameters would be needed in order to standardize the protocol.

For a long time it was suggested that furostanol saponins did not possess any biological activity except antioxidant properties and these saponins were regarded just as transport or storage forms of spirostanol glycosides. However, the antifungal activities, delivery enhancement activities and specifically the larvicidal activities of the isolated pure saponins from Balanites, clearly suggest that studies of the activity of furostanol saponins are worthy of further investigation.

Furthermore, during the course of this study, two introductory plots of *B. aegyptiaca* have been developed in two locations in Israel (one in an arid region near the Red Sea and the second in a semi-arid region near the Mediterranean Sea) with different local and international provenances. In the long run, these introductory plots could play a vital role in germplasm resources for *B. aegyptiaca*.

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Appendices

A1 – A4: Articles published or In Press

B1 – B4: Spectral data of NMR

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Variation in diosgenin level in seed kernel among different provenances of *Balanites aegyptiaca* Del (Zygophyllaceae) and its correlation with oil content

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Abstract

Balanites aegyptiaca (Zygopyllaceae) is a widely grown desert plant with multi-use potential. It is found in most of the African continent, the Middle East and South Asia however, this plant remained one of the most neglected plant species. Its seed kernel is used for oil extraction and the oil is used for human consumption and cosmetics. However the oil cake is regarded as unsuitable for feeding because of the presence of many toxic substances. In this study a spectrophotometric determination of diosgenin level and subsequent oil percentage analyses were carried out from the seed kernels of *B. aegyptiaca* collected from five Israeli provenances (Bet-Shean, Ein-gedi, Sapir, Samar and Eilat) and five international samples from Burkina Faso, Senegal, Mali, Niger and India. The result suggested that the sample of Bet Shean valley which is considered as the northern most hemisphere where *B. aegyptiaca* naturally grown contended the highest level of both diosgenin as well as oil percentage and Indian sample contained the lowest of both diosgenin and oil content. The result also showed that there is a strong positive correlation (R^2 = 0.849) between diosgenin level and oil percentage in the *B. aegyptiaca* seed kernel.

Key words: *Balanites aegyptiaca*; Provenances; Kernel cake; Diosgenin; Oil content

Introduction

Diosgenin is a steroidal sapogenin compound (Fig. 1) very useful in pharamacecutical industries as a natural source of steroidal hormones. Diosgenin is found in a few higher plant species and its medicinal interest has been increased recently (Liu et al., 2005). Recent studies have found that diosgenin can be absorbed through gut and plays an important role in the control of cholesterol metabolism (Roman et al., 1995). Other authors have also reported that it has estrogenic effects (Aradhana et al., 1992) and antitumor activities (Corbiere et al., 2003; Moalic et al., 2001). Studies have also reveled that diosgenin produces changes in the lipoxygenase activity of human erythroleukaemia cell and responsible for morphological and biochemical changes in megakaryocyte cells (Nappez et al., 1995; Beneytont el al., 1995). Furthermore, diosgenin was found the most effective as cell death inductor compared to the other two plant steroids (hecogenin and tigogenin) in the human osteosarcoma 1547 cell line (Corbiere et al., 2003). Diosgenin is generally used as starting material for partial synthesis of oral contraceptives sex hormones and other steroids (Zenk, 1978). The partial synthesis of steroids from plant based precursors has been taken a boom market since about 1960 because of the increasing demand of corticosteroids, contraceptives, sex hormone and anabolic steroids (Hall and Walker, 1991). Till now diosgenin and related steroidal saponins are obtained commercially from the tubers of various *Dioscorea* species however it is crucial to discover

new and alternative sources of these compounds because of decreasing plant resources as well as increasing demand (Savikin-Foduloic et al., 1998).

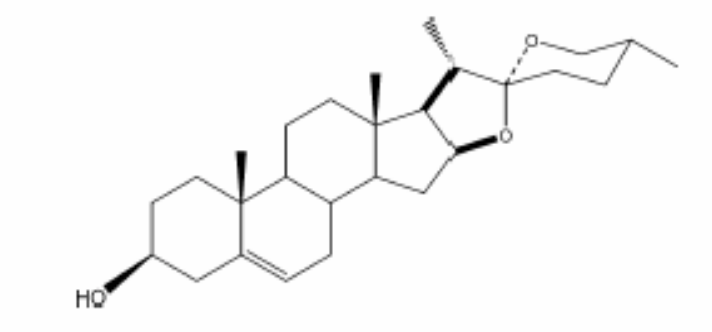

Fig 1. Chemical structure of Diosgenin

Balanites aegyptiaca Del (Zygphyllaceae) popularly called as 'desert date' is an evergreen highly drought tolerant desert plant species. It is widely grown in the Sudano-Sahielian region of Africa, the Middle East, and South Asia (Hall and Walker, 1991). *B. aegyptiaca* plant tissues have been used as various folk medicines in Africa and Asia. Although this plant has been used for various uses from ethnobotanical to fire wood however this species is considered one the most neglected tree species in the arid region (Hall and Walker, 1991). The fruit consists of epicarp (5-9%), mesocarp (28-33%), endocarp (49-54%) and kernel (8-12%). The kernel of B. *aegyptiaca* fruit is rich in oil and local people used as a source of edible oil (Newinger, 1996) however the oil cake is regarded as unsuitable for feeding because of presence of many toxic substances. Studies have shown that, B. *aegyptiaca* plant tissues content steroidal saponins with mainly diosgenin or its isomer yamogenin as sapogenin (Neuwinger, 1996). In a detailed examination, Hardman and Sofworn (1972) has reported a 2% diosgenin in seed kernel from the West African sample. Ognyanov et al. (1977) have reported a 3% sapogenin in the fruit mesocarp and 2% in seed kernel from the East African sample. Desai et al., (1978) have reported a 0.994% diosgenin from fruit pericarp; 0.94% from seed kernel and 0.45% from the oil from Indian sample.

Literature survey revealed meager studies about the diosgenin analyses in various *B. aegyptiaca* tissues with sample from mainly in African and Indian provenances but no study so far has been reported from the sample of Israeli provenances. Israel is one of the native homeland of *B. aegyptiaca* and especially the Bet-shean Valley (35° 25′ N) area is considered to be the northern most hemisphere where *B. aegyptiaca* naturally grown (Zohary, 1973). Considering the high market potential of diosgenin and vivid report of diosgenin level in *B. aegyptiaca* kernel and lack of data for broad samples including Israeli provenances this study was conducted to seek the provenances with higher diosgenin content in seed kernel. We believe that the identified provenances of *B aegyptiaca* with high level of diosgenin might help in some extent to make the *B aegyptiaca* a neglected plant species to economically competitive with tubers of Dioscorea, the traditional source of diosgenin used for the synthesis of steroid drugs and ultimately help the domestication process of *B. aegyptiaca*.

Materials and Methods

Sample collection

Ripen fruits were collected from the trees of the five *B. aegyptiaca* grown areas of Israel namely: Bet-Shean valley (close to the Sea of Galilee), Ein-Gedi (close to the Dead Sea), Sapir (Center Arava valley), Samar (South Arava valley) and Eilat (close to the Red sea) and authenticated by Prof. Uzi Plitman from the herbarium in the Hebrew University of Jerusalem. Voucher speciment (76816) was deposited in the herbarium of the Hebrew University of Jerusalem. Five other samples were also obtained from India (Jodhpur), Mali (Bamako), Niger (Zinder), Senegal (Dakkar) and Burkina-Faso (Ouaga). All abroad samples were collected from street vendors.

The epicarp (outer cover) and mesocarp (pulp) of the fruits were removed by hand and the nuts were washed by tap water. After wash, the nuts were oven dried at 70 ºC for 72 h. Decortications of the nuts were carried out by hand and released the kernel (approximately 10% of the total fresh weight). The kernel and stone ratio was 30:70 by weight. The kernels were grounded in a mortar and pistol using liquid nitrogen.

Oil extraction

Oil percentage was determined gravimetrically by dividing extracted oil by kernel weight after evaporation of solvent (AOAC, 1996; IUPAC, 1979). In brief, 3 g of pulverized kernel powder were taken in a plastic tube (50 ml capacity, centrifugable grade) and 30 ml n-hexane was added. The tubes were put over night in electric shaker (Z. Tuttnauer Ltd. Jerusalem) in high speed followed by centrifuged in 3500 rpm, 18 min., 20 C (Hermble, Germany) and supernatant were collected. In the residue two subsiding extraction were carried out using only vortexes and centrifuging. After three succeeding extraction the supernatant was clear and then all the supernatant were collected and the n-hexane was evaporated using rotary evaporator (Heta-Holten A/S, Denmark) and oil was collected. Each sample was repeated thrice.

Saponin extraction

The defatted cake (left over after oil extraction) was kept under the hood overnight. In the next day, 30 ml methanol was added to the tubes and kept over the shaker over night followed by centrifuged. The second and third extraction by methanol was also carried out as like n-hexane. At the end all supernatant of methanol extracts were pooled and the methanol was evaporated by using rotary evaporator. Finally yellowish crystal powder of crude saponins was obtained (approximately 12.2% of kernel by weight).

Diosgenin determination

Diosgenin was determined as described by Baccou et al (1977) and Uematsu et al.(2000) with some modification. Standard sapogenin (diosgenin) and *p*-anisaldehyde (4-methoxybenzaldehyde) were purchased from Sigma. Sulfuric acid and ethyl acetate were both analytical grade and obtained from Frutarom. The diosgenin level was determined by measuring absorbance at 430 nm (Fig. 2), based on the color reaction with anisaldehyde, sulfuric acid and ethyl acetate. In brief, two color developing reagent solutions were prepared: (A) 0.5 ml *p*-anisaldehyde and 99.5 ml ethyl acetate, and (B) 50 ml concentrated sulfuric acid and 50 ml ethyl acetate. 200 µg of the methanol extract of defatted kernel was taken in glass tube. For this 1 mg of defatted and methanol extract of kernel powdered was

first dissolved in 1 ml methanol and 200 µl of this solution was taken in another tube and the methanol was evaporated under reduced pressure. This residue was dissolved in 2 ml of ethyl acetate and 1 ml of each reagent A and B was added to the tube and stirred. The test tube was placed in a water bath maintaining at 60 ºC for 10 min. to develop color, then allowed to cool for 10 min. in 25 ºC. The absorbance of the color developed solution was measures in a spectrophotometer (JASCO V-530, UV/VIS). Ethyl acetate was used as control for the measurement of absorbance. As a reagent blank 2 ml ethyl acetate was placed in a tube and assayed in similar manner. For the calibration curve 2-40 µg standard diosgenin in 2 ml ethyl acetate were used (Fig 3). Each sample was repeated thrice and average was taken.

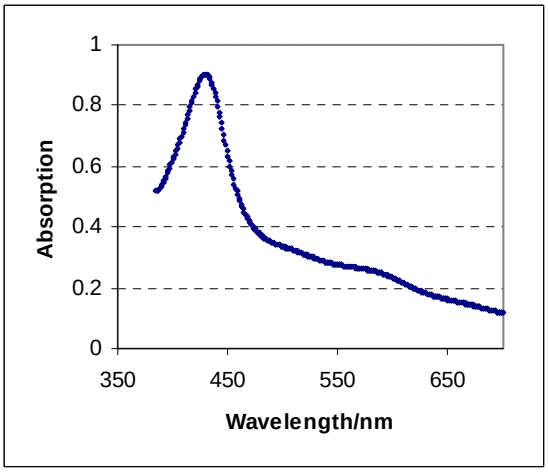

Fig 2: Absorption spectrum of diosgenin

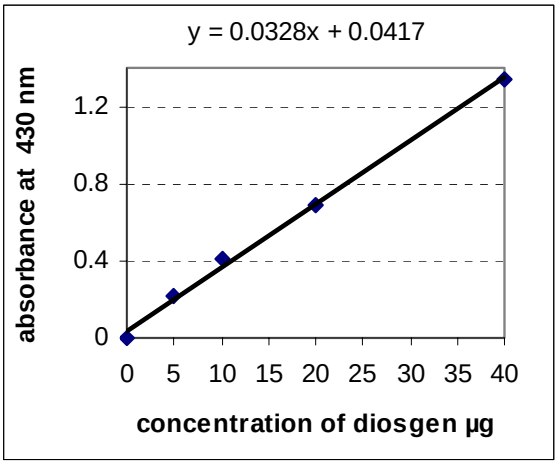

Fig 3: Calibration curve for the determination of diosgenin

Statistics

Data were statistically analyzed with JMP soft were (SAS, 2000), using Tukey-Kramer HSD test for determining significant differences among treatments at p-≤ 0.05.

Results and discussion

Since first isolation from *Dioscorea tokoro* in 1930s (Yang, 1981), diosgenin, a plant steroid (5α-spirostan-3β-ol) has profoundly been used for the different steroidal drugs. The steroidal drugs are considered to be the some of the costliest and most important medicines used throughout the world today. With recent reports of the diosgenin like the help in inducing differentiation of the erytholeukemia through changing lipoxygenase activities (Beneytout et al., 1995) and inducing apotosis and cell cycle amount in human oesterosascoma 1547 cell line (Moalic et al., 2001), the value of diosgenin further increased. Till now *Dioscorea* is the main plant this is extremely used to extract the diosgenin though there are may plant species that content diosgenin however it is crucial now to find alternate source for diosgenin production.

There have been reports that most of the *B. aegyptiaca* plant tissue including seed kernel as well as fruit and oil contain steroidal saponins. The fruit mesocarp content higher level

of diosgenin however the fruit is edible and used for different purposes like juice preparation and even preparation for alcoholic beverages and there is a little trade in kernel oil however there is almost no use of its cake. So this study was basically focused for the diosgenin in *B. aegyptiaca* seed kernel. The main objective of this study was to check the diosgenin level in various provenances grown in Israel and abroad.

Fig. 4 clearly shows that there is a big variation in percent diosgenin level among the 10 provenances that was analyzed in this study. The highest level of diosgenin was obtained in the sample from the Bet-Shean valley (2.22%) and lowest was in the sample from India (1.09%). The comparatively smaller error bars (± standard deviation) in the figure show that there was less variation within the sample. When we see the percent oil recovery in the seed kernel (Fig. 4 bottom), a similar pattern was found i.e. as like diosgenin the highest oil recovery was obtained from the Bet-Shean sample (50.22%) and lowest was in again in the Indian sample (39.20%).

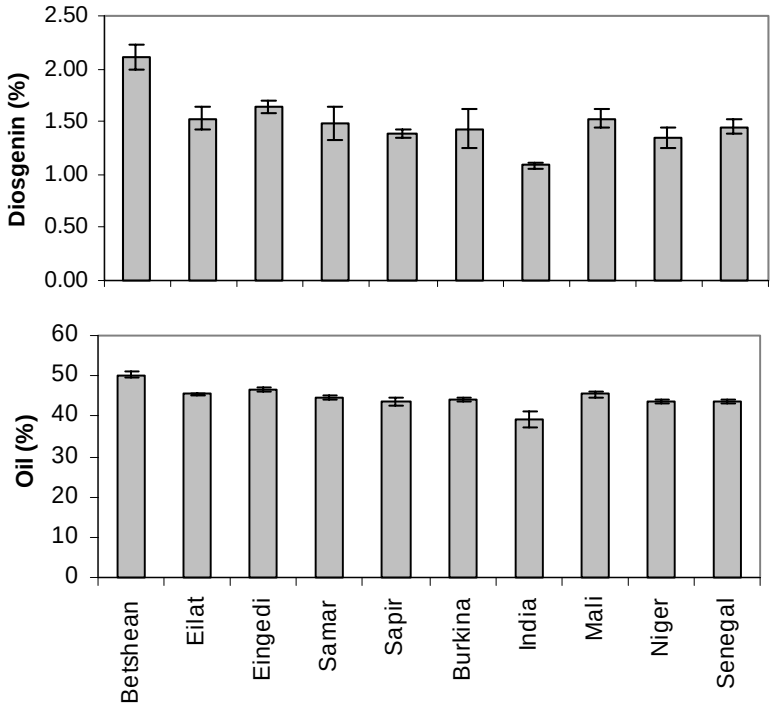

Fig 4: Percent diosgenin and oil content found in the different provenances of *B. aegyptiaca* kernel (on dry weight basis). Top: % diosgenin; Bottom: % oil. The first five provenances were from Israel. Error bars illustrate standard deviation from triplicate analysis of each sample.

Actually the total sapogenin in the *B. aegyptiaca* seed kernel in the analyzed samples would be higher because *B. aegyptiaca* also content yamogenin, an epimer of diosgenin is of equally utility to diosgenin (Fazil and Hardman, 1971). In *B. aegyptiaca* seed kernel, an approximate ratio of diosgenin to yamogenin of 70:30 has been reported (Abu-Al-Futuh, 1983). Since a pure sample of yamogenin is unavailable, so it will be reasonable to calculate the amount of total sapogenin using the ratio of diosgenin (Taylor et al. (2000). So total sapogenin combined of both diosgenin and yamogenin can be 30% higher (Table 1).

In Table 1, the total sapogenin as well as oil recovery value is presented in the regional basis. From this table we can clearly see that the Bet-Shean sample has the significantly higher level of both sapogenin and oil content compared to Israel (combined of all Israeli samples), Africa (combined of all African samples) and Indian sample. Similarly Indian sample got the significantly lower level of both sapogenin and oil content among all regions. However there was no significant different between combined Israeli and African sample in their sapogenin and oil content. Earlier studies has reported a 2% sapogenin in *B. aegyptiaca* seed kernel (Hardman and Sofowora, 1972; Ognyanov et al., 1977) in African *B. aegyptiaca* sample which lies very close to the results of this study when we calculated a combined results for Africa and even in Israel. In this study we found slightly higher level of total sapogenin in Indian sample than the earlier report of 0.99% (Desai et al., 1978). However, the Bet-Shean provenances itself shows the highest level of sapogenin (2.74%) so far reported. The differences in the sapogenin content in the African and Indian seed kernel that might be from the different determination procedure used.

Table 1. Total sapogenin (diosgenin and yamogenin) and oil recovery of the *B. aegyptiaca* seed kernel subjected to different regions. The total sapogenin value was calculated by the spectrometric value of the diosgenin.

Region	Sapogenin (%)	Oil recovery (%)
Israel (combined)	2.12 b	46.12 b
Israel (Bet-Shean only)	2.74 a	50.22 a
Africa (combined)	1.87 b	44.17 b
India	1.41 c	39.20 c

Combined value is the mean value of all samples in that region. Different letters in each column after the mean value are the significantly different at 5% level of significance according to Tukey-Cramer HSD.

Interestingly we found a positive correlation ($R^2 = 0.849$) between the diosgenin content in the seed kernel and oil content (Fig 5). Since both these parameters are the desirable characters so this findings certainly would play a vital role during the germplasm selection for the domestication of the *B. aegyptiaca*. However this rule didn't follow in the case for diosgenin content in fruit mesocarp and kernel seed oil content. In fact there was a very weak negative correlation (R^2=0.3078) in between the diosgenin content in the fruit mesocap and kernel seed oil content (results not shown).

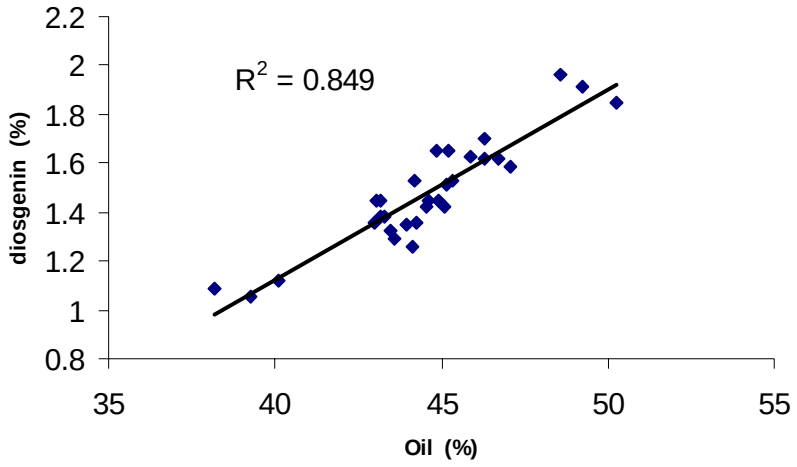

Fig 5. Relationship between oil and diosgenin content in the Balanites seed kernel (n=30). Values are taken from all 10 provinances.

Though *Dioscorea* root contents higher level of sapogenin (3-7% combined diosgenin and yamogenin) especially in its matured stage but it may take up to 3 year to be matured (Savikin-Fodulovic et al., 1998). In this circumstance, 1.08-2.2% of diosgenin (equivalent to 1.41-2.8% of sapogenin) content in *B. aegyptiaca* seed kernel is considerable amount especially from the neglected by-product of the seed kernel. One estimate shows that annually more than 4000,000 tons of *B. aegyptiaca* fruits are produced in Sudan only (Mohamed, 2002) and significant quantity from this is used for oil extraction , so this that thousands of diosgenin can be extracted from this production alone. So the seed kernel of *B. aegyptiaca* would be an alternate for diosgenin extraction in the global market. Furthermore selected provenances like Bet-Shean which content both high percentage of oil and diosgenin could be a bank of resource for germplasm for the future. Further more a nine year long trails of *B. aegyptiaca* in the Kibutz Samar of Israel clearly shows that *B. aegyptiaca* can be easily grown even in hyper arid desert condition with minimum irrigation (data not published yet).

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Bioproduction of Diosgenin in Callus Cultures of *Balanites aegyptiaca*: Effect of Growth Regulators, Explants, and Somatic Embryogenesis

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Abstract

This study describes the effects of plant growth regulators, explants and somatic embryogenesis on *in-vitro* production of the steroidal sapogenin, diosgenin in callus cultures of the *Balanites aegyptiaca* (L.) Del. (desert date). Root, shoot, hypocotyl and epicotyl callus culture of *B. aegyptiaca,* were raised on MS basal media supplemented with various combinations of either 2,4-D and NAA alone or with BAP. The diosgenin content (on dry weight basis) was found highest when calli were cultured at MS basal medium supplemented with 1.0 mg l^{-1} 2,4-D alone and/or in combination with 0.5 mg l^{-1} BAP. However the callus growth was highest in media supplemented with 2.5 or 3.0 mg l^{-1} 2,4-D. MS basal media supplemented with 2,4-D 2.5 mg l^{-1} alone and in combination with 0.5 mg l^{-1} BAP induced pre-embryogenic callus formation on root cultures. When these pre-embryogenic callus cultures were used to establish cell suspension cultures, two growth densities were obtained in embryogenic suspension cultures, inducing clusters of somatic embryos at various stages of development. The maximum number of somatic embryos were obtained at the fifth week on the medium supplemented with 1.0 mg l^{-1} 2,4-D. However, the diosgenin content in these somatic cells was found very low compared to the explants calluses. This study revealed that production of diosgenin in callus culture of *B. aegyptiaca* is possible but the amount is significantly affect by the growth regulators, type of explants and somatic embryogenesis.

Key words: *Balanites aegyptiaca*, explants, callus, embryoenesis, diosgenin, bioproduction, PGR.

Introduction

Diosgenin which is found in a very few higher plant species is a steroidal sapogenin compound which is a natural source of steroidal hormones. The medicinal property of the diosgenin has recently been increased in pharmaceutical industries (Liu et al., 2005). Studies have found that diosgenin can be absorbed through the gut and plays an important role in the control of cholesterol metabolism (Roman et al., 1995). Other authors have reported that it has estrogenic effects (Aradhana et al., 1992) and also shows antitumor activity (Moalic et al., 2001; Corbiere et al., 2003). Studies have also revealed that diosgenin produces changes in the lipoxygenase activity of human erythroleukemia cells and is responsible for morphological and biochemical changes in megakaryocyte cells (Beneytont et al., 1995; Nappez et al., 1995). Furthermore, diosgenin was found to be the most effective cell death inducer compared to the other two plant steroids (hecogenin and tigogenin) in the human osteosarcoma 1547 cell line (Corbiere et al., 2003). Diosgenin is generally used as starting material for partial synthesis of oral contraceptives, sex

hormones, and other steroids (Zenk, 1978). The partial synthesis of steroids from plant-based precursors has been a boon because of the increasing demand for corticosteroids, contraceptives, sex hormones, and anabolic steroids since about 1960 (Hall and Walker, 1991). To-date, diosgenin and related steroidal saponins were commercially obtained from the different tissues of the plant that contains diosgenin however, it is crucial to discover new and alternative sources of these compounds due to decreasing plant resources as well as increasing demand (Savikin-Foduloic et al., 1998).

Balanites aegyptiaca (L.) Delile (Zygophyllaceae), popularly known as "desert date," is a spiny evergreen tree. The tree is grown in arid regions of Africa, the Middle East, India, and Burma and is famous for various traditional folk medicines (Hall and Walker 1991). Studies have shown that steroidal sapogenins are present in all parts of *B. aegyptiaca* and that most of them are derived from diosgenin (Kamel and Koskinen 1995; Neuwinger 1996). *B. aegyptiaca* is not confined only to medicinal uses; this medium to high growth tree is used for fodder, fire wood, and timber as well. The sweet pulp fruit of *B. aegyptiaca* is edible and many food products such as juice and an alcoholic beverage are additionally produced from it. The kernel of the fruit contains a high percentage of oil which is used both for culinary purposes as well as cosmetics proposes (Chapagain and Wiesman, 2005). With increasing human pressure for its valuable uses, the tree is being extensively felled (Ndoye et al. 2003). Although the tree regenerates naturally by seed or coppice, Balanites is endangered because of the high rate of deforestation (Nour et al. 1991). In this circumstance an alternate approach would be an advantageous which could exploit this currently neglected plant and the *in- vitro* production of natural diosgenin from callus cultures might be an alternative biotechnological approach to produce diosgenin. Since the *In vitro* production of bioactive secondary metabolites is not limited by seasonal or regional restriction and can be run in controlled environment where possibility exists to screen culture with higher production (Mulabagal and Tsay, 2004). Plant species e.g., *Dioscorea floribunda*, *Solanum aculeatissimum* and *Trigonella foenum-graecum* have already been evaluated for their ability to produce sapogenin *in vitro* (Ikenaga et al., 2000; Oncina et al., 2000). Sapogenin from mature shoot derived callus in *Balanites agyptiaca* have been reported (Suthar et al., 1980). However, the development of cell lines in suspension or semi solid culture medium, which is a pre-requisite for secondary metabolite production, has been not evaluated in *B. aegyptiaca*.

In the present study an attempt was made to find out the effects of growth regulators, explants and somatic embryogenesis on sapogenin accumulation in *B. aegyptiaca* callus. The production of the sapogenin (diosgenin) by callus cultures of *B. aegyptiaca* is described.

Materials and methods

Plant material

Seeds from ripe *B. aegyptiaca* fruit were collected from the Balanites orchard maintained by Ben-Gurion University of the Negev at Kibbutz Samar in southern Israel. Authentication of the plant was carried out by Prof. Uzi Plitman from the herbarium in the Hebrew University of Jerusalem. Voucher specimen (76816) was deposited in the herbarium of the Hebrew University of Jerusalem. The seeds were surface sterilized with 90% (v/v) ethanol for 1 min, washed three times with sterilized distilled water, surface sterilized again with 3.5% aqueous sodium hypochlorite containing a few drops of Tween 20 (Sigma) for 10

min, and finally rinsed three times in sterile double-distilled water. The seeds were then placed in tubes containing enriched MS (Murashige and Skoog, 1962) medium prepared by supplementing with 3% sucrose, gelled with 0.8% (w/v) Phytagel™ (Sigma) and adjusted to pH 5.7, followed by autoclaving at 121ºC and 104 kPa for 20 min. All cultures were maintained at 24 ± 1ºC under a 16 h/8 h light/dark regime, light being provided by cool white fluorescent lamps giving a photon flux of 30 µmol m^{-2} s^{-1}.

Callus induction and somatic embryogenesis

For callus induction 20 day-old-explants of hypocotyls, epicotyls, roots and shoots were excised (4-6 mm) and placed in MS basal medium supplemented with different concentrations (1, 2.5, and 3 mg l^{-1}) of 2,4-D and (1, 2, 5 mg l^{-1}) of NAA alone and with combination of (0.5 mg l^{-1}) BAP. Calli were subculture at 3-week interval. Cell suspension cultures were established for further study of somatic embryogenesis by suspending 1 g of fresh callus (2-4 mm size) in 25 ml MS medium supplemented with (2.5 and 1.0 mg l^{-1}) 2,4-D and (2.5 and 1.0 mg l^{-1}) NAA alone and with combination of 0.5 mg l^{-1} BAP in 25 ml Erlenmeyer flasks. Cultures were grown on shaker at 120 rpm at 24 ± 1 º C. Study of somatic embryogensis were performed by a WAT-221S Image Sensor (Japan). For diosgenin determination and callus growth assessment callus cultures from media were collected after 5 weeks.

Saponin extraction

Balanites callus of the root, shoot, hypocotyls and epicotyls obtained from solid media was dried in an oven at 50 º C for 72 h and powdered mechanically. In case of the suspension culture the callus was separated from liquid media by filtration through Whatmann-1 filter paper and callus mass rinsed twice with de-ionized water. Then it was dried and powdered. The powdered was defatted by using n-hexane and the crude saponin was extracted as described by the Chapagain and Wiesman (2005).

Diosgenin determination

Diosgenin was determined as described by Baccou et al. (1977) and Uematsu et al. (2000), with some modification by Chapagain and Wiesman (2005). Standard sapogenin (diosgenin) and *p*-anisaldehyde (4-methoxy-benzaldehyde) were purchased from Sigma. Sulfuric acid and ethyl acetate were both analytical grade and obtained from Frutarom. The diosgenin level was determined by measuring absorbance at 430 nm, based on the color reaction with anisaldehyde, sulfuric acid and ethyl acetate. In brief, two color developing reagent solutions were prepared: (A) 0.5 ml *p*-anisaldehyde and 99.5 ml ethyl acetate, and (B) 50 ml concentrated sulfuric acid and 50 ml ethyl acetate. 200 µg of the crude saponin extract of callus was placed in a glass tube. To this, 1 mg of defatted crude saponin extract of callus powder was first dissolved in 1 ml methanol, and 200 µl of this solution was placed in another tube; the methanol was evaporated under reduced pressure. This residue was dissolved in 2 ml of ethyl acetate; 1 ml each of reagents A and B were added to the tube and stirred. The test tube was placed in a water bath maintained at 60ºC for 10 min. to develop color, then allowed to cool for 10 min. in 25ºC water bath. The absorbance of the color developed solution was measured in a spectrophotometer (V-530- UV/VIS, JASCO Corp., Japan). Ethyl acetate was used as a control for the measurement of absorbance. As a reagent blank, 2 ml ethyl acetate was placed in a tube and assayed in similar manner. For the calibration curve, 2–40 µg standard diosgenin in 2 ml ethyl acetate was used.

Data analysis

For each treatment at least six glass tubes (9x3 cm) that contained four explants in each were used for callus growth (as dry weight) and Diosgenin determination. Five flasks for each treatment were used for cell suspension culture. Results were statically analyzed with JMP software (SAS, 2000), using the Tukey-Kramer HSD test for determining significant differences among treatments at $p \leq 0.05$.

Results

Effect PGR and explants on callus growth

The effect of plant growth regulators (PGR) on callus growth from the root, hyptocotyl, shoot and epicotyl explants of *B. aegyptiaca* plant were assessed by measuring the dry weight (DW) of the callus on per tube basis after 5 weeks. The results are presented in Table 1. In all explants the callus DW was found increasing with increasing concentration of 2,4-D on MS basal media. In root, shoot and hypocotyl, no significant different was found in callus DW in between 2.5 mgl–1 and 3 mgl–1 2,4-D on MS basal media however in the epicotyl, the highest callus DW was found with 3 mgl–1 2,4-D which was also the highest DW among all explants used in this experiment. Same pattern of DW was also found with an addition of 0.5 mgl–1 BAP to 2,4-5 to MS basal media. In 1.0 mg 2,4-D + 0.5 mg BAP treatment the callus DW was higher in all explants compared to the 1 mg 2,4-D alone. In the root and epicotyl callus slightly higher DW was found in 2.5 mgl–1 2,4-D + 0.5 mgl–1 BAP compared to the sole 2.5 mgl–1 2,4-D but in shoot and hypocotyl, the callus DW was slightly decreased. In 3mg 2,4-D + 0.5 mg BAP the DW was decreased in all callus than the sole 2,4-D.

With supplementation of NAA on MS basal media, the DW was found much lesser than the 2,4-D supplement. The addition of 0.5 mg l–1 BAP in NAA increased the DW compared to the sole NAA however it was still less than the 2,4-D and/or 2,4-D and BAP supplement.

Effect of PGR and explants on diosgenin content

The level of diosgenin (based on the dry weight basis) was found highest in 1 mg l^{-1} 2,4-D on MS basal media to all four calluses (root, shoot, hypocotyl and epicotyl) (Table 2). With increasing 2,4-D from 1mgl–1 to 2.5 mgl–1 on MS basal media, the diosgenin content was decreased significantly. A 27, 25, 22 and 31 percent of diosgenin was decreased when 2,4-D was increased from 1.0 mgl–1 to 2.5 mgl–1 on Ms basal media, in root, shoot, hypocotyl and epicotyl callus respectively. The diosgenin level was further decreased when used 3 mgl–1 2,4-D however, it was not significant than the 2.5 mg l^{-1} concentration in all four calluses. Among the four callus, shoot derived callus contented highest level of diosgenin in all concentration of 2,4-D on MS basal media. When an addition of 0.5 mg l–1 BAP to 2,4-D was used, the level of diosgenin was found lower compared to the sole 2,4-D supplement.

In both either sole NAA at 1.0, .2.0 and 5.0 mg l^{-1} or additional 0.5 mg l^{-1} BAP on MS basal media, the diosgenin content in all callus was found less than the either sole 2,4-D or 2,4-D with BAP experiment. NAA on MS basal media gave lowest diosgenin in the callus and when additional 0.5 mgl–1 of BAP was added the diosgenin content increased. When diosgenin level with in the different concentration of NAA was compared, the diosgenin

level was significantly increased from 1mg l^{-1} to 2 mg l^{-1} on Ms basal media to all callus but when increase the NAA from 2.0 mgl–1 to 5.0 mg l^{-1} no significant increase was found in diosgenin level in any callus. In NAA+BAP experiment the level of diosgenin was found slightly increased compared to sole NAA in all callus.

A significant amount of diosgenin was detected in 2,4-D and BAP growth regulators compared to NAA and BAP treatment. Hence the effects 2,4-D and BAP growth regulators found significant for diosgenin and callus growth. Excluding root callus there was no significant difference in sapogenin accumulation amongst different explant calli. Root calli accumulate significantly lower amount of sapogenin as well as callus DW compared to other explants callus culture.

Effect of somatic embryogenesis on diosgenin content

Three different types of callus were detected on the basis of their embryogenic potential, morphology and effect of sub culturing on different culture media (Table-3). Callus type-1, showed initial high growth proliferation but with further subculture, it lost its initial growth rate and embryogenic potential (Fig 1A). Callus type-2 had intermediate characters (Fig 1B). Callus type-3 (pre-embryogenic callus) that was derived from root explants showed a suitable callus material to maintaining them for a long time period with stable growth characters i.e. proliferation and embryogenic potential (Fig 1C). They also showed a rapid induction of synchronous somatic embryogenesis (Fig-1D).

Cell suspension culture was established from pre-embryogenic callus (Callus type-3) from the root derived callus and rapid somatic embryogenesis was observed in liquid culture. After 5 weeks diosgenin was determined in cultures. The number of somatic embryos as well as diosgenin level was found highly affected by differ in the plant growth regulators (Table 4). The number of somatic embryos was found higher in 2,4-D and/or BAP supplementation on MS basal media, compared to the NAA and/or BAP. The highest number of somatic embryos (382) was found in 1.0 mg 2,4-D + 0.5 mg BAP followed by 2.5 mgl–1 2,4-D + 0.5 mgl–1 BAP (300), 1.0 mgl–1 2,4-D (290) and 2.5 mgl–1 2,4-D (245) respectively. Media containing NAA alone and with combination of BAP enhance significant amount of Diosgenin accumulation in callus culture (0.71 to 0.77 mg / g callus DW) compared to the 2,4-D alone and with combination of BAP (0.17 to 0.31). Accumulation of diosgenin was found to be decreased significantly when callus suspension culture favoured somatic embryogenesis**.**

Discussion

According to earlier reports (Suthar et al., 1980), maximum amount of diosgenin was found 0.5 mg / g of callus DW in mature shoot derived callus in *B. aegyptiaca*, while in our system *in vitro* excised shoot derived callus accumulate 2.23 mg diosgenin / g callus DW while supplemented 1 mg l^{-1} of 2,4-D on MS basal media which established the superiority of the present method. This result suggests that shoot derived calli are the most suitable starting material for obtaining diosgenin *in vitro* in *B. aegyptiaca*. The results also show that MS basal media supplemented with 2,4-D alone and with combination of BAP induced significant callus growth but reduced diosgenin accumulation in a dose dependent manner, which also confirms earlier report of *Solanum aculeatissimum* (Ikenaga et al., 2000) that also revealed that increasing concentration of 2,4-D inhibit the steroidal saponin synthesis as well as growth of callus. Our results showed that BAP was effective for diosgenin

accumulation when it is used with lower concentration of 2,4-D in most of the explants calli. The effect of NAA alone or with BAP on callus DW and diosgenin accumulation was not significant (Table and 2). In general, increasing concentration of 2,4-D showed a negative effect on diosgenin accumulation but it induced callus growth in all explants examined. Maximum amount of diosgenin accumulated (2.23 mg/ g callus DW) in shoot callus on 1 mg l^{-1} 2,4-D containing media followed by hypocotyl, epicotyl and root callus. These results strongly indicated that selection of explants and growth regulators play an important role in diosgenin production as reported earlier (Oncina et al., 2000).

A considerable difference in embryogenic potential, morphology and response to sub culture among the three types of callus cultures (Callus type-1, 2 and Callus type-3) was found. Callus type-3 (pre-embryogenic callus) continuously cultured for six months on 2.5 mg l^{-1} 2,4-D combined with 0.5 mg l^{-1} BAP, was found to maintain the initial embryogenic potential and proliferation. However it was observed that development of somatic embryo from pre-embryogenic callus and diosgenin accumulation significantly vary amongst different plant growth regulators and amount of somatic embryos in callus culture. Furthermore, in all treatments diosgenin content decreased significantly when callus suspension culture favored somatic embryogenesis. Minimum numbers of somatic embryo (32 to 68 / g calli) was obtained in media contain either NAA alone or with combination of BAP where as a range of 0.71 to 0.77 mg diosgenin / g callus DW was dictated, which was the optimum amount of diosgenin we got form callus suspension culture. Theses results predict a negative correlation between diosgenin accumulation and somatic embryogenesis.

Mass culture of cells in bioreactors has generated considerable research effort in recent decades, especially with rare and slow-growing plant species, but with little attention devoted to commercial application, aside from research done with *P. ginseng* for saponin production (Asaka et al. 1993; Wu and Lin, 2002). In this study we have achieved production and maintenance of a pre-embryogenic callus culture, an initial step in developing an industry around *B. aegyptiaca*, in our case it has been found that the stable embryogenic callus culture system of *B. aegyptiaca*. Though the level of diosgenin content was found lower however, the achievement of a high proliferation rate of embryogenic callus culture in *B. aegyptiaca*, this can open up a possible suitable system for bioproduction of diosgenin *in vitro* and this phenomenon can be further studied for the possible role of diosgenin in embryo development. Since *in vitro* production of secondary compound is an independent system in regards to the environment, this could play a vital role for the alternate production of diosgenin. This would have immense effects in future study of secondary metabolites in *Balanites aegyptiaca* which might certainly help to explore the currently most neglected desert plant.

Table1. Effect of plant growth regulators (PGR) and explants on growth (DW) of *Balanites aegyptiaca* callus.

PGR (mg / L)	Callus DW (mg / tube)			
	Root	Shoot	Hypocotyl	Epicotyl
2,4-D				
1.0	27 b	35 b	48 b	55 c
2.5	44 a	78 a	86 a	77 b
3.0	47 a	81 a	90 a	93 a
2,4-D+ BAP				
1.0 + 0.5	30 b	37 b	63 b	65 c
2.5 + 0.5	45 a	65 a	78 a	84 b
3.0 + 0.5	46 a	67 a	82 a	90 a
NAA				
1.0	11 a	19 a	42 a	41 a
2.0	15 a	23 a	51 a	44 a
5.0	12 a	20 a	52 a	47 a
NAA+ BAP				
1.0 + 0.5	25 a	26 a	50 a	58 b
2.0 + 0.5	32 a	30 a	55 a	80 a
5.0 + 0.5	31 a	31 a	60 a	74 a

Means (n=3) in each column followed by different letters are significantly different at $p \leq 0.05$.
MS basal media supplemented with different conc. of plant growth regulators (PGR) were used for callus induction from various explants. Diosgenin was determined after five weeks on culture media.

Table2. Effect of plant growth regulators (PGR) and explants on diosgenin content in *Balanites aegyptiaca* callus.

PGR (mg / L)	Diosgenin (mg / g DW)			
	Root callus	Shoot callus	Hypocotyl callus	Epicotyl callus
2,4-D				
1.0	1.68 a	2.23 a	2.14 a	2.12 a
2.5	1.22 b	1.77 b	1.67 b	1.46 b
3.0	1.18 b	1.60 b	1.57 b	1.27 b
2,4-D+ BAP				
1.0 + 0.5	1.42 a	2.11 a	1.93 a	1.91 a
2.5 + 0.5	0.68 b	1.15 b	0.86 b	1.33 b
3.0 + 0.5	0.64 b	1.08 c	0.78 b	0.75 c
NAA				
1.0	0.09 b	0.22 b	0.52 b	0.70 b
2.0	0.16 a	0.39 a	0.61 a	0.87 a
5.0	0.21 a	0.43 a	0.68 a	0.92 a
NAA+ BAP				
1.0 + 0.5	0.58 a	0.63 a	1.15 a	0.95 a
2.0 + 0.5	0.56 a	0.62 a	1.09 a	0.98 a
5.0 + 0.5	0.40 b	0.49 b	0.81 b	0.67 b

Means (n=3) in each column followed by different letters are significantly different at $p \leq 0.05$.
MS basal media supplemented with different conc. of plant growth regulators (PGR) were used for callus induction from various explants. Diosgenin was determined after five weeks on culture media.

Table 3. Effect of PGR and explants of B. aegyptiaca on production of there different types of callus. Type 1 and Type 2 callus are non embroygenic and Type 3 callus in pre-embryogenic which under go somatic embryogenesis.

PGR (mg / L)	Explants			
	Root callus	Shoot callus	Hypocotyl callus	Epicotyl callus
2,4-D				
1.0	Type-2	Type-1	Type-1	Type-1
2.5	Type-3	Type-1	Type-2	Type-2
3.0	Type-1	Type-1	Type-1	Type-1
2,4-D+ BAP				
1.0 + 0.5	Type-2	Type-1	Type-1	Type-1
2.5 + 0.5	Type-3	Type-1	Type-1	Type-2
3.0 + 0.5	Type-2	Type-1	Type-1	Type-1
NAA				
1.0	Type-2	Type-1	Type-1	Type-1
2.0	Type-2	Type-1	Type-2	Type-1
5.0	Type-2	Type-1	Type-2	Type-1
NAA+ BAP				
1.0 + 0.5	Type-2	Type-1	Type-2	Type-1
2.0 + 0.5	Type-1	Type-1	Type-1	Type-1
5.0 + 0.5	Type-1	Type-1	Type-1	Type-1

Callus cultures were selected in *Balanites aegyptiaca* on the basis of proliferation and embryogenic potential amongst different explants calli on MS basal media containing different concentration of plant growth regulators.

Table 4. Effect of plant growth regulators (PGR) on somatic embryogenesis and/or accumulation of diosgenin in cell suspension cultures of *Balanites aegyptiaca* root derived callus. **I** and **II,** 2, 4-D 1.0 and 2.5 mgl–1 ; **III** and **IV**, 2,4-D 1.0 and 2.5 mgl–1 + 0.5 mgl–1 BAP; **V** and **VI,** NAA 1.0 and 2.5 mgl–1 ; **VII** and **VII**, NAA 1.0 and 2.5 mgl–1 + 0.5 mgl–1 BAP.

PGR	Somatic embryo (No. /g calli)	Diosgenin (mg / g callus DW)
I	292 c	0.29 b
II	245 d	0.31 b
III	382 a	0.17 c
IV	300 b	0.20 c
V	32 f	0.72 a
VI	28 f	0.77 a
VII	68 e	0.73 a
VIII	56 e	0.71 a

Means in each column followed by different letter are significantly different at $p \leq 0.05$.
Different amount of somatic embryos and diosgenin level obtaining from MS basal liquid medium supplemented with different concentration of PGR. Pre-embryogenic calli (callus type-3) from root were used as source of callus for cell suspension culture.

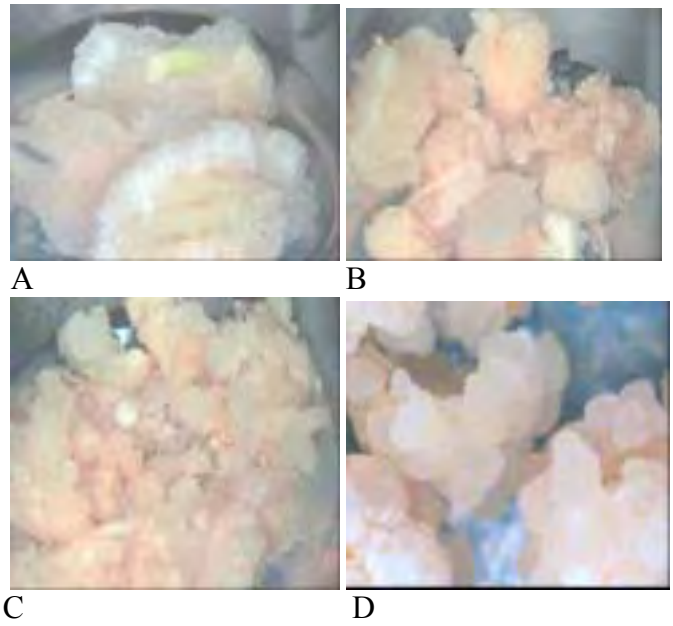

Fig 1. Different callus types of *B. aegyptiaca*. (A) Callus type-1 (B) Callus type-2 (C) Callus type-3 (pre-embryogenic callus) which under go somatic embryogenesis in MS basal liquid media with different concentration of plant growth regulators showed (D) synchronous somatic embryogenesis.

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Larvicidal effects of aqueous extracts of *Balanites aegyptiaca* (desert date) against the larvae of *Culex pipiens* mosquitoes

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Abstract

The effect of aqueous extracts of the fruit pulp, seed kernel, roots, bark, and leaves of *Balanites aegyptiaca* Del. (Zygophyllacea) against the larvae of the *Culex pipens* mosquito was investigated. Early fourth instars larvae of *Culex pipiens* mosquitoes were exposed, for up to three days, to a dilution of 0, 0.1, 0.25, 0.5, 1.0, and 2.0 percent aqueous extracts of fruit pulp, seed kernel, roots, bark, and leaves. All tested extracts showed larval mortality, however,larval mortality was greatest with the aqueous root extract. The lowest concentration of root extract (0.1%) showed 100% larval mortality after three days, whereas a 0.5% concentration of aqueous bark extract was needed for 100% larval mortality. Aqueous extracts of leaf, fruit pulp, and seed kernel showed less larval mortality compared to the root and/or bark extracts. It is suggested that all parts of the *B. aegyptiaca* contain larvicidal properties that could be developed and used as natural insecticides for mosquito control.

Key words: *Culex pipens*; mosquito; desert date; *Balanites aegyptiaca*

Introduction

Mosquitoes constitute a major public health menace as vectors of serious human diseases (El Hag et al., 1999). *Culex pipiens* (northern house mosquito) is the vector of the West Nile Virus (WNV) that causes encephalitis or meningitis. The disease affects the brain tissue and the most serious cases can result in permanent neurological damage and be fatal (Hubalek and Kaluuzka, 1999). WNV is distributed throughout Africa, the Middle East, and southern temperate and tropical Eurasia, and was recently introduced into North America as well (Campbell et al., 2002). There is no vaccine to prevent this infection, nor are there drugs to combat the disease in infected persons, so vector control is the most prevalent solution available so far for reducing morbidity. Most of the widely used vector interruption methods are synthetic insecticide-based. These synthetic insecticides not only affect the non-target population, but also constantly increase resistance to the vector (Wattal et al., 1981). The search for natural insecticides which do not have any ill effects on the non-target population and are easily degradable remains the top priority (Redwane et al., 2002).

Balanites aegyptiaca Del.*,* also known as 'desert date' in English, a member of the family Zygophyllaceae, is the one of the most common but neglected wild plant species of the dryland areas of Africa and South Asia (Hall and Walker, 1991). The tree can grow to 6–10 meters in height, is highly resistant to stresses such as sandstorms and heat waves, and grows with minimal available moisture. The tree has thick, tough glossy leaves, spiny

branches, and a double root system, and produces date-like fruits. The plants grow extensively even when neglected. One estimate is that more than 400,000 tons of Balanites fruit are produced in Sudan alone (Mohamed et al., 2000). It can successfully grow in a marginal sand dune with saline and sewage water (data not yet published). Various parts of the Balanites tree have been used for folk medicines in many regions of Africa and Asia (Hall and Walker, 1991; Iwu, 1993; Newinger, 1996; Mohamed et al., 2002). A literature survey has revealed antifeedent, antidiabetic, molluscicide, antihelminthic, and contraceptive activities in various *Balanites* extracts (Liu and Nakanishi, 1982; Jain and Tripathi, 1991; Kamel et al., 1991; Ibrahim, 1992; Rao et al., 1997). Most of the studies reported the active compounds to be saponins.

Saponins are amphiphilic glycosidic compounds with lipophilic nuclear aglycone and a hydrophilic sugar chain. Saponins are freely soluble in both organic solvents and water (Hostettman and Marston, 1995). During the course of last two decades, many plant extracts have been evaluated for their larvicidal activities (Jang et al., 2002), in order to find a method of biological control of mosquitoes; very few studies were done about the larvicidal effect of saponin-rich plants (Pelah et al., 2002). However, most of the studies focused on organic solvent extracts. Taking the bioactivity of saponin compounds into consideration, and the presence of these compounds in *Balanites aegyptiaca*, along with the severity of mosquito-borne diseases and the socioeconomic situation in Balanites grown areas, this study was carried out on the larvicidal effect of the various extracts using simple procedures, of the Balanites plant against *Culex pipens* larvae.

Materials and methods

Extracts of fruit pulp, seed kernel, leaf, root, and bark of *B. aegyptiaca* were prepared from plant material collected at the Ben-Gurion University of the Negev's Balanites plantation site at Kibbutz Samar, Israel. Extraction of all the parts was carried out using as simple a method as possible, thinking that it could be repeated any remote areas of the world where Balanites plants grow naturally. The idea is to allow for easy adoption of this methodology by the local communities. The outer cover (epicarp) of the fruit was removed by hand and the pulp was scraped manually. The endocarp (stone) of the fruits was broken manually and the seed kernel was then collected, dried, and pulverized. To prepare the extracts from leaf, bark, and root, fresh leaves, bark, and roots were collected, washed, chopped, dried, and ground to powder. Similarly, the fruit pulp was dried and powdered. Twenty grams of each ground/pulverized part were placed in separate Erlenmeyer flasks, 100 ml of tap water was added, and mixed vigorously. The mixture was kept 24 h with occasional shaking. After twenty-four hours, the mixture was filtered using a very fine muslin cloth and the final volume adjusted to 100 ml. A series of dilutions (0, 0.1, 0.2, 0.5, 1.0, and 2.0 %) was prepared using this stock solution using tap water.

Eggs of the *Culex pipens* mosquito were obtained from the Entomology Laboratory of the Israel Ministry of Health, Jerusalem, and necessary larvae were prepared in the laboratory of the Institutes of Applied Research as described by the standard WHO protocol (1973). Twenty larvae of the early fourth instars were placed in a 250 ml disposable plastic cup containing 100ml of treatment solution. After adding the larvae, the plastic cups containing the larvae were kept in the growth room maintained at room temperature. The effects of the extracts were monitored by counting the number of dead larvae each day up to three days. No adult emergence was observed, including in the control (0 %), during the three days.

The data obtained were statistically assessed for mean and standard error of the mean (±SE) using JMP software (SAS, 2000). Each treatment was conducted twice with similar results; data here represent a single independent experiment.

Results and discussion

The effects of the various aqueous extracts of *B. aegyptiaca* on the mortality of the *Culex pipiens* mosquito larvae are presented in Tables 1–5. With kernel extract treatment there was less than 50% mortality of larvae on the first day, even at the highest concentration. The second day, both 1 and 2% concentrations killed more than 50% of the larvae. The third day, both 1 and 2% concentrations killed more than 90% of the larvae but neither treatment killed 100%. A weaker pattern was shown in fruit pulp and leaf extract treatments than the kernel extract treatment. The highest concentration (2%) of both the pulp extract and leaf extract showed only 20% larval mortality the first day. Only the highest concentration of both these extracts killed approximately 90% of the larvae after 3 days. As with the kernel extract treatment, none of these treatments killed 100% of the larvae within three days. Both bark and root extract showed a very high mortality. Root extract showed the highest rate of larval mortality compared to all treatments. More than 30% of the larvae were found dead in the first day, even at the lowest concentration (0.1%) of root extract. The third day, all tested concentrations of root extract killed 100% of the tested larvae population. The first day, the bark extract was found to be even more effective than root extracts. However, at the end of the experiment (3 days), only more than 0.5% concentration of the bark extract killed 100% of the larvae. The control (0%) showed no larval mortality on any day. A gradient of increasing mortality with increasing concentration was observed in all treatments.

This work demonstrates the potency of *B. aegyptiaca* extract in the control of *Culex pipiens* mosquito larvae. Root extract seemed the most lethal, followed by bark, among the various parts tested. Earlier studies have shown these tissues of *Balanites aegyptiaca* plants contain high amounts of saponins (Liu and Nakanishi, 1982; Jain and Tripathi, 1991; Kamel et al., 1991; Farid et al., 2002), so the high mortality of various parts of the extract might be attributed to the presence of saponin compounds in the Balanites tissues. Interaction of saponin molecules with the cuticle membrane of the larvae, ultimately disarranging this membrane by the association of the saponin molecule with these membranes (Morrissey and Osbourn, 1999), could be the most probable reason for the larvae death. The deficiency of dissolved oxygen in the water due to the active presence of the antioxidant saponin molecule could not be ignored. However, the mechanism by which saponin kills the larvae is the subject of research currently under way by our team. Previous studies have shown that many plant extracts do possess insecticidal properties (Arnason et al., 1989). Our results are in line with these results. This study shows that aqueous extracts of the Balanites plant can be used as environmentally-friendly and sustainable insecticides to control mosquitoes.

Table 1. Mortality of *Culex pipiens* mosquito larvae from different concentrations of aqueous extracts of Balanites kernel. Values are the mean of 3 (n=3 ± SE).

Kernel extract concentration (%)	Mortality (%)		
	1 day	2 day	3 day
0.0	0 ± 0	0 ± 0	0 ± 0
0.1	7 ± 2	10 ±3	27 ± 2
0.2	10 ± 3	27 ± 2	43 ± 2
0.5	25 ± 3	40 ± 2	68 ± 2
1.0	30 ± 3	70 ± 2	92 ± 2
2.0	38 ± 2	73 ± 2	95 ± 2

Table 2. Mortality of *Culex pipiens* mosquito larvae from different concentrations of aqueous extracts of Balanites root. Values are the mean of 3 (n=3 ± SE)

Root extract concentration (%)	Mortality (%)		
	1 day	2 day	3 day
0.0	0 ± 0	0 ± 0	0 ± 0
0.1	33 ± 2	70 ± 3	100 ± 0
0.2	52 ± 2	90 ± 0	100 ± 0
0.5	55 ± 0	92 ±2	100 ± 0
1.0	78 ± 2	93 ± 2	100 ± 0
2.0	83 ± 2	95 ± 0	100 ± 0

Table 3. Mortality of *Culex pipinens* mosquito larvae from different concentrations of aqueous extracts of Balanites bark. Values are the mean of 3 (n=3 ± SE)

Bark extract concentration (%)	Mortality (%)		
	1 day	2 day	3 day
0	0 ± 0	0 ± 0	0 ± 0
0.1	48 ± 2	75 ± 2	80 ± 2
0.2	63 ± 2	88 ± 2	93 ± 2
0.5	72 ± 2	92 ± 2	100 ± 0
1.0	80 ± 2	95 ± 0	100 ± 0
2.0	100 ± 0	100 ± 0	100 ± 0

Table 4. Mortality of *Culex pipiens* mosquito larvae from different concentrations of aqueous extracts of Balanites fruit pulp. Values are the mean of 3 (n=3 ± SE)

Fruit pulp extract concentration (%)	Mortality (%)		
	1 day	2 day	3 day
0	0 ± 0	0 ± 0	0 ± 0
0.1	0 ± 0	0 ± 0	28 ± 2
0.2	0 ± 0	5 ± 3	32 ± 3
0.5	0 ± 0	7 ± 2	60 ± 3
1.0	5 ± 3	20 ± 3	78 ± 2
2.0	20 ± 3	35 ± 2	88 ± 2

Table 5. Mortality rate of *Culex pipiens* mosquito larvae from different concentrations of aqueous extracts of Balanites leaf. Values are the mean of 3 (n=3 ± SE)

Leaf extract concentration (%)	Mortality (%)		
	1 day	2 day	3 day
0.0	0 ± 0	0 ± 0	0 ± 0
0.1	0 ± 0	0 ± 0	12 ± 3
0.2	0 ± 0	0 ± 0	23 ± 3
0.5	7 ± 2	12 ± 2	30 ± 5
1.0	8 ± 2	25 ± 3	70 ± 5
2.0	20 ± 3	75 ± 3	92 ± 3

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Larvicidal Activity of the Fruit Mesocarp Extract of *Balanites aegyptiaca* and its Saponin Fractions against *Aedes aegypti*

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Abstract
The present study indicates the efficacy of the saponins fraction of the fruit mesocarp extract of *Balanites aegyptiaca* Del. (Zygophyllace) as a larvicidal agent against the common dengue vector – *Aedes aegypti* mosquito larvae. A series of concentrations of fruit mesocarp extract of *B. aegyptiaca* fruit, its crude saponin extract and pure saponin fraction were tested against the laboratory-grown third instars larvae and compared with their LC_{50} values. The pure saponin fraction was found most active larvicidal, followed by crude saponin extract and mesocarp extract. The LC_{50} values of pure saponin extract, crude saponin extract and mesocarp extract were 145, 315 and 935 ppm respectively. In separate experiments, concentrations of 35 ppm, 260 ppm and 850 ppm of pure saponin fraction, crude saponin extract and mesocarp extract respectively were found to inhibit the emergence of the 50% of the tested larvae population (EC_{50}). The pure saponin fraction also interfered with adult emergence.

Keywords: *Balanites aegyptiaca*; saponins; *Aedes aegypti*, dengue; larvicidal; adult emergence.

Introduction
Dengue is an acute fever which occurs as a dengue fever or dengue haemorrhagic fever (DF/DHF). It is a serious vector-borne disease caused by a virus and transmitted by *Aedes aegypti* mosquito. This globally prevailing disease has recently grown enormously. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, South-East Asia and the Western Pacific. Every year more than 100 million people residing in these areas are being infected by DF/DHF[1].

There is no vaccine to prevent dengue infection, nor are there drugs to combat the disease in infected persons, so vector control is the most opted solution available so far for reducing the morbidity. Most of the widely used vector interruption methods are synthetic insecticides-based. These synthetic insecticides not only affect the non-target population but also increase resistance to the vector[2]. In this regard, the search for natural insecticides which do not have any ill effects on non-target population and are easily degradable is of top priority these days[3].

In an earlier communication we have pointed out saponin-rich extracts from *Balanites aegyptiaca* and *Quillaja saponaria* as a possible candidate for natural bioactive agent against *Aedes aegypti* and *Culex pipeins* mosquito larvae[4]. However, high concentrations of these preparations were needed for effective control of the mosquito larvae. In continuation of our study we have investigated a saponin fraction, which in much smaller concentration, is found to be active to control the mosquito larvae. Moreover, as adult mosquitoes transmit diseases, the critical concentrations of botanicals, which inhibit 50% of the treated larval population from emerging as adults (EC_{50}), are more meaningful than the LC_{50}[5,6]. Since the determination of effective concentrations that inhibit adult emergence

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has not received the attention of many researchers, in this communication we report the efficacy of *B. aegyptiaca* saponin compared with its crude extract against the *Ae. aegypti* larvae control with concerned LC and EC values.

Materials and methods

Plant materials, extraction and isolation

Balanites aegyptiaca Del. (Zygophyllaceae) is a widely grown woody tree of the dryland areas. It is widely grown in the African continent, the Middle-East and South Asia[7]. *B. aegyptiaca* plant tissues have been used as folk medicines in Africa and Asia. The fruit consists of epicarp (5–9%), mesocarp (28–33%), endocarp (49–54%) and kernel (8–12%). One estimate shows that every year more than 4 million tons of *B. aegyptiaca* fruits are produced in Sudan alone[8].

The mesocarp (pulp) was scraped from well-ripened fruits collected from the Arava-rift valley of Israel. A voucher specimen (#76816) was deposited at the herbarium of the Hebrew University of Jerusalem. The scraped mesocarp was freeze-dried and then powdered and defatted by petroleum ether 3x1 h at 40 °C. After filtering the petroleum ether, the marc was extracted with methanol 3x1 h with mild heating. The combined methanol extract was concentrated and methanol extract of mesocarp (MCE) was obtained. In order to get the crude saponins extract the MCE was dissolved in methanol and acetone was added (1:5 v/v) to precipitate the saponins as described by Yan et al., 1996[9]. The precipitate was dried under vacuum, turning to a whitish amorphous powder named as crude saponin extract (CSE). To get the pure saponin fraction (PSF), certain amount of CSE was fractionated by applying to Merck silica gel-60 (230-400 mesh) column chromatography and eluted successfully with chloroform-methanol-water (70:30:10) as described by Favel et al., 2005[10]. Five fractions were collected and the solvents were evaporated under reduced temperature; fraction 1 was chosen based on the detection of the total saponin concentration. The total saponin concentration of each fraction was measured by spectrophotometric method as described by Baccou et al., 1977[11] and Uematsu et al., 2000[12] with some modification. The saponin fraction was named as PSF. From the fridge-dried fruit mesocarp, a yield of 63.5%, 13.3% and 4.5% (w/w) of each methanol extract (MCE), crude saponin extract (CSE) and pure saponin fraction (PSF) was obtained (Table 1).

Table 1. Yield and LC_{50} of different extract and fraction of *B. aegyptiaca* fruit mesocarp used in experiment against third instars larvae of *Ae. aegypti*

Treatment	Yield (%)	LC_{50} (ppm)
Methanolic extract of mesocarp (MCE)	63.5±3.1	935±25.3
Crude saponin extract (CSE)	13.3±0.9	315±12.9
Pure saponin fraction (PSF)	4.5±0.4	145±6.2

Each value is the mean of 3±SE (n=3). Yield represents the % (w/w) of each treatment materials obtained from the fridge-dried fruit mesocarp

Bioassay test

Eggs of the *Ae. aegypti* mosquitoes were obtained from the Entomology Laboratory of the Israel Ministry of Health, Jerusalem, and necessary larvae were reared in the laboratory

of the Institutes for Applied Research, Ben-Gurion University, as described by the standard protocol of WHO, 1973[13]. Twenty larvae of third instars were placed in a 250 ml disposable plastic cup containing 100 ml of treatment solutions. After placing the larvae the plastic cups were kept in the growth room maintained at 27±2 °C with 16 h day (light period) and 40±5 % relative humidity. A multiple 2-fold concentrations were set up to establish a working range (from 25 to 3200 ppm) in triplicate manner and LC_{50} was calculated. With each experiment, a set of control using just tap water (untreated set) was also run for comparisons.

The effects of the treatments were monitored by counting the number of dead larvae each day. For the LC_{50}, the data of 48 h was used because till that time no pupa was observed even in control treatments. During the course of the experiment, a food based on baby food was provided to the larvae. The percentage of mortality was corrected using Abbott's formula as suggested by Finney, 1971[14].

In another series of experiments, observations on the emergence and larval duration of the larvae that were reared at sub-lethal doses of the active fractions of the treatments were made and the emergence of the 50% of the test larvae (EC_{50} values) was determined using the probit programme. Each set of experiment was replicated thrice and the mean and standard error of mean was calculated.

Results and discussion

Table 1 presents the LC_{50} (ppm) after 48 h of exposure of the methanolic extract of mesocarp (MCE), crude saponin extract (CSE) and pure saponin fraction (PSF) of the *B. aegyptiaca* fruit mesocarp treatments used in the experiment against the third instars larvae of *Ae. aegypti*. The results showed that all three extracts used in the experiment were found active against the larvae; however, PSF was found to be most active. For PSF to kill 50% of the tested larvae, 145 ppm was needed, which was less than half dose of CSE and one-sixth of MCE.

The yield column shows the percentage of yield of each material in relation to the fridge-dried *B. aegyptiaca* fruit mesocarp. This indicates some proportional rate of active ingredients in the basic material.

The PSF of the methanol extract of the *B. aegyptiaca* fruit mesocarp was found very active with regard to its efficacy to inhibit adult emergence. Exposure of the early fourth instars *Ae. aegypti* larvae to 35 ppm prevented the emergence of 50% of the treated populations (Table 2), whereas a concentration of 260 ppm and 850 ppm of each saponin extract (CSE) and methanolic extract (MCE) respectively was needed to inhibit the emergence of the 50% of the tested population. This shows that in PSF only 21.2% concentration was needed no inhibit 50% emergence whereas more than 80% concentration was needed for CSE and MCE.

Table 2. EC_{50} (ppm) and their subsequent % of LC_{50} value of the different extracts and fraction of the *B. aegyptiaca* fruit mesocarp used in the experiment against third instars larvae of *Ae. aegypti*

Treatment	EC_{50} (ppm)	% of LC_{50} value
Methanolic extract of mesocarp (MCE)	850±35.8	90.9
Crude saponin extract (CSE)	260±14.3	82.5
Pure saponin fraction (PSF)	35±4.5	21.2

Each value is the mean of 3±SE (n=3)

In separate experiments when the exposure of the larvae to sub-lethal concentration of PSE (20 ppm) was carried out, that extended the duration of larval development, pupation and emergence to adult development to 7 to 8 days, compared to the larvae reared only in tap water (control) which required only 11–12 days (data not shown). The extension of the total developmental duration of *Ae. aegypti* indicated the possible presence of insect growth regulatory activities too in this fraction in sub-lethal concentration.

Conclusion

The results of this study clearly show that saponin fraction of the *B. aegyptiaca* fruit mesocarp is highly larvicidal against the *Ae. aegypti* mosquito larvae compared to crude saponin and methanolic extracts. Since the use of a 35 ppm of saponin fraction of *B. aegyptiaca* fruit mesocarp was found sufficient to inhibit the emergence of 50% of the test larvae population, this will certainly help to reduce the mosquito population drastically. Earlier studies have also indicated the possible use of saponins as a natural larvicidal agent against the mosquito; however, no study so far has reported on the saponin in relation to the inhibition of the emergence of the adult mosquito[4,15,16,17]. This low EC_{50} value of the pure saponin fraction of this experiment gives a more reliable evidence for using the saponin as a natural larvicidal agent against mosquito larvae. Since a large proportion of the population living in areas where dengue is a serious problem suffer from varying degrees of poverty, the discovery of plant-derived compounds that could help with the control or eradication of these diseases would of great value, particularly if the concerned plants were readily available to those who needed to use them. In this context, the highly bioactive compound of *B. aegyptiaca*, which are being grown widely in most of the dengue-infected areas, offer an opportunity for developing alternatives to rather expensive and environmentally hazardous insecticides. Consequently, since these plants are widely grown in rural and remote areas, their commercial exploitation would contribute towards rural economic development.

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Appendix B1

Spectral data of the main saponin compound (peak 2, tR 4.2 min) isolated from the mesocarp of *Balanites aegyptiaca* fruit.

Position	^{13}C	^{1}H	NOESY	COSY	HMBC[2]
1_{eq}	38.6	1.88	$H1_{ax}$ -$H2_{eq}$ -H9-$H11_{eq}$-$H11_{ax}$-H19	$H1_{ax}$-$H2_{eq}$-$H2_{ax}$	H19
1_{ax}		1.08	$H1_{eq}$-$H2_{eq}$$H2_{ax}$-H3-H9	$H1_{eq}$-$H2_{eq}$-$H2_{ax}$	
2_{eq}	30.8	1.92	$H1_{eq}$-$H1_{ax}$-$H2_{ax}$-H3	$H1_{eq}$-$H1_{ax}$-$H2_{ax}$-H3-$H4_{eq}$	$H1_{eq}$-$H1_{ax}$-H3
2_{ax}		1.61	$H1_{eq}$-$H1_{ax}$-$H2_{eq}$-H3-$H4_{ax}$-H19	$H1_{eq}$-$H1_{ax}$-$H2_{eq}$-H3	
3	79.4	3.59	$H1_{ax}$-$H2_{eq}$-$H2_{ax}$-$H4_{eq}$-$H4_{ax}$	$H2_{eq}$-$H2_{ax}$-$H4_{eq}$-$H4_{ax}$	$H1_{ax}$-$H2_{ax}$-$H4_{eq}$-H1'
4_{eq}	39.6	2.44	H3-$H4_{ax}$-H6-H19	$H2_{eq}$-H3-$H4_{ax}$	H6-H19
4_{ax}		2.29	$H2_{ax}$-H3-$H4_{eq}$-H6-H19	H3-$H4_{eq}$-H6-$H7_{eq}$-$H7_{ax}$	
5	142	…	….	…	$H1_{eq}$-$H4_{eq}$-$H7_{eq}$-H19
6	122.7	5.38	$H4_{eq}$-$H4_{ax}$-$H7_{ax}$-$H7_{eq}$	$H4_{ax}$-H7eq-$H7_{ax}$	$H4_{eq}$-$H7_{eq}$
7_{eq}	32.8[a]	2.00	H6-$H7_{ax}$-H8	$H4_{ax}$-H6-$H7_{ax}$-H8	H6-H8
7_{ax}		1.55	H6-$H7_{eq}$-H9-H14	$H4_{ax}$-H6-$H7_{eq}$-H8	
8	32.8[a]	1.67	$H7_{eq}$-$H11_{ax}$-$H15_{\beta}$-H18-H19	$H7_{eq}$-$H7_{ax}$-H9-H14	H6-$H7_{eq}$-H9-H14
9	51.8	0.97	$H1_{eq}$-$H1_{ax}$-$H7_{ax}$-$H12_{ax}$-H14	H8-$H11_{eq}$-$H11_{ax}$	$H7_{ax}$-H8-$H11_{eq}$-$H11_{ax}$- $H12_{eq}$-H19
10	38.1	---	---	---	$H1_{eq}$-$H1_{ax}$-$H4_{eq}$- H6-H8-H9-H19
11_{eq}	22.0	1.57[c]	$H1_{eq}$ - $H11_{ax}$-$H12_{eq}$-$H12_{ax}$-H19	H9-$H11_{ax}$-$H12_{ax}$-$H12_{eq}$	
11_{ax}		1.52	$H1_{eq}$ –H8-$H11_{eq}$-H12eq-$H12_{ax}$-H19	H9-$H11_{eq}$-$H12_{ax}$-$H12_{eq}$	
12_{eq}	40.9	1.78[d]	$H1_{eq}$- $H11_{ax}$-$H12_{eq}$-H18-H20-H21	$H11_{eq}$-$H11_{ax}$-$H12_{ax}$	$H11_{eq}$-$H11_{ax}$-H17a-H19
12_{ax}		1.20	H9-$H11_{eq}$-$H11_{ax}$-$H12_{eq}$-H14	$H11_{eq}$-$H11_{ax}$-$H12_{eq}$	
13	41.9	…	…	…	H14-H15α-H16a-H17a-H17b-H20a-H20b
14	57.8	1.14	$H7_{ax}$-H9-$H12_{ax}$-$H15_{\alpha}$-$H15_{\beta}$ -H16b	H8-$H15_{\alpha}$-$H15_{\beta}$	H8-$H12_{eq}$-$H15_{\alpha}$-$H15_{\beta}$ -H18
15_{α}	33.2	1.97	H14-$H15_{\beta}$-$H16_{\beta}$-H16a-H16b	H14-$H15_{\beta}$-H16a-H16b	H14
15_{β}		1.27	H8-H14-$H15_{\alpha}$-H16b	H14-$H15_{\alpha}$-H16a-H16b	
16a	82.5	4.37	$H15_{\alpha}$-H17a	$H15_{\alpha}$-$H15_{\beta}$-H17a	$H15_{\alpha}$-$H15_{\beta}$-H17a
16b		4.56	H14- $H15_{\alpha}$-$H15_{\beta}$-H17b	$H15_{\alpha}$-$H15_{\beta}$-H17b	
17a	65.2	1.73	H16a-H21	H16a-H20b	$H15_{\alpha}$-H18-H20b-H21
17b		1.77[d]	H16b-H1-H20a-H21	H16b-H20a	
18	16.8	.83	H8-$H12_{eq}$-H17b-H20a-H20b	…	H14-H17a-H17b
19	19.9	1.05	$H1_{eq}$-$H2_{ax}$-$H4_{eq}$-$H4_{ax}$-H8-$H11_{eq}$-$H11_{ax}$	…	$H1_{eq}$-$H1_{ax}$-H9
20a	41.0	2.1	H17b-H18-H21	H17b-H21	H17b-H18-H21-H23
20b	41.4	2.18	H18	H17a-H21	
21	16.3	1.00	H17a-H17b-H20a-H23	H20a-H20b	$H12_{eq}$-H17a-H20a-H20b
22	114	…	…	…	H20b-H21-H23a-$H23_{b}$- $H24_{a}$a-$H24_{b}$a

	112				H20a-H21-H23$_b$-H24$_b$b
23$_a$	31.5	1.68	H21-H23$_b$	H23$_b$-H24$_a$a-H24$_b$a	H20b-H24$_a$a-H24$_b$a
23$_b$		1.76	H23$_a$	H23$_a$-H24$_a$a-H24$_b$a	
24$_a$a	29	1.25	H23$_a$-H23$_b$-H24$_b$a-H25	H23$_a$-H23$_b$-H24$_b$a-H25	H23$_a$-H23$_b$-H26$_a$-H26$_b$-H27
24$_b$a		1.5	H24$_a$a	H23$_a$-H23$_b$-H24$_a$a-H25	
24$_a$b	28.7	1.31	H24$_b$b-H25	H24$_b$b-H25	H23$_a$-H23$_b$-H26$_a$-H26$_b$-H27
24$_b$b		1.62	H24$_a$b-H25	H24$_a$b-H25	
25	35.1	1.74	H26$_a$-H26$_b$	H24$_a$b-H24$_b$b-H26$_a$-H26$_b$-H27	H23$_a$-H23$_b$-H24$_a$a- H24$_b$a-H26$_a$-H26$_b$-H27
26$_a$	76.1	3.34[e]	H24$_a$b-H24$_b$b-H25-H26$_b$	H25-H26$_b$	H24$_a$a-H24$_b$a-H25-H27
26$_b$		3.79	H24$_a$b-H24$_b$b-H25-H26$_a$	H25-H26$_a$	H24$_a$a-H24$_b$a-H25-H27
27	17.5	0.95	H25	H25	H24$_a$a-H24$_b$a-H24$_a$b-H24$_b$b-H26[a]-H26$_b$
1’	100.5	4.51	H3-H4$_{eq}$-H4$_{ax}$-H2’-H3-H4-H5’	H2’	H3-H2’-H3’-H5’
2’	78.7	3.42	H1’-H3’-H4’-H1’’’-H5’’’	H1’-H3’	H1’-H3’-H1’’’
3’	77.9[b]	3.65[f]	H2’-H4’-H5’	H2’-H4’	H1’-H2’-H4’
4’	81.0	3.54	H2’-H5’-H6$_a$’-H6$_b$’-H1’’	H3’-H5’	H3’-H5’-H6$_a$’-H6$_b$’-H1’’
5’	76.3	3.39[g]	H1’-H3’-H4’-H6$_a$’-H6$_b$’	H4’-H6$_a$-H6$_b$	H1’-H3’-H4’-H6$_a$’-H6$_b$’
6$_a$’	62.0	3.83	H4’-H5’- H6$_b$’	H5’-H6$_b$’	H4’
6$_b$’		3.87[h]	H4’-H5’-H6$_a$’	H5’-H6$_a$’	
1’’	104.7	4.39	H4’-H6$_a$’-H6$_b$’-H2’’-H3’’-H4’’	H2’’	H4’-H2’’-H3’’-H5’’
2’’	75.1	3.20	H4’-H1’’-H3’’-H4’’	H1’’-H3’’	H3’’
3’’	78.0[b]	3.36	H1’’-H2’’-H4’’-H5’’	H2’’-H4’’	H2’’-H4’’-H5’’
4’’	71.5	3.28	H1’’-H2’’-H3’’-H5’’-H6$_a$’’-H6$_b$’’	H3’’-H5’’	H2’’-H3’’-H5’’-H6$_a$’’-H6$_b$’’
5’’	78.0[b]	3.24	H3’’-H4’’-H6$_a$’’-H6$_b$’’	H4’’-H6$_a$’’-H6$_b$’’	H3’’-H4’’-H6$_a$’’-H6$_b$’’
6$_a$’’	62.5	3.66[f]	H4’’-H5’’-H6$_b$’’	H5’’-H6$_b$’’	H4’’-H5’’
6$_b$’’		3.88[h]	H4’’-H5’’-H6$_a$’’	H5’’-H6$_a$’’	
1’’’	102.1	5.24	H2’-H2’’’-H3’’’-H5’’’	H2’’’	H2’-H5’’’
2’’’	72.3	3.89[h]	H1’’’-H3’’’	H1’’’-H3’’’	H1’’’-H3’’’-H4’’’-H5’’’
3’’’	72.4	3.66[f]	H1’’’-H2’’’-H4’’’-H5’’’	H2’’’-H4’’’	H1’’’-H2’’’-H4’’’-H5’’’
4’’’	74.0	3.39[g]	H3’’’-H5’’’-H6’’’	H3’’’-H5’’’	H2’’’-H3’’’-H5’’’-H6’’’
5’’’	69.8	4.12	H2’-H1’’’-H3’’’-H4’’’-H6’’’	H4’’’-H6’’’	H1’’’-H3’’’-H4’’’-H6’’’
6’’’	18	1.23	H4’’’-H5’’’	H5’’’	H4’’’-H5’’’
1’’’’	104.7	4.23	H2’’’’-H3’’’’-H5’’’’	H2’’’’	H26$_a$-H26$_b$-H2’’’’-H3’’’’-H5’’’
2’’’’	75.2	3.18	H1’’’’-H3’’’’	H1’’’’-H3’’’’	H3’’’’
3’’’’	71.8	3.34[e]	H1’’’’-H2’’’’-H4’’’’	H2’’’’-H4’’’’	H2’’’’-H4’’’’-H5’’’’
4’’’’	78.2[b]	3.65[f]	H3’’’’-H5’’’’	H3’’’’-H5’’’’	H2’’’’-H3’’’’-H5’’’’-H6$_a$’’’’-H6$_b$’’’’
5’’’’	78.2[b]	3.34[e]	H4’’’’-H6$_a$’’’’-H6$_b$’’’’	H4’’’’-H6$_a$’’’’-H6$_b$’’’’	H3’’’’-H4’’’’-H6$_a$’’’’-H6$_b$’’’’
6$_a$’’’’	62.9	3.66[f]	H5’’’’-H6$_b$’’’’	H5’’’’-H6$_b$’’’’	…
6$_b$’’’’		3.86[h]	H5’’’’-H6$_a$’’’’	H5’’’’-H6$_a$’’’’	

Appendix B2

Spectral data of the main saponin compound (peak 4, tR 7.2 min) isolated from the mesocarp of *Balanites aegyptiaca* fruit.

Position	^{13}C	^{1}H	NOESY	COSY	HMBC[2]
1_{eq}	38.8	1.88	$H1_{ax}$ -$H2_{eq}$ - $H2_{ax}$ -H3-H9-$H11_{eq}$- $H11_{ax}$-H19	$H1_{ax}$-$H2_{eq}$-$H2_{ax}$	H19
1_{ax}		1.08	$H1_{eq}$-$H2_{eq}$-H3-H9-H11	$H1_{eq}$-$H2_{eq}$-$H2_{ax}$	
2_{eq}	30.8	1.93	$H1_{eq}$-$H1_{ax}$-$H2_{ax}$-H3- H19	$H1_{eq}$-$H1_{ax}$-$H2_{ax}$-H3- $H4_{eq}$	$H1_{eq}$-$H1_{ax}$-H3- $H4_{eq}$
2_{ax}		1.61	$H1_{eq}$-$H2_{eq}$-$H4_{ax}$-H19	$H1_{eq}$-$H1_{ax}$-$H2_{ax}$-H3	
3	79.4	3.59	$H1_{eq}$ -$H1_{ax}$- $H2_{eq}$ -$H4_{eq}$-$H4_{ax}$-H6-H1	$H2_{eq}$-$H2_{ax}$-$H4_{eq}$-$H4_{ax}$	$H1_{ax}$-$H2_{ax}$-$H4_{eq}$-H1'
4_{eq}	39.6	2.44	H3-$H4_{ax}$-H6-H19	$H2_{eq}$-H3-$H4_{ax}$	H6-H19
4_{ax}		2.29	$H2_{ax}$-H3-$H4_{eq}$-H6-H19	H3-$H4_{eq}$-H6-$H7_{eq}$-$H7_{ax}$	
5	141.9	…	….	…	$H1_{eq}$-$H4_{eq}$-$H7_{eq}$-H19
6	122.7	5.37	H3-$H4_{eq}$-$H4_{ax}$-$H7_{ax}$-$H7_{eq}$-H19-H1'	$H4_{ax}$-H7eq-$H7_{ax}$	$H4_{eq}$-$H7_{eq}$
7_{eq}	33.2	2.00	H6-$H7_{ax}$-H8- $H15_{\beta}$	$H4_{ax}$-H6-$H7_{ax}$-H8	H6-H8
7_{ax}		1.56[b]	H6-$H7_{eq}$-H8-H9-H14-$H15_{\alpha}$	$H4_{ax}$-H6-$H7_{eq}$-H8	
8	32.8	1.65	H6- $H7_{eq}$- $H7_{ax}$ -$H15_{\beta}$-H18-H19	$H7_{eq}$-$H7_{ax}$-H9	H6-$H7_{eq}$- $H7_{ax}$ -H9-H14
9	51.8	0.97	$H1_{eq}$-$H1_{ax}$-$H7_{ax}$- $H11_{eq}$ - $H12_{eq}$ -$H12_{ax}$-H14	H8-$H11_{eq}$-$H11_{ax}$	$H1_{ax}$ - $H1_{eq}$ -$H7_{ax}$-H8-$H11_{eq}$-$H11_{ax}$- $H12_{eq}$-H14-H19
10	38.1	---	---	---	$H1_{eq}$-$H1_{ax}$-$H4_{eq}$- H6-H8-H9-H19
11_{eq}	22.0	1.56[b]	$H1_{eq}$ - $H1_{ax}$-H9-$H11_{ax}$ -$H12_{eq}$-$H12_{ax}$—H18-H19	H9-$H11_{ax}$-$H12_{eq}$-$H12_{ax}$	H9-$H12_{eq}$- $H12_{ax}$
11_{ax}		1.51	$H1_{eq}$ -$H11_{eq}$-$H12_{eq}$-H18-H19	$H11_{eq}$ –$H11_{ax}$-$H12_{ax}$	
12_{eq}	40.9	1.78[c]	$H11_{eq}$- $H11_{ax}$-$H12_{ax}$-H18	$H11_{eq}$-$H11_{ax}$-$H12_{ax}$	H18
12_{ax}		1.20	H9-$H11_{eq}$-$H12_{eq}$-H14-H17a	$H11_{eq}$-$H11_{ax}$-$H12_{eq}$	
13	41.9	…	…	…	$H12_{eq}$ - $H12_{ax}$ H14-H15α-H17a-H17b-H18-H20a-H20b
14	57.8	1.14	$H7_{ax}$-H9-$H12_{ax}$-$H15_{\alpha}$-$H15_{\beta}$-H16a-H17b-H18	H8-$H15_{\alpha}$-$H15_{\beta}$	H8-$H15_{\alpha}$-$H15_{\beta}$-H18
15_{α}	33.0	1.97	$H7_{ax}$-H14-$H15_{\beta}$-$H16_{\beta}$-H16a-H16b	H14-$H15_{\beta}$-H16a-H16b	H8-H14
15_{β}		1.27	H8-H14-$H15_{\alpha}$-H16a-H18	H14-$H15_{\alpha}$-$H16_{a}$-$H16_{b}$	
16a	82.5	4.37	H14-$H15_{\alpha}$- $H15_{\beta}$ H17a-OMe	$H15_{\alpha}$-$H15_{\beta}$-H17a	$H15_{\alpha}$-$H15_{\beta}$-H17a
16b	82.3	4.57	$H15_{\alpha}$- -H17b	$H15_{\alpha}$-$H15_{\beta}$-H17b	$H15_{\alpha}$-$H15_{\beta}$-H17b
17a	65.1	1.73	H14-H16a-H18-H20b-H21	H16a-H20b	$H12_{ax}$ -$H15_{\alpha}$-H18-H20b-H21
17b	64.1	1.78[c]	H16b-H18- H21	H16b-H20a	H18-H20-H21
18	16.9	0.83	H8- $H11_{eq}$ - $H11_{ax}$ -H14- $H15_{\beta}$ –H17a-H17b-H19-H20a-H20b	…	$H2_{ax}$ -H14-H17a-H17b
19	19.9	1.05	$H1_{eq}$-$H2_{eq}$ -$H2_{ax}$-$H4_{eq}$-$H4_{ax}$-H6-H8- $H11_{eq}$-$H11_{ax}$-H18	…	$H1_{eq}$-$H1_{ax}$-H9
20a	41.0	2.10	H18-H21	H17b-H21	H17a-H17b-H18-H21
20b	41.2	2.18	H17a-H18-H21-H23b-H24a-H24b-H25	H17a-H21	H18-H21-H23a-H23b
21	16.2	1.00	H17a-H17b-H20a-H20b	H20a-H20b	H17a-H17b-H20b
	16.0				H17a-H17b-H20a
22	114	…	…	…	H17a-H20b-H21-H23a-$H23_{b}$- $H24_{a}$-$H24_{b}$

	112.0				H15 $_{\beta}$ –H17a-H20a-H21
23_a	31.4	1.64	$H23_b$	$H23_b$-$H24_a$-$H24_b$	H20b-$H24_a$-$H24_b$
23_b		1.83	$H23_a$-$H24_a$-H27	$H23_a$-$H24_a$-$H24_b$	
24_a	29.0	1.15	H20b-$H23_b$-$H24_b$-H27	$H23_a$-$H23_b$-$H24_b$-H25	$H23_a$-$H23_b$-$H26_a$-$H26_b$-H27
24_b	28.7	1.61	H20b-$H24_a$	$H23_a$-$H23_b$-$H24_a$-H25	$H23_a$-$H23_b$-$H26_a$-$H26_b$-H27
25	35.0	1.75	$H26_a$-H27	$H24_a$-$H24_b$-$H26_a$- H27	$H23_a$-$H23_b$-$H24_a$- $H24_b$- $H26_a$-$H26_b$-H27
26_a	76.3	3.38	H25-$H26_b$-H1""	H25-$H26_b$	$H24_a$-$H24_b$-H25-H27-H1""
26_b		3.56	H25-$H26_a$	H25-$H26_a$	
27	17.3	0.95	H25	H25	$H24_a$-$H24_b$-H25-26_b
	17.4				
OMe	47.7	3.15	H16a	…	….
1’	100.5	4.51	H3-H6-H2’-H3'-H5’-H6'-$H6_b$'-H1'"	H2’	H3-H2’-H3’-H5’
2’	78.6	3.42	H1’-H3’-H4’- H5’-H6'- H1’’’-H5'"	H1’-H3’	H1’-H3’-H1’’’
3’	77.8[a]	3.65[d]	H1’-H2'-H4’-H5’-$H6_a$'-$H6_b$'	H2’-H4’	H1’-H2’-H4’-H5'
4’	81.1	3.56	H2’-H3'-H5’-$H6_a$’-$H6_b$’-H1’’	H3’-H5’	H1'-H3’-H5’-$H6_a$’-$H6_b$’-H1’’
5’	76.3	3.39[e]	H1’-H2'-H3’-H4’-$H6_a$’-$H6_b$’	H4’-$H6_a$-$H6_b$'	H1’-H4’-$H6_a$’-$H6_b$’
6_a’	62.0	3.83	H1'-H3'-H4’-H5’- $H6_b$’-H1"	H5’-$H6_b$’	H4’-H5'
6_b’		3.88[h]	H1'-H3'-H4’-H5’-$H6_a$’-H1"	H5’-$H6_a$’	
1’’	104.3	4.46	H4’-$H6_a$’-$H6_b$’-H2’’-H3’’-H4’’-H5"	H2’’	H4’-H2’’-H3’’
2’’	74.1	3.41	H1’’-H3’’-H4’’	H1’’-H3’’	H3’’
3’’	87.7	3.52	H1’’-H2’’-H4’’-H5’’-H1""	H2’’-H4’’	H1"-H2’’-H4’’-H1’’""
4’’	77.7[a]	3.37[e]	H1’’-H2’’-H3’’-H5’’-$H6_a$’’-$H6_b$’’	H3’’-H5’’	H1’’-H3’’-H5’’-$H6_a$’’-$H6_b$’’
5’’	78.2[a]	3.66[d]	H1"-H3’’-H4’’-$H6_a$’’-$H6_b$’’	H4’’-$H6_a$’’-$H6_b$’’	H1"-H4’’-$H6_a$’’-$H6_b$’’
6_a’’	62.4	3.65[d]	H4’’-H5’’-$H6_b$’’	H5’’-$H6_b$’’	H4’’
6_b’’		3.89[f]	H4’’-H5’’-$H6_a$’’	H5’’-$H6_a$’’	
1’’’	102.1	5.24	H1'-H2’-H2’’’-H3’’’-H4'"-H5’’’	H2’’’	H2’-H2'"-H5’’’
2’’’	72.2	3.90[f]	H1’’’-H3’’’-H5'"	H1’’’-H3’’’	H1’’’-H3’’’
3’’’	72.4	3.66[d]	H1’’’-H2’’’-H4’’’-H5’’’	H2’’’-H4’’’	H2’’’-H4’’’-H5’’’
4’’’	74.0	3.39[c]	H1'"-H3’’’-H5’’’-H6’’’	H3’’’-H5’’’	H2’’’-H3’’’-H5’’’-H6’’’
5’’’	69.7	4.13	H2’-H1’’’-H2’’’-H3'"-H4’’’-H6’’’	H4’’’-H6’’’	H1’’’-H2'"-H3’’’-H4’’’-H6’’’
6’’’	18.0	1.24	H4’’’-H5’’’	H5’’’	H4’’’-H5’’’
1’’’’	104.6	4.24	$H26_a$-H2’’’’-H3""-H5’’’’	H2’’’’	$H26_a$-$H26_b$-H2’’’’-H3’’’’-H5’’’
2’’’’	75.2	3.18	H1’’’’-H3’’’’	H1’’’’-H3’’’’	H1""-H3’’’’
3’’’’	77.8[a]	3.35[g]	H1’’’’-H4’’’’-H5’’’’	H2’’’’-H4’’’’	H1""-H2’’’’-H4’’’’-H5’’’’
4’’’’	71.7	3.29[h]	H3’’’’-H5’’’’	H3’’’’-H5’’’’	H3’’’’-H5’’’’-$H6_a$’’’’-$H6_b$’’’’
5’’’’	77.9a	3.25[e]	H1’’’’-H4""-$H6_a$’’’’-$H6_b$’’’’	H4’’’’-$H6_a$’’’’-$H6_b$’’’’	H1""-H2""-H3’’’’-H4’’’’-$H6_a$’’’’-$H6_b$’’’’
6_a’’’’	62.9	3.67[d]	H5’’’’-$H6_b$’’’’	H5’’’’-$H6_b$’’’’	H4""-H5""
6_b’’’’		3.87[h]	H5’’’’-$H6_a$’’’’	H5’’’’-$H6_a$’’’’	
1’’’’’	106.0	4.5	H3"-H2""-H3""-$H5_a$’’’’’-$H5_b$""	H2’’’’	H3"-H2""-H3’’’’’-$H5_a$’’’"-$H5_b$""
2’’’’’	75.3	3.27[h]	H1’’’’’-H3’’’’’-H4""	H1’’’’’-H3’’’’’	H1""-H3’’’’’
3’’’"	78.4[a]	3.34[g]	H1’’’"-H2’’’"-H4’’’"	H2’’’"-H4’’’"	H2’’’"-H4’’’"-$H5_a$’’’"-$H5_b$""
4’’’’’	71.0	3.51	H2""-H3’’’’’-H5’’’’’	H3’’’’’-$H5_a$’’’’’-$H5_b$""	H3’’’’’-$H5_a$’’’’’-$H5_b$’’’’’
5a’’’’’	67.2	3.24[h]	H1’’’’’-H4""-$H5_b$’’’’’	H4’’’’’-$H5_b$’’’’’	H1""-H3’’’’’-H4’’’’’
5b’’’’’		3.91[i]	H1’’’’’-H4""-$H5_a$’’’’’	H4’’’’’-$H5_a$’’’’’	

Appendix B3

Spectral data of the saponin compound (peak 5, tR 8.2 min) isolated from the mesocarp of *Balanites aegyptiaca* fruit.

Position	^{13}C	^{1}H	NOESY	COSY	HMBC[2]
1_{eq}	38.6	1.88	$H1_{ax}$ -$H2_{eq}$ - $H2_{ax}$- $H11_{ax}$- $H11_{eq}$- - H19	$H1_{ax}$-$H2_{eq}$-$H2_{ax}$	$H2_{eq}$- $H2_{ax}$-H3-H6-H9 H19
1_{ax}		1.08	$H1_{eq}$-$H2_{eq}$-H3-H9	$H1_{eq}$-$H2_{eq}$-$H2_{ax}$	
2_{eq}	30.8	1.92	$H1_{eq}$-$H1_{ax}$-H3-H19	$H1_{eq}$-$H1_{ax}$-$H2_{ax}$-H3- $H4_{eq}$	$H1_{eq}$-$H1_{ax}$-H3-$H4_{eq}$
2_{ax}		1.61	$H1_{eq}$-$H1_{ax}$-$H2_{eq}$-H19	$H1_{eq}$-$H1_{ax}$-$H2_{eq}$-H3	
3	79.4	3.59	$H1_{ax}$-$H2_{eq}$-$H4_{ax}$	$H2_{eq}$-$H2_{ax}$-$H4_{eq}$-$H4_{ax}$	$H1_{eq}$ -$H1_{ax}$-$H2_{ax}$-$H4_{eq}$- $H4_{ax}$ -H1'
4_{eq}	39.6	2.44	H3-$H4_{ax}$-H19-H1'	$H2_{eq}$-H3-$H4_{ax}$	$H2_{eq}$ - $H1_{ax}$-H6
4_{ax}		2.29	$H2_{ax}$-H3-$H4_{eq}$-H6-H19-H1'-H5'''	H3-$H4_{eq}$-H6-$H7_{eq}$-$H7_{ax}$	
5	142	…	….	…	$H1_{eq}$-$H4_{eq}$- $H4_{ax}$ -$H7_{eq}$- $H7_{ax}$ H19
6	122.7	5.38	$H4_{eq}$-$H4_{ax}$-$H7_{ax}$-$H7_{eq}$-H19	$H4_{ax}$-H7eq-$H7_{ax}$	$H1_{ax}$-$H4_{eq}$-$H7_{eq}$- $H7_{ax}$
7_{eq}	33.2	2.00	H6-$H7_{ax}$-H8	$H4_{ax}$-H6-$H7_{ax}$-H8	H6-H8-H9
7_{ax}		1.55[b]	H6-$H7_{eq}$-H8-H9	$H4_{ax}$-H6-$H7_{eq}$-H8	
8	32.8	1.65	$H7_{ax}$- $H7_{eq}$-$H15_{\beta}$-H18-H19	$H7_{eq}$-$H7_{ax}$-H9-H14	$H7_{eq}$- $H7_{ax}$ -H9-H14
9	51.7	0.97	$H1_{eq}$-$H1_{ax}$-$H7_{ax}$-H14	H8-$H11_{eq}$-$H11_{ax}$	$H1_{ax}$-$H7_{ax}$-H8-$H11_{eq}$-$H11_{ax}$- $H12_{eq}$-H19
10	38.1	---	---	---	$H1_{eq}$-$H1_{ax}$-$H4_{eq}$- H8-H9-H19
11_{eq}	22.0	1.57[b]	$H1_{eq}$ - $H11_{ax}$-$H12_{eq}$-$H12_{ax}$.H18-H19	$H11_{ax}$-$H12_{ax}$-$H12_{eq}$	H9
11_{ax}		1.51	$H1_{eq}$ –$H11_{eq}$-H12eq-H18- H19	$H11_{eq}$-$H12_{ax}$-$H12_{eq}$	
12_{eq}	40.0	1.78	$H11_{eq}$- $H11_{ax}$-$H12_{ax}$-H14-H18-H21	$H11_{eq}$-$H11_{ax}$-$H12_{ax}$	H17a-H17b-H18
12_{ax}	40.9	1.20	H9-$H11_{eq}$- $H12_{eq}$-H14-H17a	$H11_{eq}$-$H11_{ax}$-$H12_{eq}$	
13	41.9	…	…	…	$H12_{eq}$ -H14-H15α-H16a-H17a-H17b-H18-H20a-H20b-H21
14	57.8	1.14	$H7_{ax}$-H9-$H12_{ax}$-$H15_{\alpha}$-$H15_{\beta}$--H16b-H17a-H17b-H20b	H8-$H15_{\alpha}$-$H15_{\beta}$	H8-$H15_{\alpha}$-$H15_{\beta}$-H18
15_{α}	33.0	1.97	H14-$H15_{\beta}$-H16a-16b	H14-$H15_{\beta}$-H16a-H16b	H14
15_{β}		1.27	H8-H14-$H15_{\alpha}$-H18-H20a	H14-$H15_{\alpha}$-H16a-H16b	
16a	82.5	4.37	H14-$H15_{\alpha}$-H17a-OMe	$H15_{\alpha}$-$H15_{\beta}$-H17a	$H15_{\alpha}$-$H15_{\beta}$-H17a-H17b
16b	82.3	4.56	H14- $H15_{\alpha}$.H17b	$H15_{\alpha}$-$H15_{\beta}$-H17b	
17a	65.1	1.73	$H12_{ax}$-H14-H16a-H18-H20a-H20b-H21	H16a-H20b	$H15_{ax}$-H18-H20a-H20b-H21
17b	64.0	1.77	H14-H16b-H18-H20a-H21	H16b-H20a	
18	16.9	0.83	H8-H11eq-H11ax- $H15_{\beta}$ -H17a-H17b-H19-H20a-H20b-H21	…	H17a-H17b
19	19.9	1.05	$H1_{eq}$-$H2_{eq}$ –$H2_{ax}$-$H4_{eq}$-$H4_{ax}$-H8-$H11_{eq}$-$H11_{ax}$-H18	…	$H1_{eq}$-$H1_{ax}$-H9
20a	41.2	2.10	$H15_{\beta}$ –H17a-H17b-H18-H21	H17a-H21	H17a-H17b-H21
20b		2.18	H14-H17b-H18-H21	H17b-H21	
21	16.2	1.00	H17a-H17b-H18-H20a-H20b	H20a-H20b	H17a-H17b-H20a-H20b
22	114	…	…	…	H20a-H21-$H23_{a}$-$H23_{b}$- $H24_{a}$a-$H24_{b}$a-OMe

	112				H20b-H24$_a$a-H24$_b$b
23$_a$	31.4	1.64	H23$_b$- H24$_a$a -H24$_b$a	H23$_b$-H24$_a$a-H24$_b$a	H20b-H24$_a$a-H24$_b$a-H25
23$_b$		1.82	H23$_a$-H27-OMe	H23$_a$-H24$_a$a-H24$_b$a	
24$_a$a	29	1.15	H23$_a$-H24$_b$a-H24$_b$a-H25	H23$_a$-H23$_b$-H24$_b$a-H25	H23$_a$-H23$_b$-H25-H26$_a$-H26$_b$-H27
24$_b$a		1.59	H23$_a$-H24$_a$a	H23$_a$-H23$_b$-H24$_a$a-H25	
24$_a$b	28.7	1.27	H24$_b$b-H25	H24$_b$b-H25	H23$_a$-H23$_b$- H25-H26$_a$-H26$_b$-H27
24$_b$b		1.62	H24$_a$b-H25	H24$_a$b-H25	
25	35.0	1.74	H26$_a$-H26$_b$-H27	H24$_a$b-H24$_b$b-H26$_a$-H27	H26$_b$- H23$_a$-H23$_b$-H24$_a$a- H24$_b$a- H24$_a$b-H26$_b$b-H26a-H26$_b$-H27
26$_a$	76.0	3.39	H25-H26$_b$-H27	H25-H26$_b$	H24$_a$a-H24$_b$a-H24 $_b$b -H25
26$_b$		3.73	H25-H26$_a$-H27	H25-H26$_a$	H27-H1""
27	17.3	0.94	H25-H26 $_a$- H26$_b$	H25	H24$_a$a-H24$_b$a-H24$_a$b-H24$_b$b-
	17.4				H26$_a$-H26$_b$
OMe	47.7	3.14	H16$_a$-H23B	….	H20b-H21
1’	100.5	4.51	H3-H4$_{eq}$-H4$_{ax}$-H6-H2’-H3'-H4-H1"’	H2’	H3-H2’-H3’-H5’
2’	78.7	3.42	H1’-H3’-H4’-H1’’’-H5’’’	H1’-H3’	H1’-H3’-H4'-H1’’’
3’	77.9[a]	3.65[c]	H1'-H2’-H4’-H5’	H2’-H4’	H1’-H2’-H4’-H5'
4’	81.0	3.55	H1'-H2’-H3'-H5’-H6$_a$’-H6$_b$’-H1’’-H2"	H3’-H5’	H2'-H3’-H5’-H6$_a$’-H6$_b$’-H1’’
5’	76.3	3.39[d]	H1’-H3’-H4’-H6$_a$’-H6$_b$’	H4’-H6'$_a$-H6$_b$'	H1’-H3’-H4’-H6$_a$’-H6$_b$’
6$_a$’	62.0	3.83[e]	H4’-H5’- H6$_b$’	H5’-H6$_b$’	H4’-H5'
6$_b$’		3.87[f]	H4’-H5’-H6$_a$’	H5’-H6$_a$’	
1’’	104.7	4.40	H4’-H2"-H3’’-H6$_a$’’-H6$_b$"	H2’’	H4’-H2’’-H3’’
2’’	75.2	3.21	H4’-H1’’-H3’’-H4’’	H1’’-H3’’	H3’’-H4"
3’’	77.9[a]	3.36	H1’’-H2’’-H4’’	H2’’-H4’’	H1"-H2"-H4’’-H5’’
4’’	71.7	3.28	H2’’-H3’’-H6$_a$’’-H6$_b$’’	H3’’-H5’’	H2’’-H3’’-H5’’-H6$_a$’’-H6$_b$’’
5’’	77.8[a]	3.24	H4’’-H6$_a$’’-H6$_b$’’	H4’’-H6$_a$’’-H6$_b$’’	H1"-H3"-H4’’-H6$_a$’’-H6$_b$’’
6$_a$’’	62.9	3.65[c]	H4’’-H5’’-H6$_b$’’	H5’’-H6$_b$’’	H4’’-H5’’
6$_b$’’		3.86[h]	H4’’-H5’’-H6$_a$’’	H5’’-H6$_a$’’	
1’’’	102.1	5.24	H1'-H2’-H2’’’-H3’’’-H5’’’-H6’"	H2’’’	H2’"-H5’’’-H6’"-H2'
2’’’	72.2	3.89	H1’’’-H3’’’	H1’’’-H3’’’	H1’’’-H3’’’-H4’’’
3’’’	72.4	3.65[c]	H1’’’-H2’’’-H4’’’-H5’’’	H2’’’-H4’’’	H1’’’-H2’’’-H4’’’-H5’’’
4’’’	74.0	3.39[d]	H3’’’-H6’’’	H3’’’-H5’’’	H2’’’-H3’’’-H5’’’-H6’’’
5’’’	69.8	4.13	H4$_{ax}$-H1’’’-H3’’’-H4’’’-H6’’’	H4’’’-H6’’’	H1’’’-H3’’’-H4’’’-H6’’’
6’’’	18.0	1.24	H1’"-H4’’’-H5’’’	H5’’’	H4’’’-H5’’’
1’’’’	104.6	4.24	H2’’’’-H3’’’’	H2’’’’	H26$_b$-H2’’’’-H3’’’’
2’’’’	75.1	3.18	H1’’’’-H3’’’’	H1’’’’-H3’’’’	H1’"-H3’’’’
3’’’’	71.4	3.34[g]	H1’’’’-H2’’’’-H4’’’’-H5’"	H2’’’’-H4’’’’	H2’’’’-H4’’’’-H5’’’’
4’’’’	78.2[a]	3.65[c]	H3’’’’-H5’’’’	H3’’’’-H5’’’’	H1’"-H2’’’’-H3’’’’-H5’’’’-H6$_a$’’’’-H6$_b$’’’’
5’’’’	78.2[a]	3.33[g]	H3’"-H4’’’’	H4’’’’-H6$_a$’’’’-H6$_b$’’’’	H1’"-H3’’’’-H4’’’’H5’"- H6$_a$’’’’-H6$_b$’’’’
6$_a$’’’’	62.5	3.33[g]	H4’"-H5’’’’-H6$_b$’’’’	H5’’’’-H6$_b$’’’’	H5’"
6$_b$’’’’		3.83[e]	H4’’’’-H5’"-H6$_a$’’’’	H5’’’’-H6$_a$’’’’	

אפיון ספוניני התמר המדברי (זאקום מצרי) ופעילויותיהם הביולוגיות

עבודה למילוי חלק הדרישות לתואר

דוקטור לפילוסופיה

מאת:

בישנו פ. צ׳אפג׳אן

הוגש לסנט

אוניברסיטת בן-גוריון בנגב

פברואר 2006

באר-שבע

אפיון ספוניני התמר המדברי (זאקום מצרי) ופעילויותיהם הביולוגיות

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אוניברסיטת בן-גוריון בנגב

אושר ע״י המנחה:_____

אושר ע״י דיקן ביה״ס ללמודי מחקר מתקדמים ע״ש קרייטמן _____

פברואר 2006

באר-שבע

עבודה זו נעשתה בהנחיית המנחה:

פרופ' זאב ויסמן

המעבדה לביוטכנולוגיה של שמנים צמחיים,

המחלקה להנדסה ביוטכנולוגית,

הפקולטה להנדסה,

אוניברסיטת בן-גוריון בנגב

תקציר

עץ התמר המדברי (זאקום מצרי) הינו ירוק-עד השייך למשפחת הזוגניים. הזאקום גדל לרוב באזורים הצחיחים של אפריקה, המזרח התיכון, ודרום אסיה. ישראל נחשבת כאזור הצפוני ביותר בו גדלים עצי הזאקום בטבע. בישראל, הזאקום נפוץ באופן טבעי באזור עין גדי, לאורכה של הערבה ובבקעת בית שאן. לזאקום שמושים רבים—מרפואה עממית ועד דלק לבעירה. חלקים מסוימים מעץ זה שמשו כתרופות עממיות באפריקה ואסיה. מחקרים מוקדמים הראו שלחומרים ספונינים תרומה מרכזית למרבית השימושים הרפואיים. שמן הזקום נחשב מקור חשוב וטוב לתמרוקים, ואפילו האצולה המצרית הקדומה השתמשה בו. יתר על כן, הזאקום מאד מותאם לתנאים הסביבתיים באזורים צחיחים, היכן שלעצים אחרים קשה להסתגל. למרות הקשת הרחבה של השמושים האפשריים של עץ זה והצלחתו האקולוגי, הזאקום הינו אחד ממיני העצים המוזנחים ביותר באזורים הצחיחים וטרם תורבת.

הספונינים שייכים לקבוצת חומרים מורכבת ומגוונת מבחינה כימית. שמם נובע מיכולתם ליצור צורה יציבה, קצף דמוי-סבון בתמיסות מימיות. בצמחים, הספונינים מתפקדים כמטבוליט שניוני ומהווים חלק ממנגנון ההגנה הראשוני של הצמח בפני עקות סביבתיות שונות ומגוונות. מבחינה כימית, ספונינים הינם גליקוזידים הקשורים לשלד טבעתי בעל אופי ליפידי (אגליקון). עד כה בודדו מצמחים רבים וזוהו ספונינים שסווגו לשלוש קבוצות: תלת-טרפנואידים, סטרואידים ואלקלואידים סטרואידים. בעקבות שיפורים טכנולוגיים ואנליטיים משמעותיים שחלו בשנים האחרונות שהביאו להבהרת מגוון התכונות המיטיבות שלהם ואפשרויות יישומם, גברה ההתעניינות בספונינים. מחקרים חדשים מציעים שספונינים מעורבים בהפעלת מערכת החיסון, עוזרים בהגנת הגוף נגד סוגי סרטן שונים ומסייעים להתמודדות עם הכולסטרול הרע שבגוף. דווח שספונינים מסייעים להפחית את כמות השומנים בדם, ואף מפחיתים את רמת הגלוקוז בדם. מאמץ מיוחד מוקדש כיום לקביעת ערכים פרמקולוגיים ותזונתיים עבור רמת הספונינים הנדרשת לגוף האדם. ההכרה בחשיבותם של הספונינים עודדה את המאמצים לאיתור מקורות צמחיים חדשים וחלופיים לסיפוק הדרישה לספונינים. עד לעת הזו, ספונינים סטרואידיים הופקו באופן מסחרי ממגוון טיפוסים של המין Dioscorea. אולם

לאחרונה החל מאמץ לגלות מקורות צמחיים חדשים וחלופיים לחומרי טבע אלו.

עד כה, דווח על מציאת ספונינים רק בטיפוסי זאקום מאפריקה והודו בלבד. המטרה הראשונה של המחקר הנוכחי היתה לאפיין את הספונינים הנוצרים ברקמות השונות של טיפוס הזאקום המצרי הנפוץ בישראל. לצורך השגת מטרה זו, השתמשנו בחומר צמחי שנאסף מחלקת אוסף טיפוסי הזאקום המצרי של המעבדה לביוטכנולוגיה של שמנים, שהוקמה בשנת 1998 בסמר בדרום הערבה. פיתחנו שיטה להפקה, בידוד וזיהוי כימי אנליטי של מרבית הספונינים המיוצרים ונאגרים ברקמות ציפת הפרי, בזרעים ובשורשים של טיפוס הזאקום המצרי הנפוץ בישראל. מיצוי מתנולי נמצא היעיל ביותר להפקה אנליטית של הספונינים מן הרקמות השונות. הבידוד וההפרדה של הספונינים השונים נעשה בכרומטוגרפיה נוזלית בלחץ גבוה (HPLC). בגלל בליעת האור האולטרה סגולי המועטת של ספונינים אלו, נאלצנו להשתמש בגלאי אינדקס הרפרקציה של החומרים. פרופיל המטבוליטים שהתקבל בגלאי זה הושווה לפרופיל המתקבל על ידי גלאי של ספקטרומטרית המסות (MS) של הספונינים השונים. טביעת האצבע הכימית של כל ספונין שהופרד בשיטה זו, נקבעה על ידי מערכת ה- MS/MS. באופן זה זיהינו את מרבית החומרים הספונינים שנוצרים בכל אחת מן הרקמות שנבדקו. על מנת לאמת באופן סופי את המבנה הכימי של הספונינים שזוהו באמצעות ספקטרומטרית המסות, בדקנו אותם במערכת מתקדמת של NMR (800MHz).

נמצא שכל הספונינים הנוצרים רקמות הזאקום המצרי מורכבים משלד אגליקוני, הנקרא דיאוסגנין שמשקלו המולקולרי 414 דלתון. משקלו המולקולרי של הספונין העיקרי בציפת הפרי הוא 1064 דלתון. לפחמן המצוי בעמדה 26 של האגליקון קשורה יחידה אחת של גלוקוז. לפחמן המצוי בעמדה 3 של האגליקון קשורה שרשרת פחמנים המורכבת מגלוקוז-גלוקוז-ראמנוז. משקלם המולקולרי של הספונינים האחרים בציפת הפרי הוא: 1078, 1196, 1210, 1046 דלתון. משקלו המולקולרי של הספונין העיקרי בזרעי הזאקום המצרי הוא 1210 דלתון. ספונין זה הנוצר גם בציפת הפרי, אך ברמה נמוכה יותר. הוא דומה לספונין העיקרי של ציפת הפרי (1064 דלתון), אך מכיל עוד יחידת קסילוז בשרשרת הסוכרית הקשורה לפחמן המצוי בעמדה 3 של האגליקון. ספונין זה מכיל גם יחידת מתיל בעמדה 22 של האגליקון. משקלם המולקולרי של הספונינים האחרים ברקמת הזרע הוא: 1196, 1064, 1224,

1078, 1046 דלתון. משקלו המולקולרי של הספונין העיקרי בשורשי הזאקום המצרי הוא 1196 דלתון. ספונין זה דומה לספונין העיקרי של הזרע (1210 דלתון), אך חסר את יחידת המתיל שבעמדה 22 של האגליקון. משקלם המולקולרי של הספונינים האחרים ברקמת הזרע הוא: 1340, 1064, 1224, 1530, 1586, 1572, 1516 דלתון.

מטרה חשובה נוספת בעבודה זו היתה לאפיין את הפעילויות הביולוגיות של הספונינים שהופקו וזוהו מרקמות הזאקום. להשגת מטרה זו מוקדה העבודה על בחינת ההשפעה של סדרת תכשירים ספונינים שבודדו מרקמות זאקום כנגד שני מיני יתושים מעבירי מחלות ידועים (*Aedes aegypti* ו- *Culex pipiens*), וכנגד טווח רחב של פטריות התוקפות צמחים. נמצאו ראיות מוצקות ליכולתם של ספונינים מזאקום מצרי לקטול את זחלי יתושים שני המינים שנבדקו. נמצא גם קשר בין המבנה הכימי של הספונינים לבין יעילותם בקטילת זחלי היתושים. ככלל נראה שתכשירי ספונינים שהכילו קבוצת מתיל בעמדה 22 של האגליקון היו יעילים יותר מחסרי המתיל. נמצא גם שנוכחות היחידה הסוכרית של קסילוז בשרשת הצדדית הקשורה לעמדה 3 של האגליקון הפחיתה את יעילות התכשירים בקטילת זחלי יתושים. לגבי פעילות הספונינים על עיכוב התפתחות פטריות תוקפות צמחים נמצאה שונות רבה בהשפעתם. בעוד שנצפתה פעולת עיכוב חזקה כנגד הפטריה *Pithium ultimum*, כנגד הפטריה *Alternaria solani* נצפתה פעולת עיכוב בינונית בלבד. כנגד הפטריות *Fusarium oxysporum*, *Verticillium dahlia*e ו- *Collectrotrichum coccodes* נצפתה פעולת עיכוב חלשה אם בכלל.

במטרה להגביר את הבנתנו את יחסי הגומלין בין מולקולות הספונינים וקרומי הצמח, התמקדנו בלימוד השפעתם על הובלת 2,4-D(^{14}C) דרך ממברנות של קוטיקולות מבודדות מעלים של *Citrus grandis*. נמצא שתכשירי ספונינים שהופקו מרקמות זאקום מצרי זרזו משמעותית את קצב המעבר של 2,4-D דרך הקוטיקולות המבודדות בהשוואה לבקורת. נמצאו הבדלים בין תכשירי הספונינים שהוכנו מרקמות שונות של זאקום מצרי. התכשיר הספוניני שהוכן מציפת הפרי זרז את קצב המעבר של החומר הביולוגי דרך הקוטיקולה באופן מובהק יותר מהתכשירים שהוכנו מרקמות אחרות.

ההסבר המוצע להשפעות הביולוגיות של תכשירי הספונינים שהוכנו מזאקום מצרי הן במישור הביוטי והן במישור הא-ביוטי קשור בזיקה החזקה של

הספונינים להתקשר אל פיטוסטרולים המצוים הן בממברנות של פתוגנים והן בממברנת הקוטיקולה הצמחית. תפקידם של הפיטוסטרולים בהקשר זה הוא לייצב את הממברנות. בהקשרם לפיטוסטרולים בממברנה, הספונינים גורמים לערעורה. נמצא שעוצמת ההשפעה של הספונינים על קטילת זחלים, עיכוב פטריות וזרוז קצב המעבר דרך ממברנת הקוטיקולה תלויה בריכוז הספונינים שבתכשיר. בריכוזים נמוכים יחסית הספונינים גורמים לערעור מתון והפיך של הממברנה, ואילו בריכוזים גבוהים הפגיעה בממברנה הינה בלתי הפיכה. נראה שישנה חשיבות הן למבנה הכימי המרחבי של הספונינים והן למבנה של הפיטוסטרולים בהקשר ליכולת האינטראקציה ביניהם.

אופיים הכימי האמפיפטי של חומרים ספונינים גורם להם להתארגן בצורה אנרגטית יעילה, וליצור מיצלות בסביבה מימית. אפיון בעזרת מיקרוסקופ אלקטרוני חודר (TEM) הראה בברור שתכשירי הספונינים שהוכנו מזאקום מצרי מכילים וזיקולות ננומטריות רבות. בעזרת שימוש במערכת פיזור אור דינמית (DLS), נמצא שגודלם הממוצע של הוזיקולות הספוניניות הוא בסקאלה של מאות בודדות של ננומטרים. התגלה קשר בין מבנה הספונינים העיקריים לבין גודלן של הוזיקולות המתקבלות. בעוד שבתכשיר הספוניני שהוכן מציפת הפרי גודלן הממוצע של הוזיקולות כ- 170 ננומטר, בתכשירים שהוכנו מרקמות זרע או שורש התגלו וזיקולות בקוטר ממוצע של כ- 500 ננומטר. נראה שלוזיקולות ולגודלן ישנה חשיבות מרובה ביכולת שלהן לבצע את האינטראקציה עם הפיטוסטרולים שבממברנות. יכולת זו עשויה להיות מעורבת בהשפעותיהם הביולוגיות רחבות ההיקף של הספונינים.

מבין כל המקומות בהם נמצאים הזקום, השוואה כמותית של רמת הספונינים בטיפוסי זאקום מצרי שנאספו מאזורים שונים ברחבי העולם העלתה, שזרע טיפוס הזאקום מאזור בית שאן בישראל הוא העשיר ביותר בספונין (2.74% DW). הזרע של טיפוס הזאקום שנאסף מאזור רג׳סתאן בהודו הכיל את הרמה הנמוכה ביותר של ספונינים (1.41% DW). נמצא יחס חיובי חזק (R^2=0.849) בין רמת הספוגנין לכמות השמן בזרע.

בעבודה זו הראנו גם את פוטנציאל הייצור של ספונינים בתרבית רקמה של זאקום מצרי, ויחסי הגומלין בין הרכב חומרי הצמיחה שבתרבית לבין רמת ייצור הספונינים.

לסיכום, תוצאות מחקר זה עשויות לסייע לקדם את ההבנה של היבטים הקשורים בספונינים ככלל, ובהקשר לזאקום המצרי בפרט. עבודה זו עשוייה להביא להעלאת המודעות לפוטנציאל הטמון בעץ השמן העתיק, הזאקום המצרי. בעקבות כך צפויות להתבצע פעולות הקשורות בתרבותו ופיתוחו באזורים צחיחים שונים בעולם ויישומו בתעשייה הביוטכנולוגית.