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ARTICLES

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Full Length Research Paper

Analysis of bioactive chemical components of two medicinal plants (*Coriandrum sativum* and *Melia azedarach*) leaves using gas chromatography-mass spectrometry (GC-MS)

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The main objective of this study was to determine the phytochemical composition in the leaves of *Coriandrum sativum*, using methanolic extraction and report the main functional components by using IR technique. The phytochemical compounds in the extract were then screened by GC-MS method. Seven bioactive phytochemical compounds were identified in the methanolic extract of *C. sativum*: 1,6-octadien-3-ol, 3,7-dimethyl, 1,6-octadien-3-ol,3,7-dimethyl, 2-aminobenzoate, bicyclo[2.2.1]heptan-2-one,1,7,7-trimethyl., geranyl vinyl ether, 9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol., ascorbic acid 2,6-dihexadecanoate and 7aH-cyclopenta[a] cyclopropa[f]cycloundecene. Thirteen bioactive phytochemical compounds were identified in the methanolic extract of *Melia azedarach*. In the present investigation, a variety of compounds have been detected in *M. azedarach* including trichloromethane, propanedioic acid, diethyl ester, 2-pyrrolidinyl-methylamine, butanedioic acid, diethyl ester, 2-piperidimethanamine, butanedioic acid, hydroxyl-, diethyl ester, 2,5-dimethylhexane-2,5-dihydroperoxide, dithiocarbamate, s-methyl-,n-(2-methyl-3-oxobutyl), triethyl citrate, y-sitosterol, ethyl 9,12,15-octadecatrienoate, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, and octadecane, 3-ethyl-5-(2-ethylbutyl). It contains chemical constitutions which may be useful for various herbal formulation as anti-inflammatory, analgesic, antipyretic, cardiac tonic and antiasthamatic. *C. sativum* is highly active against *Aspergillus terreus* 6.01 ± 0.200. Bioactive compounds of *C. sativum* and *M. azedarach* were assayed for *in vitro* antibacterial activity against *Staphylococcus aureus, Proteus mirabilis, Pseudomonas aerogenosa, Escherichia coli* and *Klebsiella pneumonia* using the diffusion method in agar. The zone of inhibition was compared with different standard antibiotics. The diameters of inhibition zones ranged from 5.60 ± 0.320 to 1.96 ± 0.200 mm for all treatments.

Key words: Anti-bacterial, antifungal activity, *Coriandrum sativum*, GC-MS analysis, *Melia azedarach*, phytochemicals.

INTRODUCTION

Coriander is an annual popular culinary medicinal plant with a distinctive pungent, fatty, and aldehydic aroma (Msaada et al., 2009). Coriander is recognized as one of the most important spices in the world and is of great significance in international trade (Msaada et al., 2007). The bioactive non-nutrient plant compounds in fruit, vegetables, grains, and other plant foods have been linked to reductions in the risk of major chronic diseases

(Altameme et al., 2015). Coriander has been cultivated since ancient times and is originally from the Mediterranean and Middle Eastern region and grows extensively in India, Russia, Central Europe, Asia and Morocco. The Coriander essential oil is generally obtained by steam distillation of the dried fully ripe fruits (seeds) and oil has a characteristic odor of linalool and a mild, sweet, warm and aromatic flavor (Ramadan and Moersel, 2006). The seeds have medical uses and traditionally applied for curing digestive disorder, pain in joints and rheumatism. Stomachic, spasmolytic, carminative, diarrhoea and dyspepsia of various origin's coriander are also used in aromatherapy (Gil et al., 2002; Eikani et al., 2007; Grosso et al., 2008, Hussein et al., 2015; Imad et al., 2015). It is used to treat female diseases such as menoxenia, ovulation type dysfunctional uterine bleeding (Paarakh, 2009). It is used for treating leucorrhea; spermatorrhea. Coriander fruit possess stimulant and carminative properties (Khare, 2007). The fruits are used as astringent, anthelmintic, emollient, stomachic, antibilious, digestive, appetizer, constipating, diuretic, antipyretic, refrigerant, tonic, expectorant, anodyne, antidiabetic and dyspepsia (Paarakh, 2009; Hameed et al., 2015a). *Melia azedarach*, family Meliaceae is from west Asia (Sumathi, 2013). It is a moderate-sized deciduous tree 9 to 12 m in height dark grey and a cylindrical bole. *M. azedarach* is traditionally been used as anthelmintic, astringent and stomachic agent. It is widely distributed in Himalayan region. The leaves are bi- or trip innate, pinnate opposite or alternate, ovate orlanceolate, serrate, acuminate, glabrous on both surfaces (Bergsson et al., 2002; Dawson et al., 2002; Lee et al., 2002; Hameed et al., 2015b). *M. azedarach* is used for the treatment of inflammations, leprosy and cardiac disorders. Its fruits and leaves extracts possess antiviral, antifertility activity, ovicidal and larvicidal activity (Wandscheer et al., 2004; Corpinella et al., 2007; Mandal and Dhaliwal, 2007). The plant possesses antioxidant, antimalarial, antihepatotoxic, antibacterial, antiparasitic, and antiulcer properties (Dai et al., 1999; Devi et al., 2001; Bahuguna et al., 2009; Samudram et al., 2009; Nahak et al., 2010; Hameed et al., 2015c). The aim of the study was to investigate the presence of phytochemical compounds from the leaves of *Coriandrum sativum* and *M. azedarach* by using gas chromatography-mass spectrometry and evaluation antibacterial activity.

MATERIALS AND METHODS

Collection and preparation of plant material

The leaves were purchased from a local market in Hilla city, middle of Iraq. After thorough cleaning and removal of foreign materials, the leaves were stored in airtight container to avoid the effect of humidity and then stored at room temperature until further use (Jasim et al., 2015).

Preparation of sample

About 20 g of the plant sample powdered were soaked in 120 ml methanol for 18 h in a rotatory shaker. Whatman No.1 filter paper was used to separate the extract of plant. The filtrates were used for further phytochemical analysis. It was again filtered through sodium sulphate in order to remove the traces of moisture.

Gas chromatography - Mass spectrum analysis

GC-MS (Agilent 7890 A) was used in this study to identify the components present in the extract (Mohammed and Imad, 2013; Kareem et al., 2015). About 1 µL of the methanol extract was injected into the GC-MS using a micro syringe (Imad et al., 2015a). Each of the peaks in the chromatogram represented the signal created when a compound eluted from the Gas chromatography column into the detector. The x-axis showed the retention time and the y-axis measured the intensity of the signal to quantify the component in the sample injected. As individual compounds eluted from the gas chromatographic column, they entered the electron ionization (mass spectroscopy) detector, where they were bombarded with a stream of electrons causing them to break apart into fragments. The fragments obtained were actually charged ions with a certain mass (Imad et al., 2015b). The mass/charge (M/Z) ratio obtained was calibrated from the graph obtained, which was called as the mass spectrum graph which is the fingerprint of a molecule. Before analyzing the extract using gas chromatography and mass spectroscopy, the temperature of the oven, the flow rate of the gas used and the electron gun were programmed initially. The temperature of the oven was maintained at 100°C. Helium gas was used as a carrier. The flow rate of helium was set to 1 ml per minute. The electron gun of mass detector liberated electrons having energy of about 70 eV. The column employed here for the separation of components was Elite 1 (100% dimethyl poly siloxane). The identity of the components in the extracts was assigned by the comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library (Muhanned et al., 2015; Imad et al., 2015c).

Determination of antibacterial activity of crude bioactive compounds of *C. sativum* and *M. azedarach*

Proteus mirabilis, Escherichia. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae and *Staphylococcus aureus* were swabbed in Mueller Hinton agar plates. 60 µL of plant extract was loaded on the bored wells. The wells were bored in 0.5 cm in diameter. The plates were incubated at 37°C for 24 h and examined. After the incubation the diameter of inhibition zones around the discs was measured.

Determination of antifungal activity

Five-millimeter diameter wells were cut from the agar using a sterile cork-borer, and 50 µl of the samples solutions (*C. sativum* and *M.*

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azedarach) was delivered into the wells. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganisms. Methanol was used as control. Amphotericin B and fluconazole were used as reference antifungal agents. The tests were carried out in triplicate. The antifungal activity was evaluated by measuring the inhibition-zone diameter observed after 48 h of incubation (Huda et al., 2015; Ameera et al., 2015).

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) and differences among the means were determined for significance at $p < 0.05$ using Duncan's multiple range test using SPSS software) version 9.1.

RESULTS AND DISCUSSION

Gas chromatography and mass spectroscopy analysis of compounds was carried out in methanolic leaves extract of *C. sativum* and *M. azedarach* shown in Tables 1 to 2. The GC-MS chromatogram of the twenty peaks of the compounds detected is shown in Figures 1 and 2. Chromatogram GC-MS analysis of the methanol extract of *C. sativum* showed the presence of 20 major peaks and the components corresponding to the peaks were determined as follows. The first set up peak was determined to be 1,6-octadien-3-ol,3,7-dimethyl (Figure 2). The second peak was indicated to be 1,6-octadien-3-ol,3,7-dimethyl, 2-aminobenzoate (Figure 3). The next peaks was considered to be bicyclo[2.2.1]heptan-2-one,1,7,7-trimethyl, geranyl vinyl ether, 9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol, ascorbic acid 2,6-dihexadecanoate, 7ah-cyclopenta[a] cyclopropa[f]cycloundecene (Figures 4 to 9). Coriander oil may have future use as a free radical scavenger, preventing oxidative deterioration in foods. In a report by Ramadan and Moersel (2006) coriander oil was shown to have greater activity against the radical generating activity of 1,1-diphenyl-2- picrylhydrazyl in several oils. Recently, Coriander oil has been reported to possess many medicinal properties, including antimicrobial properties against selected pathogenic (Martins et al., 2003; Ishikawa et al., 2003) antioxidant (Quynh et al., 2009), antidiabetic (Pourmortazavi and Hajimirsadeghi, 2007), anticancer and antimutagenic activities (Mohammad et al., 2011). *Coriandrum sativum* can act as source for oleic acid as the percentage found was 38.55% and soxhlation method can be used to extract it from the fruit (Padmaa, 2014). Shahidi (2008) reported that their synergistic effects are rendered by a combination of phytochemicals present in source materials, and complementary nature of phytochemicals from different sources are important factors to consider in the formulation of functional foods and in the choice of a healthy diet.

Medicinal plants are used in traditional treatments to cure variety of diseases (Hariprasad and Ramakrishnan, 2011). Various scientific studies reported the analgesic, anticancer, antiviral, antimalarial, antibacterial, antifeedent and antifertility activity of this plant (Vishnukanta, 2008; Sen and Batra, 2011). Gas chromatography and mass spectroscopy analysis of compounds was carried out in methanolic leaves extract of *M. azedarach* as shown in Table 2. Chromatogram GC-MS analysis of the methanol extract of *M. azedarach* showed the presence of 13 major peaks and the components corresponding to the peaks were determined as follows. The first set up peak was determined to be Trichloromethane (Figure 10). The second peak indicated to be Propanedioic acid and diethyl ester (Figure 11). The next peaks considered to be 2-pyrrolidinyl-methylamine, butanedioic acid, diethyl ester, 2-piperidimethanamine, butanedioic acid, hydroxyl, diethyl ester, 2,5-dimethylhexane-2,5-dihydroperoxide, dithiocarbamate, s-methyl-,n-(2methyl-3-oxobutyl), triethyl citrate, y-sitosterol, ethyl 9,12,15-octadecatrienoate, hexadecanoic acid,2-hydroxy-1-(hydroxymethyl) ethyl ester, and octadecane, 3-ethyl-5-(2-ethylbutyl) (Figures 12 to 22). Plant based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects. Continued further exploration of plant derived antimicrobials is needed today.

Antibacterial and antifungal activity

K. pneumoniae, P. aeroginosa, E. coli, S. aeureus and *P. mirabilis* were five clinical pathogens selected for THE antibacterial activity maximum zone formation against *E. coli* (Table 3). Methanolic extraction of plant showed notable antifungal activities against *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus flavus* and *Aspergillus fumigates* (Table 4). *C. sativum* and *M. azedarach* was very highly active against *A. terreus* (6.01 ± 0.200). *Aspergillus* was found to be sensitive to all test medicinal plants and mostly comparable to the standard reference antifungal drug amphotericin B and fluconazole to some extent.

Conclusion

C. sativum and *M. azedarach* are native plant of Iraq. Thus, the GC-MS analysis of methanolic leaves extract of *C. sativum* and *M. azedarach* showed a highly complex profile containing approximately 20 components. This study may be useful to explore the pharmacological and biosynthetic activity of the plants further.

Conflict of interests

The authors did not declare any conflict of interest.

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The authors thank Dr. Abdul-Kareem Al-Bermani,

Table 1. Major phytochemical compounds identified in methanolic extract of *Coriandrum sativum* leaves.

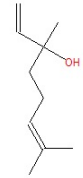
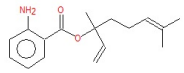
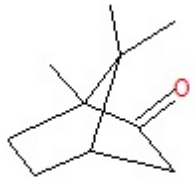
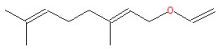
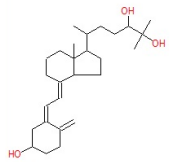
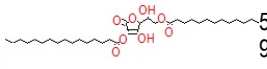
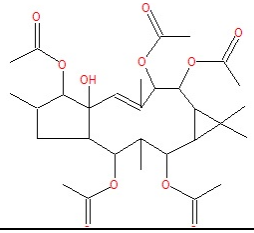
Phytochemical compound	RT (min)	Formula	Molecular weight	Exact mass	Chemical structure	MS Fragmentation	Pharmacological actions
1,6-Octadien-3-ol,3,7-dimethyl	4.3	C10H18O	154	154.1358		55,71,80,93.107,121,136,154	Anti-inflammatory and anti-cancer properties.
1,6-Octadien-3-ol,3,7-dimethyl, 2-aminobenzoate	4.643	C17H23NO2	273	273.1729		55,69,80,93,105,121,136,154	Anti-inflammatory, antiseptic, anti-depressant
Bicyclo[2.2.1]heptan-2-one,1,7,7-trimethyl.	5.908	C10H16O	152	152.1201		55,81,95,137,152	Immune enhancement and anti-microbial activity
Geranyl vinyl ether	8.534	C12H20O	180	180.1514		53,69,93,136,152,178	anti-microbial, anti-cancer and anti-malaria
9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol.	10.248	C27H44O3	416	416.329		55,69,91,118,136,158,176,189,207,221,253,281,291	Anti-cancer, anti-inflammatory, and hepatoprotective
Ascorbic acid 2,6-dihexadecanoate	15.212	C38H68O3	652	652.4914		57,73,85,98,115,129,143,157,185,199,213,256,299,327,396,414	Antimetastatic and anti-invasive and antioxidant action
7aH-Cyclopenta[a] cyclopropa[f]cycloundecene	19.52	C30H44O11	580	580.2884		55,69,111,149,237,281,358,400,46	Immune enhancement and anti-micro-organism.

Table 2. Major phytochemical compounds identified in methanolic extract of *Melia azedarach* leaves.

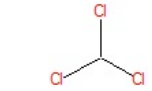
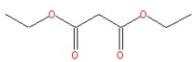
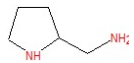
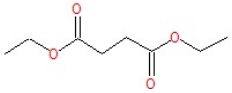
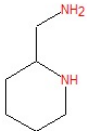
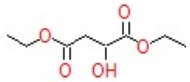
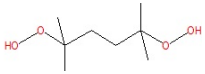
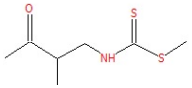
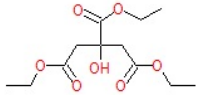
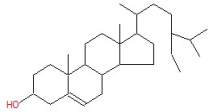
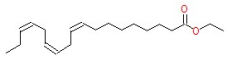
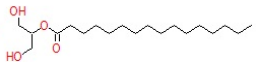
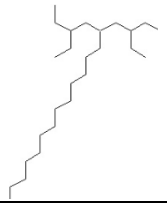
Phytochemical compound	RT(min)	Formula	Molecular weight	Exact mass	Chemical structure	MS Fragment -ions	Pharmacological actions
Trichloromethane	4.58	CHCL3	117	117.9438		50,59,70,83,118	anti-virus, anti-cancer, anti-mutagenic, anti-allergic and anti-ulcer
Propanedioic acid, diethyl ester	4.683	C7H12O4	160	160.0376		53,60,70,88,115,133, 160	anti-inflammatory
2-pyrrolidinyl-methylamine	5.233	C5H12N2	100	100.1		55,77,84,99	anti-Allergic
Butanedioic acid, diethyl ester	6.39	C8H14O4	174	174.0892		55,73,84,101,129,147 ,174	antimicrobial, antispasmodic and anti-inflammatory effects
2-Piperidimethanamine	6.537	C6H14N2	114	114.1157		56,67,84,96,114	anti-depressant activity and anti-tumor
Butanedioic acid, hydroxyl-, diethyl ester	7.27	C8H14O5	190	190.8141		60,71,75,89,102,117, 145	anti-ulcer
2,5-Dimethylhexane-2,5-dihydroperoxide	8.391	C8H18O4	178	178.1502		55,69,75,85,95,111,1 27,144,,178	anti-aging agents and anti-oxidant
Dithiocarbamate,S-methyl-,N-(2methyl-3-oxobutyl)	10.056	C7H13NOS2	191	191.0439		57,85,143,191	anti-cancer agents
Triethyl citrate	12.122	C12H20O7	276	276.1209		60,69,87,115,129,157 ,167,185,203,213,231	Anti-Ulcer and anti-inflammatory agent
Y-Sitosterol	13.044	C29H50O	414	414.3862		55,69,81,119,145,161 ,213,255,303,329,345 ,381,396,414	Anti-inflammatory activity
Ethyl 9,12,15-octadecatrienoate	17.106	C20H34O2	306	306.2559		55,67,79,95,121,135, 191,221,261,306	antioxidant, anti-inflammatory, antimicrobial and pesticide
Hexadecanoic acid,2-hydroxy-1-(hydroxymethyl)ethyl ester	20.001	C19H38O4	330	330.277		57,74,84,98,112,134, 154,182,213,239,275, 270,299,330	antioxidant, anti-inflammatory and anthelmintic activities

Table 2. Contd.

Octadecane, 3-ethyl-5-(2-ethylbutyl)	20.596	$C_{26}H_{54}$	366	366.422552		57,71,85,99,127,183, 197,225,281,295,337, 364	anti-oxidant and anti-inflammatory effect

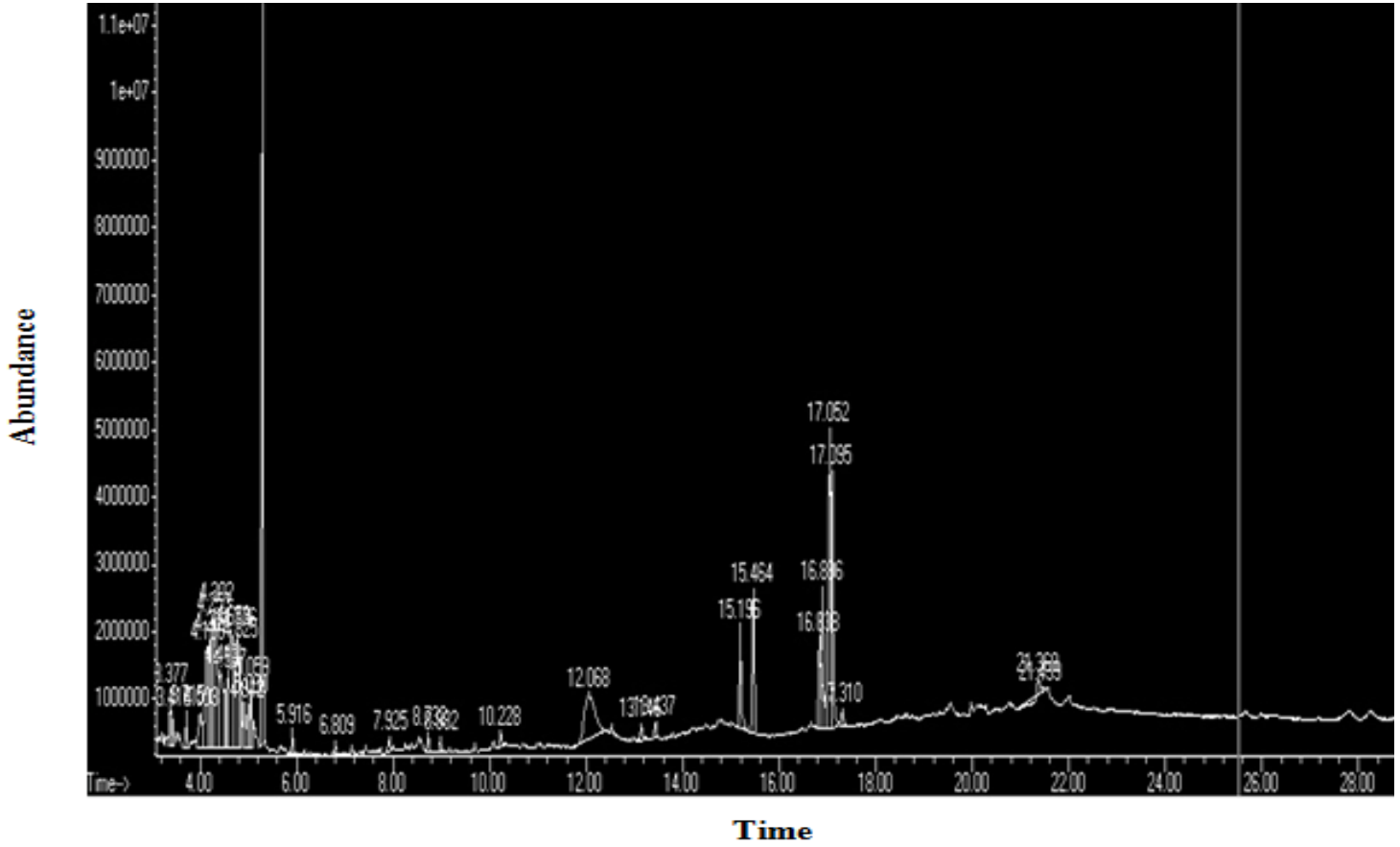


Figure 1. GC-MS profile of methanolic seeds extract of *Coriandrum sativum*.

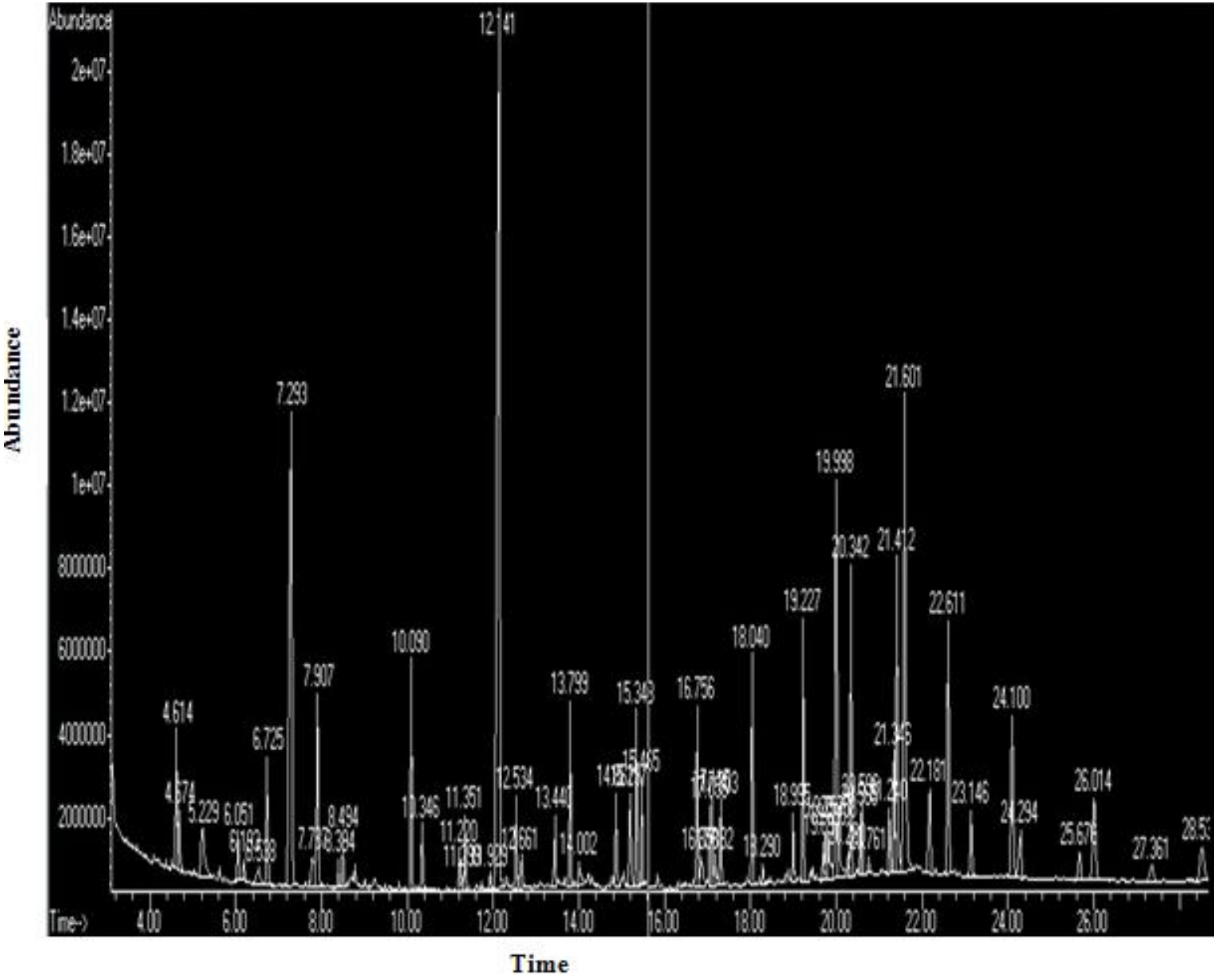


Figure 2. GC-MS chromatogram of methanolic extract of *Melia azedarach*.

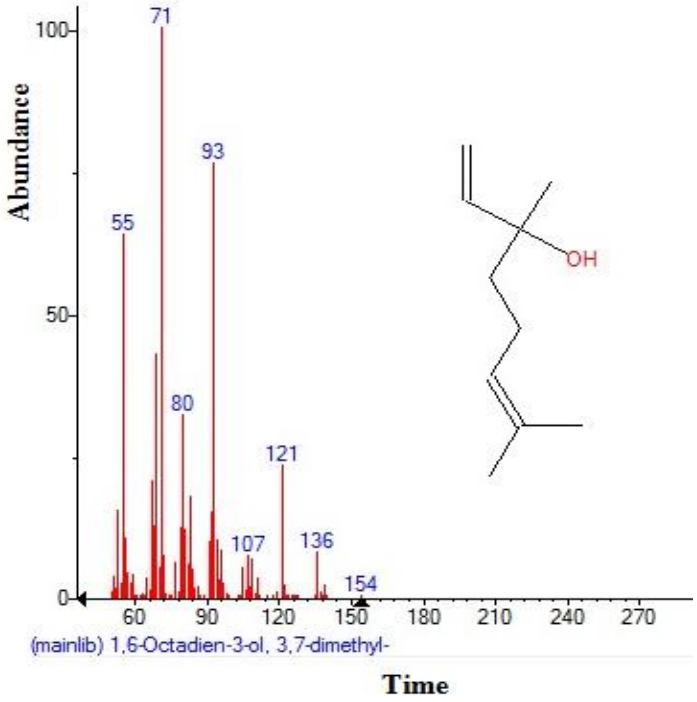


Figure 3. Structure of 1,6-Octadien-3-ol,3,7-dimethyl present in the methanolic leaves extract of *Coriandrum sativum* by using GC-MS analysis.

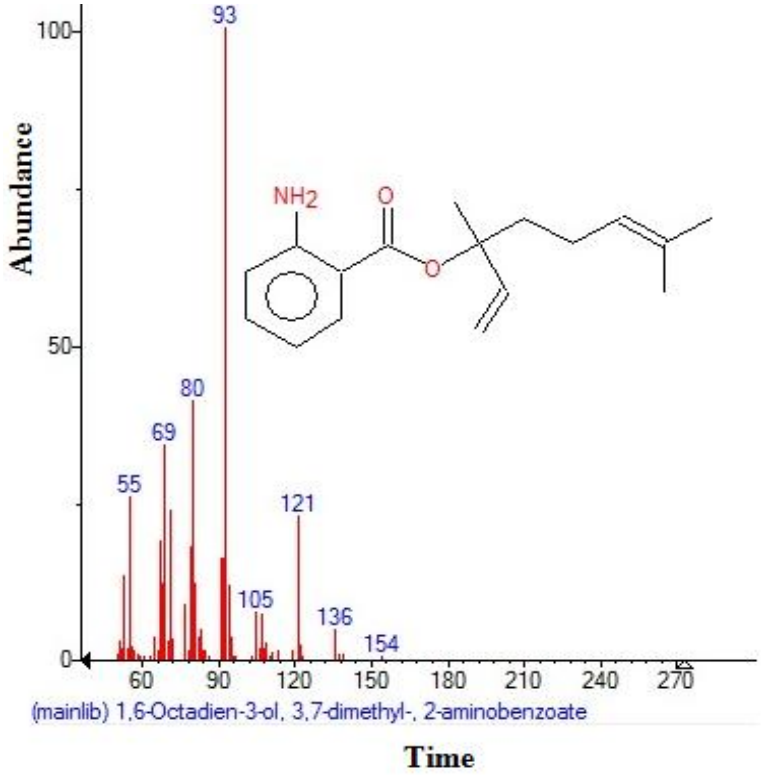


Figure 4. Structure of 1,6-Octadien-3-ol,3,7-dimethyl, 2-aminobenzoate present in the methanolic leaves extract of *Coriandrum sativum* by using GC-MS analysis.

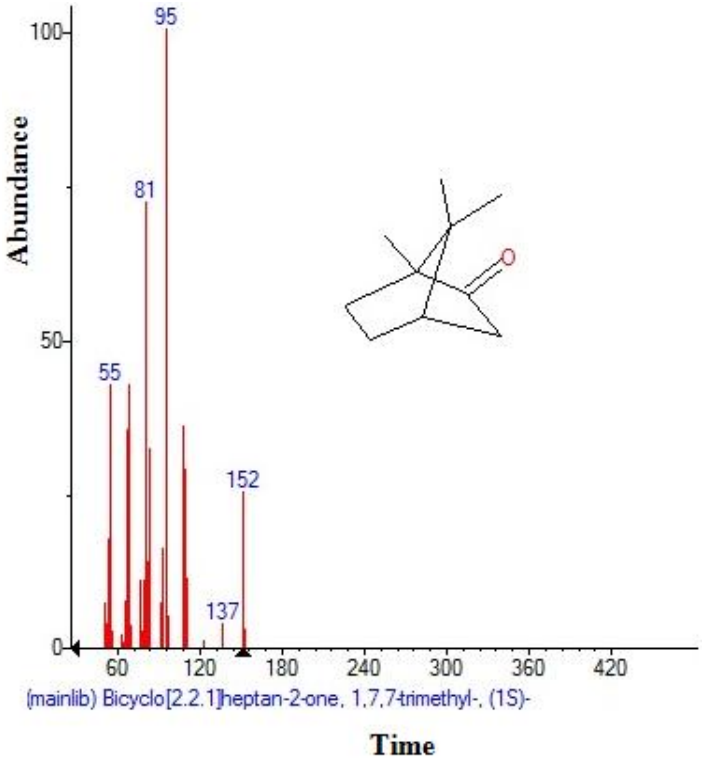


Figure 5. Structure of Bicyclo[2.2.1]heptan-2-one,1,7,7-trimethyl present in the methanolic leaves extract of *Coriandrum sativum* by using GC-MS analysis.

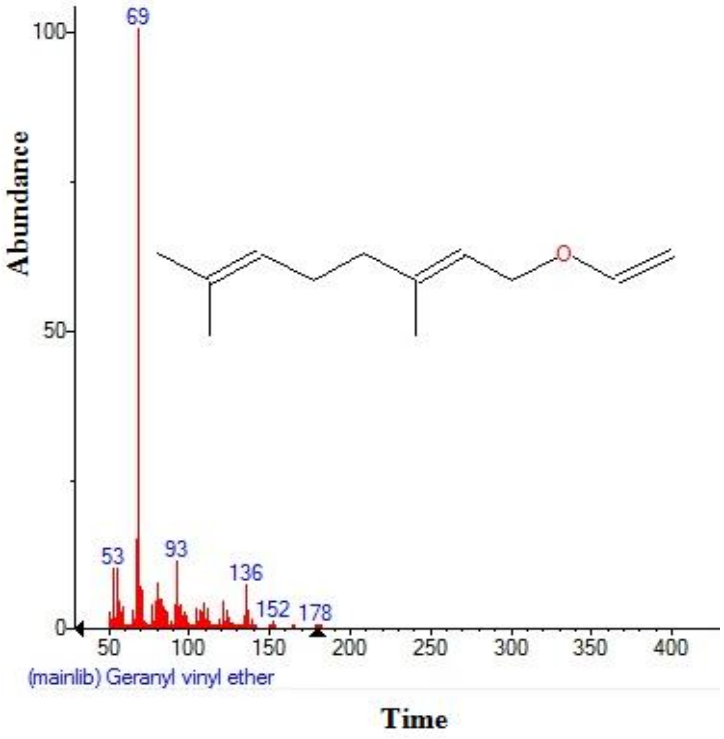


Figure 6. Structure of Geranyl vinyl ether present in the methanolic leaves extract of *Coriandrum sativum* by using GC-MS analysis.

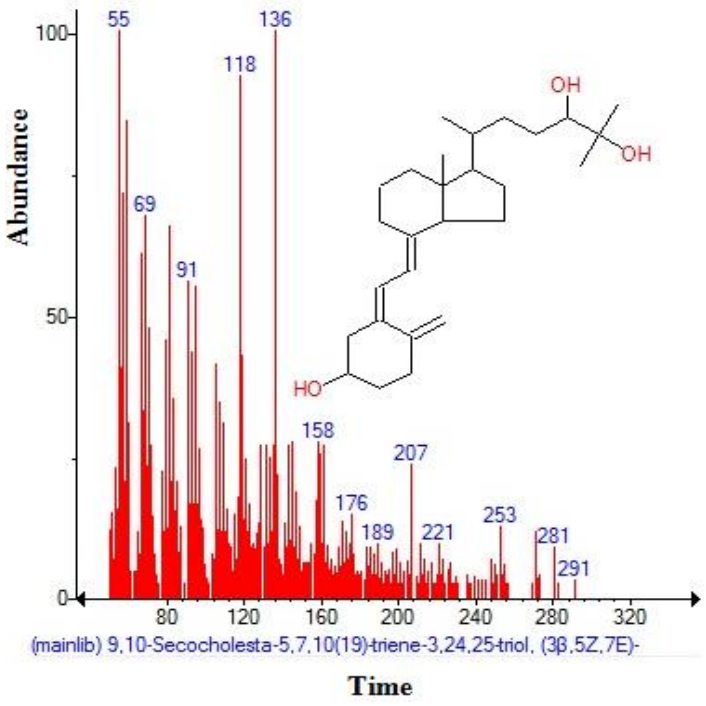


Figure 7. Structure of 9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol present in the methanolic leaves extract of *Coriandrum sativum* by using GC-MS analysis.

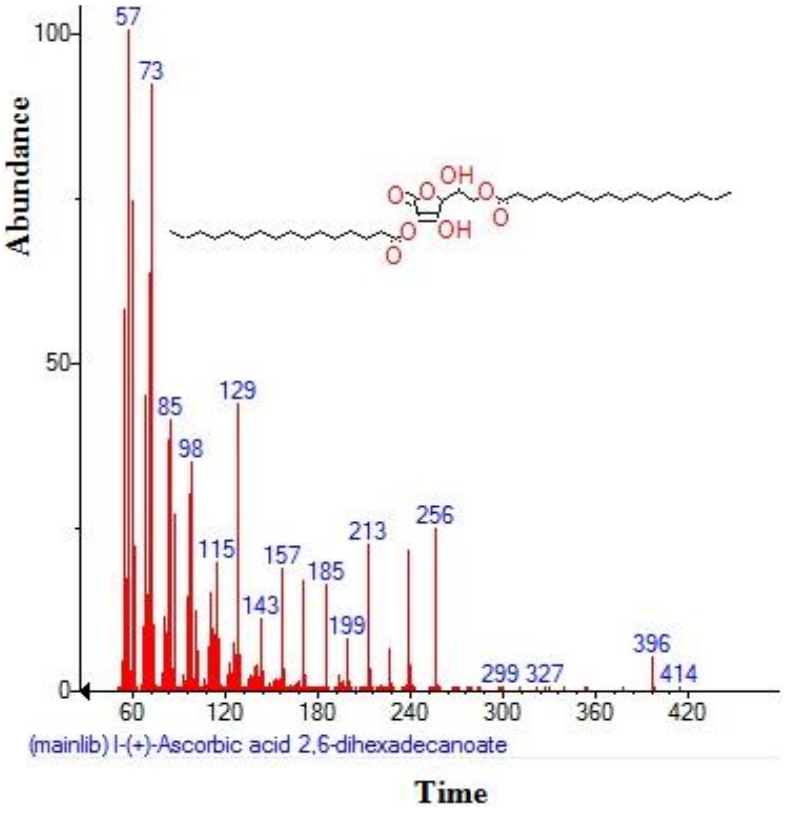


Figure 8. Structure of Ascorbic acid 2,6-dihexadecanoate present in the methanolic leaves extract of *Coriandrum sativum* by using GC-MS analysis.

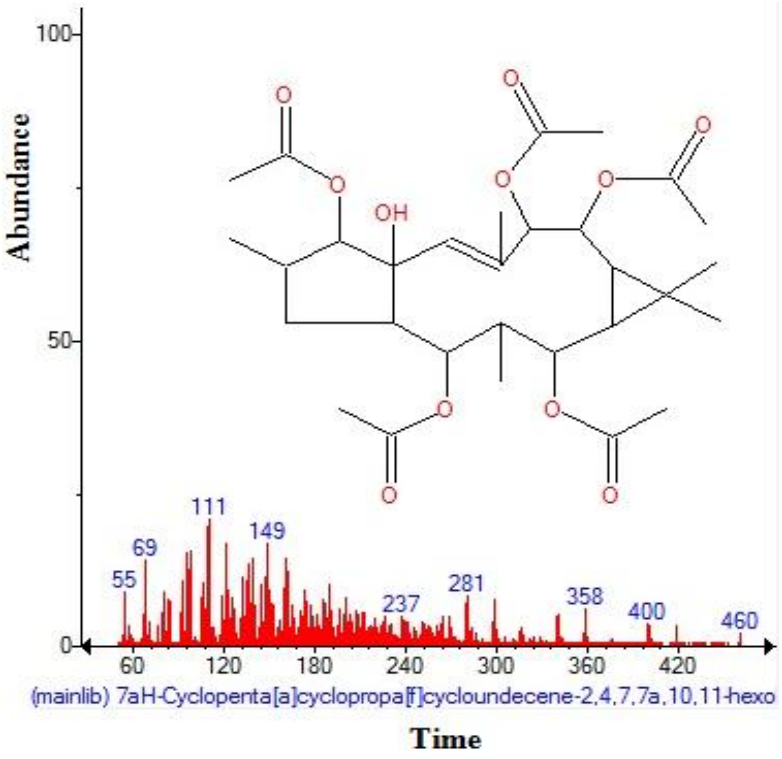


Figure 9. Structure of 7aH-Cyclopenta[a]cyclopropa[f]cycloundecene present in the methanolic leaves extract of *Coriandrum sativum* by using GC-MS analysis.

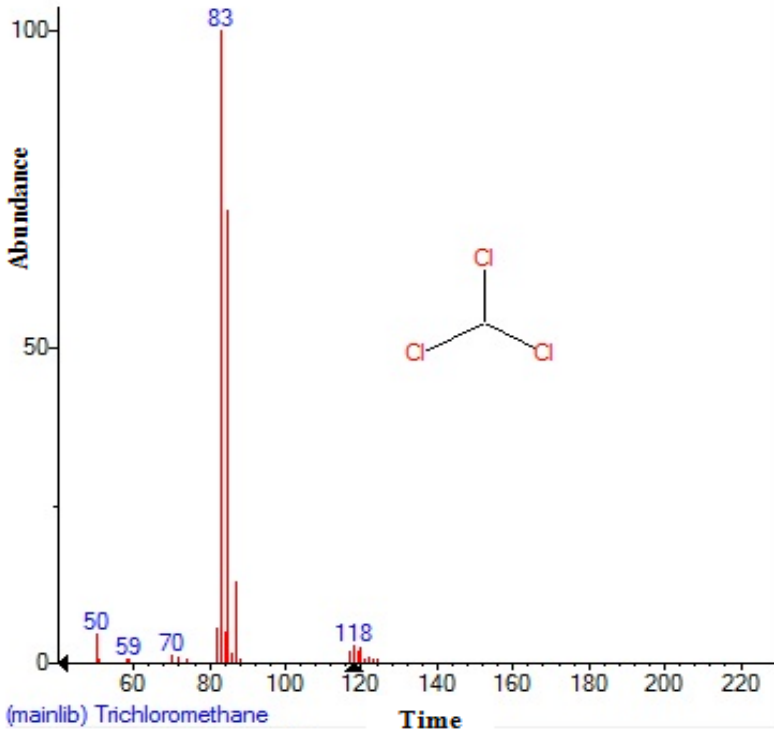


Figure 10. Mass spectrum of trichloromethane with retention time (RT) = 4.580.

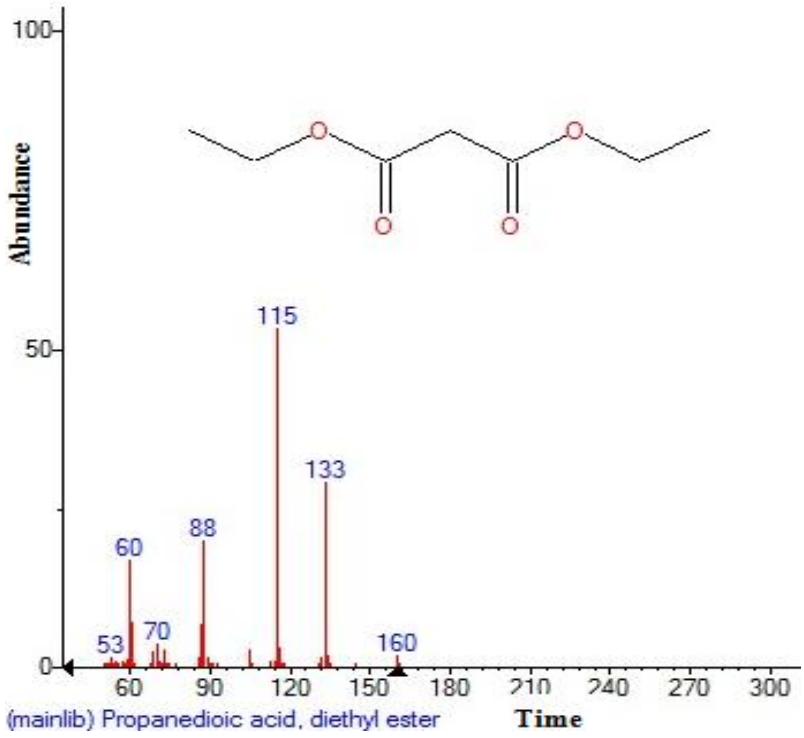


Figure 11. Mass spectrum of propanedioic acid, diethyl ester with retention time (RT) = 4.683.

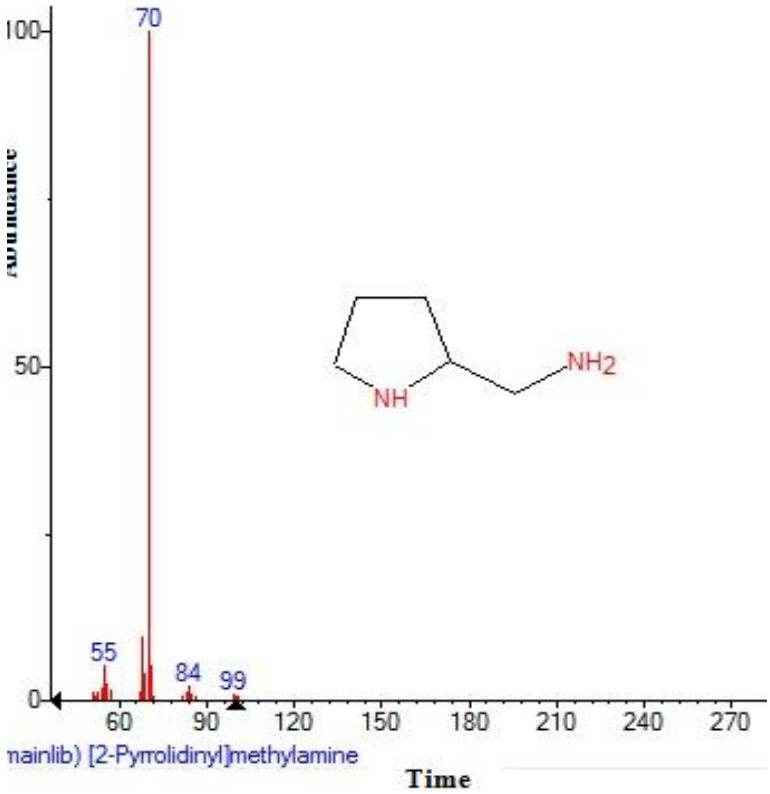


Figure 12. Mass spectrum of 2-pyrrolidinyl-methylamine with retention time (RT) = 5.233.

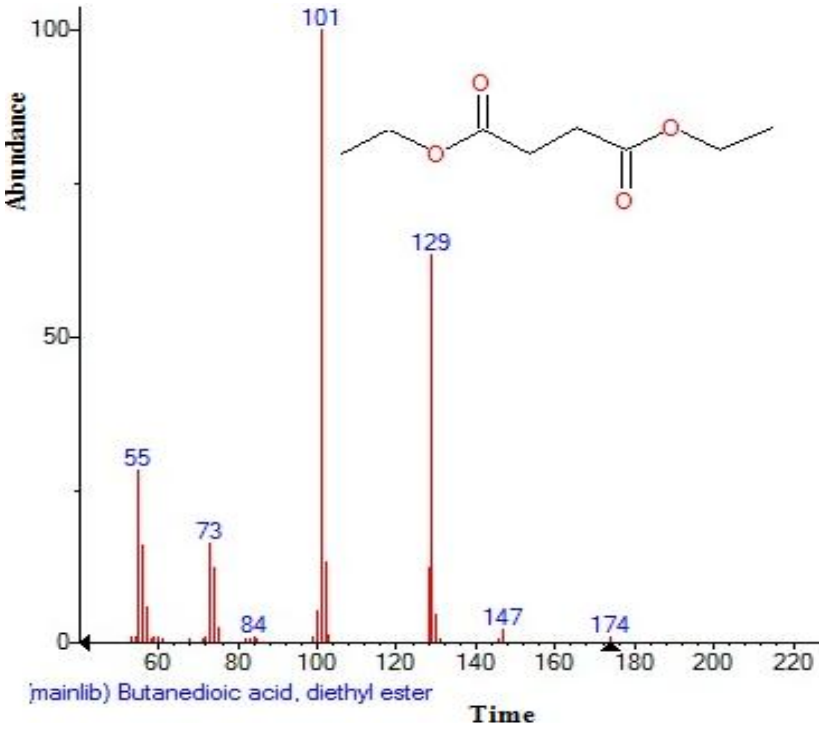


Figure 13. Mass spectrum of butanedioic acid, diethyl ester with retention time (RT) = 6.39.

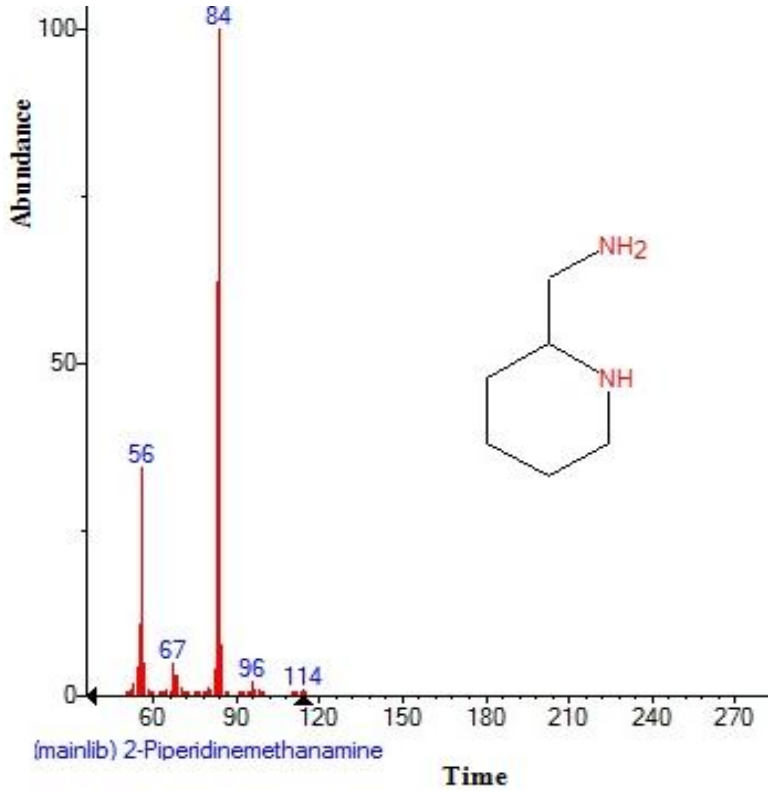


Figure 14. Mass spectrum of 2-piperidimethanamine with retention time (RT) = 6.537.

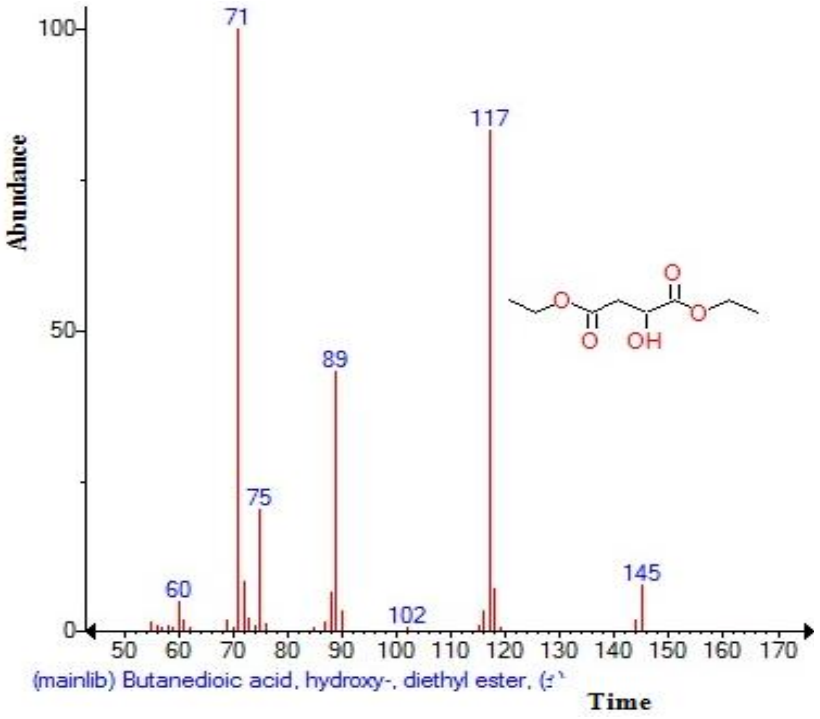


Figure 15. Mass spectrum of butanedioic acid, hydroxyl-, diethyl ester with retention time (RT) = 7.270.

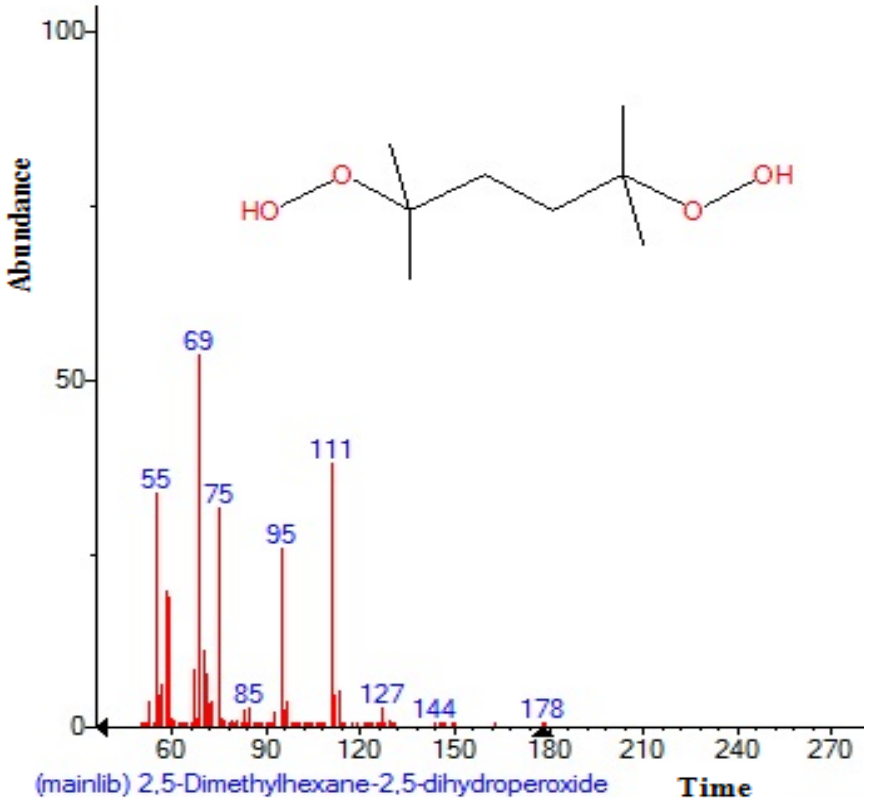


Figure 16. Mass spectrum of 2,5-dimethylhexane-2,5-dihydroperoxide with retention time (RT)= 8.391.

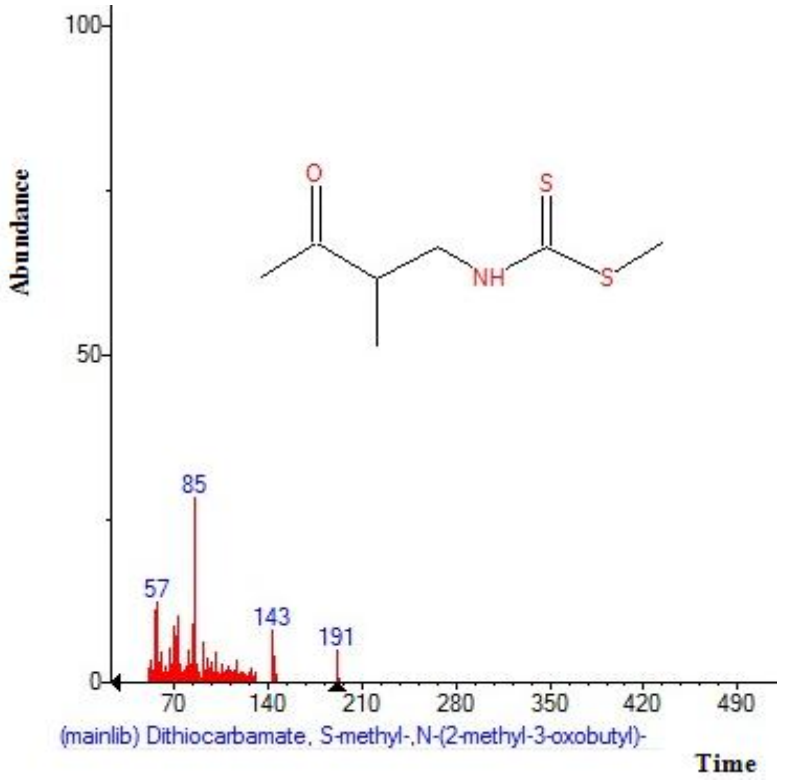


Figure 17. Mass spectrum of dithiocarbamate, S-methyl-,N-(2methyl-3-oxobutyl) with retention time (RT) = 10.056.

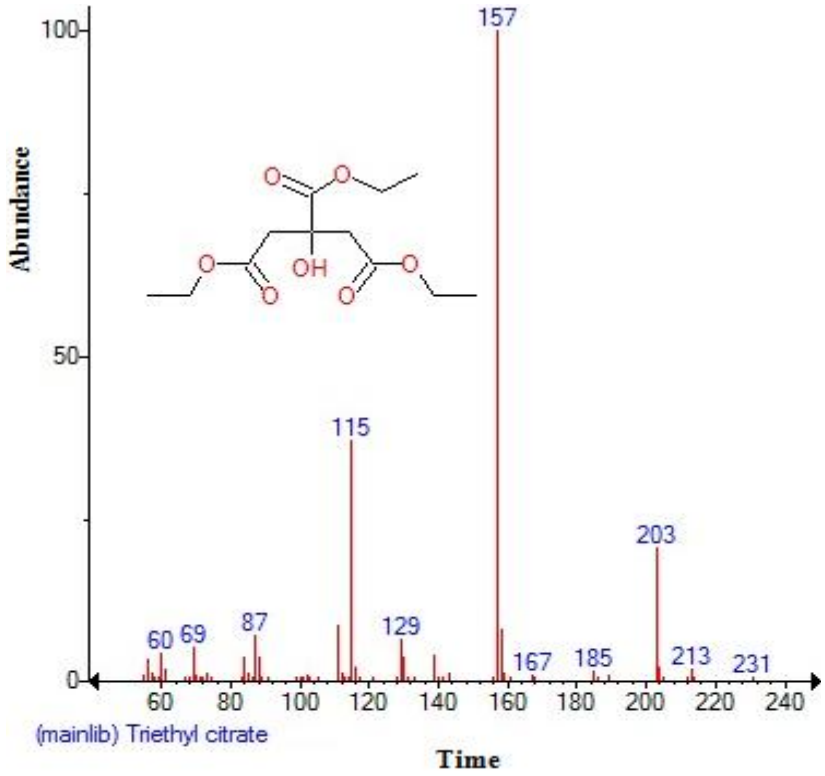


Figure 18. Mass spectrum of Triethyl citrate with Retention Time (RT) = 12.122.

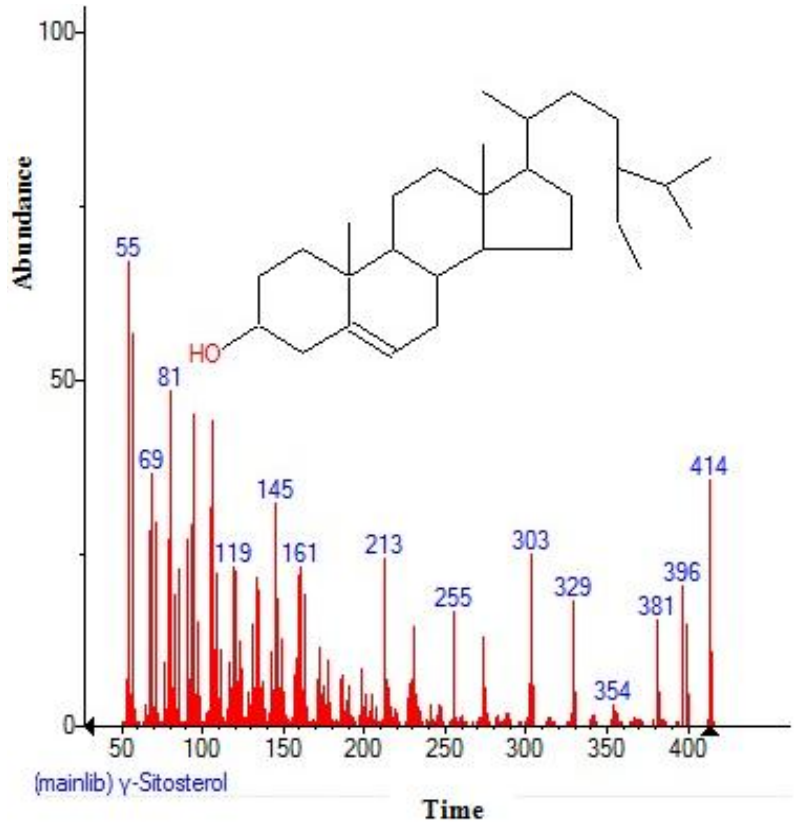


Figure 19. Mass spectrum of Y-sitosterol with retention time (RT) = 13.044.

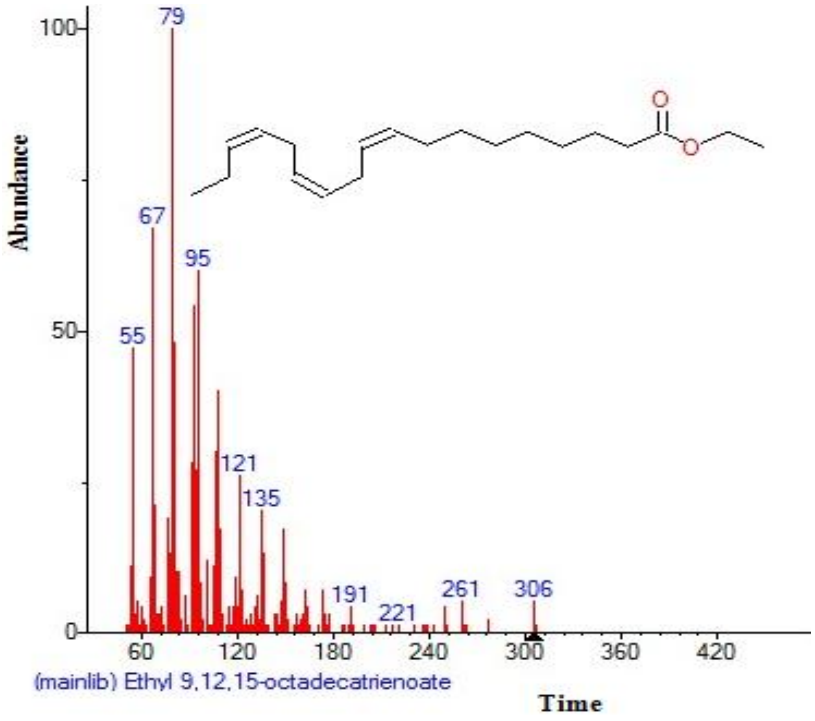


Figure 20. Mass spectrum of ethyl 9,12,15-octadecatrienoate with retention time (RT) = 17.106.

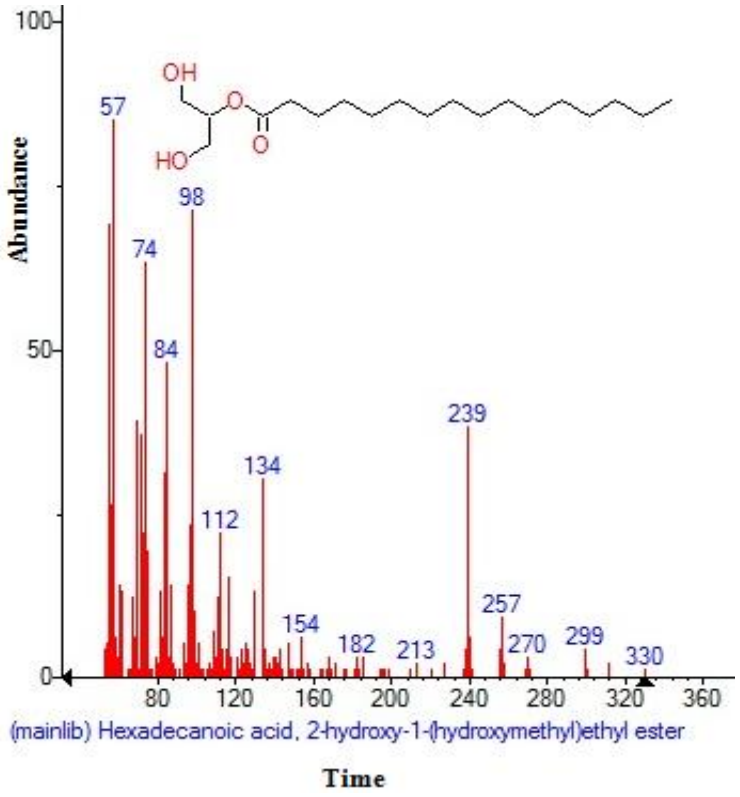


Figure 21. Mass spectrum of hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester with retention time (RT) = 20.001.

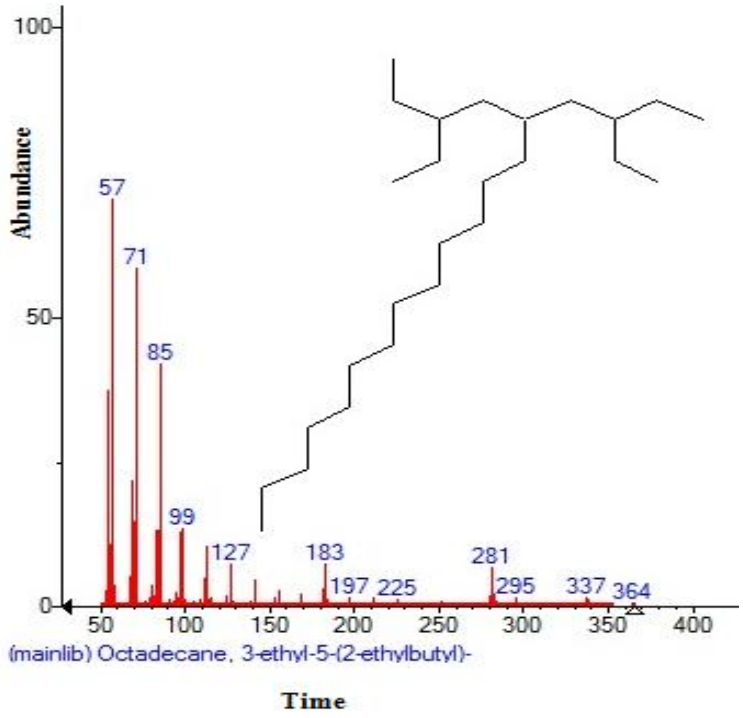


Figure 22. Mass spectrum of octadecane, 3-ethyl-5-(2-ethylbutyl) with retention time (RT) = 20.59.

Table 3. Zone of inhibition (mm) of test bacterial strains to *Coriandrum sativum* and *Melia azedarach* bioactive compounds and standard antibiotics.

Bacteria	Plants/antibiotics			
	Coriandrum sativum	***Melia azedarach***	**Rifambin**	**Cefotoxime**
Pseudomonas aeroginosa	4.08±0.120	3.99±0.110	1.96±0.200	2.99±0.160
Escherichia coli	5.60±0.320	4.11±0.200	2.91±0.310	2.99±0.620
Klebsiella pneumonia	3.00±0.510	3.99±0.170	3.23±0.300	2.00±0.310
Staphylococcus aureus	1.97±0.810	3.04±0.240	2.08±0.220	3.68±0.230
Proteus mirabilis	3.30±0.860	2.80±0.300	2.00±0.250	2.90±0.520

Table 4. Zone of inhibition (mm) of *Aspergillus spp.* test to *Coriandrum sativum* and *Melia azedarach* bioactive compounds and standard antibiotics.

Plant/Antibiotics	***Aspergillus niger***	***Aspergillus terreus***	***Aspergillus flavus***	***Aspergillus fumigatus***
Coriandrum sativum	3.00±0.180	6.01±0.200	5.94±0.300	5.08±0.140
Melia azedarach	2.07±0.100	5.01±0.310	4.66±0.130	6.00±0.130
Amphotericin B	2.05±0.120	4.00±0.340	4.06±0.200	4.36±0.180
Fluconazol	4.07±0.311	2.96±0.155	3.00±0.265	4.90±0.620
Control	0.00	0.00	0.00	0.00

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Full Length Research Paper

In vitro regeneration of *Calophyllum brasiliense* Cambess: A valuable medicinal tree

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***Calophyllum brasiliense* (Calophyllaceae) is a medicinal tree known mainly for producing calanolides, secondary metabolites against HIV-1 reverse transcriptase. This wild plant is listed as threatened and despite its outstanding medicinal value, no studies have been conducted on its propagation or preservation. This study standardized a procedure for the micropropagation of *C. brasiliense* with nodal segments from *in vitro* seedlings. The *in vitro* seed germination was 48.6%. The nodal explants displayed a high percentage of shoot induction (77.5%), shoots per segment (6.9), nodes per shoot (3.8), leaves per shoot (8.0) and shoot length (4.2 cm) when 0.5 mg L^{-1} indole-3-butyric acid plus 0.1 mg L^{-1} thidiazuron were used. Furthermore, maximum shoot rooting (63.5%) and root length (2.2 cm) were recovered using 1.0 mg L^{-1} indole-3-acetic acid. More than three-quarters of the acclimatized plants (77.5%) grew successfully in pots. Thus, this study developed an *in vitro* propagation protocol for *C. brasiliense* that can be used as a potential resource for restoring wild populations or performing phytopharmacological studies.**

Key words: Seed germination, plant growth regulators, micropropagation, nodal segments, medicinal plants.

INTRODUCTION

Calophyllum brasiliense Cambess is a medicinal tree belonging to the family Calophyllaceae (APG, 2009) that is distributed mainly in the rainforests of Latin America from Brazil to Mexico (Bruneton, 1993). Of the *Calophyllum* genus, only *C. brasiliense* exists in Mexico, and its members are restricted and scattered throughout small tropical areas. The species is commonly known as ocú, bari, or leche maria, and traditionally, it has been used

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Abbreviations: BA, 6-Benzyladenine; **IBA,** indole-3-butyric acid; **TDZ,** thidiazuron; **MS,** Murashige and Skoog; **PGRs,** plant growth regulators; **IAA,** indole-3-acetic acid.

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in many ways, such as wood, fodder, dye extracts, soap, biofuels and medicine (Stevens, 1980). Current studies have demonstrated that *C. brasiliense* contains a variety of phytochemicals, including xanthones, coumarins, chalcones, flavonoids and triterpenes, which possess antibacterial (Pretto et al., 2004), anticancer (Ito et al., 2002), antiparasitic (Brenzan et al., 2008) and antiviral activity (Huerta-Reyes et al., 2004). In addition, this species has been highlighted as an important resource of calanolides, dipyranocoumarins that inhibit the reverse transcriptase of human immunodeficiency virus type 1 (HIV-1) (Huerta-Reyes et al., 2004). Due to excessive collection for its medicinal properties and attractive wood, plus its recalcitrant seed's low viability, its population has decreased drastically (Afolayan and Adebola, 2004; Sorol et al., 2015). Despite its significant medical importance, no studies have developed an efficient system of micropropagation as an alternative for the conservation and restoration of *C. brasiliense*.

In this regard, plant tissue culture is an important tool in plant biotechnology, making it possible to clone plants (George et al., 2008). As this tool is applied to more species, it becomes more efficient and effective and more widely applied to the propagation and preservation of exceptional species as future resources (Pence, 2014). For instance, studies on *Callophyllum apetalum* found high *in vitro* multiplication (74%) with 1 mg L^{-1} 6-benzyladenine (BA) and 2 mg L^{-1} indole-3-butyric acid (IBA) to induce shoots and shoot rooting, respectively (Nair and Seeni, 2003). A similar study carried out by Thengane et al. (2006) found the maximal multiple shoots of *Calophyllum inophyllum* with 0.2 mg L^{-1} thidiazuron (TDZ). Moreover, when they rooted shoots with 0.50 or 5 mg L^{-1} IBA alone or in combination with 0.5 mg L^{-1} BA, were observed results of rooting 52%. Although, these species belong to the same genus, the best method of micropropagating a new plant, such as *C. brasiliense*, must usually be determined experimentally (Gahan and George, 2008).

This study germinated seeds *in vitro* and established a micropropagation system for *C. brasiliense* via nodal segments from germinated plantlets.

MATERIALS AND METHODS

Seeds source and disinfection

We collected mature *C. brasiliense* seeds in November 2013 from San Andres Tuxtla, State of Veracruz, Mexico. The endocarp and tegument were removed prior to disinfection and the seeds were superficially disinfected using a soap solution for 5 min. Then, they were immersed for 1 h in 27% (v/v) BRAVO® 720 solution as antifungal for 1 h. After they were immersed in a 4.2% (v/v) sodium hypochlorite solution supplemented with Tween-20 (three drops per 100 ml) for 1 h. Finally, under aseptic conditions, the seeds were rinsed three times with sterilized distilled water.

Culture medium and incubation conditions

MS (Murashige and Skoog, 1962) culture medium was supplemented with 3% sucrose (w/v), 100 mg L^{-1} citric acid, 150 mg L^{-1} ascorbic acid, 250 mg L^{-1} polyvinylpyrrolidone as an antioxidant solution and 0.18% of phytagel (w/v) (Sigma, St. Louis, MO, USA) to germinate the seeds. The pH of the culture medium was adjusted to 5.8, and the medium was sterilized at 121°C and 15 psi for 18 min. The disinfected seeds were placed in glass test tubes containing 15 ml of culture medium without plant growth regulators (PGRs) to germinate *in vitro*. Nodal explants from 6-month-old plants were cut in a Petri dish containing the same antioxidant sterile solution mentioned above to prevent browning of the tissue. Explants were placed into Gerber-type flasks (four explants per flask) containing 25 ml of culture medium with PGRs to induce *in vitro* rooting and shoots. To induce shoots, various concentrations of PGRs purchased from Sigma-Aldrich Co. were added: IBA (0.0 and 0.5 mg L^{-1}) in combination with BA (1.0 and 2.0 mg L^{-1}) or TDZ (0.1 and 1.0 mg L^{-1}). To induce roots, 1.0 and 2.0 mg L^{-1} of IBA or IAA was added. All the cultures were incubated at 25 ± 2°C with a photoperiod of 16 h with white fluorescent light (60 µmol m^{-2} s^{-1}). When the seedlings reached approximately 4 cm in height, they were transferred to polyethylene bags containing a mix of agrolite, peat moss and soil (1:1:1).

Statistical analysis

The statistical analyses were performed with SAS 9.0 software. All data were subjected to an analysis of variance followed by the Tukey-Kramer multiple media comparison test with a significance of 0.05. All the laboratory experiments were done in duplicate with three replicates. A completely randomized experimental design was used.

RESULTS AND DISCUSSION

Seed germination

After 12 days of culture, 48.6% of the seeds had germinated (Figure 1a). Although, the germination percentage was low (less than 50%), this percentage was slightly higher than the germination percentage (36%) previously reported in *ex vitro* conditions (Bernabé-Antonio et al., 2010). The seedlings cultured *in vitro* exhibited good development after 6 months (Figure 1b). The low percentages of germination may be due to *C. brasiliense* seeds being recalcitrant (that is, intolerant to desiccation and low temperatures), and thus, tending to lose their viability rapidly (Sorol et al., 2015). In this work, the seeds were collected after their physiological maturity, and the embryos may have lost moisture before sowing *in vitro*. In addition, the seeds are likely to have a chemical latency that prevents germination. For example, when *Garcinia* spp. seeds (a species taxonomically close to *C. brasiliense*) were germinated under *in vitro* conditions with BAP (2.5 mg L^{-1}) 100% germination was achieved (Mohan et al., 2012).

Shoot proliferation

All culture media containing PGRs induced shoots (Figure 1c) and explant browning was prevented with an antioxidant solution. Significant differences ($P \leq 0.5$) were

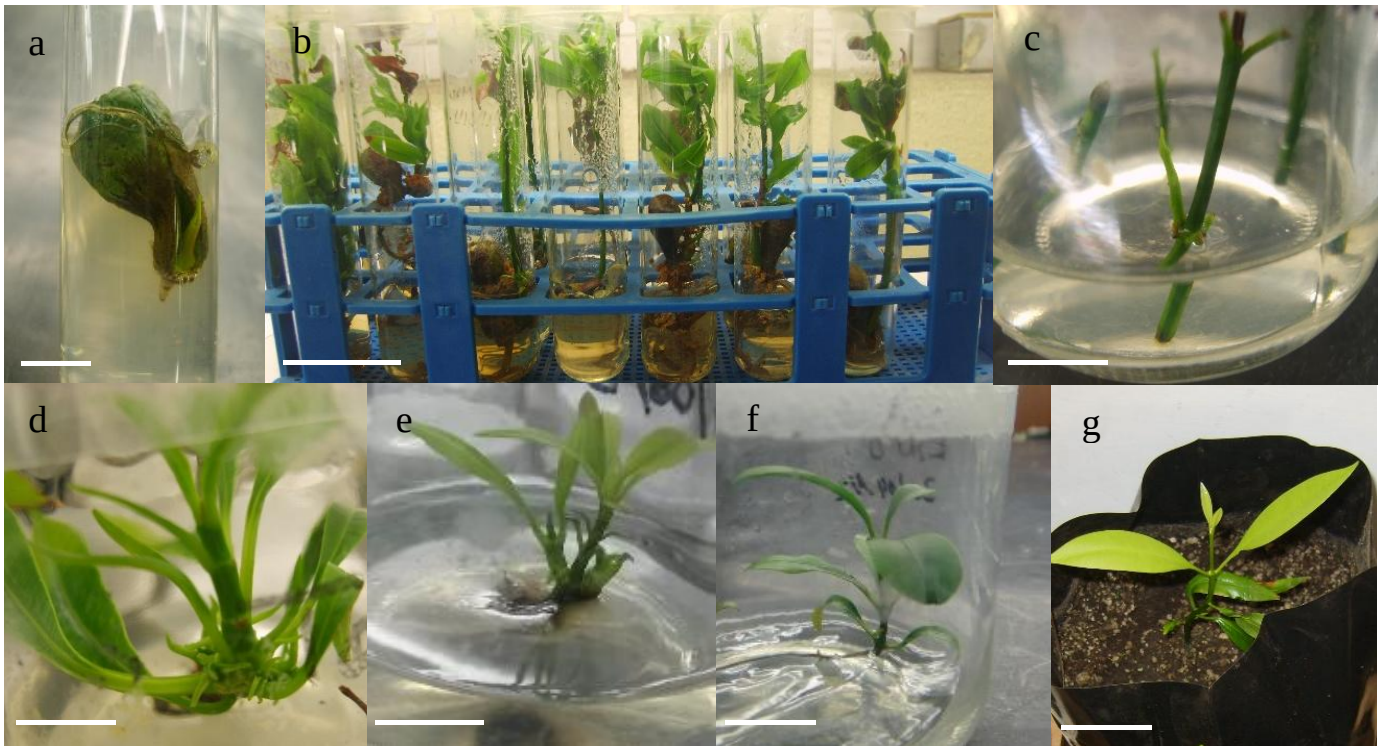

Figure 1. Germination and micropropagation of *C. brasiliense* in MS medium: (a) seed germination at 12 days; (b) seedling at 6 months; (c) nodal segment showing shoot induction after 2 weeks of culture; (d) shoot proliferation from nodal segments with 0.5 mg L^{-1} IBA plus 0.1 mg L^{-1} TDZ at 3 months; (e) shoot elongation from (d) without plant growth regulators; (f) shoot rooting with IAA (1 mg L^{-1}); (g) shoot acclimatization at 6 months. Bars: 1 cm (a), (c)–(f); 5 cm (b), (g). IBA, indole-3-butyric acid; TDZ, thidiazuron; IAA, indole-3-acetic acid.

Table 1. Effect of auxins and cytokinins (mg L^{-1}) on shoot proliferation of *C. brasiliense* through nodal segments in MS medium.

Auxin	Cytokinin		Shoot induction (%)	Number of shoots per segment	Number of nodes per shoot	Number of leaves per shoot	Shoot length (cm)
IBA	BA	TDZ					
Control			0.0 ± 0.0^{e}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}	0.0 ± 0.0^{e}	0.0 ± 0.0^{c}
0.0	1.0		25.7 ± 2.3^{b}	5.8 ± 0.3^{b}	2.5 ± 0.7^{b}	4.6 ± 1.1^{c}	1.3 ± 0.4^{b}
0.0	2.0		31.2 ± 3.9^{c}	5.8 ± 0.1^{b}	2.5 ± 0.7^{b}	4.6 ± 0.2^{c}	2.3 ± 0.4^{b}
0.5	1.0		36.9 ± 5.3^{c}	2.1 ± 0.2^{c}	3.6 ± 0.9^{a}	6.5 ± 0.5^{b}	3.5 ± 0.3^{a}
0.5	2.0		34.3 ± 4.4^{c}	2.1 ± 0.5^{c}	3.6 ± 0.9^{a}	7.5 ± 3.5^{a}	3.1 ± 0.1^{a}
0.0		0.1	34.2 ± 5.0^{c}	2.1 ± 0.3^{c}	2.1 ± 0.1^{b}	2.0 ± 0.1^{d}	1.1 ± 0.2^{b}
0.0		1.0	22.1 ± 2.0^{d}	1.3 ± 0.3^{c}	2.0 ± 0.0^{b}	2.0 ± 0.4^{d}	1.5 ± 0.1^{b}
0.5		0.1	77.5 ± 5.1^{a}	6.9 ± 0.4^{a}	3.8 ± 0.4^{a}	8.0 ± 0.2^{a}	4.2 ± 0.1^{a}
0.5		1.0	69.6 ± 3.7^{b}	5.3 ± 0.6^{b}	3.7 ± 0.8^{a}	6.9 ± 2.0^{b}	3.5 ± 0.3^{a}

Means ± standard deviation followed by different letters are significantly different at $P \leq 0.05$ according to the Tukey-Kramer multiple media comparison test. BA: 6-benzyladenine; IBA: indole-3-butyric acid; TDZ: thidiazuron.

found in all the variables evaluated (Tables 1 and 2). The cytokinin TDZ was observed to be more suitable than BA when they were combined with IBA. The treatment 0.50 mg L^{-1} IBA combined with 0.1 mg L^{-1} TDZ resulted in the maximum shoot induction (77.5%), shoots per segment (6.9), nodes per shoot (3.8), leaves per shoot (8.0) and shoot length (4.2 cm) (Figure 1d). When the shoots were excised from the proliferous node and grown in MS medium without PGRs, they showed good development (Figure 1e). TDZ is a cytokinin-like substance for woody plant tissue culture and facilitates the efficient micropropagation of many recalcitrant woody species when low concentrations (0.22 mg L^{-1} TDZ) are used (Huetteman and Peece, 1993). In this work, a decrease in the values of the variables was observed when 1.0 mg L^{-1} TDZ was used. Similar studies were carried out by

Table 2. Effect of IBA and IAA auxins on shoot rooting of *C. brasiliense* in MS medium.

Auxins (mg L^{-1})		Shoot rooting (%)	Number of roots per shoot	Root length (cm)
IBA	IAA			
Control		0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
1.0		32.2 ± 5.2^{b}	2.0 ± 0.2^{a}	1.2 ± 0.4^{b}
2.0		22.1 ± 1.8^{b}	1.5 ± 0.7^{a}	1.6 ± 2.2^{b}
	1.0	63.5 ± 3.4^{a}	1.1 ± 0.5^{b}	2.2 ± 0.7^{a}
	2.0	57.5 ± 2.4^{a}	1.0 ± 0.1^{b}	1.6 ± 0.6^{b}

Means ± standard deviation followed by different letters are significantly different at $P \leq 0.05$ according to the Tukey-Kramer multiple media comparison test. IBA, indole-3-butyric acid; TDZ, thidiazuron; IAA, indole-3-acetic acid.

Thengane et al. (2006) that reported concentrations of 0.2 mg L^{-1} TDZ were used to induce the largest number of shoots in *C. inophyllum* and Akbaş et al. (2009) that observed similar concentrations of BA (1 mg L^{-1}) were suitable for inducing shoots from *Amygdalus communis* nodal segments. Furthermore, the highest frequency of organogenetic response of explants were obtained on shoot buds of *Rumex tianschanicus* x *Rumex patientia* cultured with low concentrations of IAA (0.17 mg L^{-1}) or 2.2 mg L^{-1} BA (Ślesak et al., 2014). These results suggest that the organogenetic response depends on genotype (Ćosić et al., 2015). Although, general methodologies can be established for plant tissue culture, even closely related varieties of plants can differ in their culture requirements. Therefore, the best method of micropropagating a new plant, such as *C. brasiliense*, must usually be determined through experimentation (Gahan and George, 2008).

Shoot rooting

Significant differences ($P \leq 0.5$) were found in shoot rooting from *C. brasiliense* (Table 2). IBA and IAA induced rhizogenesis in shoots (Figure 1f); however, the rooting percentages with IBA were lower than those with IAA (Table 2). The highest percentage of shoot rooting (63.5%) occurred with 1.0 mg L^{-1} IAA, but this value was not statistically different when 2.0 mg L^{-1} IAA was added to the culture medium. In addition, 1.0 mg L^{-1} IAA exhibited the maximum root length (2.2 cm), whereas the largest number of roots was displayed in the presence of 1.0 mg L^{-1} IBA. The seedlings that exhibited the greatest height were acclimatized in pots and 77.5% of the plants survived transplantation after 3 months (Figure 1g). Thengane et al. (2006) demonstrated that roots were more easily induced with IBA (0.5 mg L^{-1}) without supplementation of any cytokinin in *C. inophyllum*. Likewise, Thengane et al. (2006) found a higher percentage of acclimatization when using only IBA. A similar *C. apetalum* study reported improved shoot rooting and acclimatization with larger concentrations of IBA (2.0 mg L^{-1}) (Nair and Seeni, 2003). In contrast, some species, such as *Hibiscus cannabinus* L., showed reduced rooting in explants when cytokinins or auxins were added (Ayadi et al., 2011). Contrary to these reports, IAA auxin was the most suitable for rooting shoots of *C. brasiliense* in this study.

Conclusions

This study is the first investigation of the regeneration of *C. brasiliense* and can be directly used as a potential tool for replenishing the plant's declining populations in the wild. About 50% of *in vitro* seed germination was achieved in this work. Using nodal explants, a high percentage of induction and shoot proliferation was achieved with 0.5 mg L^{-1} IBA plus 0.1 mg L^{-1} TDZ and the maximum shoot rooting was seen with 1 mg L^{-1} IAA. In addition, 77.5% of the plants grew easily in potted conditions. However, further detailed research is needed to improve the shoot multiplication and rooting ability of *C. brasiliense*. Further studies are needed to determine whether the seedlings grown *in vitro* retain the ability to produce calanolides or other biologically active compounds.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Gene mining a marama bean expressed sequence tags (ESTs) database: Embryonic seed development genes and microsatellite marker identification

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Tylosema esculentum **(marama bean) is one of the underutilized legumes that have potential to provide protein and fatty acids to ensure food security in dry parts of Southern Africa. In order to establish rapid domestication programs for the plant, it is important to explore the plant's genome and identify functional genes molecular markers like microsatellites in order to develop molecular tools. With the advent of high-throughput sequencing technologies and associated bioinformatics methods, expressed sequence tags (ESTs) have been developed for many plant species. These are being developed as an economic means of obtaining large numbers of gene sequences. The aim of this study was to identify genes with important roles for valuable agronomic traits and microsatellite sequences for marama bean. The authors reported the identification of genes associated with embryonic development and microsatellite sequences. The future direction will entail characterization of these genes using gene over-expression and mutant assays.**

Key words: Namibia, simple sequence repeats (SSR), data mining, homology searches, bioinformatics, *Tylosema esculentum*.

INTRODUCTION

In order to meet the future food and nutrition demands of an increasing population in southern Africa, and to make optimal use of marginal land, there is need to start research on little known edible plant species that offer great potential. *Tylosema esculentum* (Marama bean) is one of those research neglected plants. Marama bean is found in Namibia and Botswana in large populations and small populations in Gauteng, South Africa (Chingwaru et al., 2011). Marama bean is a species in the legume family that produces pods and bean-like seeds perennially. It is native to dry areas of Kalahari agro-ecological zones with little seasonal rainfall. It is particularly important in subsistence agriculture (Müseler and Schönfeldt, 2006). These neglected crops are usually accepted by the local population and better adapted to existing environmental conditions. The potential to provide a more stable food supply for a drought stricken Africa has been reported (Müseler and Schönfeldt, 2006). The plant is a nutritional and valuable food source and can be successfully used in programs

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specifically aimed at improving household and food security and in programs aimed to improve protein deficiency in southern Africa.

T. esculentum is a non-nodulating, undomesticated tuber-producing legume, abundant in protein, oil and starch (Takundwa et al., 2010). The bean and tuberous root extracts of the plant have also been used as medicine (Chingwaru et al., 2011). Despite abundance of protein, oil and starch, the plant has low yields, producing one or two seeds per pod. With the advent of bioinformatics, researchers have sequenced some legume genomes. The prominent ones are soybean (*Glycine max*), barrel medic (*Medicago truncatula*) and birdsfoot trefoil (*Lotus japonicus*), common bean (*Phaseolus vulgaris*), mungbean (*Vigna radiata*), red bean (*Vigna acutifolius*), narrow-leafed lupin (*Lupinus angustifolius*), wild peanut (*Arachis duranensis* and *Arachis ipaensis*), pigeon pea (*Cajanus cajan*) and chickpea (*Cicer arietinum*). The impact of these assembled, annotated genomes has been enormous. These genome sequences are useful for genome comparisons and to transfer information from these biological models to other crop species and vice versa (Cannon et al., 2009). Besides the genome sequencing of some legumes, researchers have also analyzed and exploited ESTs of some plant species in order to understand them better. These powerful tools are used to gain further insight in the molecular manifestations of growth, development, ripening and survival of the organism studied. ESTs have proven to be an economically feasible alternative for gene discovery in species lacking a draft genome sequence (Matukumalli et al., 2004), such as the *T. esculentum*.

An expressed sequence tag (EST) is a short sub-sequence of cDNA derived from cellular mRNA and thus represents part of a protein-coding gene (expressed genes). ESTs are short (200-800 nucleotide bases in length); unedited, randomly selected single-pass sequence reads derived from cDNA libraries (Nagaraj et al., 2006). EST libraries have been developed for plant species such as tomato, apple, rice, grape and citrus (Gonzalez-Ibeas et al., 2007). However, amongst the comprehensive ones are *Arabidopsis thaliana* and *Oryzae sativa* which are the common models for analysis (Gonzalez-Ibeas et al., 2007). Bioinformatics tools can be used to identify and dissect biological processes that are of great technological importance such as flavor development and fruit ripening through the analysis of ESTs (Gonzalez-Ibeas et al., 2007). Gene mining can be used to select candidate genes that are associated with traits of interest (Frank et al., 2004; Higgs and Attwood, 2005). The EST collections can also be used to develop microarrays to identify genes expressed during plant developmental stages and/or responding to environmental stimuli as well as to gain deeper understanding of the common regulatory mechanisms amongst diverse fruit species and ripening physiological patterns (Gonzalez-Ibeas et al., 2007; Fei et al., 2004). Some previous studies have used this analysis to identify genes involved in fruit ripening and pathogen defense (Gonzalez-Ibeas et al., 2007).

T. esculentum has no genome draft. Nonetheless, due to its economic and agricultural potential, it is imperative to explore what genes and microsatellites can be efficiently and rapidly mined and identified. Delayed or inefficient analysis due to tool constraints or lack thereof may impede development of potential products such as molecular markers, beneficial genes and useful biochemical pathways. The objectives of this study were to identify genes and microsatellites represented in the ESTs library developed for marama bean.

MATERIALS AND METHODS

ESTs generation and bioinformatic analyses

RNA was extracted from the embryogenic axis of germinating marama bean seeds using a Qiagen RNA extraction kit (Qiagen, Germany) and this RNA was used to construct the ESTs library using an oligo-dT primer based cDNA synthesis kit (Roche, Germany). Pyro-sequencing with 454 Sequencing technology was used to directly sequence the resultant derived cDNAs without using vectors. For the analysis of datasets, a Window 7 professional, 32-bit operating system and Intel (R) Celeron (R) CPU at 1.80 GHz computer was used together with an internet connection. *T. esculentum* ESTs datasets were analyzed using on-line detached programs. There were two EST datasets that were analyzed: the marama bean single reads and the marama contigs datasets. On average, the ESTs were between 50 and 276 bp for the single reads and 100 and 718 bp for the contigs.

The single reads dataset contained 13,582 sequences which were multiple aligned using ClustalW (www.clustalw.com). This was the preliminary processing to ensure minimum redundancy of sequences. Sequences (20) were aligned at a time. After multiple sequence alignment, 10,660 sequences remained. The sequences clustered as similar scored 90% or higher. The longest sequence of each batch was selected for downstream processing.

A BLASTn search was run against the non-redundant nucleotide database of NCBI's Genebank (www.ncbi.nlm.nih.gov/BLAST/). Default search parameters were used. After the BLASTn, a tBLASTx search was done on the sequences that produced significant alignment hits. Non-plant genes and similarity alignments with E-value >0.01 were disregarded.

The marama contigs were also processed similarly, multiple aligned using ClustalW and then searched against the *Arabidopsis* database, using the default TAIR BLASTn search parameters (www.arabidopsis.org/BLAST/). The sequences before and after multiple alignment were 924 contigs. The alignments with E-value < 0.5 were considered significant. Contig sequences (50) were analyzed. The single reads that gave significant similarities were scanned for SSRs using an SSR search tool (SSRIT) (www.gramene.org/db/markers/ssrtool).

RESULTS

After the analysis of 3247 out of 10660 sequences in the single reads dataset, 227 genes and proteins were identified to be of plant origin. The genes identified were

found to be involved in essential cellular and metabolic processes in other various plants (Table 1). These were classified as housing keeping genes (79% of the total predicted proteins) and those that did not exhibit high frequencies are classified as specialized (29% of the total predicted proteins) (Figure 1). It was also observed that some of the important putative marama bean genes that were identified and are worth investigating were similar to rps 2; disease resistance; retrotransposons B_{39}_yara_autonomous TY1-type, glycosyltransferase CAZy family GT_{47}; tRNA-Lys (trnK) gene intron and maturase K (matK) gene; centromeric retrotransposon Pisat1-6 mutant gag-pol polyprotein gene; inverted repeat B; RING/FYVE/PHD zinc finger superfamily and transposable element gene. Tables 2 and 3 show the genes that were identified with BLASTx from the single reads data base and TAIR BLASTn from the large contigs, respectively. Table 4 shows the microsatellite repeats that could be mined in the GRAMENE database using SSRIT microsatellite search tool.

For the large contigs dataset, 50 out of 924 sequences were searched against the *Arabidopsis* database and 34 genes with high similarities were found. In this study, microsatellite sequences were identified and genes associated with these SSR markers were identified to be closest to CBL interacting protein kinase (MTR_2g049790) with (CT) repeats; mitochondrion like with (GA) repeats; NA Damage-repair/toleration protein DRT111 and chloroplastic gene with (TC) repeats and lastly galactosyl transferase 11-like gene with a (TTG) repeats.

DISCUSSION

The objectives of this study were to identify genes and microsatellites from the EST single reads and contigs libraries as the first approach of identifying functional genes in marama bean at the embryonic seed stage. The plant lacks a genome draft and therefore has an unknown genome size. Due to the potential of the plant and the endeavors to domesticate it, functional genomic information is necessary to identify and map biochemical pathways and also to design primers for microsatellites. Genes (180) and proteins were identified in the single reads dataset that are involved in photosynthetic and energy processes. Genes (47) from the single reads dataset and the 34 genes identified in the contigs dataset are involved in processes such as transcription, transport, cellular communication, disease resistance and DNA repair.

Within all the genes identified in both the single reads and contig datasets, 7 genes identified have important uses in plant disease resistance as well as in plant biotechnology. For instance, rps2 gene is involved in disease resistance, while retrotransposons and transposons can be used in mutagenesis and plant evolutionary studies (Kumar and Bennetzen, 1999). In this study, the longest marker identified contains three base repeats and the rest contain two bases (dinucleotide repeats). Some genes associated with markers are involved in cellular transport and DNA repair such as DNA repair protein RAD51 homolog 2-like. It still remains to be evaluated how useful will these markers be in the selection and breeding of marama bean with desired superior traits. Similar studies have been done on plant to develop and use microsatellite markers for genetic variation analysis in the Namibian germplasm within and between populations using ESTs. The markers are now available for use in efforts of domestication and conservation. Takundwa et al. (2010) stated that it is desirable to isolate and characterize more DNA markers in the plant for more productive genetic studies such as genetic mapping, marker associated selection and gene discovery. In a study by Bombarely et al. (2010), ESTs were generated and analyzed in the evaluation of *Fragaria xananassa* at a genetic and molecular level. The analysis of the transcription analysis generated knowledge and molecular tools that would be essential in ongoing breeding programs and had also allowed the development of molecular markers that have been applied to germplasm characterization. ESTs have also been used in studies of plants such as tomato to understand tissue specific genes and biological responses in fruit ripening (Fei et al., 2004), and the fruit traits were studied using ESTs for melon (*Cucumis melo*). The genes of interest were the genes in the essential traits such as fruit development, fruit maturation and disease resistance, and to speed up the process of breeding new and better adapted melon varieties, such genes are yet to be studied in marama bean.

Conclusion

This study has demonstrated the first significant progress in the identification of genes using EST database gene mining for advancing molecular breeding and biotechnological crop improvement for this species, *T. esculentum*. If a sequence is known, microsatellites and markers can be identified, and then marama bean-specific primers can be developed. Genes that have been identified in marama bean are involved in energy generation, disease resistance, transcription, maturation and DNA repair.

There are a lot more genes to be discovered and studied beyond what this study has discovered for marama bean. In marama bean, traits of interest are, but not limited to increasing number of seeds per pod produced by the plant, selecting for early flowering and early germination (Takundwa et al., 2010). In breeding programs, traits of interest can be linked to markers, which can be used for marker associated selection which is time-saving than traditional breeding. The legumes are remarkably well positioned in the genomic era.

Table 1. NCBI BLASTn search outputs against a NR nucleotide database: marama bean single reads dataset.

Protein/gene	Number of hits	Identity (%)	EST length (bp)	E-value	Species	Accession number
Chloroplast	55	92	268	1.00E-177	*Eleutherococcus senticosus*	JN637765.1
Plastid	15	98	100	1.00E-11	*Quercas rubra*	JX970937.1
Mitochondrion (Mitochondrial DNA)	53	100	84	5.00E-06	*Carica papaya*	EU431224.1
ycf2	7	100	192	2.00E-72	*Lacistema robustum*	JX6643392.1
ATP synthase subunit α (atpA)	7	98	115	3.00E-09	*Medicago truncatula*	XM003638699.1
Uncharacterized	5	78	269	3.00E-17	*Glycine max*	XM003545696.1
Putative β-1,3 galactosyltransferase 11-like	1	92	240	4.00E-28	*Glycine max*	XM003526636.1
S-adenosyl-L-homocysteine hydrolase	1	88	149	2.00E-20	*Beta vulgaris*	AB221012.1
Glutamic acid rich-protein-like	1	91	84	6.00E-17	*Cicer arietinum*	XM004498226.1
GC-rich-sequence DNA-binding factor 1-like	1	96	97	2.00E-16	*Glycine max*	XM003528521.1
Chloroplast partial PsA gene for photosystem I P700 chlorophyll a apoprotein A1	1	94	126	1.00E-24	*Vitis riparia*	HF585117.1
α-tubulin 7	1	100	121	3.00E-06	*Salix arbutifolia*	KC238445.1
Mitochondrial, ATP 1, NAD 4 genes for hypothetical protein, ATP synthase subunit 1, NADH dehydrogenase subunit	3	98	241	8.00E-61	*Solanum melongena*	AB762698.1
polygalacturonase-like	1	97	116	2.00E-17	*Glycine max*	XM003551901.1
Psa B	1	98	181	3.00E-19	*Erythroxylum areolatum*	JX662950.1
wbABI 3 mRNA for ABI-3 homolog	2	85	231	7.00E-16	*Psophacarpus tetragonolobus*	AB164427.1
Serine hydroxymethyl transferase 3	3	91	163	4.00E-22	*Glycine max*	NM001250562.1
ATP synthase subunit β (atp β)	6	98	126	9.00E-30	*Averrhoa carambola*	JX663789.1
Photosystem II D2 protein & photosystem II CP43 protein genes (psb D & psb C)	2	98	76	1.00E-09	*Petermannia cirrosa*	AY465689.1
NADH dehydrogenase subunit 5 gene (nad 5)	1	100	119	8.00E-24	*Anthericum ramosum*	JX182968.1
Ndh B (Ndh B)	1	94	116	5.00E-26	*Drypetes roxburghii*	JX664317.1
Ribulose biphosphate carboxylase large chain (rbcL) (1,5 bisphosphate)	3	99	203	2.00E-100	*Tylosema esculentum*	AJ584710.1
Ribosomal protein S4 mitochondrial-like (rps4)	1	99	256	7.00E-106	*Cicer arietinum*	XM004488640.1
RNA polymerase β chain (rpo C2)	6	96	153	1.00E-41	*Quillaja saponaria*	EU002536.1
rpl 14	1	96	132	1.00E-27	*Pera bumeliifolia*	JX664267.1
18S ribosomal RNA	2	99	178	2.00E-60	*Metanarthecium luteoviride*	AB679366.1
rPOB subunit (RNA polymerase B)	2	95	246	7.00E-41	*Podocalyx loranthoides*	JX663494.1

Table 1. Contd.

trns-trnG intergenic spacer and tRNA-Gly (trnG gene)	1	95	221	1.00E-18	*Cercis racemosa*	JN942525.1
tRNA-Lys (trnK) gene intron and maturase K (matK) gene	1	99	255	2.00E-36	*Tylosema fassoglense*	JN881458.1
Photosystem I assembly protein ycf4 (ycf4) gene	1	96	252	5.00E-87	*Tephrosia rhodesica*	HM048910.1
Photosystem II CP43 chlorophyll apoprotein (psb C) gene	2	96	192	4.00E-56	*Cornus florida*	GQ998106.1
Glucan endo-1,3-beta-glucosidase 4-like	1	83	214	2.00E-32	*Vitis vinifera*	XM002283512.1
SRG-1-like protein	3	77	201	2.00E-14	*Fragaria vesca*	XM004303154.1
Cytochrome C heme attachment protein (ccsA gene)	1	94	214	1.00E-32	*Berberidopsis corallina*	GQ997938.1
Endoglucanase 11-like	1	85	206	1.00E-33	*Glycine max*	XM003518482.1
Manganese-dependent ADP-ribose/CDP-alcohol diphosphate like	1	86	154	4.00E-16	*Fragaria vesca sub.sp vesca*	XM004299102.1
Centromeric retrotransposon Pisat1-6 mutant gag-pol polyprotein gene	1	84	185	4.00E-27	*Pisum sativum*	GU136552.1
Mitogen-activated protein kinase 19-like	1	88	256	6.00E-47	*Cicer arietinum*	XM004488631.1
Glycosyltransferase, CAZy family GT47	1	89	142	1.00E-08	*Populus trichocarpa*	XM002313394.1
Mitochondrial voltage-dependent anion-selective channel	1	93	159	1.00E-17	*Phaseolus coccineus*	DQ072165.1
Retrotransposon B39_yara_autonomous-Ty1-type	1	89	182	5.00E-08	*Arachis ipaensis*	KC608799.1
PsA	1	97	69	8.00E-06	*Pera bumeliifolia*	JX664222.1
putative Pentatricopeptide repeat-containing protein Atg68930-like	1	89	138	8.00E-21	*Fragaria vesca*	XM004298286.1
5S rRNA gene	1	100	185	2.00E-16	*Beta vulgaris*	Z25803.1
Psb D gene	1	97	268	2.00E-60	*Phyllanthus urinaria*	JX662334.1
CBL-interacting protein kinase (MTR_2g049790)	1	94	191	3.00E-33	*Medicago truncatula*	XM003595548.1
Heterogeneous nuclear ribonucleoprotein D-like	1	91	202	6.00E-22	*Glycine max*	NM001252787.2
Phosphoenolpyruvate-carboxylase pepc1 isoform	2	88	239	1.00E-12	*Vicia faba*	AJ011302.1
DNA repair protein RAD51 homolog 2-like	1	96	201	1.00E-09	*Glycine max*	XM003547460.1
psb E-Pet L intergenic spacer	1	80	246	3.00E-29	*Pronus virginiana*	DQ826228.1
Histone-lysine N-methyltransferase EZA1-like	1	90	165	8.00E-11	*Cucumis sativus*	XM004164933.1
Uridine nucleosidase 1-like	1	86	215	1.00E-18	*Glycine max*	NM001255381.2
Histone H3 1-like variant 1	1	87	239	7.00E-31	*Callithrix jacchus*	XM002746095.1
DNA damage-repair/toleration protein DRT111, chloroplastic-like	1	85	241	8.00E-46	*Vitis vinifera*	XM002281707.1

Table 1. Contd.

Putative 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase 3	1	84	266	6.00E-42	*Faqus sylvatica*	DQ166521.1
U-box domain-containing-protein 4-like	1	82	254	5.00E-41	*Glycine max*	XM003551125.1
Thylakoid structural protein (Psb B gene)	1	96	256	4.00E-91	*Ceratophyllum sp SM-2010*	GU902269.1
Rps 2 (rps 2 gene)	1	98	255	1.00E-08	*Passiflora ciliata*	JX663163.1
Magnesium transporter MRS2-1-like	1	91	191	2.00E-50	*Glycine max*	XM003543660.1
PetB (petB gene)	1	92	285	2.00E-87	*Caloncoba echinata*	JX663902.1
Nucleobase-ascorbate transporter 1-like	1	90	215	1.00E-32	*Cicer arietinum*	XM004501987.1
Inverted repeat B (transposon boundary in chloroplast)	1	91	258	6.00E-86	*Rhodeleia championii*	EF207455.1
UDP-arabinose 4-epimerase 1-like	1	89	142	6.00E-22	*Glycine max*	XM003546247.1
Photosystem Q (B) protein-like	1	96	247	1.00E-92	*Cicer arietinum*	XM004515165.1
NdhI (ndhI)	1	93	237	2.00E-16	*Vismia ferruginea*	JX662090.1
Acetyl-CoA carboxylase carboxyltransferase β	1	88	260	2.00E-62	*Camellia oleifera*	FJ965289.1

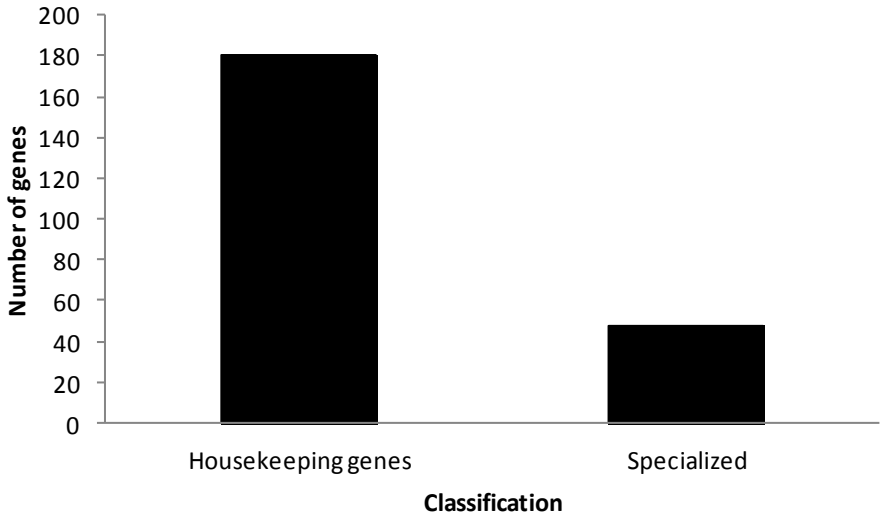

Figure 1. The overall classification of the genes identified (in single reads dataset) as housekeeping genes or specialized.

Future perspectives

In the future, it will be important to identify and characterize more genes and traits, and to extend new genomic tools to orphan species like marama bean. Some of the most critical work does not only rely on new high-throughput sequencing or genomic technologies. This includes characterizing and managing germplasm collections and breeding lines in many species; developing mapping populations for various traits of interest in less-studied species. Working with indigenous farmers ensures that the by-product of centuries of conservation and indigenous knowledge are not lost. Investigating protocols for hybrid seed production in

Table 2. NCBI tBLASTx search results against NR nucleotide marama bean database from single reads.

Protein/gene	Identity (%)	Number of positive hits	EST length (bp)	E-Value	Species	Accession
Chloroplast	81	80	260	1.00E-29	*Camellia cuspidata*	KF156833.1
Plastid	100	100	100	0.026	*Quercus rubra*	JX970937.1
PsbE-PetL Intergenic spacer	64	83	246	2.00E-08	*Prunus virginiana*	DQ826228.1
Plastid Genes	98	100	192	2.00E-27	*Acrotrema costatum*	HQ664618.1
Chloroplast	60	63	115	4.80E-02	*Berberis bealei*	KF176554.1
Centromeric retrotransposon PiSat 1-6 mutant gag-pol polyprotein gene	68	84	269	2.00E-25	*Pisum sativum*	GU136552.1
Mitochondrial sequence	100	100	268	2.00E-24	*Cucumis melo subsp.melo*	JF412793.1
Mitochondrial orf227, atp1, nad4 genes for hypothetical protein, ATP synthase subunit 1, NADH dehydrogenase subunit 4	86	94	241	2.00E-21	*Solanum melongena*	AB762698.1
Tubulin alpha- 4 chain-like	84	85	121	3.50E-02	*Glycine max*	XM003555953.1
psaA (psaA gene)	100	100	126	5.00E-07	*Turnera ulmifolia*	JX664233.1
GC -rich sequence DNA-Binding Factor 1-like	89	89	97	6.00E-04	*Glycine max*	XM003528521.1
Glutamic acid-rich protein-like	100	100	84	4.90E-02	*Cicer arietinum*	XM004498226.1
RNA for putative adenosylhomocysteinase	97	96	149	3.00E-10	*Trifolium pratense*	AB236805.1
Putative beta-1,3-galactosyltransferase sqv-2	89	94	240	1.00E-14	*Ricinus communis*	XM002509867.1
nad 5	92	95	119	2.00E-06	*Lygodium flexuosum*	AJ131135.1
Chloroplast	89	91	268	4.00E-47	*Trachelium caeruleum*	EU090187.1
Ndh B (ndh B) gene	86	86	116	1.00E-07	*Drypetes roxburghii*	JX664317.1
NAD(P)H-quinone oxidoreductase chain 4 chloroplastic-like	97	96	214	6.00E-13	*Cicer arietinum*	XM004516889.1
Chloroplast	94	94	258	4.00E-26	*Lotus japonicus*	AP002983.1
Unknown	80	88	237	2.00E-21	*Lotus japonicus*	BT146355.1
18S ribosomal RNA gene	94	95	178	1.00E-23	Marine streptophyte	EU143544.1
5S ribosomal RNA and nontranscribed spacer	55	70	185	9.00E-06	*Trevesia baviensis*	AY304751.1
Ribosomal protein S4 mitochondrial-like	99	98	256	3.00E-42	*Cicer arietinum*	XM004488640.1
Rpl 14 (rpl) gene	92	92	132	7.00E-10	*Averrhoa carambola*	JX664237.1
tRNA-Lys (trnK) gene, intron; and maturase K (matK) gene	91	93	255	2.00E-11	*Bauhinia scandens*	JN881423.1
Chromosome POP064-N07	89	92	191	7.00E-16	*Populus trichocarpa*	AC209224.1

Table 2. Contd.

Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	100	100	239	5.00E-39	*Caesalpinia sp.*	AB586306.1
Putative Pentatricopeptide repeat-containing protein At1g68930-like	93	100	139	1.00E-11	*Vitis vinifera*	XM002267577.1
Mitogen-activated protein kinase 19-like	95	98	256	3.00E-20	*Cicer arietinum*	XM004488631.1
Manganese-dependent ADP-ribose/CDP-alcohol diphosphatase-like	80	87	154	3.00E-15	*Cicer arietinum*	XM004501808.1
Endoglucanase 11-like	80	87	206	1.00E-18	*Glycine max*	XM003518482.1
SRG1- like protein	68	76	201	2.00E-17	*Glycine max*	XM003547143.1
Protein	76	91	214	5.00E-26	*Populus trichocarpa*	XM002297602.1
Chloroplast	94	97	285	2.00E-36	*Turbina corymbosa*	KF242504.1
Chloroplast	92	96	192	3.00E-25	*Operculina macrocarpa*	KF242502.1
Serine hydroxymethyltransferase (SHMT2)	96	100	163	1.00E-11	*Populus tremuloides*	EF148390.1
ATP synthase beta chain (atpB) gene	88	92	126	4.00E-06	*Cystopteris pellucida*	JN168037.1
Photosystem II protein D1 (psb A) gene	100	100	247	6.00E-36	*Chlamydomonas reinhardtii*	FJ458214.1
Photosystem I assembly protein ycf4 (ycf4) gene	97	96	252	4.00E-35	*Liquidambar styraciflua*	GQ998510.1
Photosystem II CP47 protein (psbB) gene, Photosystem II subunit (psbT) and photosystem II subunit (psbN) and photosystem II subunit (psbH) genes	97	97	256	4.00E-40	*Thalassia testudinum*	HQ901410.1
RNA polymerase beta subunit (*rpoB*) gene	84	88	246	3.00E-17	*Urginavia altissima*	JQ274454.1
RpoC2 (*rpoC2*) gene	100	100	153	6.00E-18	*Scyphostegia borneensis*	JX662688.1
U-box domain-containing protein 4-like	75	84	254	3.00E-29	*Glycine max*	XM003538281.1
Phospho-2-dehydro-3-deoxyheptonate aldolase 2, chloroplastic like	90	93	266	6.00E-20	*Glycine max*	XM003545637.1
Uridine nucleosidase 1-like	74	80	215	6.00E-13	*Vitis vinifera*	XM002283117.2
Putatative enhancer of zeste, ezh	68	72	165	1.00E-15	*Ricinus communis*	XM002515233.1
Histone H3 type 2	100	100	239	3.00E-20	*Culex quinquefasciatus*	XM001862639.1
DNA-damage-repair/toleration protein DRT111, chloroplastic like	81	90	241	1.00E-07	*Vitis vinifera*	XM0022881707.1
DNA repair protein RAD51 homolog 2-like	54	67	201	2.30E-01	*Solanum lycopersicum*	XM004251232.1
Phosphoenolpyruvate carboxylase (*PepC*-large) gene	92	100	239	4.00E-09	*Gaertnera paniculata*	AF333864.1

Table 2. Contd.

Heterogeneous nuclear ribonucleoprotein D-like	93	92	202	3.00E-11	*Glycine max*	NM001252787.2
UDP-arabinose 4-epimerase 1-like	93	92	142	5.00E-07	*Glycine max*	XM003546247.1
Nucleobase-ascorbate transporter 1	80	92	215	3.00E-22	*Arabidopsis thaliana*	NM126592.2
Magnesium transporter MRS2-1-like	85	89	191	4.00E-20	*Glycine max*	XM003543660.1
probable Polygalacturonase-like	95	100	116	2.00E-03	*Setaria italica*	XM004951228.1
B3 domain-containing transcription factor ABI3-like	81	93	231	4.00E-10	*Vitis vinifera*	XM003632349.1
Uncharacterized	82	89	181	5.00E-08	*Cicer arietinum*	XM004496867.1

Table 3. TAIR BLASTn search outputs.

Contig number	Gene/protein	E- Value	Identity (%)	Marama bean Contig size (bp)	Accession Number
contig00001	Unknown Protein	0.064	100	132	AT5G28910.2
contig00003	Homeo doman glabrous 2	0.026	95	203	AT1G05230.4
contig00005	RING/FYVE/PHD Zinc finger superfamily protein	0.33	100	170	AT3G47550.6
contig00008	RNA Binding (RRM/RBD/RNP motifs) family protein	0.055	100	115	AT5G16260.1
contig00009	GDP-D-mannose 3',5'-epimerase	0.24	100	445	AT5G28840.2
contig00010	thalianol synthase 1 (THAS 1)	0.45	100	225	AT5G48010
contig00013	Nucleoporin, Nup133/Nup155 - like	0.095	95	188	AT2G05120.2
contig00014	phosphotidyl serine synthase family protein	9.00E-05	96	174	AT1G15110.2
contig00015	Putatatvie lysine decarboxylase family protein (LOG 1, ATLOG 1)	0.002	100	225	AT2G28305.1
contig00016	Laccase	0.097	92	193	AT5G01190.1
contig00018	putative methyl transferase family protein	0.095	100	188	AT5G06050.1
contig00020	prenylated RAB acceptor 1.B5 (PRA1.B5)	0.45	100	221	AT5G01640.1
contig00021	Cytochrome P450 superfamily protein (CYP81D1)	0.17	100	221	AT3G28740.1
contig00024	Tudor/PWWP/MBT domain containing protein	0.33	100	412	AT2G48160.1
contig00026	photosystem II reaction center protein B (PSBB)	5.00E-51	87	203	ATCG00680.1
contig00027	high affinity K+ transporter 5 (HAK5, ATHAK5)	0.011	100	229	AT4G13420.1
contig00030	Transcription factor Jumonsi (jmj) family protein/zinc finger (C5HCZ type) family protein	0.38	100	132	AT2G38950.1
contig00031	phosphatidic acid phosphohydrolase 2 (PAH 2)	0.18	100	230	AT5G42870.2
contig00032	plastid - encoded CLP p (CLPP 1, PCPLPP)	0.11	100	155	ATCG00670.1
contig00033	phytoene desaturation (POS1, HPD)	0.4	100	140	AT1G06570.2
contig00034	ATP synthase subunit 1 (ATP1)	2.00E-39	95	119	ATMG01190.1
contig00035	NRAMP metal ion transporter family protein (NRAMP5, ATNRAMP5)	0.092	100	128	AT4G18790.1
contig00037	F - Box and associated interaction domains- containing protein	0.41	100	143	AT5G62660.1
contig00038	Transposable element gene	0.16	100	208	AT3G44000.1
contig00040	Galactose Oxidase/ kelch repeat superfamily protein	0.037	100	193	AT1G55270.1
contig00041	lysm domain GP1-anchored protein 2 precursor (LYM2)	0.34	100	120	AT2G17120.1
contig00043	photosystem II reaction center protein N (PSBN)	0.01	87	209	ATCG00700.1

Table 3. Contd.

contig00044	2 - oxoglutarate (20G) and Fe (II) - dependent oxygenase superfamily protein	0.17	100	219	AT3G18210.2
contig00045	Reticulon family protein	0.17	100	224	AT4G28430.1
contig00046	pseudogene, similar to NADH dehydrogenase	2.00E-59	90	219	AT2G07709.1
contig00047	F-Box/ RN1- like domains- containing protein	0.18	90	229	AT1G16930.1
contig00048	s-locus lectin protein kinase family protein	0.37	95	131	AT5G35370.1
contig00049	chloroplast ribosomal protein S14 (RPS14)	7.00E-21	93	122	ATCG00330.1

Table 4. SSRs identified: di- to tri-nucleotide (2-3) repeat motifs search outputs on GRAMENE database for 66 sequences in single reads.

Sequence	Motif	Number of repeats	SSR start	SSR end	Sequence length
024042_2232_1498	CT	5	181	190	191
031209_2673_1063	GA	4	51	58	84
026256_2398_2536	TC	4	166	173	241
003796_2321_0642	TTG	4	207	218	240

various legumes; and working to maintain and develop under-studied legumes for use in diverse, challenging growing environments around the globe is a responsibility to help diversity crops for a changing world climate (Cannon et al., 2009).

The rapid increment in the information and data generation in plant science, demands for tools and methods in data management, visualization integration, analysis, modeling and prediction has also increased (Useche et al., 2001, Rhee et al., 2006; Frank et al., 2004). In this regard, bioinformatic analysis is a utility. This specific knowledge can then be used to produce stronger, more drought resistant crops and improve the quality of livestock, making them healthier, more disease resistant and more productive (Singh et al., 2011).

Conflict of interests

The authors did not declare any conflict of interest.

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Multiploidy occurrence in tomato calli from anther culture

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Anther culture has long been used for the production of fully homozygous lines in order to produce, mainly, doubled haploid plants, which are of great interest in plant breeding. For tomato, a recalcitrant species for androgenesis production protocols have not been standardized. It is known that the genotype, anther size, the developmental stage of the microspore, and the medium composition are some factors that can influence the calli production. The present study aimed to adapt flow cytometry methodology to verify the microsporogenesis phases of anthers in order to assess the anther responsiveness of different tomato genotypes in an androgenesis-induction culture medium and to analyze the DNA ploidy level of calli produced by flow cytometry. Anthers from flower buds of length 1.0 to 5.9 mm, corresponding to the size range as analyzed by flow cytometry and cytogenetic methods, were inoculated into Murashige and Skoog (MS) basal medium containing the growth regulators 6-(*y*,*y*-dimethylallylamino) purine and indole-3-acetic acid. The obtained calli were subsequently analyzed by flow cytometry to determine the DNA ploidy level. Surprisingly, despite no pretreatment with microtubule-depolymerizing agents, five classes of multiploid calli were observed, as follows: class I (2C-4C-8C-16C), class II (2C-4C-8C-16C-32C), class III (4C-8C), class IV (4C-8C-16C) and class V (8C-16C-32C). Multiploid calli were identified in short-term (two month) culturing, suggesting that the variable culture duration did not directly influence the occurrence of endoreduplication. In this work, this type of somaclonal variation has been reported for the first time in tomato anther culture, and their possible origin has been discussed.

Key words: Callogenesis, flow cytometry, polyploidy, *Solanum lycopersicum*, somaclonal variation.

INTRODUCTION

In vitro androgenesis is a remarkable example of cell totipotency in plants (Reynolds, 1997; Góralski et al., 2005), and it can induce male gametophyte cells or their precursors to deviate their route toward a sporophytic

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Abbreviations: FCM, Flow cytometry; **PMCs,** pollen mother cells.

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program, resulting in the development of haploid embryos (Góralski et al., 1999; Bárány et al., 2005; Seguí-Simarro, 2010). Haploid plants are interesting in fundamental research owing to the development of sporophytes with the gametophytic number of chromosomes. Nevertheless, their main practical utility is because of the generation of doubled haploid (DH) plants obtained by duplication of the chromosomes of haploid lineage either spontaneously or through induction by a microtubule-depolymerizing agent (Murovec and Bohanec, 2012). The DH generation is an exciting and powerful tool of pure line production (100% homozygous) for breeding programs (Kasha and Maluszynski, 2003; Dunwell, 2010; Germanà, 2010, 2011). Despite the knowledge obtained through model systems to study androgenesis such as in rapeseed (*Brassica napus* L.), tobacco (*Nicotiana tabacum* L.), or barley (*Hordeum vulgare* L.), the production of DH plants by androgenesis remains to be effectively standardized in tomato (*Solanum lycopersicum*, 2n = 2x = 24 chromosomes). The main hindrance in standardization is probably the recalcitrant nature of the crop (Bal and Abak, 2007; Seguí-Simarro and Nuez, 2007; Seguí-Simarro et al., 2011). *S. lycopersicum* has great economic importance in the horticultural industry (Wang et al., 2005), and it is considered as a good model for genetic studies of the Solanaceae family members (Hay et al., 2004; Koo et al., 2008).

The genotype dependency is a critical factor affecting the response in tomato androgenesis (Asoliman et al., 2007), and it has been reported that different cultivars of the same species exhibit diverse responses to another culture. For example, Zagorska et al. (1998) tested the androgenetic ability of 85 tomato cultivars, of which 53 were responsive and produced calli and a smaller number of cultivars developed regenerants. According to Seguí-Simarro and Nuez (2007), tomato genotypes can be divided into two categories: those inducible to form callus from meiocytes and those inducible to form embryos from microspores. Another critical aspect in androgenesis induction is the stage of microspore development at the time of anther excision and plating for *in vitro* culturing (Seguí-Simarro and Nuez, 2008a). In nearly all responsive crops, including model species such as rapeseed and tobacco, the inducible stage revolves around the first pollen mitosis, that is, from vacuolate microspore to early bicellular pollen (Touraev et al., 2001). In most tomato, the meiocyte is the most suitable developmental stage for androgenesis induction (Zamir et al., 1980; Summers et al., 1992; Shtereva et al., 1998; Brasileiro et al., 1999; Seguí-Simarro and Nuez, 2005; 2007). However, according to Seguí-Simarro and Nuez (2005), the stage with highest response in terms of callus generation in tomato is metaphase I to telophase II.

Hence, it is necessary to identify the appropriate anther developmental stage. Generally, traditional cytogenetic procedures such as squashing have been used for this purpose (Seguí-Simarro and Nuez, 2005). However, an accurate determination of microspore/pollen stage requires a complex cytogenetic analysis. Nevertheless, external morphological indicator, such as the corolla length, has been used because it is a simple procedure for measuring large-scale cases, although it is a less reliable method (Dunwell, 2010). An alternative method used to determine the microspore/pollen development stage is flow cytometry (FCM), which is a practical and efficient methodology for quantify (screening) nuclear DNA content. This technique allows analyzing the individual nuclei faster than the cytogenetic traditional methods. Moreover, this methodology can be also used for evaluating the DNA ploidy level of calli, and these results can be related to any effect on androgenetic potential of anthers. The loss of embryogenic and organogenic competence is one of the major problems encountered when developing *in vitro* regeneration systems for propagation and genetic manipulation of plants (Winkelmann et al.,1998).

Considering that different genotypes can trigger different androgenetic responses, the difficulty of selecting responsive anthers to the culture and solving why calli have not develop in embryogenic calli, the present study focused on (i) the introduction of a procedure to select microsporogenesis stages is potentially more responsive for callus induction, associating cytogenetic, and FCM technique; (ii) the use of a culture medium previously described as effective in the literature for callus induction in different genotypes; (iii) evaluation of responsiveness of different genotypes by callus production, and (iv) screening of the DNA ploidy level profile in the calli.

MATERIALS AND METHODS

Plant materials

Flower buds belonging to four genotypes of *S. lycopersicum*: 'Debora', 'Santa Clara', 'Alambra', and 'Pizza Doro' were provided by Dr. Derly J. H. da Silva, Vegetable Germplasm Bank Curator [Universidade Federal de Viçosa (UFV), Minas Gerais, Brazil]. Flower buds were collected in the morning at the experimental field of UFV; some of the collected buds were immediately fixed in a solution of methanol:acetic acid (3:1 v/v) for cytogenetic and FCM analyses, while the remaining were used for anther culturing. The fixative was changed three times, and the samples were stored at -20°C (Carvalho and Saraiva, 1997). The experiments were conducted at the Laboratório de Citogenética e Citometria, Departamento de Biologia Geral, UFV.

Monitoring of anther maturation by FCM

A total of 15 flower buds (1.0 to 6.0 mm) of all genotypes were removed from the donor plants in the morning, and their anthers were classified according to their size as small (0.5 to 0.7 mm), medium (0.8 to 1.5 mm), or large (1.6 to 1.9 mm). Four anthers of each bud were subjected to FCM procedure (total: 60 anthers) and one anther was reserved for cytogenetic analysis (total: 15 anthers). First, the anthers were softened by enzymatic maceration

at 36°C as follows: the anthers were placed on a 70 µm mesh nylon filter submerged into a 2.0 mL microtube (Eppendorf) containing enzyme solution consisting of 2% cellulase (obtained from *Trichoderma viride*; Onozuka Yakult Honsha Co®), 1% cellulase (obtained from *Aspergillus niger*; Sigma®), 2.5% macerozyme (Yakult Honsha Co®), 2.5% pectinase powder (Sigma®), and 1% hemicellulase. The enzymatic maceration process was performed in two steps: the anthers were maintained in the enzymatic solution for 2 h and the softened anthers were macerated with a pin head so as to release the pollen mother cells (PMCs), followed by incubation of the PMCs in the same digestion solution for 30 min. Finally, the tubes were centrifuged for 5 min at 100 ×*g*, and the enzyme solution was discarded. Subsequently, the pellet was resuspended in 1 mL of OTTO-I lysis buffer (Otto, 1990) and the solution was homogenized with 12 pulses (approximately 1 s per pulse) by using a commercially available mini-handheld mixer to provide nuclei suspensions (Silva et al., 2010). The suspensions were filtered through 30 µm mesh nylon filter and centrifuged for 5 min at 100 ×*g*. The supernatant was discarded, and the pellet was resuspended in 100 µL of OTTO-I and incubated for another 10 min at the room temperature.

The nuclei suspension was stained with 1.5 mL of the OTTO-II buffer (Otto, 1990) supplemented with 1.5 mM 4′6′-diamino-2-phenylindole (DAPI; Sigma®), filtered through a 20 µm mesh nylon filter, and maintained in the dark for 30 min. The nuclei suspensions were analyzed by PAS-III flow cytometer (15-01-1000; Partec GmbH) equipped with a UV lamp (with emission at 378 nm), excitation filters (KG 1, BG 38, and UG 1), a GG 435 long-pass barrier filter for blue fluorescence, and a TK 420 nm dichroic mirror to supply epi-illumination. The equipment was carefully calibrated and aligned using microbeads and standard solutions as per the manufacturer's recommendations. The FlowMax software (Partec GmbH) was used to process the data. The instrument gain was set up in such a way that the G_0/G_1 peak was positioned on channel 200 using nuclear suspension from leaves of the same plant.

Cytogenetic analysis

The anther that was separated in each bud was macerated in the same enzymatic solution used in the FCM analysis. Each anther was slightly fragmented with the tip of a scalpel blade in 50 µL of 45% acetic acid solution (v/v). The excessive material (wall anther) was removed from the slide and, on a hot plate, the solution containing the anther cell suspension was spread on the slide using a hair dryer. The slides were stained with 4.0 mM DAPI (Sigma®) and observed under the Olympus TM BX-60 fluorescence microscope.

Anther culture

Flower buds of *S. lycopersicum* genotypes 'Debora', 'Santa Clara', 'Alambra', and 'Pizza Doro' were sorted manually according to the size, from 1.0 to 6.0 mm at an interval of 1.0 mm. Next, the buds were pre-treated with 1% polyvinylpyrrolidone (PVP; Sigma®) solution in order to minimize the oxidation of the anthers and were maintained at 4°C for 48 h. After a cold shock, buds were sterilized under laminar flow hood with 70% (v/v) ethanol (Merck®) for 20 s, followed by immersion in 1.0% (v/v) solution of sodium hypochlorite (Merck®) for 5 min, and washed three times (3 min, each) with autoclaved distilled water (Zagorska et al., 1998). The anthers were aseptically removed; four anthers were inoculated into 60 × 15 mm Petri dishes (J. Prolab®) containing callus induction medium, and one anther was reserved for cytogenetic analysis to determine the stage of meiotic development. Induction medium consisted of MS salts and vitamins (Murashige and Skoog, 1962; Sigma®) supplemented with 2.8 g L^{-1} phytagel, 20 g L^{-1} sucrose, 0.1 g L^{-1} myo-inostol, 0.04 g L^{-1} cysteine, 1 mg L^{-1} 6-(*y*,*y*-dimethylallylamino) purine (2iP; Sigma®) and 2 mg L^{-1} indole-3-acetic acid (IAA; Sigma®). The pH was adjusted to 5.7 prior to autoclaving. The growth regulators were filter-sterilized and added after autoclaving of the medium (adapted from Shtereva et al., 1998).

Petri dishes were sealed and kept in darkness for 1 month at 25 ± 2°C and subsequently transferred to a 16/8 h light/dark photoperiod regime with 36 µmol m^{-2} s^{-1} light radiation. Subcultures were made at 30 day intervals. The percentage of callus production was evaluated 60 days after inoculation. Anthers that did not respond to culture were discarded. The experiment followed a completely randomized design in a factorial 4 × 5, with a total of 20 treatments, corresponding to four genotypes and five bud sizes. Each treatment consisted of six Petri dishes containing four explants, and the parameter evaluated was the average of explants exhibiting calli. The data were submitted to analysis of variance (ANOVA) and Tukey's test at 5% probability level, both performed by using the statistical program Genes (Cruz, 1997).

Calli screening by FCM

We analyzed the ploidy level of young calli (2 month old) by FCM, on six calli in each treatment, one per plate, except the plates where the anthers did not respond to induction of callus. Random pieces of each callus were removed and processed to release the nuclei, as described by Galbraith et al. (1983). Approximately 50 mg of callus was chopped in 100 µL of OTTO-I lysis buffer, followed by the addition of 400 µL of the same buffer. Then, the nuclei suspension was adjusted to 1 mL with the same buffer, filtered through 30 µm-mesh nylon filter into 2-mL microcentrifuge tubes, and centrifuged at 100 ×*g* for 5 min. The pellet was then incubated in 100 µL of OTTO-I lysis buffer for 10 min. The nuclei suspension was stained with 1.5 mL of the OTTO-II buffer supplemented with 1.5 mM DAPI (Sigma®), filtered through the 20 µm-mesh nylon filter, and maintained in the dark for 30 min. The suspension was analyzed by the Partec PAS® flow cytometer. For DNA ploidy level characterization of the calli, nuclear suspension from leaves of the correspondent plants (2C ploidy standard) was used to determine the G_0/G_1 peak. The peak relative to the 2C standard nuclei was set to channel 200. The histograms were obtained from the semi-logarithmic scale (log X-axis).

Cytological analysis

In order to confirm the nuclei isolation efficiency and to eliminate the possibility of nuclei aggregate formation, slides were prepared after the cytometric analysis. Nuclei suspensions were transferred to 2 mL of the microcentrifuge tubes and centrifuged at 100 ×*g* for 5 min. The supernatant was discarded, and 30 µL of the pellet were resuspended and then placed on a glass slide and covered with a coverslip. The images were captured by the DP71 digital camera coupled to the Olympus TM BX-60 fluorescence microscope using WU filter.

RESULTS

Monitoring of anther maturation by FCM and cytogenetic methods

For the first time, FCM approach was used to screen the meiotic progression in tomato anthers. Anthers of different sizes were evaluated by FCM in the four genotypes of tomato. The generated histograms showed

coefficient of variation (CV) of < 5%, which is considered acceptable in plant FCM studies (Doležel and Bartoš, 2005). Histograms with low CV indicate that nuclei suspensions have intact, isolated, and stoichiometrically stained nuclei. Among the 60 anthers of different sizes analyzed, two histogram patterns were identified. The small anther profile consists of two histogram peaks, corresponding to the nuclei with 2C and 4C DNA content (Figure 1a). The large anther profile showed three peaks (1C, 2C and 4C ploidy level) (Figure 1b). Differences in the relative nuclear DNA content in PMCs and microspores within anthers were clearly evident from FCM analysis; young anthers (small) did not show n cells. In parallel to FCM analysis, cytogenetic analysis was performed to identify the meiotic phases correlated to the evaluated anther sizes. The developmental stages of microsporogenesis demonstrated that the (a) anthers of 0.5 to 0.7 mm size contained cells at interphase and prophase I; (b) anthers of 0.8 to 1.5 mm size contained meiocytes at metaphase I, anaphase I, telophase I, and meiosis II phases; and (c) anthers of > 1.6 mm size presented cells as tetrads and microspores (data not shown).

Anther culture

The four genotypes used in this study were tested for their androgenetic response by anther culture under the same experimental conditions. The FCM results in anther contributed to the choice of flower buds of the optimal size for inoculation of anthers, as suggested in the literature. Thus, anthers containing meiocytes (1.0 to 6.0 mm sized flower bud) were inoculated into the induction medium. Calli formation occurred 30 days after the beginning of each culturing. During the initial culturing, in the dark, the calli presented whitish color, which turned to green under 16/8 h photoperiod. Some shoots were formed on the generally compact calli (Figure 2). The evaluation of genotype × anther size interaction showed significant differences in some treatments for callus production, as can be visualized in Table 1. The callus production for ‘Pizza Doro’ genotype was influenced by the anther size (2 to 3 mm), and the genotype ‘Alambra’ influenced both 4 to 5 and > 5 mm anther lengths.

Calli screening by FCM

The procedure adopted here for nuclei extraction from calli was effective. The microscopic examination of nuclei suspensions revealed isolated nuclei, with only few nuclear aggregates. Moreover, the FCM analyses results were consistent in showing low CVs. Surprisingly, these FCM analyses showed that all calli were multiploid, a term suggested here to denote calli with a complex type of mixoploidy, presenting multiple DNA ploidy levels in the same calli and different ploidy levels in different calli. These multiploid calli were divided in five classes of DNA ploidy levels: class I (2C-4C-8C-16C), class II (2C-4C-8C-16C-32C), class III (4C-8C), class IV (4C-8C-16C-32C), and class V (8C-16C-32C) (Figure 3). We have presented ‘Débora’ FCM data to illustrate the multiploid event; however, the same classes were observed for the other genotypes as well. Each analyzed callus presented one of these classes. The calli showed the following distribution: 44.4% of the total belonging to class II, 27.78% to class I, 16.67% to class IV, and 5.55% to classes III and V. Class II was also analyzed cytologically to examine the nuclear size profile. Figure 4 illustrates five nuclear types in the ascending order of size that was compatible with five types of DNA ploidy levels as detected by FCM.

DISCUSSION

In this study, we show that flow cytometry can be a useful tool to screen the developmental stage of anthers. Brasileiro et al. (1999) also analyzed the correlation between tomato anther length and meiotic phase. These authors reported that flower buds of size 5.0 to 6.0 mm and anthers of size 2.0 to 2.3 mm showed cells in meiosis. Furthermore, Seguí-Simarro and Nuez (2005) observed that meiocytes were concentrated in the buds of length 4.0 to 5.9 mm. However, according to Zamir et al. (1980), anther and flower bud sizes are not directly correlated to the microsporogenesis stage. In the present study, different developmental stages were identified for similar bud sizes in the same plant, anthers in the same flower, and even within a single anther. Furthermore, the difference in the results of the present study and other literature reports can be attributed to the different genotypes analyzed in each experiment as well as the environmental and physiological conditions in which the plants were grown. The cytogenetic and FCM techniques allowed differentiation of the anthers at the interphase/prophase and the tetrad/microspore stages. Small anthers at the interphase/prophase I displayed a histogram profile similar to that of the standard tomato leaf, whereas large anthers in the tetrad stage and microspore stages showed a peak corresponding to the haploid DNA content of microspores. Based on these results, flower buds of size 1.0 to 6.0 mm and anther size similar to that analyzed by FCM and cytogenetic procedures were inoculated into the induction medium.

The FCM analysis presented here represents a relatively simple, rapid, and reliable method to analyze and discriminate the development stage of anthers. The sample preparation in this study requires a small amount of anthers and the analysis is rapid. Moreover, a significant number of nuclei can be measured in a few minutes, which makes the results statistically robust. This procedure is interesting to accelerate the meiotic

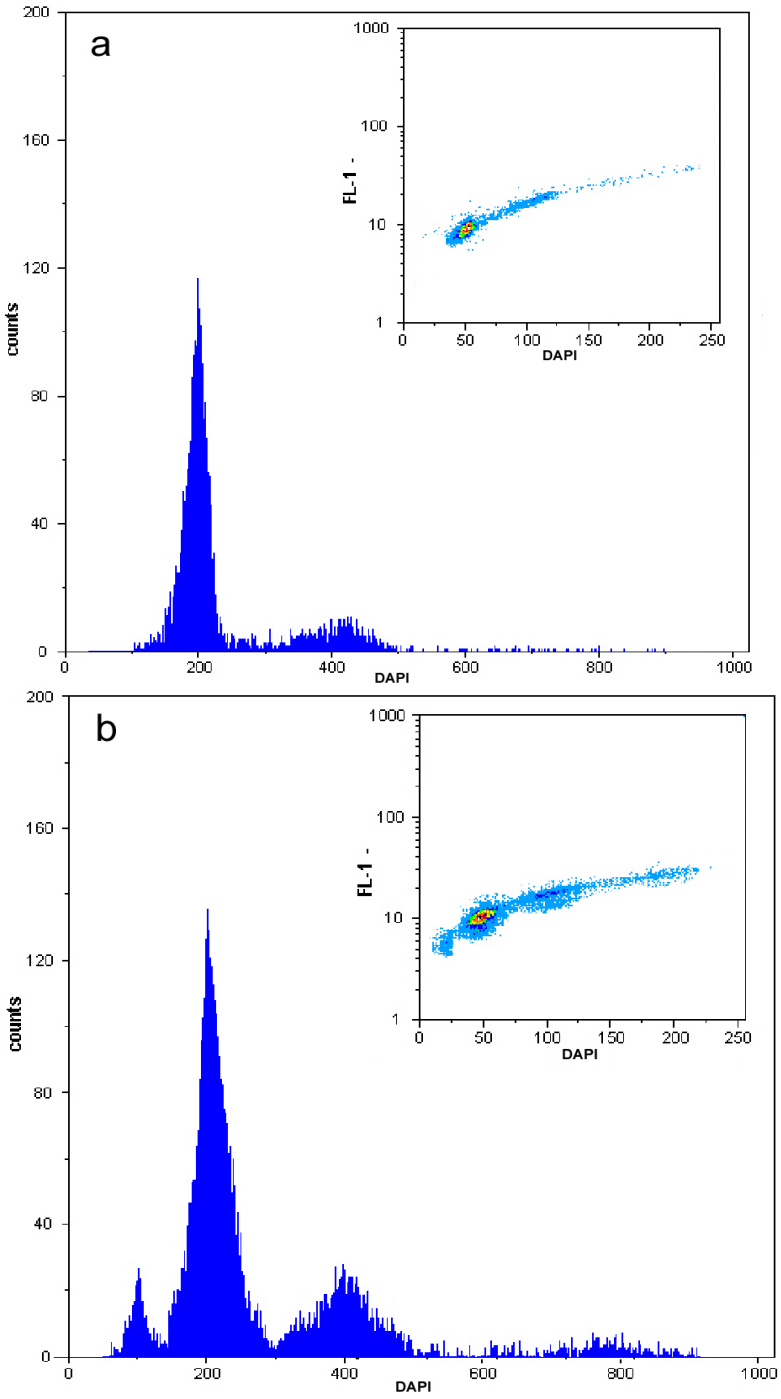


Figure 1. Representative FCM histograms of nuclei isolated from DAPI-stained tomato 'Debora' anthers. a) Small anthers (0.5 to 0.7 mm) with G_0/G_1 peak of anther wall cells and microspore mother cells set to channel 200. The G_2/M peak of tapetal cells, G_2 microspore mother cells and meiocytes at prophase I is represented in channel 400. b) Large anthers (1.6 to 1.9 mm) with G_0/G_1 peak of anther wall cells and meiocytes II set to channel 200. The G_2/M peak of anther wall cells and meiocytes I is represented in channel 400. The peak in channel 100 corresponds to haploid nuclei released from tetrads. The G_2/Mpeak in channel 800 corresponds to endoreduplicated tapetal cells. In the upper right panel of both histograms, density dot plots (cytograms) show the distribution frequencies of nuclei corresponding to the peaks based on two fluorescence parameters.

Figure 2. Calli originated from anther culture of tomato 'Debora'. Left: initial callus maintained in the dark. Center: callus transferred to light, showing greenish color and early shoot. Right: shoot at a more advanced developmental stage. The brownish calli pieces correspond to vestiges of anthers in oxidation process. Scale bar = 5 mm.

Table 1. Average number of anthers per Petri dish showing calli in accordance with the flower bud size at 60 days after inoculation.

Budsize (mm)	Genotype			
	Débora	Santa Clara	Alambra	Pizza Doro
1-2	0.667^{Aa}	1.667^{Aa}	0.00^{Ab}	0.00^{Ab}
2-3	1.667^{Aa}	2.50^{Aa}	2.667^{Aa}	3.00^{Aa}
3-4	1.33^{Aa}	1.00^{Aa}	2.33^{Aa}	1.33^{Ab}
4-5	0.667^{Ba}	1.00^{Ba}	3.33^{Aa}	1.33^{Bb}
≥ 5	0.00^{Ba}	1.50^{Aa}	2.33^{Aa}	1.00^{Bb}

*Means followed by the same capital letters at the horizontal group are statistically homogeneous as determined by Tukey's test at 0.05% probability level.

analysis, especially in plants in which no correlation exists between bud size or anthers with the meiotic stage. We have identified the response of four different tomato genotypes with different anther sizes in relation to calli production that were induced in an induction medium previously tested. Most studies on tomato anther culture have reported the occurrence of callogenesis ((Zamir et al., 1980; Shtereva et al., 1998; Brasileiro et al., 1999; Seguí-Simarro and Nuez, 2007; Corral-Martínez et al., 2005; 2011). However, considering the difficulty in achieving plant regeneration from calli in the test species besides the high recalcitrance of tomato to another culture (Seguí-Simarro and Nuez, 2005; Corral- Martínez et al., 2011), very little progress has been made in this context, with no standardized method being available for the generation of haploid and DH tomato plants (Seguí-Simarro and Nuez, 2007).

Various culture media have been tested for the induction of callus in tomato anthers. In this study, the induction medium suggested by Shtereva et al. (1998) was efficient for calli production in four genotypes of tomato anthers tested. Using the same medium, Asoliman et al. (2007) obtained a high frequency of somatic embryos from tomato anther culture, whereas Seguí-Simarro and Nuez (2005) obtained a high percentage of calli. Identification of the microsporogenic phase is considered as an important step for the successful anther culture. In this study, anthers were selected according to the size of flower buds and analyzed by FCM and cytogenetic procedures. In order to select the desired anther phase, Seguí-Simarro and Nuez (2005) and Góralski et al. (2005) identified the stage most responsive to androgenesis in tomato and maize plants, respectively, based on the relationship between *in vitro* anther development and cell characterization of the microspore/pollen.

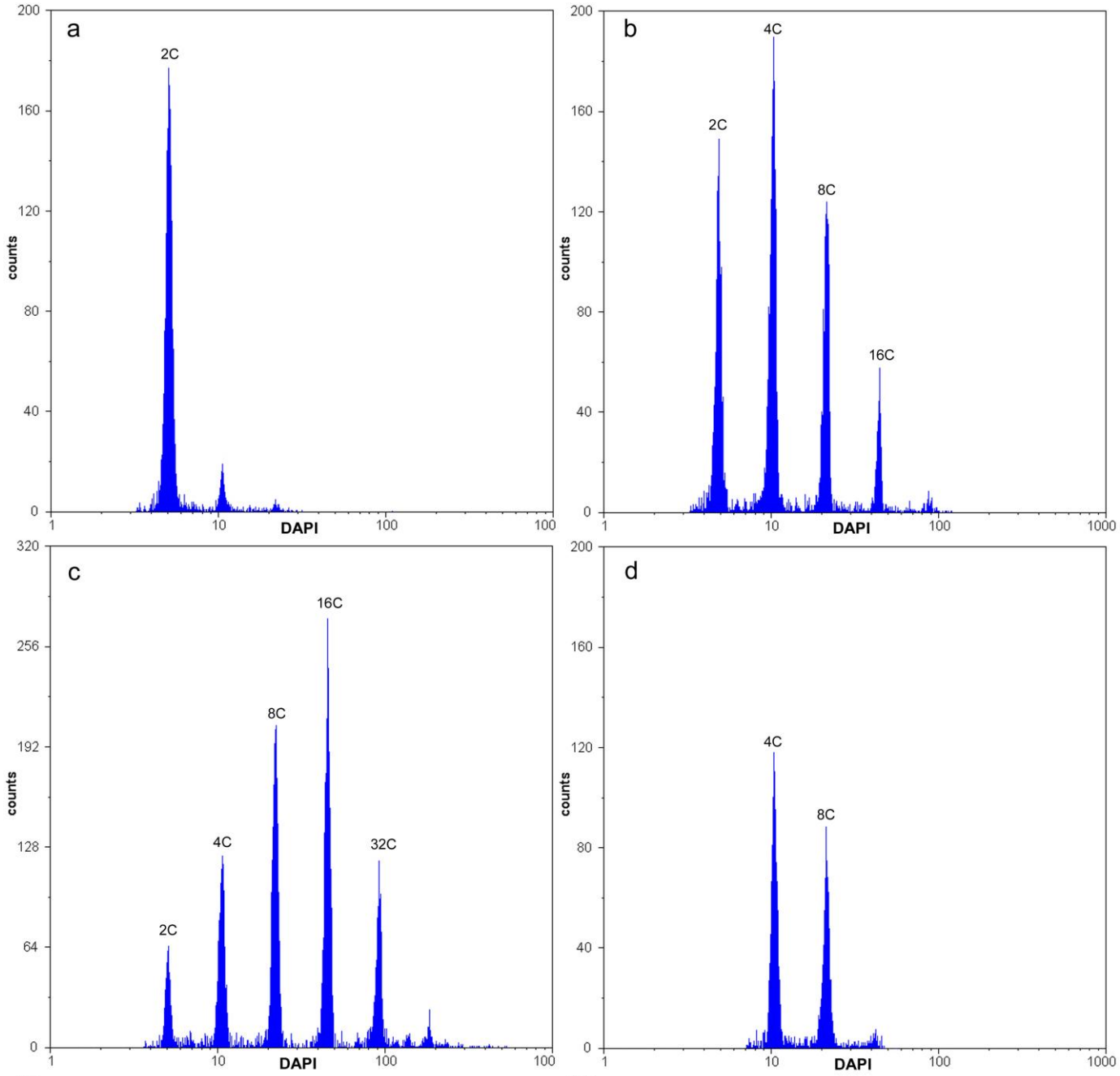
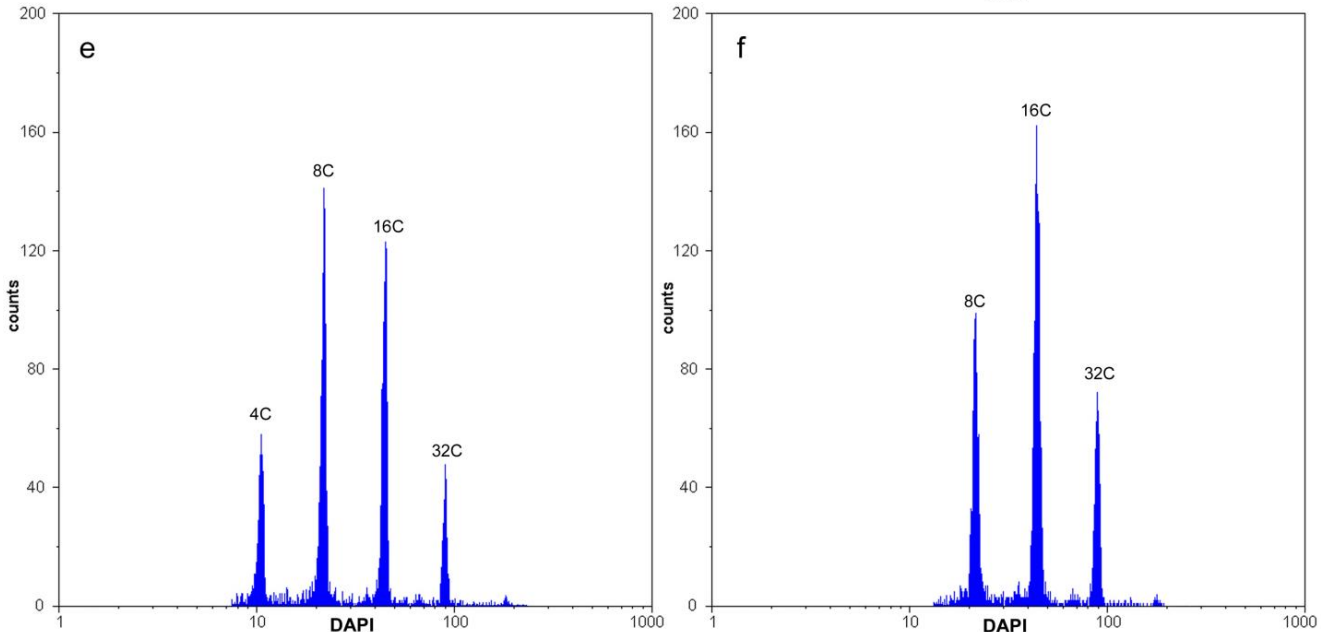


Figure 3. Representative histograms, in semi-logarithmic scale (log X-axis), of tomato 'Debora' nuclei stained with DAPI. The X-axis represents the relative DNA content; the Y-axis indicates the number of examined particles. The channel numbers are proportional to the log of fluorescence intensities. a) Donor plant leaf used as diploid DNA content standard. (b-f) Multiploid calli originated from anthers. The last peak with unidentified ploidy corresponds to the G_2/M phase. The various peaks corresponding to multiploid calli were identified by comparison of their nuclear DNA content with that of the diploid leaf.

Our results show that anthers in meiosis, up to anaphase II, were equally responsive for callus induction. Anthers containing microspores, obtained from buds with length ranging from 5.0 to 5.9 mm, did not respond to *in vitro* induction. Other studies corroborated that meiocytes is the most responsive stage for tomato anther culture (Zamir et al., 1980; Summers et al., 1992; Brasileiro et al., 1999; Seguí-Simarro and Nuez, 2005). Gresshoff and Doy (1972), Summers et al. (1992) and Brasileiro et al. (1999) reported that anthers showed higher frequencies of callus production during prophase I. Nevertheless, Seguí-Simarro and Nuez et al. (2005) identified



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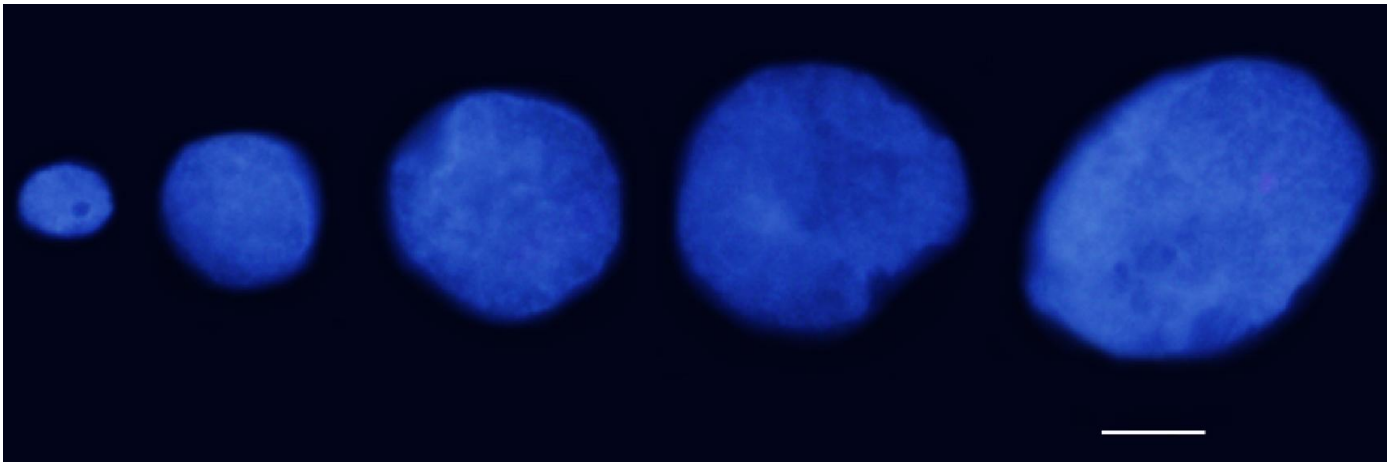

Figure 4. Cytological analysis of class II (2C-4C-8C-16-32C) nuclear suspension showing well-preserved, isolated, DAPI-stained nuclei. Scale bar = 10 µm.

callusogenic response in meiocytes from metaphase I until telophase II, whereas Shtereva et al. (1998) identified the response from prophase to telophase II as being optimal for tomato anther implantation. In most species, including model systems such as rapeseed and tobacco, the most sensitive period for inductive treatment is that near the first haploid mitosis, that is, from the vacuolated microspore to bicellular pollen stage (Touraev et al., 2001). Bal and Abak (2005) reported that the uninucleate stage was found to be suitable for the production of multicellular structure (proembryo) from tomato-isolated microspores. Occasional shoots were observed in this study, but these shoots did not regenerate into plants, probably owing to the recurrent polyploidization noted in calli. The ploidy was examined in an attempt to find out the reason of compact callus without embryogenic competence.

According to Ochatt et al. (2000), the increase in the DNA ploidy levels in calli can significantly complicate the regeneration of plants. The low efficiency of polyploidy calli regeneration is related to the loss of totipotency due to polyploidization (Colijn-Hooymans et al., 1994).

According to Phillips et al. (1994), changes in DNA ploidy levels are the most common somaclonal variations in tissue cultures. In fact, cell polyploidization in plant tissue culture has been described in numerous experimental systems. Park et al. (2010) cultivated *Doritaenopsis* leaf, root tip and somatic embryo *in vitro* in order to investigate their degrees of endoreduplication and found that somatic embryos showed the highest level (2C-64C). Several possible origins for somaclonal variations have been suggested, among which culture time is notable. Here, it was observed that this variable did not affect polyploidization in the calli, which presented populations of polyploid cells after two months of inoculation. In contrast, Salas et al. (2011) observed that culture time was the main factor influencing chromosome doubling in embryos derived from eggplant anther culture. Moreover, Song et al. (2000), Hao and Deng (2002) and Zhang et al. (2006) reported an increase in chromosome variations throughout subculturing.

The *in vitro* environment itself is an important source of stress. Though initial culture conditions promote cell proliferation, they may also cause defects in normal cytokinesis (Seguí-Simarro and Nuez, 2008b). In the present study, we used the culture medium suggested by Shtereva et al., (1998) assuming that the medium used successfully by the authors would be adequate for the genotypes used in this study. Thus, an interaction among growth regulators added to the culture medium and the genotypes of the donor plants may have induced endoreduplication cycles, resulting in high DNA ploidy levels in calli arising from tomato anther culture. It has been reported that the influence of growth regulators on calli induction depends on the donor plant genotype (Shtereva et al., 1998), representing one of the main factors that determine the type and frequency of genetic alterations (Jain, 2001). Seguí-Simarro and Nuez (2008a) also revealed that growth regulators induce increased DNA ploidy levels in microspores during anther culturing.

In our study, no population of haploid nuclei was observed in calli analysis by FCM, but multiploid nuclei were observed. According to Salas et al. (2011), this multiploidy could have somatic origin, as reported for anther culture of eggplant and the related species. In addition, Levenko et al. (1977) mentioned that cells of the anther wall contribute to the formation of mixoploid calli in tomato plants. Corral-Martínez et al. (2011) further showed that up to 83% of the calli produced from tomato anthers were derived from anther wall tissue. Nevertheless, Seguí-Simarro and Nuez (2007) and Corral-Martínez et al. (2011) also reported calli with haploid DNA content. Moreover, we suggested the possibility of the multiploidy to be meiotic cell lineage origin, whereas only anthers with meiocytes II were responsive. However, anthers with meiocytes after phase II possess somatic tissues capable of induction and which have not responded. This study allowed us to conclude that: (i) the association of cytogenetic and FCM techniques were effective in correlating the anther size with the meiotic developmental stage. Therefore, this method was considered to be adequate for the identification of the developmental stages of microsporogenesis that are most responsive to callus induction on a large scale; (ii) Anthers containing meiocytes in the stages of prophase I up to anaphase II (2.0 to 3.9 mm) showed a higher percentage of callus formation. However, (iii) no haploid material was obtained in all genotypes tested; (iv) multiploid calli were identified in the short-term culture (two months), suggesting that this variable (time of culturing) was not directly responsible for recurrence of endoreduplication. The occurrence of these endoreduplication cycles in tomato anther culture has been reported for the first time in the present paper. Most reports of anther culture reported that the DNA ploidy levels were not exceptionally high. Since we used different genotypes in a culture medium considered suitable for haploid production, our results indicate a greater importance of the interaction between genotype and growth regulators in the process of obtaining haploids that promoted calli with different and high DNA ploidy levels.

Conflict of interests

The authors did not declare any conflict of interest.

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Extraction and physicochemical characterization of a potential multifunctional pharma-excipient from crab shell wastes

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An efficient and beneficial waste disposal mechanism is highly desired. This study was carried out to extract and characterize a potential multifunctional pharmaceutical excipient from crab shell wastes. Shells of *Pachygrapsus mamoratus* were obtained from Oron, a coastal town in Akwa Ibom State of Nigeria. Chitin was extracted from the powdered shell by deproteination and demineralization; and chitosan was derived by alkaline deacetylation of the chitin. The polymer was subjected to Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC). It was also evaluated for flow properties, pH and hydration and swelling characteristics. The shells gave a yield of 17% chitosan. FTIR analysis of the polymer showed C-H bond of substituted cyclic hydrocarbon, cyclic C-N bond, C-O bond of glucose molecules, C-H bond of side chain –CH_2OH, presence of β-ester linkage, N-H of amides and bonded and free O-H groups. The last transition in the thermogram of chitosan was a polymer degradation exotherm with a peak at 337.9°C. The chitosan had higher bulk density, higher flow rate, lower Carr's index and lower Hausner's ratio compared to sodium carboxymethylcellulose. It also had lower hydration and swelling capacities. Therefore, the crab shell-derived chitosan has better thermal stability, better flow properties but poorer swelling properties compared to sodium carboxymethylcelluose.

Key words: Crab shell, chitosan, physicochemical characteristics, pharmaceutical excipient.

INTRODUCTION

The different approaches to domestic solid waste management can be arranged in decreasing order of preference and efficiency as: waste prevention, re-use, recycling, composting, land filling, dumping and burning (USEPA, 2014). Extraction of chitosan from crab shells for pharmaceutical use is as efficient as waste recycling. Chitosan is obtained by deacetylation of chitin, a component of shells of crustaceans such as shrimps and

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Abbreviations: HR, Hausner's ratio; **CI,** Carr's index; **FTIR,** fourier transform infrared; **DSC,** differential scanning calorimetry; **SCMC,** sodium carboxymethylcellulose; **BD,** bulk density; **TD,** tapped density; **MSC,** moisture sorption capacity; **HC,** hydration capacity; **SC,** swelling capacity.

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crab (Wang et al., 2006). Chitin is similar to cellulose, the difference being that the C-2 hydroxyl groups are replaced by acetamido residue. It consists mainly of unbranched chains of β-(1,4)-acetamido-2-deoxy-D-glucan (also known as β-(1,4)-N-acetyl-D-glucosamine). Thus, chitin is a polymer of N-acetyl-D-glucosamine with β-1,4 linkages. It is practically insoluble in water. Chitosan, also known as soluble chitin, is chemically known as 2-amino-2-deoxy-β-D-glucopyranose. It is composed randomly of β-(1,4)- linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine. The amino group in chitosan has a pKa of 6.5 which leads to a protonation of acidic to neutral solution. This makes chitosan water-soluble and a bioadhesive which readily bonds to negatively charged surfaces such as mucosal membrane (Sonia and Sharma, 2011). Chitosan has found a wide application as a drug delivery agent (Hu et al., 2013). It is used as a binder in wet granulation (Kepsutlu et al., 1999), as a drug-carrier in microparticle system, in preparation of hydrogels, as a bioadhesive polymer, as a site-specific drug delivery agent such as to the stomach and colon, as a biodegradable polymer for implants, for hormone delivery and for slow release of drugs from tablets and granules (Yin et al., 2009). It is also used as diluents in direct compression and as a viscosifier. It is used as permeation or absorption enhancer for poorly permeable drugs such as neomycin and cimetidine and for transport of polar drugs across epithelial surfaces (Yin et al., 2009). It is therefore, a multifunctional pharmaceutical excipient. Moreover, a high variety of properties are achieved when chitosan is admixed to amphiphilic compounds (Chiappisi and Gradzielski, 2015).

Crabs are important seafood in coastal areas and they constitute important source of protein in these areas. Disposal of the shell wastes is however, a continuous environmental burden (Burrow et al., 2007). The fact that these wastes can be utilized as sources of chitin and chitosan which are widely utilized in pharmaceutical, medical and environmental fields has provided a more interesting solution to seafood waste accumulation.

The objectives of this study were: to provide a means of disposing the seafood wastes in the coastal towns of Nigeria, to extract chitin and convert it to chitosan; and to carry-out physicochemical characterization of the chitosan. The physicochemical characteristics of the chitosan will be compared with those of sodium carboxymethylcellulose (SCMC), the two substances being cellulose-related. Sodium carboxymethylcellulose is a cellulose derivative with many applications as a pharma-excipient.

MATERIALS AND METHODS

Materials

Sodium hydroxide pellets (BDH Chemicals, England), 36% v/v hydrochloric acid (BDH Chemicals, England) and distilled water which was prepared in the Process Laboratory of Department of Pharmaceutics and Pharmaceutical Technology, University of Uyo, Uyo, Nigeria.

Crab shell collection and blending

Shells of *Pachygrapsus mamoratus* were obtained from Oron, a coastal town in Akwa Ibom State, Nigeria. They were sun-dried for five days to remove moisture from the shells. The dried shells were crushed using mortar and pestle and then powdered using laboratory blender (Christison, United Kingdom).

Extraction of chitin

Chitin extraction was carried-out using a modified form of the method described by Burrow et al. (2007). The process involved two steps namely: deproteination and demineralization.

Deproteination

A 75 g sample of the powdered exoskeleton was weighed and then divided into three equal parts and placed in three different 250 ml beakers labeled A B, C so as to obtain results in triplicates. Each of the samples was treated with 100 ml of 4% w/v NaOH solution and boiling for 1 h in order to dissolve the proteins and sugar. The system was allowed to cool and the supernatant was decanted off. The sediment was dried on cardboard paper for 30 min at room temperature. The powder was further crushed to pieces in a mortar and then screened through a 0.5 mm sieve.

Demineralization

The resulting powder was demineralized with 135 ml of 1% w/v HCl and was left overnight. This was done to remove the calcium carbonate content of the sample. The demineralized crab shell was then treated for 1 h with 50 ml of 2% w/v NaOH to decompose the albumen into water soluble amino acid which was drained off. The sample was washed with deionized water and then air-dried.

Conversion of chitin to chitosan

The chitin was converted to chitosan by deacetylation process using the method described by Burrow et al. (2007). It was carried out by adding 100 ml of 50% w/v NaOH to each sample and then heating at 100°C for 2 h in a water bath. The samples were then removed and cooled for 30 min at room temperature. Afterward, each sample was washed continuously with 50% w/v NaOH and filtered to retain the solid matter which is chitosan. Each sample of the chitosan was placed in a 250 ml beaker and air-dried for 3 h and then oven-dried at 120°C for 24 h to obtain dry chitosan.

Physicochemical characterization

The Fourier transform infrared spectrum and differential scanning thermogram of the chitosan were obtained while the other physicochemical properties were determined and compared with those of sodium carboxymethylcellulose.

Fourier transform infrared (FTIR) spectroscopy

A sample of the chitosan was prepared in a potassium bromide disk

in a hydrostatic press at 6 to 8 tons pressure. The FTIR spectrum was recorded at scanning range of 350 to 5,000 cm^{-1} using a spectrophotometer (model 8400S, Shimadzu Corporation, Kyoto - Japan).

Differential scanning calorimetry (DSC)

DSC analysis of chitosan was carried out on a 1 mg sample in an A1 40 µL crucible using a DSC – 204FI machine (NETZSCH Co., Germany). The scanning was done at 20°C/min heating rate over a temperature range of 0 to 500°C under nitrogen environment.

Angle of repose

For the angle of repose, 20 g powder of each polymer was poured inside a funnel of orifice diameter 0.75 cm clamped at a height of 10 cm from the table surface. The powder was allowed to flow freely and the angle of repose, Θ, was calculated using the equation

$$\Theta = Tan^{-1} (2h/D) \quad (1)$$

Where, h = height of heap and D is the diameter.

Flow rate

A 20 g sample was placed in a flow rate machine (Erweka, GMBH, Germany). The time of flow was determined and the flow rate was calculated.

Bulk and tapped densities

A 20 g sample was placed in a 50 ml measuring cylinder and the bulk volume was taken. The system was tapped 100 times after which the volume was retaken. The bulk density (BD) and tapped density (TD) were calculated as the ratio of mass to the corresponding volume. The Carr's index (CI) and Hausner's ratio (HR) were calculated using Wells and Aulton (2007) equations:-

$$CI = \frac{TD - BD}{TD} \; X \; 100\% \quad (2)$$

$$HR = \frac{TD}{BD} \quad (3)$$

pH

The pH of 2% w/v dispersion of each polymer was determined 24 h after preparation using a pH meter.

Moisture content

A 2 g sample of polymer was weighed and transferred into an electronic moisture analyzer (Type MB 35, OHAUS, Switzerland) and the percent moisture content was determined.

Moisture sorption capacity (MSC)

The moisture sorption capacity was determined by gravimetric method. A 2 g sample of polymer was placed in a desiccator containing distilled water (relative humidity 100%) for 5 days after which it was reweighed. The percentage moisture uptake was determined using equation 4 (Beristain et al., 2006).

$$MSC = M_m / M_d \times 100\% \quad (4)$$

Where, M_m is the mass of moisture absorbed and M_d is the mass of the dry polymer.

Hydration and swelling capacities

Compacts (500 mg) weight (W_d) of the polymers were prepared by compressing the powder using a tableting machine (Shanghai Tiaxiang and Chenta Pharmaceutical Machinery Co., China) fitted with a 12 mm flat faced punch and die. The thickness 'h' and diameter '2r' of the compacts were measured with the aid of micrometer screw gauge. The compacts were placed on glass plates and transferred into a beaker containing 60 ml of distilled water. At 10 min intervals, the glass plates with the hydrated compacts were removed, dried by blotting with tissue paper and then weighed using the method of Builders et al. (2009) with slight modification. The process was continued until a constant weight (W_e) of the hydrated mass was obtained. The hydration capacity (HC) was determined using equation 5.

$$HC = W_e / W_d \quad (5)$$

The thickness 'h' and diameter '2r' of the hydrated mass at constant weight were also measured. Volumes of compacts were calculated using Equation 6.

$$V = \pi r^2 h \quad (6)$$

The swelling capacity (SC) was calculated as the ratio of the final volume of swollen mass to the initial volume of the compact.

Statistical analysis

Data, obtained in triplicates from evaluations were expressed as mean values ± standard error of the mean. Statistical analysis was done using Student's t-test. Significance of difference was taken at *p - values* less than 0.05.

RESULTS

The crab shells gave a yield of 17 ± 0.5% chitosan. The FTIR spectrum of the polymer (chitosan) is shown in Figure 1. The absorption peaks were observed at 399.28, 447.50, 675.11, 858.35, 1035.81, 1471.74, 1643.41, 2287.65, 3362.04, 3834.61 and 4488.50 cm^{-1}. The DSC thermogram is shown in Figure 2. It showed a diffuse endotherm over the temperature range of 30 and 85°C (with peak at 54.8°C), followed by a sharp endotherm which peaked at 107.2°C. This was followed by another endotherm which peaked at 182.6°C and subsequently an exotherm which peaked at 337.9°C. The areas of the four transitions were 632.5, 771.5, 1647.0 and 1849.0 J/g, respectively. The flow properties of sodium carboxymethylcellulose and those of chitosan are presented in Table 1. There were significant differences

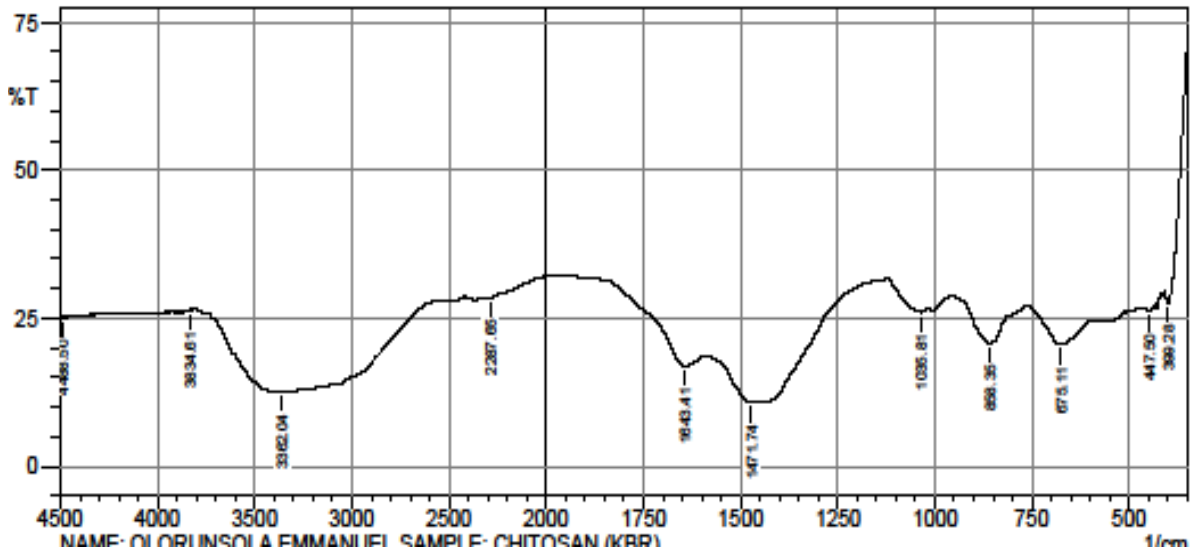

Figure 1. Fourier transform infrared spectrum of chitosan.

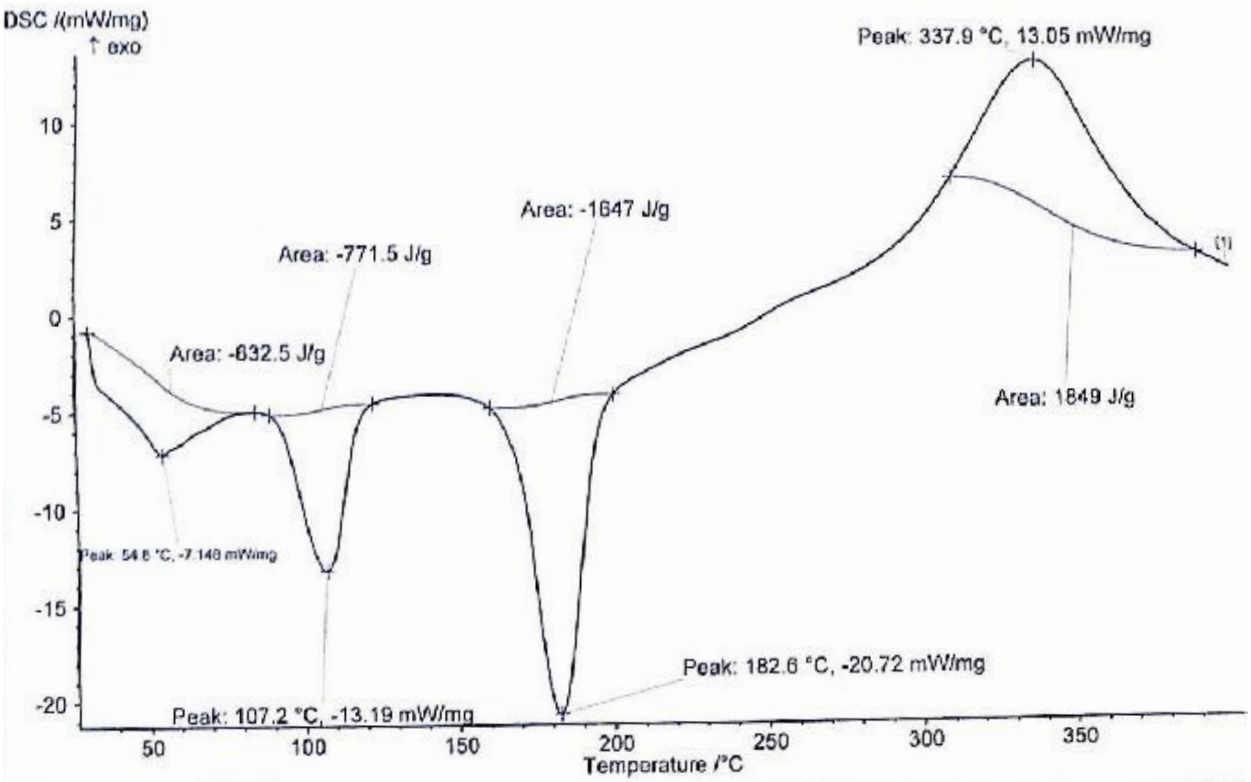

Figure 2. Differential scanning thermogram of chitosan.

in all the flow parameters of the two polymers. Chitosan had lower angle of repose, higher flow rate, higher bulk density, higher tapped density, lower Carr's index and lower Hausner's ratio. The pH, hydration and swelling characteristics of the two polymers are presented in Table 2. There were significant differences in these parameters determined for the two polymers. Chitosan had higher pH, lower moisture content, lower moisture sorption capacity, lower hydration capacity and lower swelling capacity.

Table 1. Flow properties of the polymers.

Parameter	Sodium carboxymethylcellulose	Chitosan	p-value
Angle of repose (°)	50.12 ± 0.36	35.32 ± 0.56	< 0.05
Flow rate (g/min)	9.15 ± 0.06	19.23 ± 1.12	< 0.005
Bulk density (g/ml)	0.20 ± 0.01	0.59 ± 0.02	< 0.005
Tapped density (g/ml)	0.31 ± 0.01	0.75 ± 0.03	< 0.005
Carr's Index (%)	35.48 ± 0.40	21.33 ± 0.15	< 0.025
Hausner's Ratio	1.55 ± 0.00	1.27 ± 0.02	< 0.05

Table 2. pH, hydration and swelling characteristics of the polymers.

Parameter	Sodium carboxymethylcellulose	Chitosan	p-value
pH of 2 % w/v dispersion	5.27 ± 0.03	12.30 ± 0.88	< 0.005
Moisture content (%)	10.31 ± 0.33	6.64 ± 0.45	< 0.025
Moisture sorption capacity	96.42 ± 2.66	33.65 ± 1.77	< 0.005
Hydration capacity	4.98 ± 0.05	1.15 ± 0.06	< 0.0005
Swelling capacity	4.22 ± 0.03	1.40 ± 0.05	< 0.005

DISCUSSION

Absorption peaks of FTIR below 600 cm^{-1} are not used for characterization (Coutt, 2008). Therefore, the peaks at 399.28 and 447.50 cm^{-1} cannot be assigned to specific functional groups. The peak at 675.11 cm^{-1} can be attributed to C-H bending vibration of substituted cyclic hydrocarbon while that at 858.35cm^{-1} can be assigned to cyclic C-N stretching. The absorption peak at 1035.81 cm^{-1} can be assigned to C-O bending of glucose molecule. Those at 1471.74 and 1643.41 cm^{-1} can be assigned to C-H bending of side chain $–CH_2OH$ and presence of β-esters. The absorption peak at 3362.04 cm^{-1} fell within the range of 3300 and 3500 cm^{-1} which is characteristic of N-H of amides while those at 3834.61 and 4488.50 cm^{-1} correspond to stretching vibration of bonded and free O-H groups (Coutts, 2008). All these absorption peaks are typical of chitosan molecule. The various absorption peaks of sodium carboxymethylcellulose were differently assigned to C-H bending of substituted cyclic hydrocarbon, C-H stretching of cyclic hydrocarbon, O-H bending and C-O stretching of alcohol and O-H stretching of bonded and free hydroxyl groups (Olorunsola et al., 2014). Hence, the major difference in the functional groups of chitosan and sodium carboxymethylcellulose is the acetamido residue of chitosan.

The diffuse endotherm of chitosan which peaked at 54.8°C can be ascribed to loss of the absorbed water (Horvat et al., 2005); and 632.5 J of heat per gram sample of the polymer was absorbed in the process. The second transition (with peak at 107.2°C) is a relaxation endotherm (Chung et al., 2002) with enthalpy of 771.5 J/g. Endothermic relaxation is a second order reaction just like glass transition (Horvat et al., 2005). The third endotherm can be ascribed to polymer melting. The area under the curve (164.7 J/g) represents the latent heat of melting while the peak (182.6°C) represents the melting point of the polymer (Builders et al., 2009). The last transition, a diffuse exotherm can be ascribed to the polymer degradation (Iqbal et al., 2013). The exothermic transition temperature of chitosan is higher than that of SCMC. Hence, it has a better thermal stability. The significantly higher bulk density of chitosan is an indication that it has better packing and/or higher density. Chitosan has a significantly higher flow rate ($P< 0.005$). The angle of repose of SCMC which is greater than 50° suggests a very poor flow while that of chitosan which fell between 30 and 40° suggests a fair flow (Wells and Aulton, 2007). The Carr's index of SCMC which is greater than 35% and the Hausner's ratio which is greater than 1.5 suggest that the polymer has a poor flow. On the other hand, the Carr's index of chitosan which was 21.33% and the Hausner's ratio of 1.27 suggest that the polymer possesses a fair flow (Wells and Aulton, 2007). Therefore, chitosan possesses a better flow and may be a better diluent in direct compression.

The pH of sodium carboxymethylcellulose is in the acidic region while that of chitosan is in the basic region. Sodium carboxymethylcellulose has a pKa of 4.3 (Tian et al., 2006). Being a weak acid, it will undergo full ionization and exhibit maximum solubility at high pH (Aulton, 2007). Hence, it will form better hydrogel at low pH. Conversely, chitosan has a pKa of 6.5 (Sonia and Sharma, 2011) and being a basic polymer, it will undergo full ionization and exhibit maximum solubility at low pH (Aulton, 2007). This could be responsible for the better activity of chitosan as a permeation enhancer at low pH

(Yin et al., 2009). The protonation constant and solubility of chitosan has been shown to vary with the degree of deacetylation (Wang et al., 2006).

Sodium carboxymethylcellulose with higher moisture content is also characterized by a higher moisture sorption capacity. Hence, even though it contains more water, it has the ability to absorb more if exposed to a humid condition. However, it had been shown that the level of hygroscopy of the polymer does not mean poor stability (Tian et al., 2006). Therefore, it might be difficult to compare the stability of the two polymers based on hygroscopy alone. The lower hydration capacity ($P < 0.0005$) and lower swelling capacity ($P < 0.005$) of chitosan are indication that the polymer is not a strong hydrogel as SCMC. It is however, important to note that while SCMC will improve drug delivery by acting mainly as a bioadhesive agent, chitosan will improve drug delivery both as a bioadhesive agent and by temporarily increasing intestinal permeability (Kos et al., 2008).

Conclusion

The extraction of chitosan from crab shell wastes has a dual benefit. It serves as a means of disposing seafood wastes and as a means of generating a multifunctional pharmaceutical excipient. The chitosan possesses better thermal stability, higher bulk density, better flow properties but weaker swellability compared to sodium carboxymethylcellulose.

Conflict of interests

The authors did not declare any conflict of interest.

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