Novel Cycloartan derivative with genetic and metabolic profiling of two Crassulaceae species

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ABSTRACT

Crassula tetragona L. and Crassula ovata (Mill). are ornamental species of family Crassulaceae. Although this family is known for its high medicinal values, however, there is no much work considering the two species. Hence, this study represents the first comparative investigation of the genetic and metabolic profiling of the aerial part of both species. In this study, an examination of the genetic properties of both plants were accomplished, quantitative estimation of the main chemical classes of both species were also performed, investigation of the lipoidal matter of the plants and the major compounds of the methylene chloride (MeCl) fractions were isolated and identified using 1D and 2D NMR. Our results proved the genetic difference using RAPD and ISSR techniques of both plants. Estimation of triterpene was 63.18 µg/100 g and 87.06 µg/100 g, ursolic acid equivalent, in C. tetragona and C. ovata respectively. The unsaponifiable matter (USM) of n-hexane extract of C. tetragona and C. ovata revealed the presence of 32 hydrocarbons with the presence of n-tricontane as the major hydrocarbon in both species, in addition to seven steroidal components in both species. The investigation of fatty acid methyl ester (FAME) revealed the presence of 12 components in C. tetragona and 9 components in C. ovata, and a novel triterpene, namely, 28 Methyl-5α-cycloart12, 20, 24-trien-15-β-Ol was isolated and identified from MeCl together with 5 known compounds.

INTRODUCTION

For many years, natural products had a significant role in the cure of widespread ailments and have improved the quality of human beings lives. (Akanda et al., 2014). Crassula is one of the largest genus of family Crassulaceae that comprises about 150 species (El-Hawary et al., 2016). Botanical description of closely allied plant species in these days is greatly supported via investigation of various powerful genetic principles such as DNA fingerprinting. It is reported as a favourable tool for the authentication of medicinal plant species and especially...
useful in varieties or species that can't be distinguished morphologically and/or phytochemically (Kady et al., 2015). DNA fingerprinting following successful PCR amplification of tandemly repeated sequences is well-known as polymorphic and widespread in plant genomes (Mcgregor et al., 2000). Thus, PCR based on various techniques which include Random Amplified polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) are playing great rule in plant improvement (Manimekalai et al., 2006). The purpose of the present investigation was to evaluate the benefit of the RAPD and the ISSR marker systems to distinguish the two Crassula species, and it is considered the first assessment of genomic DNA of the two species as well as to figure out the dissimilarity in their chemical composition by determination of the lipid content with respect to their chemical composition, the mineral content and amino acid composition and identification of their terpenoidal compounds using GLC as well as quantitative determination of triterpenes, and isolation from the methylene chloride fraction. There was no much work concerning the genus Crassula or species tetragona.
Table 1: List of the primer names and their nucleotide sequences used in the study

| NO. | Name | Sequence (5’-3’) |
|-----|------|-----------------|
| 1   | OP-A02 | GTG ATC GCA G     |
| 2   | OP-B02 | CTC ACC GTC C     |
| 3   | OP-B07 | GTG ACC CCT C     |
| 4   | OP-C12 | GGA CCC AAC C     |
| 5   | OP-C19 | GAC GGA TCA G     |
| 6   | OP-D01 | ACC GCG AAG G     |
| 7   | 14A   | CTC TCT CTC TCT CTC TGC |
| 8   | 44B   | CAC ACA CAC ACA GT |
| 9   | 49A   | CAC ACA CAC ACA AG |
| 10  | HB-09 | GTG TGT GTG TGT GC |
| 11  | HB-14 | CTC CTC CTC GC   |
| 12  | HB-15 | GTG GTG GTG GC   |

Figure 3: Ursolic acid standard calibration curve

nor ovata, so we will shed light on both plants’ constituents using different techniques.

MATERIALS AND METHODS

Plant Material

C. tetragona and Covata collected from EL Orman botanical garden, Giza, Egypt and identified by Dr. Mohamed Gibali (senior botanist, National Research Center, Cairo, Egypt).

Genetic profiling

Was accomplished at Biotechnology Research Lab, Horticulture Research Institute, Agriculture Research Center.

DNA isolation and sample preparation

The plant material (0.2 g) was kept in 1.5 ml microfuge tubes for DNA extraction utilizing the method illustrated by Williams et al. (Williams et al., 1990). Plant tissue was crushed with liquid nitrogen, 400 μl of AP1 (Activator protein-1) buffer and 4 μl stock solution of RNase (100 mg/ml) then vortexed strongly, admixture was incubated and mixed during incubation. Then, 130 μl of AP2 (Activator protein-2) buffer was combined to the lysate, mixed and incubated, Lysate was administrated to the Qiashredder spin and was added in centrifugation for 2 min. Typically, Then, 0.5 volume of AP3 buffer and 1 volume of ethanol (96-100%) were combined to the cleared lysate and mixed. Then, the mix was applied over DNeasy Tiny spin column. Then, centrifuged. Then, 500 μl buffer AW (Column wash buffer) was combined to the column of DNeasy and centrifuged. Then, 500 μl AW buffer was combined to a column of DNeasy and centrifuged. A column of DNeasy was then moved to a 1.5 ml microfuge tube and 100 μl of preheated (65ºC) AE buffer was pipetted precisely onto the column of DNeasy sheath. Then, incubated and centrifuged to elute. A fresh microfuge can be applied for primary elute. Otherwise, the microfuge cylinder can be used again for the next elution stage to mix the elutes.

Oligonucleotide primers (RAPD and ISSR PCR analysis)

Two methods were accomplished for worthy statistical inquiry. The DNA magnifications were achieved in an automatic thermal round (model Techno 512) automated for one round at 94º C for 4 min displaced by 45 rotations of 1 min at 94º C, 1 min at 37º C, and 2 min at 72º C. the reaction was lastly kept at 72º C for 10 min. PCR amplification was done using six random deca-mer arbitrary primers and six ISSR primers produced by (Operon Biotechnologies, Inc. Germany).

Polymerase chain reaction (PCR)

The PCR amplification in both RAPD and ISSR anal
Table 2: The ISSR and RAPD analysis of the *Crassula* species

| Primer Band no. | *M.W* | Species | Primer Band no. | *M.W* | Species |
|----------------|-------|---------|-----------------|-------|---------|
| OP-A02 1       | 1384.174 | 1 | 0 | OP-B02 1 | 901.139 | 1 | 0 |
| 2              | 1323.045 | 0 | 1 | 2 | 795.516 | 1 | 1 |
| 3              | 1091.958 | 1 | 1 | 3 | 699.542 | 1 | 1 |
| 4              | 891.114 | 1 | 1 | 4 | 596.271 | 1 | 1 |
| 5              | 766.560 | 0 | 1 | 5 | 490.733 | 1 | 1 |
| 6              | 677.020 | 1 | 1 | 6 | 308.675 | 1 | 1 |
| 7              | 618.542 | 0 | 1 | 7 | 287.770 | 1 | 1 |
| 8              | 552.496 | 1 | 1 | 8 | 239.620 | 1 | 1 |
| 9              | 522.166 | 0 | 1 | 9 | 226.018 | 1 | 1 |
| 10             | 475.272 | 1 | 1 | 10 | 223.392 | 1 | 1 |
| 11             | 434.220 | 1 | 1 | Total 10 | 9 |
| 12             | 405.775 | 1 | 1 | OP-C12 1 | 1200.895 | 0 | 1 |
| 13             | 383.500 | 0 | 1 | 2 | 1079.554 | 0 | 1 |
| 14             | 354.354 | 1 | 1 | 3 | 959.462 | 0 | 1 |
| 15             | 331.141 | 1 | 1 | 4 | 839.851 | 0 | 1 |
| 16             | 312.963 | 1 | 1 | 5 | 746.424 | 1 | 1 |
| 17             | 299.141 | 0 | 1 | 6 | 655.862 | 1 | 1 |
| 18             | 278.495 | 1 | 1 | 7 | 554.775 | 0 | 1 |
| 19             | 257.329 | 0 | 1 | 8 | 504.444 | 1 | 1 |
| 20             | 235.102 | 1 | 1 | 9 | 453.475 | 1 | 1 |
| 21             | 214.795 | 0 | 1 | 10 | 370.671 | 1 | 1 |
| 22             | 200.724 | 0 | 1 | 11 | 333.218 | 1 | 1 |
| Total          | 13 | 21 | Total 12 | 8 | 10 |

| OP-B07 1      | 539.609 | 1 | 0 | Total 14 | 13 |
| 2              | 485.517 | 1 | 1 | OP-C19 1 | 1191.589 | 0 | 1 |
| 3              | 432.100 | 1 | 1 | 2 | 1052.081 | 0 | 1 |
| 4              | 397.376 | 1 | 1 | 3 | 955.396 | 0 | 1 |
| 5              | 369.457 | 1 | 1 | 4 | 857.204 | 1 | 1 |
| 6              | 347.274 | 1 | 1 | 5 | 766.020 | 1 | 1 |
| 7              | 312.463 | 1 | 1 | 6 | 654.950 | 1 | 1 |
| 8              | 252.959 | 1 | 1 | 7 | 476.869 | 1 | 1 |
| 9              | 232.630 | 1 | 1 | 8 | 415.996 | 1 | 0 |
| 10             | 201.091 | 1 | 1 | 9 | 401.226 | 0 | 1 |
| 11             | 184.931 | 1 | 1 | 10 | 391.672 | 1 | 0 |
| 12             | 151.358 | 1 | 1 | 11 | 382.345 | 0 | 1 |
| 13             | 143.833 | 1 | 1 | 12 | 359.989 | 0 | 1 |
| 14             | 134.706 | 1 | 1 | 13 | 314.036 | 1 | 1 |
| Total          | 14 | 13 | Total 14 | 12 | 13 |
ysis was made conferring to the method described by Williams et al. (John GK Williams et al., 1990). PCR was achieved in 30-µl volume tubes contained dNTPs (2.5 mM) 3.00 µl, MgCl₂ (25 mM) 3.00 µl, Buffer (10 x) 3.00 µl, Primer (10 pmol) 2.00 µl, Taq DNA polymerse (5U/µl) 0.20 µl, Template DNA (25 ng) 2.00 µl, H₂O (distilled water) 16.80 µl.

Total triterpenes
Spectrophotometric method using vanillin reagent for total saponin content (Hiai et al., 1976). Briefly, one mg of the ethanol extract of both plants were separately dissolved in 10 ml 80% aqueous methanol to obtain a final concentration of 100 µg/ml. 1 ml of the sample solution was moved, and 0.25 ml of vanillin reagent (8%, w/v in absolute alcohol) were placed in an ice bath, and then 2.5 ml of 72% v/v of sulphuric acid was slowly added to the wall of each test tube and left for 3 min. Finally, the test tubes were warmed in a water bath at 60 °C for 10 min and cooled in an ice bath. The absorbance of the color produced was measured at λ max 544 nm against a blank prepared by the same procedure except ursolic acid. The total triterpene content of the analyzed sample was expressed as µg of ursolic acid /mg of sample.

Lipid content Investigation

Preparation of lipoidal matter
Round 50 g of the air-dried powder of each plant was extracted with n-hexane till exhaustion (no residue was left after evaporation of the last 5 ml of extract). Each extract was filtered, and the filtrate was evaporated under reduced pressure and temperature at about 60 °C. The residue left was kept for an examination of total lipoidal matter (3 g and 2.89 g) for C. tetragona and C. ovata respectively.

Preparation of unsaponifiable Matter (USM)
One gram of each residue of n-hexane was saponified separately by heating under reflux state for 24 hours with a combination of alcoholic potassium hydroxide 5% solution (200 ml) and benzene (20 ml). The heated mixtures were concentrated, diluted with 100 ml distilled water and extracted with consecutive portions of ether (each of 50 ml) till exhaustion, then washed with water several times till the washed extracts were neutral to litmus paper, dehydrated over anhydrous sodium sulphate and the solvent was distilled off. Each residue of unsaponifiable matter (USM) obtained was waxy, yellowish-white in color and weighed 0.83 g and 0.67 g for plants of C. tetragona and C. ovata respectively. (Vogel, 1957)

Isolation of the fatty acids from the saponifiable
The alkaline aqueous solution was left after extraction of USM, acidified with 10% hydrochloric acid and the liberated fatty acid were extracted with ether (5 x 50 ml) the mixed ethereal extract was washed with water till neutral to litmus, dried over anhydrous sodium sulphate, evaporated till dryness under reduced pressure. The fatty acids, isolated from the Hexane extracts of the plants of *C. tetragona* and *C. ovata*, amounted to 0.19 g and 0.3 g respectively.

**GLC analysis of the USM and FAME**

Detection of hydrocarbon and sterol content of USM was held out on GLC Hewlett Packard. HP-6890, G.C network GC system equipped with an FID detector. The analysis was achieved on an HP-5 column; using N2 as a carrier gas, injection temperature 240°C, detector temperature 280°C. Aliquots, 2 μL each, of 10% chloroform solutions of the analyzed USM and reference samples were co-chromatographed and rough identification of the hydrocarbons and sterols of the USM was held out by a comparison of their retention time with the available reference compounds. Total fatty acids fractions for both species were imperiled to methylation. The fatty acids methyl esters were examined by GLC under the same conditions like USM except for detector temperature 300 °C. Identification of the fatty acids methyl esters was carried out by direct comparison of retention time of each of the separated components with those of available reference fatty acid methyl esters analyzed under the same conditions.
Table 3: The ISSR and RAPD analysis of the Crassula species. (Continued from Table 2)

| Primer | Band no. | *M.W | Species | Primer | Band no. | *M.W | Species |
|--------|----------|------|---------|--------|----------|------|---------|
| OP-C19 | 1        | 1432.484 | 0 | 1 | 1435.551 | 1 | 1 |
|        | 2        | 1304.511 | 1 | 1 | 1304.005 | 1 | 1 |
|        | 3        | 1201.948 | 0 | 1 | 1201.764 | 1 | 0 |
|        | 4        | 996.785  | 1 | 0 | 996.574  | 1 | 0 |
|        | 5        | 918.416  | 1 | 1 | 918.204  | 1 | 0 |
|        | 6        | 836.367  | 0 | 1 | 836.155  | 1 | 0 |
|        | 7        | 767.612  | 1 | 1 | 767.406  | 1 | 0 |
|        | 8        | 674.930  | 0 | 1 | 674.721  | 1 | 0 |
|        | 9        | 651.655  | 1 | 0 | 651.447  | 1 | 0 |
|        | 10       | 607.486  | 1 | 0 | 607.277  | 1 | 0 |
|        | 11       | 515.718  | 0 | 1 | 515.508  | 1 | 0 |
|        | 12       | 469.646  | 0 | 1 | 469.437  | 1 | 0 |
|        | 13       | 442.964  | 0 | 1 | 442.755  | 1 | 0 |
|        | 14       | 403.391  | 1 | 1 | 403.182  | 1 | 0 |
|        | 15       | 376.049  | 1 | 1 | 376.840  | 1 | 0 |
|        | 16       | 358.859  | 1 | 0 | 358.650  | 1 | 0 |
|        | 17       | 322.999  | 1 | 0 | 322.790  | 1 | 0 |
|        | 18       | 271.017  | 1 | 1 | 271.807  | 1 | 0 |
|        | 19       | 214.482  | 0 | 1 | 214.273  | 1 | 0 |
|        | 44B      | 1        | 484.3110 | 1 | 484.1020 | 1 | 0 |
|        | 2        | 427.8500 | 1 | 1 | 427.6410 | 1 | 1 |
|        | 3        | 413.9391 | 0 | 1 | 413.7301 | 1 | 0 |
|        | 4        | 374.8611 | 1 | 0 | 374.6521 | 1 | 0 |
|        | 5        | 336.6781 | 1 | 0 | 336.4691 | 1 | 0 |
|        | 6        | 302.3851 | 1 | 0 | 302.1761 | 1 | 0 |
|        | 7        | 267.1331 | 1 | 0 | 267.9241 | 1 | 0 |

ISOLATION AND IDENTIFICATION

Sample Preparation

Fresh plants of *C. tetragona* and *C. ovata* were subjected to air drying, and the dried plants were percolated with absolute ethanol till exhaustion, the crude extracts in each case were suspended in distilled water, and the aqueous suspension of each plant was successively extracted by a partition with methylene chloride and n-butanol, the methylene chloride fraction was subjected for isolation.

Chemicals and equipments

Silica gel for column chromatography, CC (E. Merck, Darmstadt, Germany). Pre-coated silica plates 60 F 254 (20×20 cm) (E. Merck, Darmstadt, Germany). Silica gel for VLC (E. Merck, Darmstadt, Germany). The chromatograms were sprayed with p-anisaldehyde/sulfuric acid spray reagent. $^1$H-NMR (300 MHz) and $^{13}$C-NMR (75 MHz) were measured on a Varian Mercury-VX-300 instrument (Varian Medical Systems, Inc., Cary, NC). The NMR spectra were recorded in CDCl$_3$ and chemical shifts were given in (ppm) relative to TMS as an internal standard.
**RESULTS AND DISCUSSION**

**DNA Profile**

The data represented in this paper were important in that RAPD and ISSR fingerprinting has been efficacious in distinguishing variation in a species thought being devoid of a molecular variant (Raina et al., 2001). The efficiency of a molecular marker technique depends on the amount of polymorphism it can detect among the set of attainments under investigation. In the present study, ISSR and RAPD primers not only generated the highest number of polymorphic markers, but the polymorphism was sufficiently well distributed to enable discrimination between accessions by each primer pair. Table 1

**The RAPD Technique**

In order to evaluate the efficiency of RAPD-PCR fingerprinting for the two species, the DNA was used as a template for the 6 RAPD primers banding pattern. Total of 94 bands template for all primers, 41 of them were polymorphic (43.617%). The highest band number was produced with primer OP-A02 (22 bands), while the lowest was produced with primer OP-B02 (10 bands). Primer OP-D01 showed the highest polymorphic percent (68.421%) while primer OP-B07 showed the lowest polymorphic percent (7.143%). Figure 1, Tables 2, 3 and 4.

**The ISSR Technique**

The results of ISSR fingerprinting by using 6 ISSR primers banding pattern showed 60 bands for all primers, 28 of them were polymorphic (46.666%). The highest band number was produced with primer HB-15 (15 bands) while the lowest was produced with primer 44B (7 bands). Primer HB-14 showed the highest polymorphic percent (81.818%) while the primer, while primer HB-15 showed the lowest polymorphic percent (13.333%). Figure 2, Tables 2, 3 and 4.

**Total triterpenes**

Total triterpene was extracted according to the spectrophotometric method using vanillin reagent for total saponin content found to be 63.18889 µg/100µg ± 0.01 in *C. tetragona* and 87.06667µg/100µg ± 0.01 in *C. ovata*. The triterpene content of the tested sample, expressed as usorolic acid equivalent, was deduced from the pre-established calibration curve in and calculated using the following equation:

\[ y = 0.0046x + 0.0027 \]

\[ R^2 = 0.9619 \]

Where, \( y = \) absorbance, \( x = \) corresponding concentration (µg/ml) and \( R^2 = \) correlation coefficient.

**Lipid content investigation**

Investigation of the unsaponifiable matters of *n*-hexane extract of *C. tetragona* revealed the presence of 13 identified hydrocarbons with the presence of *n*-trictrane (57.65%) as the major hydrocarbon. In addition to Four identified steroidal components; with the presence of campesterol (19.77%)

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### Table 4: The ISSR and RAPD analysis of the Crassula species. (Continued from Table 2)

| Primer | Band no. | *M.W | Species | Primer | Band no. | *M.W | Species |
|--------|----------|------|---------|--------|----------|------|---------|
| HB-15  | 1        | 1306.502 | 0 | 1      | HB-14  | 1119.368 | 0 | 1      |
| 2      | 1125.641 | 1 | 1 | 2      | 1055.422 | 1 | 0      |
| 3      | 1006.623 | 1 | 1 | 3      | 884.680 | 0 | 1      |
| 4      | 946.029 | 1 | 1 | 4      | 854.001 | 0 | 1      |
| 5      | 846.002 | 0 | 1 | 5      | 682.938 | 0 | 1      |
| 6      | 737.995 | 1 | 1 | 6      | 546.140 | 0 | 1      |
| 7      | 646.448 | 1 | 1 | 7      | 508.918 | 1 | 1      |
| 8      | 585.320 | 1 | 1 | 8      | 415.037 | 1 | 1      |
| 9      | 536.593 | 1 | 1 | 9      | 343.825 | 0 | 1      |
| 10     | 498.070 | 1 | 1 | 10     | 320.392 | 1 | 0      |
| 11     | 393.398 | 1 | 1 | 11     | 288.202 | 0 | 1      |
| 12     | 365.155 | 1 | 1 | 12     | 279.022 | 1 | 1      |
| 13     | 299.362 | 1 | 1 | 13     | 243.400 | 1 | 1      |
| 14     | 279.022 | 1 | 1 | 14     | Total  | 4 | 9      |
| 15     | 243.400 | 1 | 1 | 15     | Total  | 13 | 15     |
### Table 5: Constituents identified by GLC analysis of the USMs of *C. tetragona* and *C. ovata*

| No. | Common name    | Crassula tetragona | Crassula ovata | Percentage Crassula tetragona | Percentage Crassula ovata |
|-----|----------------|--------------------|----------------|-----------------------------|---------------------------|
| 1   | n-Dodecane     | 10.319             | ---            | 0.70100                     |                           |
| 2   | n-Tridecane    | 11.787             | 11.787         | 0.49644                     |                           |
| 3   | n-Pentadecane  | 12.678             | 0.25354        |                            |                           |
| 4   | n-Hexadecane   | 14.193             | 14.541         | 0.17590                     | 1.09287                   |
| 5   | n-Heptadecane  | 15.217             | ---            | 0.44748                     |                           |
| 6   | n-Octadecane   | 15.348             | 0.51380        | 0.43427                     |                           |
| 7   | n-Nonadecane   | 17.783             | 0.37024        | 0.22519                     |                           |
| 8   | Unknown        | ---                | 19.348         | 0.06483                     |                           |
| 9   | n-Heneicosane  | 20.019             | 20.037         | 0.19979                     | 0.21879                   |
| 10  | n-Tricosane    | 22.088             | 22.093         | 0.21175                     | 0.14178                   |
| 11  | n-Tetracosane  | 23.073             | 23.083         | 0.18471                     | 0.48431                   |
| 12  | Unknown        | ---                | 23.426         | 0.1339                      |                           |
| 13  | n-Pentacosane  | 23.98              | 23.697         | 0.30808                     | 3.8624                    |
| 14  | Unknown        | ---                | 23.994         | 0.3856                      |                           |
| 15  | -Hexacosane    | 24.94              | 24.910         | 2.67690                     | 1.3898                    |
| 16  | n-Heptacosane  | 25.77              | 25.770         | 1.22838                     | 2.1925                    |
| 17  | Unknown        | ---                | 26.113         | 3.5036                      |                           |
| 18  | Unknown        | 26.09              | ---            | 0.25318                     |                           |
| 19  | n-Octacosane   | 26.80              | 26.686         | 11.7514                     | 5.3741                    |
| 20  | n-Nonacosane   | 27.67              | 27.651         | 1.91295                     | 2.3535                    |
| 21  | n-Tricontane   | 29.46              | 29.114         | 57.6510                     | 57.441                    |

No. of Total identified hydrocarbons: 13

% Identified hydrocarbon: 77.4384

% Identified sterols & triterpenes: 22.5616

% Total identified components: 97.4768
as the major steroidal compound. On the other hand, investigation of the unsaponifiable matters of n-hexane extract of C. ovata revealed the presence of 15 identified hydrocarbons with "n"-tricontane (57.44%) as the major hydrocarbon. In addition to Three identified steroidal components; with the presence of β-sitosterol (13.98%) as the major steroidal compound. Table 5.

FA metabolism have significant physiological consequences for many medical disorders such as obesity, cardiovascular disease, and diabetes mellitus (Bollinger et al., 2013).

Investigation of FAME revealed the presence of 9 identified components in C. tetragona and 5 identified components in C. ovata, respectively. Table 6 showed that C. ovata has a higher percent of total unsaturated fatty acid 73.42% than that in C. tetragona 32.56%.

Meanwhile, the major unsaturated fatty acid is Oleic /Elaidic (30.90%) found in C. tetragona and Arachidonic acid (73.42%) in C. ovata. Arachidonic acid is the most abundant prostaglandin precursor (or its precursor, linoleic acid) (Ramwell, 1981).

C. tetragona contains the monounsaturated fatty acid Oleic acid (omega-9); which has great importance to protects cell membranes from free radicals (Haug et al., 2007). Moreover, both species showed the presence of different long-chain mono and polyunsaturated fatty acids. The very long-chain polyunsaturated fatty acids (PUFAs) (C-18–C-22) have great importance in modern nutrition as they possess a protective effect on the cardiovascular system, inflammatory diseases and cancer (Gogus and Smith, 2010). The unsaturated
| NO. | Common name | Crassula tetragona | Crassula ovata | Crassula tetragona | Crassula ovata |
|-----|-------------|--------------------|---------------|-------------------|---------------|
| 1.  | Unknown     | ——                | 19.006        | ——                | 0.11277       |
| 2.  | Unknown     | ——                | 20.861        | ——                | 0.26171       |
| 3.  | Unknown     | ——                | 40.225        | ——                | 0.50010       |
| 4.  | Pentanoic   | ——                | 44.264        | ——                | 1.30029       |
| 5.  | Pentadecanoic | 27.790            | ——             | 5.0229            | ——            |
| 6.  | Unknown     | ——                | 47.009        | ——                | 2.20781       |
| 7.  | Tricosanoic | ——                | 48.106        | ——                | 2.1123        |
| 8.  | Myristic    | 22.620            | ——             | 3.0843            | ——            |
| 9.  | Palmitic    | 30.083            | ——             | 21.505            | ——            |
| 10. | Unknown     | 30.314            | ——             | 2.2773            | ——            |
| 11. | Stearic     | 33.164            | ——             | 5.5531            | ——            |
| 12. | Unknown     | 33.777            | ——             | 3.2061            | ——            |
| 13. | Behenic acid| 41.137            | 41.220         | 12.3766           | 9.41384       |
| 14. | Lignoceric acid | 50.646            | 50.870         | 11.9169           | 10.66501      |

| NO. of identified Saturated fatty acids | 6 | 4 |

| % Identified Saturated fatty acids | 73.42612 |

| No. of identified Unsaturated fatty acids | 3 |

| % Identified Unsaturated fatty acids | 73.42612 |

| % Total identified components | 96.9171 |

Rt* = Retention time
### Table 7: $^1$H NMR and $^{13}$C spectral data of compounds 1, 2, 3, 4, 5 and 6 in CDCl$_3$

| Pos | $\delta$C (ppm) | $\delta$H (ppm) | $\delta$H (ppm) | $\delta$H (ppm) | $\delta$H (ppm) | $\delta$H (ppm) | $\delta$H (ppm) | $\delta$C (ppm) |
|-----|----------------|----------------|----------------|----------------|----------------|----------------|----------------|---------------|
| 1.  | 31.92          | 1.18-1.20      | ---            | ---            | ---            | ---            | ---            | 38.5          |
|     | (CH$_2$)       |                |                |                |                |                |                |               |
| 2.  | 30.39          | 1.70-1.66      | ---            | ---            | 29.7           | ---            | ---            | 22.8          |
|     | (CH$_2$)       |                |                |                |                |                |                |               |
| 3.  | 78.82          | 3.22 (dd)      | 3.95           | 3.55           | 80.3           | 3.31 (m)      | 5.0 (m)       | 72.0          |
|     | (CH)           |                | (1H, m)        |                |                |               |               |               |
| 4.  | 40.47          | ---            | ---            | ---            | 39.4           | ---            | ---            | 37.5          |
|     | (C)            |                |                |                |                |                |                |               |
| 5.  | 47.15          | 1.22           | 5.04           | ---            | 47.1           | ---            | 5.30          | 46.1          |
|     | (CH)           |                |                |                |                |                | (br.s)        |               |
| 6.  | 20.91          | 1.00           | ---            | ---            | 26.8           | ---            | ---            | 17.7          |
|     | (CH$_2$)       |                |                |                |                |                |                |               |
| 7.  | 28.09          | 1.94           | ---            | ---            | 28.0           | ---            | ---            | 32.0          |
|     | (CH$_2$)       |                |                |                |                |                |                |               |
| 8.  | 47.99          | 1.49           | ---            | ---            | 47.8           | ---            | ---            | 39.3          |
|     | (CH)           |                |                |                |                |                |                |               |
| 9.  | 20.00          | ---            | ---            | ---            | 20.9           | ---            | ---            | 47.9          |
|     | (C)            |                |                |                |                |                |                |               |
| 10. | 26.05          | ---            | ---            | ---            | 25.4           | ---            | ---            | 36.6          |
|     | (C)            |                |                |                |                |                |                |               |
| 11. | 26.51          | 2.09           | ---            | ---            | ---            | ---            | ---            | 22.7          |
|     | (CH$_2$)       |                |                |                |                |                |                |               |
| 12. | 124            | 5.02           | ---            | ---            | ---            | ---            | 5.19 (d)      | 124.5         |
|     | (CH)           |                |                |                |                |                |                |               |
| 13. | 140            | ---            | ---            | ---            | 45.3           | ---            | ---            | 139.2         |
|     | (C)            |                |                |                |                |                |                |               |
| 14. | 48.80          | ---            | ---            | ---            | 48.8           | ---            | ---            | 40.0          |
|     | (C)            |                |                |                |                |                |                |               |
| 15. | 79.03          | 3.12 (dd)      | ---            | ---            | 31.9           | ---            | ---            | 28.3          |
|     | (CH)           |                |                |                |                |                | (2.04 (td. J=6. 7, 14.5) |               |
| 16. | 28.09          | 0.92           | ---            | ---            | 26.4           | ---            | ---            | 26.7          |
|     | (CH$_2$)       |                |                |                |                |                |                |               |
| 17. | 55.30          | 0.67           | ---            | ---            | 52.1           | ---            | ---            | 33.9          |
|     | (CH)           |                |                |                |                |                |                |               |
| 18. | 15.38          | 0.69           | 0.67           | 1.27 (3H, 18.0 s) | ---            | 0.57 (s)      | ---            | 47.9          |
|     | (CH$_3$)       |                |                |                |                |                |                |               |

*Continued on next page*
| Pos | δC (ppm) | δH (ppm) | δH (ppm) | δC (ppm) | δH (ppm) | δH (ppm) | δC(ppm) |
|-----|----------|----------|----------|----------|----------|----------|----------|
| 19. | 29.68    | 0.26-0.49| 1.18     | 0.27     | 29.3     | ---      | 1.04(s)  |
|     | (CH2)    |          |          | (1H, d, J=4, 19a) |          |          |          |
|     |          |          |          | 0.50(1H, d, J=4, 19b) |          |          |          |
| 20. | 156(C)   | ---      | ---      | 35.6     | ---      | ---      | ---      |
| 21. | 109 (CH2)| 4.6 (d)  | 0.97     | 0.80(3H, 20.9 | ---      | 0.88(3H, --- | 40.0     |
|     |          | 4.5 (br.s)|          | d, J=6.3) |          | d, J=9.6) |          |
| 22. | 34.33 (CH2)| 1.35 | ---      | 34.6     | ---      | ---      | ---      | 38.5     |
| 23. | 48.00 (CH)| 1.47     | 31.6     | 0.86     | ---      | 0.90 (s) | 29.3     |
| 24. | 125 (CH)  | 5.04 (tt)| 2.24     | 1.22     | 41.0     | 0.85     | 0.91 (s) | 16.0     |
|     |          | (1H, d, J=7.08) |          | (1H, d, J=7.08) |          | (1H, d, J=7.08) |          |
| 25. | 130 (C)  | ---      | ---      | ---      | 154.3    | 0.87(s)  | 1.33 (s) | 14.2     |
| 26. | 25.77 (CH3)| 1.6  | 0.93     | 1.7      | 15.2     | 1.12 (s) | 0.79    | 0.92(s)  |
|     |          | (3H, s)  |          | (3H, s)  |          | (3H, d, J=6.3) |          |
| 27. | 17.67 (CH3)| 1.53 | 0.80     | 4.48     | 111.5    | 0.97(s)  | 0.82    | 0.92(s)  |
|     |          | (3H, s)  | (1H, brs, H-27a) |          | (1H, brs, H-27b) |          | (1H, d, J=6.3) |          |
|     |          |          | 4.94     | 1.53     | 111.5    | 0.97(s)  | 0.82    | 0.92(s)  |
| 28. | 16.14 (CH3)| 0.96 | 0.78     | 0.90     | 19.2     | 0.71(s)  | ---      | 0.89 (s) | 27.8     |
|     |          | (3H, s)  | (3H, s28) |          |          | (3H, s)  |          |          |
| 29. | 14 (CH3) | 0.73     | ---      | 0.81(3H, 14.1 s) | 1.71 (s) | 0.84 (3H, t) | 1.27 (d, J=6.3) | 22.3   |
| 30. | 25.44 (CH3)| 0.88 | ---      | 1.18(3H, 25.0 s) | 4.6(d)  | ---      | 1.29 (d, J=6.3) | 22.3   |
fatty acids have a great role in decreasing the risk of certain cancers, as colon cancers, breast and prostate (Lunn and Theobald, 2006).

Table 6 showed that C. tetragona has a higher percent of total saturated fatty acid 59.46% than that in C. ovata. 23.49%; the major saturated fatty acid is palmitic acid (21.50%) in C. tetragona and lignoceric acid (10.66%) in C. ovata. Saturated fatty acids have altered effects on the concentration of plasma lipoprotein cholesterol fractions. For example, myristic (C14:0) and palmitic (C16:0) acids increase LDL cholesterol (C et al, 2003) those fatty acids were found in both plants.

Previous studies stated that oleic acid has a role in preventing inflammation of skeletal muscle cells (Coll et al, 2008). purified palmitoleic acid has shown anti-inflammatory activity (Bernstein et al, 2014). Moreover both linoleic acid and γ-linolenic acid play an important role in inflammation suppression through two distinct pathways; the first one is through competitive inhibition of the activity of cyclooxygenase and lipoxygenase enzyme, resulting in decrease the production of pro-inflammatory substances prostaglandins and leukotrienes; while the second mechanism aid in the biosynthesis of prostaglandin E1 that has an inhibitory effect on pro-inflammatory cells (C et al, 2003).

Isolation

A phytochemical investigation was performed on the MeCl extract of the aerial parts of C. tetragona and C. ovata. Six compounds were isolated by different isolation methods illustrated in (Figure 4). Structure elucidation was achieved by NMR and mass spectrometry (Table 7). Compound 1 was found to be a novel cycloarten derivative isolated as 28 Methyl-5α-cycloart12, 20, 24-trien-15β-ol, namely meliloffinaside. In addition to 5 known compounds, namely Campesterol (2) (L et al, 2012), Cyclolaudenol (3) (Ezzat et al, 2016), Lupeol (4) (L et al, 2012), β-sitosterol (5) (L et al, 2012; Pant et al, 2013; ?) and α-amyrin (6) (L et al, 2012; Vázquez et al, 2012). Shown in (Figure 5 ) and (Table 7 ). Compound 1 was isolated as yellowish-white microcrystalline powder; gave a purple colour with p-anisaldehyde/H2SO4. It also gave positive Liebermann-Burchard (Liebermann, 1889) and Salkowski (McNamara et al, 2006) (Judith R. McNamara, 2006 #1) tests. The molecular formula of compound 1 was deduced as C30H48O2 with a molecular ion peak at (440+2H). In addition to the following characteristic peaks at 411 (50%), 393 (62%), 365 (27%) and 286 (59%), compared with cycloarten mass fragmentation (L et al, 2012) Shown in (Figure 6 ). The cycloarten nucleus was confirmed from the 1H NMR and 13CNMR data (Table 7, Figures 7 and 8 ). All carbons and protons positions were confirmed by HSQC shown in Figure 9 The long-range coupling was observed in HMBC between Me-29 (δH 0.73), Me-30 (δH 0.88), H-2 (δH 1.70), H-5 (δH 1.22) and C-3 (δC 78.82) this suggested that the hydroxyl group attached to C-3, the long-range coupling between H-17 (δH 0.67), Me-18 (δH 0.69), H-8 (δH 1.49), H-16 (δH 0.89) and C-15 (δC 79.03) and this suggested that the second hydroxyl group attached to C-15, the correlation between H-12 (δH 5.02) and C-17 (δC 55.30), C-10 (δC 26.05), C-11 (δC 26.51), and the correlation between C-13 (δC 140) with H-11 (δH 2.09) and H-16 (δH 0.92) suggested that the position of the double bond at C-12 and C-13, the correlation between C-20 (δC 156) with H-21 (δH 4.6) and H-28 (δH 0.96) and the long-range coupling between C-21 (δC 109) with H-22 (δH 1.35) suggested that the position of the second double bond at C-20 and C-21 and the last correlation between C-24 (δC 125) with H-28 (δH 0.96), Me-26 (δH 1.6), and Me-27 (δH 1.53) in addition to the long-range coupling between C-24 (δC 130) with Me-26 (δH 1.6), and Me-27 (δH 1.53) suggested that the position of the last double bond at C-24 and C-25, shown in Figure 10. This compound was a novel compound identified as 28 Methyl-5α-cycloart12, 20, 24-trien-15β-ol.

CONCLUSION

This is the comparative study that shed light on the C. tetragona and C. ovata in the comparative demonc DNA investigation using 6 Primers for RAPD, and ISSR techniques showed a similarity in both species by 56.4% and 53.4% respectively. And on making a quantitative estimation showed the highest triterpen content. The investigation of the unsaponifiable matters of n-hexane extract of the species revealed the presence of 22 hydrocarbons and n-tricontane was the major hydrocarbon. In addition to seven steroidal components with the presence of campesterol as the major steroidal compound in C. tetragona and the presence of β-sitosterol as the major steroidal compound in C. ovata. The investigation of FAME revealed the presence of 21 components in C. tetragona and C. ovata, and the major unsaturated fatty acid is Oleic/Elaidic (30.90%) found in C. tetragona and Arachidonic acid (73.42%) in C. ovata. And a novel triterpene, namely, 28 Methyl-5α-cycloart12, 20, 24-trien-15β-Ol was isolated and identified from MeCl together with 5 known compounds.
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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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