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Bioactivity Guided Investigation of *Caryota mitis & Caryota urens* Chemopreventive Activity *via In Vitro* and *In Silico* Studies

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Abstract

The study highlights for the first time the potential role of Caryota mitis and Caryota urens (family Arecaceae) in cancer chemoprevention as NQO1 enzyme inducers. Among the tested leaf extracts of different solvent polarities, *C. urens* petroleum ether leaf extract showed the most potent induction of NQO1 enzyme activity (4.79 times to vehicle control) via DCPIP assay and a significant difference was observed at concentration 25 µg/mL via NQO1 western blot analysis. First comparative GC/MS analysis of both species' petroleum ether leaf extracts was conducted to reveal for 35 vs 34 compounds in *C. mitis* and *C. urens* unsaponifiable matter, respectively, and 29 vs 15 fatty acids as methyl esters, respectively. Isolation attempts from the active *C. urens* extract resulted in the identification of five compounds i.e. oleanolic acid (T1), ursolic acid (T2), α-tocopherol (T3), palmitic and stearic acids (T4 & T5) using different spectroscopic techniques; where T1 and T3 are first time to be identified in *C. urens*. Assessment of the isolated compounds' chemopreventive activity was conducted via in silico molecular modeling against KEAP-1 –Nrf2 complex involved in of NQO1 where T1, T2 and T3 showed promising docking score of -7.7, -7.9 and -7.05 kcal/mol, respectively, compared to -8.2 kcal/mol for 4'-bromoflavone as a positive control.

Keywords: Caryota, NQO1 inducers, Nrf2/Keap-1 docking, chemoprevention, GC/MS, triterpenes

Introduction

The field of cancer chemoprevention, *i.e.* preventing cancer from developing, is now widely evolving in an attempt to counteract the steady increase in global cancer morbidity and mortality [1].

Various natural or synthetic chemopreventive agents are used in cases of high risk of developing cancer *e.g.*, family history, or for preventing its recurrence after treatment. Induction of phase II detoxifying enzyme NAD(P)H: quinone oxidoreductase 1 (NQO1) is among the established mechanisms that mediates for cancer chemoprevention [2].

Family Arecaceae has been investigated for its role in cancer chemoprevention through *in vivo* and *in vitro* studies. Wide range of its members proved their ability to prevent the progress of carcinogenesis *via* different mechanisms of action, including NQO1 enzyme induction [3-8]. Nevertheless, genus *Caryota* has little or no reported information on its efficacy in this field. *C. mitis* and *C. urens* palms are worldwide distributed and traditionally used in treatment of various ailments as mentioned in the Ayruvedic medicine [9-11], additionally; *C. urens* is involved in beverage and sweetener preparations inferring its edibility [12].

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In this study, NQO1 enzyme was selected as a marker for tracing the cancer chemopreventive effect of *C. mitis* and *C. urens* successive leaf extracts *via* mechanism-based kinetic colorimetric assay using 2,6-dichlorophenolindophenol redox dye (DCPIP assay). Results were confirmed by western blot technique for the active samples upon serial dilution, against DMSO vehicle and 4'-bromoflavone positive control. Western blot is considered a reliable and powerful tool of choice for detecting a particular protein since the technique depends on specific antigen-antibody interaction upon the separation of proteins into individual bands [13].

This was followed by a phytochemical investigation of the lipoidal content in petroleum ether extracts found most active in both species *via* GC/MS analysis and chromatographic separation. Five non-polar compounds were isolated including 2 triterpenoids that are reported for the first time in *C. urens* leaves. Spectral analyses used in compounds identification included high resolution ESI-MS, ^{1}H and ^{13}C-NMR.

Finally, *in silico* molecular modelling study was conducted in an attempt to assess the chemopreventive effect of the isolated constituents. Kelch-like ECH-associated protein 1 (KEAP1) was selected as the target protein for the docking study. since interacting with KEAP-1 activates the release and translocation of Nrf2 factor into the nucleus leading to the up regulation of the cytoprotective phase II enzymes such as NQO1 and hemeoxygenase (HO) [14].

The study provides the first insight into *C. mitis* and *C. urens* potential role as cancer chemo-preventive agents through a definitive mechanism of action, and attempts to correlate it with their chemical reservoir.

Experimental

1.1. Plant material

Caryota mitis and C. urens leaves were collected during March-April 2016 from Orman and Mazhar botanical gardens, Giza, Egypt. The plant material was kindly identified by Agricultural engineer Therese Labib, consultant of plant taxonomy at the Ministry of Agriculture and ex-director of Orman botanical garden, Egypt. Plant specimens are deposited at Faculty of Pharmacy- Cairo University (voucher No.: 15-12-2016)

1.2. Chemicals and reagents

Petroleum ether 60-80 ° C, ethanol, benzene and acetic acid were purchased from Adwic (Cairo, Egypt). Diethyl ether, acetone, ethyl acetate, methanol and chloroform were obtained from Sigma Aldrich (St. Louis, MO, USA). *n*-Butanol was obtained from Alpha chemicals (Cairo, Egypt). All cell culture reagents were purchased from Lonza (Verviers, Belgium). Reagents for NQO1 assay were purchased from Serva electrophoresis GmbH (Heidelberg, Germany). Silica gel for vacuum liquid chromatography was obtained from Merck (Darmstadt, Germany) and reversed phase silica (RP-silica) in ready-packed guard cartridges were obtained from Alltech Agilent (St. Clara, CA, USA)

1.3. Extracts preparation:

C. mitis and *C. urens* leaf powder (500 g) were successively macerated on cold with solvents of increasing polarities namely; petroleum ether, chloroform, ethyl acetate and 70% ethanol to yield 22 g, 13 g, 5 g and 60 g, respectively, for *C. mitis* extracts and likewise; 25 g, 7 g, 3 g and 80 g, respectively, for *C. urens* extracts.

1.4. Determination of NQO1 enzyme induction

1.4.1. Hepa-1c1c7 cell culture

Murine hepatoma cell line Hepa-1c1c7 was maintained as monolayer culture in α- modified Minimum Essential Medium Eagle (α-MEME) supplemented with 10% (v/v) heat-and charcoal–inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulphate in humidified incubator (Sartorius CMAT, Germany, 5% CO_2/95% air). At about 80% confluence, cells were routinely sub-cultured with Trypsin EDTA solution.

1.4.2. Assessment of the induction of NQO1 in Hepa-1c1c7 cells

In a preliminary screening experiment, the induction of NQO1 by extracts from the two plants in

Hepa-1c1c7 cells was assessed. Briefly, cells (3×10^5 cells/mL) were seeded onto 6-well plates and left overnight to adhere and form semi-confluent monolayers. Monolayers were treated with either vehicle (final concentration 0.5% v/v DMSO), plant extracts (100 µg/mL) for additional 24 h. In parallel, sulforaphane (5 µM) was used as positive control for NQO1 induction. After aspiration of treatment media, monolayers were washed with ice-cold Dulbecco's PBS (2 mL/well). Cells were then scrapped in ice-cold lysis buffer (25 mM Tris-Cl, pH 7.4, 250 mM sucrose and 5 µM FAD) and transferred to labelled microcentrifuge tubes. Cell suspensions were then sonicated on ice for 5 s (20% amplitude). Sonicates were then centrifuged (15,000 ×g for 10 min) and the supernatants were aliquoted and stored at − 80 °C freezer until assayed.

1.4.3. Kinetic DCPIP reduction assay for NQO1 enzymatic activity

The dicoumarol-sensitive NQO1 activity was measured in cell lysate and reaction rates were normalized to the total protein contents as determined by Bradford assay [15]. Briefly, the reaction mixture contained the following reagents in a final volume of 500 µL: 25 mM Tris buffer (pH 7.4), 0.7 mg/mL bovine serum albumin, 0.2 mM β-NADH, 20 µM 2,6-dichlorophenolindophenol (DCPIP) in the absence (total reductase activity) or the presence of 20 µM dicoumarol (NQO1 inhibitor). The kinetic determination of enzyme activity was carried out using UV-visible spectrophotometer, monitoring the enzymatic reduction of DCPIP at 600 nm within 1 min and a linear reaction was observed. The enzyme activity was normalized to 1 mg of total proteins and expressed as ΔA/sec/mg protein. The induction was expressed as fold of specific NQO1 activity of the tested samples over vehicle.

1.4.4. Western blot analysis for determination of NQO1 protein expression

Hepa-1C1C7 overnight cultures (seeded at zero time as 3×10^5 cells/well-6 well plates) were treated with either vehicle (0.5% DMSO) , positive control (4´-bromoflavone) or petroleum ether extracts (final concentrations of 6.25, 25 and 100 µg/mL). Treatment was performed for 24 h after which cell lysates were prepared as mentioned in section 2.4.2. Samples (fixed amount of 25 µg total proteins/lane as determined using a Thermo Nanodrop spectro-photometer) were resolved under denaturing conditions by electrophoresis (SDS-PAGE) on 12.5% acrylamide/bisacrylamide gel (200 Volts for 1 h). Resolved proteins were then transferred to nitrocellulose membranes at 90 V for 90 min. Uniform transfer were routinely confirmed using Ponceau S staining [16, 17]. The membranes were then blocked in 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at 25°C and then probed overnight (4°C) with primary antibodies against NQO1 (abcam, UK) with gentle shaking. After three washes in TBST (10 min each), membranes were probed with appropriate secondary antibodies for 1 h at 25°C, washed three times in TBST and then developed using enzyme chemiluminescence (ECL). Protein bands were visualized on developed membranes using a chemiluminescence imager (UVP, UK). Densitometric analysis of obtained NQO1 bands was performed using Visionworks LS software (Analytik Jena, Germany) and normalized as fold of NQO1 protein expression over vehicle control.

1.4.5. Statistical analysis

Significance of induction in NQO1 protein expression upon treatment with extracts and isolated compounds relative to vehicle control-treated cells were assessed *via* one way ANOVA using GraphPad Prism V 6.0 software (San Diego, USA). Data were expressed as Mean +/- SEM and considered significantly different at least when $P< 0.05$.

1.5. Gas chromatography/mass spectrometry (GC/MS) analysis of petroleum ether leaf extracts

Petroleum ether leaf extract (3 g) from each species was saponified by refluxing with 100 mL alcoholic KOH for 6 h. The unsaponifiable matter (1.3 g) was extracted with ether, evaporated and dried with anhydrous sodium sulphate while fatty acids were methylated through refluxing with 50 mL methanol mixed with 0.25 mL sulphuric acid for 3 h. [18]. Fatty acid methyl esters (FAME, 1 g) were extracted with ether, washed with distilled water, evaporated and dehydrated over anhydrous sodium sulphate [19]. Both samples were analysed *via* GC/MS adopting conditions stated in Farag *et al* [20]. The identification of compounds was accomplished by comparing their retention time and mass spectral data with Wiley and

Wiley/NIST mass spectral database and other literature [21]

1.6. Investigation of C. urens petroleum ether leaf extract for isolation and identification of non-polar compounds

Caryota urens petroleum ether leaf extract (20g) was fractionated *via* vacuum liquid chromatography (VLC) containing silica for TLC as stationary phase (1:30) and eluted with gradient mobile phase starting with 100% hexane followed by 25% gradual increment in polarity by the addition of dichloromethane, ethyl acetate and methanol. Sub fractions were screened on TLC developed in solvent systems: S1= benzene - acetone (9:1 v/v), S2= chloroform - methanol (9.5:1 v/v) and S3= benzene - ethyl acetate (6:2 v/v) then further fractionated and purified through preparative TLC and ready-packed cartridges with reversed phase silica (stationary phase) eluted by solvent systems S1 and S2 to yield 5 compounds (T1-T5).

1.7. Spectroscopic analyses

Mass spectra of isolated compounds in negative ionization mode were obtained using Orbitrap high resolution UPLC/PDA/ESI-MS/MS (Thermo Fisher Scientific, Germany) equipped with a heated electrospray ion source (negative spray voltage of 3 kV, capillary temperature of 300 °C, source heater temperature of 250 °C, FTMS resolution of 30.000). The MS system was coupled to an UPLC system (DionexUltiMate 3000, Thermo Fisher Scientific), equipped with a RP-18 column (particle size 1.8 µm, pore size 100 Å, 150 × 1 mm ID, Acquity HSS T3, Waters; column temperature of 40 °C) and a photodiode array detector (220–600 nm, Thermo Fisher Scientific). Samples were eluted in isocratic and linear gradient elution techniques using eluents A (acetonitrile) and B (water) both containing 0.1% (v/v) formic acid. Acquired NMR data included ^{1}H and ^{13}C spectra were recorded on an Agilent (Varian) VNMRS 400 NMR spectrometer operating at a proton and carbon NMR frequencies of 399.94 and 100.56 (MHz), respectively.

1.8. 3 D-molecular docking simulation of isolated compounds

The isolated flavonoids (ligands) and 4'-bromoflavone (control) were retrieved from PubChem and modelled for its potential inhibitory action on Kelch-like ECH-associated protein 1 (KEAP1) PDB ID: 4IQK. Ligands binding study was carried out using Maestro 11.9 software. Energy minimization of ligands was optimized and ligand preparation was carried out using LigPrep 2.4 software. Docking of ligands was achieved using Schrodinger 16.4 software Glide's Extra Precision (XP) [22]**.** The size of grid box for each protein was set to 20 Å.

Results and Discussion

1.9. In vitro Determination of cancer chemo-preventive activity:

1.9.1. DCPIP assay for monitoring NQO1 enzyme induction

Successive polar and non-polar leaf extracts were assayed at a dose of 100 µg/mL to determine their potential role as inducers of the chemopreventive marker NQO1. As shown in Figure 1, petroleum ether leaf extracts of both species showed the highest enzyme activity with an induction of 4.79 and 2.75 times the control by *C. urens* and *C. mitis*, respectively, inferring the positive role of their nonpolar/lipoidal constituents as NQO1 inducers. These were followed by *C. urens* chloroform and *C. mitis* ethanol extracts that recorded 50% and 41% increased activity above the control representing the activity of semipolar and polar constituents, respectively.

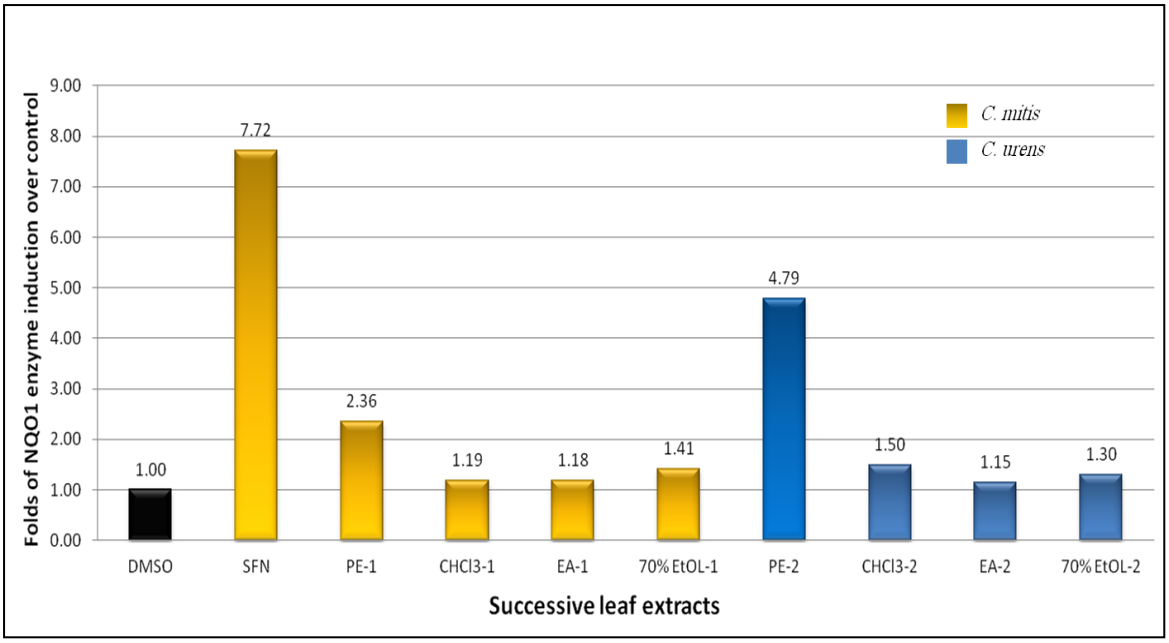

Fig. 1. Folds of increase in NQO1 enzyme activity, relative to control, induced by *C. mitis* and *C. urens* successive leaf extracts at 100 µg/mL using sulforaphane (SFN) positive control. PE: petroleum ether, CHCl3: chloroform, EA: ethyl acetate, EtOL: ethanol.

1.9.2. Western blot analysis of active extracts

Western blot confirmed the inducing activity of both species' petroleum ether leaf extracts upon their serial dilution (6.25-100 µg/mL). Significant difference was observed at concentration 25 µg/mL that induced NQO1 enzyme by ≈ 2.5 times the vehicle (DMSO) control (**Fig. 2**), presenting them as promising chemopreventive agents and prompting their further chemical investigation.

1.10. Lipoidal matter investigation of C. mitis and C. urens petroleum ether leaf etracts via GC/MS analysis

Previously, *C. urens* leaf and fruit samples were subjected to GC/MS and GLC analyses that enabled the identification of various constituents [23,24] while little is known about *C. mitis* non polar content. In an attempt to investigate the lipid content in a comparative manner, unsaponifiable matter and FAME were analysed via GC/MS.

The analysis of unsaponifiable matter in *C. mitis* and *C. urens* detected 35 and 34 compounds constituting 76.49% and 85%, respectively (**Table 1**). Phenyl hydrocarbons that are incorporated as raw materials in cosmetics and cleaning products [25, 26] were the major detected class in both species forming 67.35% and 68.39% of the total identified compounds in *C. mitis* and *C. urens*, respectively. Organosulfur compound was detected in *C. mitis* at peak 5 identified as isopropyl pentyl sulfide at M+ 146 and fragments at m/z 103, m/z 75, m/z 70, m/z 47 and m/z 43 matching literature [27]. Several alkyl sulfides are known to mediate for the chemopreventive effect via different mechanisms [28] but yet no studies were reported on the herein identified sulfide. Other detected compounds in both species included butylated hydroxytoluene, butylated hydroxyl anisole and tocopherols (peaks 8, 10, 34 & 36) which are well-known antioxidants and were previously reported as NQO1 inducers [28-30]

On the other hand, 29 vs 15 fatty acids were detected as methyl esters in *C. mitis* and *C. urens* (**Table 2**) constituting 85.44% and 98.9%, respectively. Methyl hexadecanoate (or palmitic acid methyl ester) was the major fatty acid in both species representing 19% and 32.5%, respectively, followed by the methyl esters of 9-octadecenoic or oleic acid (11.8% & 21.7%), octadecanoic or stearic acid (8.8%

and 12.6%) and in *C. mitis* 9,12-octadecadienoate or linoleic acid (9.05%).

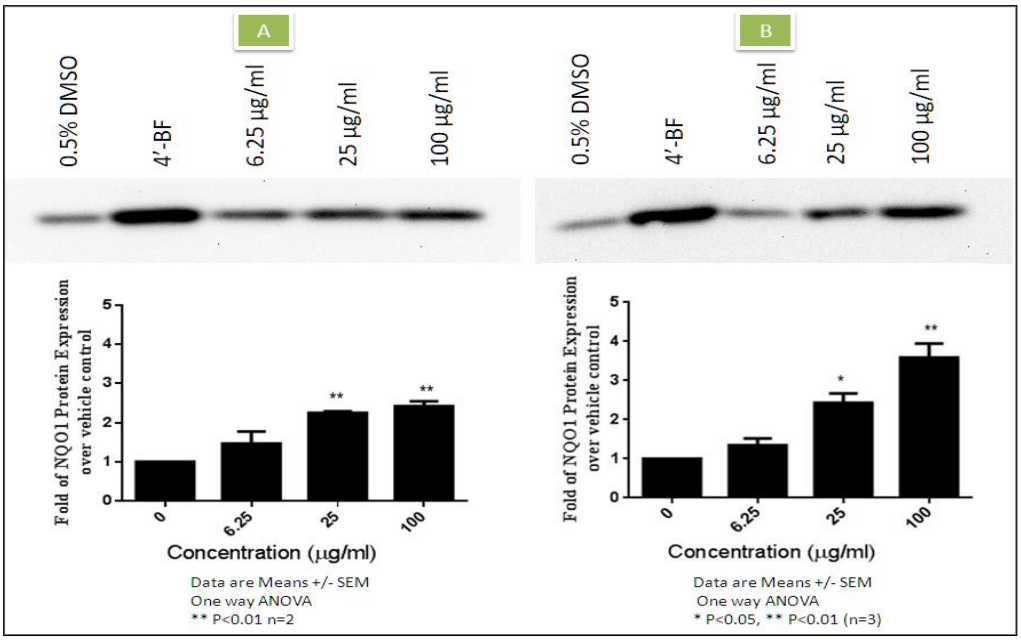

Fig. 2. Western blots detecting NQO1 protein expression levels induced by *C. mitis* (A) and *C. urens* (B) petroleum ether leaf extracts at increasing concentrations of 6.25, 25.00 and 100.00 µg/mL and their calculated folds of induction

1.11. Isolation and structural elucidation of non-polar compounds from C. urens petroleum ether leaf extract using MS and NMR

Caryota urens petroleum ether leaf extract showed the most potent induction of NQO1 enzyme relative to the other leaf extracts, and thus was selected for further fractionation in an attempt to correlate its chemical content with the observed activity. Chromatographic isolation and spectral analyses resulted in identifying 5 compounds (**Fig.3**), i.e. 3 triterpenoids (T1-T3) and 2 fatty acids (T4 & T5), including 2 compounds that are first to be reported in *C. urens* leaves (T1 & T3) [31-33]. T2, T4 and T5 were previously reported in *C. urens* and their spectra matched published literature [23, 34-37].

1.11.1. Spectral data of T1-T5

1H and 13C -NMR data of T1-T3 (in CDCl3, ppm) are presented in **Table 3**.

T1; **Oleanolic acid** (white ppt, 14 mg): HR-ESIMS data: $[M-H]^-$ 455.3514, $C_{30}H_{47}O_3^-$ (degree of unsaturation=7); fragments at *m/z* 437.3610, $C_{30}H_{45}O_2^-$, *m/z* 410.3481, $C_{29}H_{46}O^-$; *m/z* 407.3305, $C_{29}H_{43}O^-$, *m/z* 391.2982 $C_{28}H_{39}O^-$, *m/z* 392.3580, *m/z* 246.6653, *m/z* 202.8421 and *m/z* 189.1228.

T2; **Ursolic acid** (white ppt, 12 mg): $[M-H]^-$ 455.3535, $C_{30}H_{47}O_3^-$ (degree of unsaturation =7)

T3; **α-Tocopherol** (oily residue, 14 mg): $[M-H]^-$ 429.3717, $C_{29}H_{49}O_2^-$; fragments at *m/z* 205.1222, $C_{13}H_{17}O_2^-$ and *m/z* 165.0911, $C_{10}H_{13}O_2^-$, 151.0747; $C_9H_{11}O_2^-$

T4 and **T5; Palmitic and Stearic acids** (white ppt, 8-9 mg): ^{1}H-NMR data (400 MHz, $CDCl_3$): δ0.8-0.9 (*t, ω*-methyl H-16/18), δ1.25-1.45 (*m,* methylenes –CH_2), δ1.6-2 (*m, β*-CH_2), δ2.4-2.5 (*t, α*-CH_2). GC/EI-MS of methylated derivatives: *m/z*: 43, 74, 87, 143, 185, 199, 227 (**T4**), 255 (**T5**).

1.11.2. Spectral-based identification of isolated compounds

HR-MS data of compound T1, identified as oleanolic acid, showed molecular ion at $[M-H]^-$ 455.3514, $C_{30}H_{47}O_3^-$ that lost water and carboxylic moieties upon fragmentation at *m/z* 437.3610, $C_{30}H_{45}O_2^-$ and *m/z* 410.3481, $C_{29}H_{46}O^-$, respectively. Other detected fragments at *m/z* ≈ 392, 247, 203 and 189 matched reported data for RDA fragmentation of

δ^{12}-unsaturated triterpenes as oleanolic or ursolic acids (**Fig.4 & 5**) [38-40]. This was confirmed by a characteristic base peak at *m/z* 407.3305, $C_{29}H_{43}O^-$ corresponds to $[M\text{-}H\text{-}HCHO\text{-}H_2O]^-$ and further losing methane group to yield *m/z* 391.2982, $C_{28}H_{39}O^-$ (**Fig.4 &5**) [40, 41].

^{1}H-NMR analysis is the tool of choice for distinguishing between oleanane, ursane and lupane triterpene nucleui. Herein, T1 showed the presence of *multiplet* signal at δ3.65 ppm for the hydroxylated methine H-3, a *triplet* for vinylic proton H-12 at δ5.12 ppm and angular methyl *singlets* at δ 1.22 (H-23), 1.16 (H-24), 0.86 (H-25), 1.02 (H-26, H-30), 1.26 (H-27) and 0.92 (H-29) ppm (**Table 3**) confirming T1 to be oleanolic acid.

This was confirmed by ^{13}C-NMR analysis that exhibited 30 carbon signals in accordance with published literature (**Table 3**). Assignments included signals for 7 methyls, 10 methylenes, 3 methines and 6 quaternary carbons in addition to 4 characteristic peaks at δ79.05, δ122.67, δ143.60 and δ180.84 ppm assigned for the oxygenated C-3, olefenic methine C-12, olefenic quaternary C-13 and the carboxylic C-28 carbon atoms, respectively [31,32,42].

Olenaolic acid was reported in family *Arecaceae* [43], but to our knowledge this is the first report for its isolation from genus *Caryota***.** Various biological activities were attributed to oleanolic acid that included antitumour, antimicrobial, antituberculosis, antidiabetic and anti-inflammatory among others [44-46].

On the other side, ^{1}H-NMR data of T2 showed signals for two of the angular methyls as *doublets* at δ0.94 ppm (H-29) and δ0.9 ppm (H-30) that indicated ursane-type nucleus, in addition to the 5 *singlets* at δ1.15 (H-23), δ0.92 (H-24), δ0.79 (H-25), δ0.93 (H-26) and δ1.0 (H-27) ppm and signals at δ3.25 ppm and δ5.26 ppm for the hydroxylated methine H-3 and vinylic proton H-12, respectively (**Table 3**). Thus together with its HR-MS data confirmed to be ursolic acid which is frequently reported for its simultaneous natural occurring with oleanolic acid due to similar structural features that consider them isomers [45]. Ursolic acid was previously isolated and identified in *C. urens* petroleum ether leaf extract [23]. It's reported to possess the same biological and beneficial health effects as oleanolic acid *i.e.* treatment of infectious diseases, antituberculosis, immune-modulatory, antitumor, and antimicrobial activities [32,44,47]. Interestingly, a combination of both oleanolic and ursolic acid was assessed for their antibacterial activity against *Mycobacterium tuberculosis* H37Rv and drug resistant clinical strain (MDR) of tuberculosis. The mixture showed *in vitro* and *in vivo* synergistic effect through acting on defined pathways as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) *via* NF-kB transactivation, suggesting for their promising immunomodulatory effect that could be harnessed in the treatment of other diseases [44,47].

With regards to compound T3, identified as α-tocopherol (vitamin E), the molecular ion at $[M\text{-}H]^-$ 429.3717, $C_{29}H_{49}O_2^-$, lost a hexadecanoyl hydrocarbon side chain upon fragmentation to yield *m/z* 205.1222, $C_{13}H_{17}O_2^-$ followed by sequential loss of methyl groups to yield base peak at *m/z* 165.0911, $C_{10}H_{13}O_2^-$ and a minor peak at *m/z* 151.0747; $C_9H_{11}O_2^-$ (**Fig.6**).

^{1}H-NMR analysis provided the main signals characterizing vitamin E (**Table 3**). This included 3 *singlets* for the methyl protons attached to the benzylic ring at C-5, C-7 and C-8 appeared downfielded at δ2.04, δ2.09 and δ2.16 ppm, respectively, and H-4 *triplet* at δ2.6 ppm. In addition, the side chain methyl protons were detected upfielded at δ0.9-0.95 ppm [34, 48].

^{13}C-NMR analysis further confirmed and annotated the 29 carbons of vitamin E (**Table 3**) in good accordance with published data [48]. The most noticeable were 3 oxygenated carbons at δ140 and δ149 ppm for the benzylic sp2 carbons C-6 and C-9, respectively and an aliphatic C-2 at δ75 ppm. In addition, the quaternary benzylic carbons C-5, C-7 and C-8 were assigned at δ123, δ124.9 and δ126 ppm, respectively, and a C-10 methine at δ117 ppm.

GC/MS analysis of *C. urens* petroleum ether leaf extract (**Table 1**) detected vitamin E and it's well known in F. Arecaceae [49,50] but first to be reported in *Caryota* species.

Compounds T4 and T5 were precipitated out of subfractions as white flakes. ^{1}H-NMR analysis showed a typical pattern of fatty acid signals *viz*. *triplet* at δ2.4-2.5 *ppm* for the *alpha* protons adjacent to the carboxylic group, *multiplet* at δ1.6-2 *ppm* for the *beta* methylene and a *triplet* for the terminal methyl protons at δ0.8-0.9 *ppm* [34,35].

Upon methylation, GC/MS analysis showed base peak at m/z 74 in both compounds and molecular ions were detected at m/z 227 and m/z 255 for T4 and T5, respectively. According to NIST and Wiley library, T4 and T5 were identified to be palmitic and stearic acids, two of the major fatty acids detected in *C. urens* as determined by GC/MS analysis (**Table 2**).

Table 1: GC/MS analysis results of the unsaponifiable matter in *C. mitis* and *C. urens* petroleum ether leaf extracts

No.	Compound	R_t	M.wt	Base peak	M.F.	*C.mitis*	*C.urens*
						Area %	
1	Ethyl hexanol	18.8	130	57	$C_8H_{18}O$	0.15	0.13
2	2,2-Diethoxyethylamine	23.9	133	103	$C_6H_{15}NO_2$	0.34	(-)
3	n-Dodecane	24.3	170	57	$C_{12}H_{26}$	0.13	0.14
4	n-Tridecane	27.2	184	57	$C_{13}H_{28}$	0.13	0.21
5	Isopropyl pentyl sulfide	29.4	146	103	$C_8H_{18}S+$	0.07	(-)
6	n-Tetradecane	30.0	198	57	$C_{14}H_{30}$	0.11	0.17
7	4-Phenyl nonane	31.2	204	91,133	$C_{15}H_{24}$	0.08	0.08
8	Butylated hydroxyanisole	31.6	180	165	$C_{11}H_{16}O_2$	(-)	0.12
9	2-Phenyl nonane	32.6	204	105	$C_{15}H_{24}$	0.02	(-)
10	Butylated hydroxytoluene	32.8	220	205	$C_{15}H_{24}O$	0.16	1.29
11	5-Phenyl decane	33.5	218	91, 147	$C_{16}H_{26}$	2.01	0.77
12	4-Phenyl decane	33.8	218	91, 133	$C_{16}H_{26}$	2.15	1.55
13	3-Phenyl decane	34.3	218	91, 119	$C_{16}H_{26}$	2.35	4.4
14	2-Phenyl decane	35.3	218	105	$C_{16}H_{26}$	3.02	5.6
15	5-Phenyl undecane	36.0	232	91, 147	$C_{17}H_{28}$	7.2	5.24
16	4-Phenyl undecane	36.3	232	91,133	$C_{17}H_{28}$	4.04	2.3
17	3-Phenyl undecane	36.8	232	91,119	$C_{17}H_{28}$	5.2	9.19
18	2-Phenyl undecane	37.8	232	105	$C_{17}H_{28}$	6.2	8.04
19	5-Phenyl dodecane	38.3	246	91,147	$C_{18}H_{30}$	7.02	2.31
20	4-Phenyl dodecane	38.6	246	91, 133	$C_{18}H_{30}$	3.46	1.36
21	3-Phenyl dodecane	39.1	246	91, 119	$C_{18}H_{30}$	3.87	6.88
22	2-Phenyl dodecane	40.1	246	105	$C_{18}H_{30}$	4.9	3.65
23	6-Phenyl tridecane	40.3	260	91, 161	$C_{19}H_{32}$	3.01	2.81
24	5-Phenyl tridecane	40.5	260	91,147	$C_{19}H_{32}$	2.05	(-)
25	4-Phenyl tridecane	40.8	260	91,133	$C_{19}H_{32}$	2.78	2.45
26	3-Phenyl tridecane	41.3	260	91, 119	$C_{19}H_{32}$	3.69	5.77
27	2-Phenyl tridecane	42.1	260	105	$C_{19}H_{32}$	4.3	5.99
28	Octadecanol	43.5	270	83	$C_{18}H_{38}O$	0.09	0.29
29	Isophytol	45.2	296	71	$C_{20}H_{40}O$	(-)	0.16
30	Phytol	45.9	296	71	$C_{20}H_{40}O$	4.99	7.45
31	Geranyl linalool	47.1	290	69	$C_{20}H_{34}O$	0.13	(-)
32	1-Docosene	47.2	308	97	$C_{22}H_{44}$	(-)	0.38
33	n-Eicosanol	48.9	298	43	$C_{20}H_{42}O$	(-)	0.62
34	β-or γ-Tocopherol	50.6	416	57,83,97	$C_{28}H_{48}O_2$	0.03	0.33
35	n-Tetracosanol	52.2	354	83,57	$C_{24}H_{50}O$	0.04	0.81
36	α-Tocopherol (Vitamine E)	53.8	430	165,430	$C_{29}H_{50}O_2$	0.34	0.33
37	n-Heptacosane	55.4	380	57	$C_{27}H_{56}$	0.29	0.55
38	n-Octacosane	57.2	394	57	$C_{28}H_{58}$	0.11	0.03
39	Squalene	57.5	410	69	$C_{30}H_{50}$	1.94	3.51
40	Stigmasterol	58.8	412	55, 207	$C_{29}H_{48}O$	0.09	0.09
Total identified compounds						**76.49**	**85**
Non oxygenated compounds						**70.11**	**73.38**
Oxygenated compounds						**6.38**	**11.62**
Straight chain hydrocarbons						**0.77**	**1.48**
Phenyl hydrocarbons						**67.35**	**68.39**
Alcohols						**0.13**	**1.72**
Terpenoids						**7.06**	**11.12**
Others						**1.18**	**2.29**

Table 2: GC/MS analysis results of the fatty acid methyl esters in *C. mitis* and *C. urens* petroleum ether leaf extracts

No.	Compound	M.wt.	Base peak	M.F.	*C. mitis*		*C. urens*	
					R_t	A%	R_t	A%
1	Methyl decanoate	186	74	$C_{11}H_{22}O_2$	10.0	1.25	-	-
2	Dimethyl octanedioate	202	74, 129	$C_{10}H_{18}O_4$	10.9	0.22	-	-
3	Methyl dodecanoate	214	74	$C_{13}H_{26}O_2$	11.4	3.17	7.7	3.9
4	Dimethyl nonanedioate	216	152	$C_{11}H_{20}O_4$	11.6	0.92	-	-
5	Methyl tridecanoate	228	74	$C_{14}H_{28}O_2$	12.1	0.38	-	-
6	Methyl tetradecanoate	242	74	$C_{15}H_{30}O_2$	12.8	5.09	9	5.43
7	Dimethyl undecanedioate	244	91	$C_{13}H_{24}O_4$	13.0	0.44	-	-
8	Methyl- 4,8,12-trimethyl tridecanoate	270	87	$C_{17}H_{34}O_2$	13.1	0.54	9.3	0.2
9	Methyl pentadecanoate	256	74	$C_{16}H_{32}O_2$	13.6	1.33	9.7	1.17
10	Methyl-5,9,13-trimethyl tetradecanoate	284	74	$C_{18}H_{36}O_2$	14.0	0.58	10.1	0.2
11	Methyl-9-hexadecenoate	268	41	$C_{17}H_{32}O_2$	14.4	0.78	10.7	0.45
12	Methyl hexadecanoate	270	74	$C_{17}H_{34}O_2$	14.8	19.00	11.2	32.53
13	Methyl-10-heptadecenoate	282	41	$C_{18}H_{34}O_2$	15.6	0.34	-	-
14	Methyl heptadecanoate	284	74	$C_{18}H_{36}O_2$	16.0	3.18	11.9	3.02
15	Methyl 9,12-octadecadienoate	294	81	$C_{19}H_{34}O_2$	17.2	9.05	13.1	3.6
16	Methyl-9-octadecenoate	296	55	$C_{19}H_{36}O_2$	17.3	11.86	13.2	21.70
17	Methyl-10-octadecenoate	296	41	$C_{19}H_{36}O_2$	17.5	0.31	-	-
18	Methyl octadecanoate	298	74	$C_{19}H_{38}O_2$	17.7	8.85	13.7	12.63
19	Methyl 7,10-octadecadienoate	294	67	$C_{19}H_{34}O_2$	18.0	0.21	-	-
20	Methyl- 3,7,11,15-tetramethylhexadec-2-enoate	324	43, 114	$C_{21}H_{40}O_2$	18.6	0.22	-	-
21	Methyl-17-methyl octadecanoate	312	74	$C_{20}H_{40}O_2$	19.7	0.60	-	-
22	Methyl-11-eicosenoate	324	41	$C_{21}H_{40}O_2$	21.77	0.28	-	-
23	Methyl eicosanoate	326	74	$C_{21}H_{42}O_2$	22.5	3.14	18	4.4
24	Methyl heneicosanoate	340	74	$C_{22}H_{44}O_2$	25.5	0.52	-	-
25	Methyl docosanoate	354	74	$C_{23}H_{46}O_2$	28.8	5.62	22.8	5.46
26	Methyl tricosanoate	368	74	$C_{24}H_{48}O_2$	32.0	1.55	25.3	0.95
27	Methyl tetracosanoate	382	74	$C_{25}H_{50}O_2$	35.4	4.49	28.7	3.35
28	Methyl pentacosanoate	396	74	$C_{26}H_{52}O_2$	38.6	0.55	-	-
29	Methyl hexacosanoate	410	74	$C_{27}H_{54}O_2$	42.7	0.96	-	-
Total identified compounds						**85.44**		**98.9**
Unsaturated FA						**23.06**		**25.66**
Saturated FA						**62.38**		**73.24**
Branched FA						**1.94**		**0.4**
Dicarboxylic FA						**1.58**		**-**

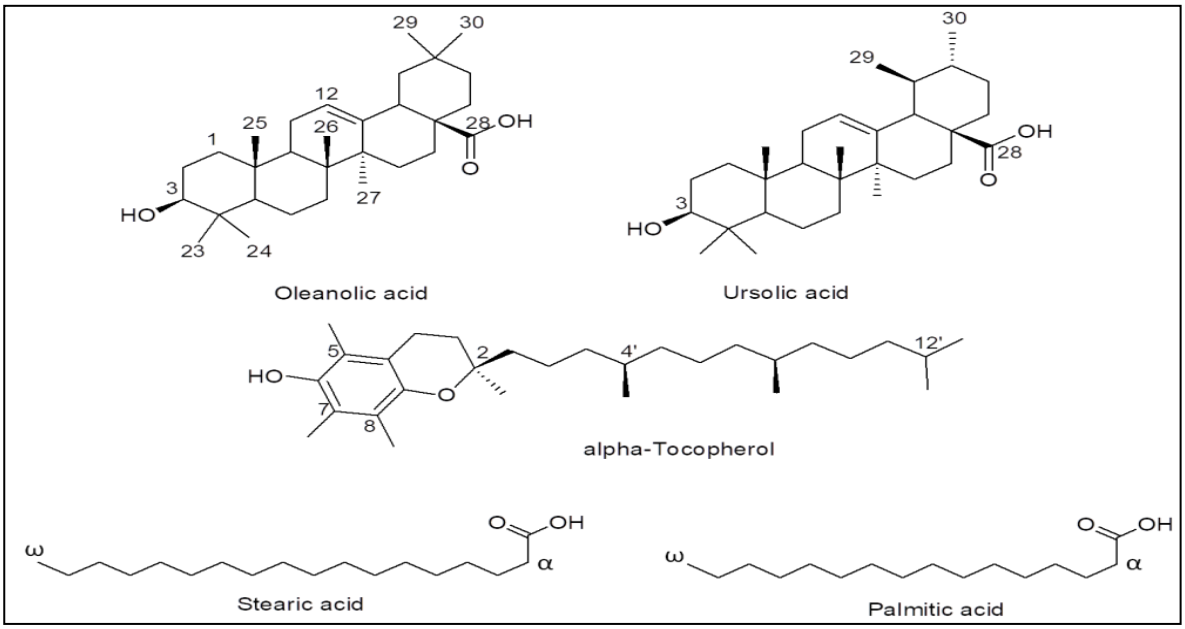

Fig. 3. Chemical structures of the isolated compounds from C. urens petroleum ether leaf extract

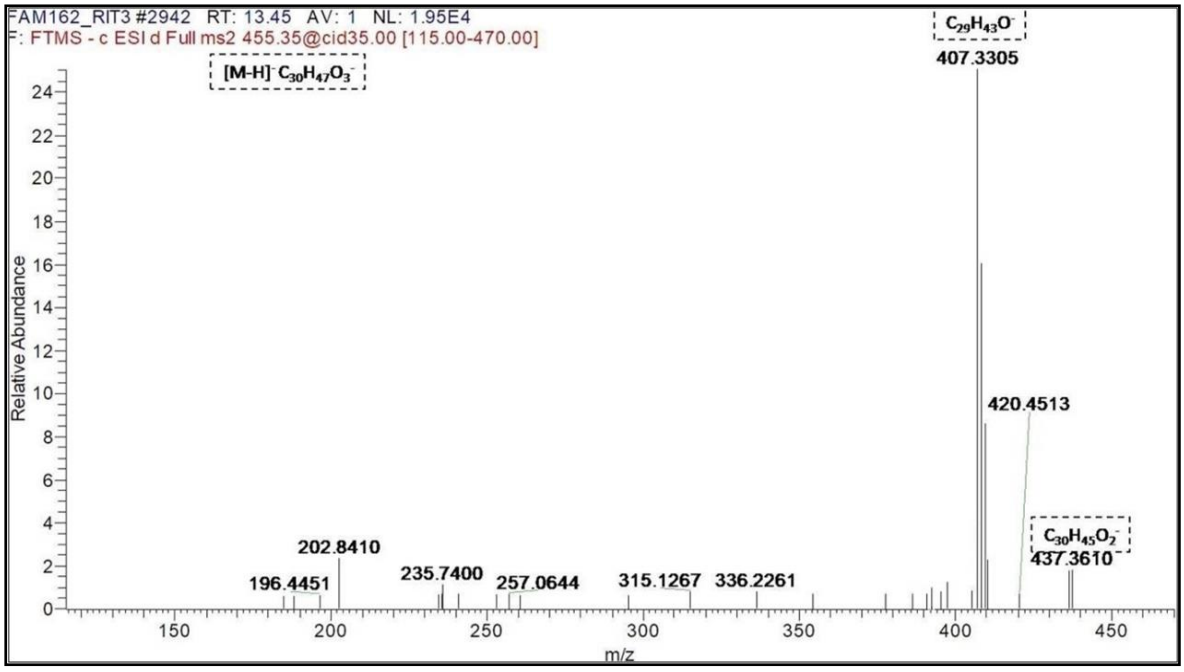

Fig. 4. MS spectra of compound (T1) with predicted formulae in negative ionization mode

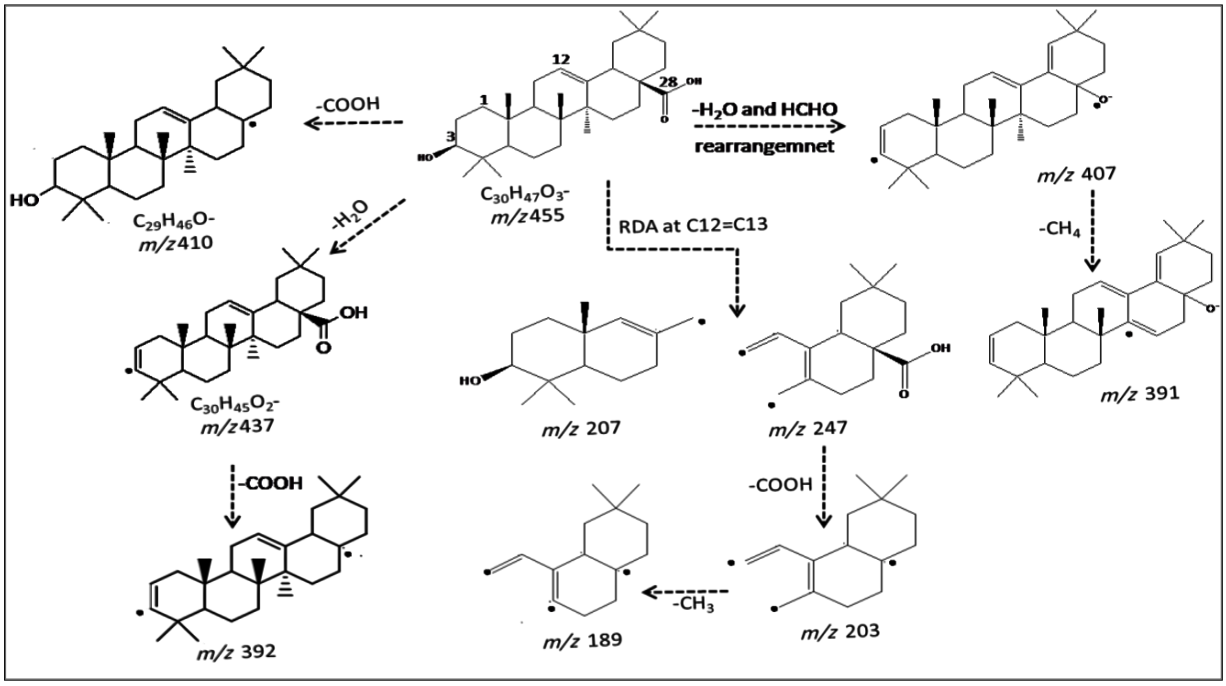

Fig. 5. Scheme of the suggested fragmentation pathway of compound (T1) according to the fragments predicted formulae obtained via UPLC/HR-orbitrap-ESI-MS

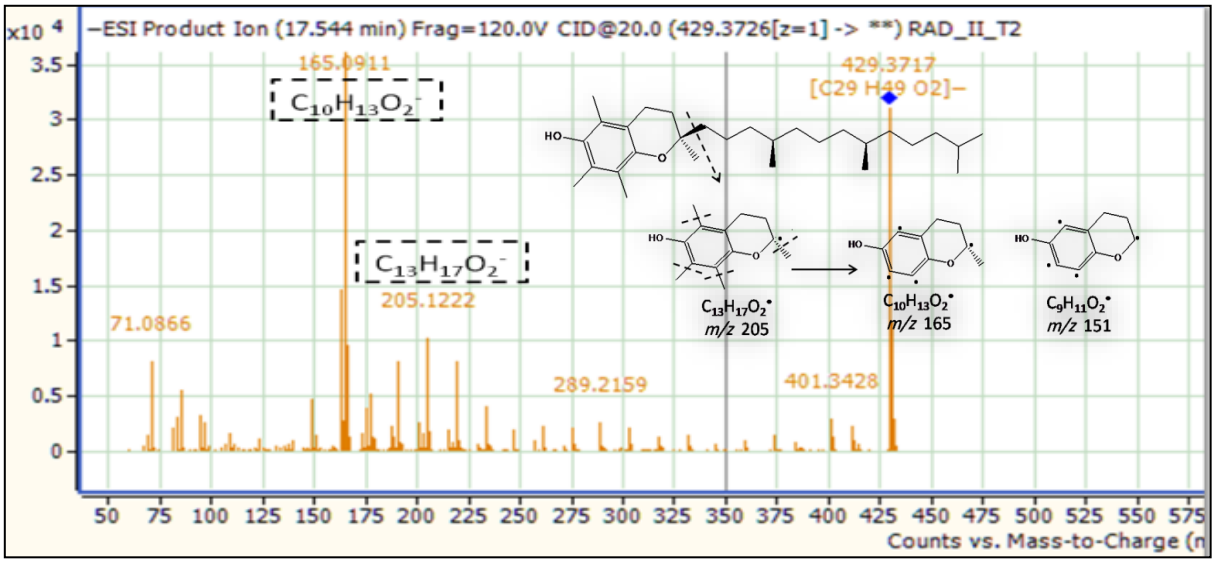

Fig. 6. MS spectra of compound (T3) with predicted formulae in negative ionization mode

Table 3: NMR chemical shifts (ppm) of compounds T1-T3 isolated from *C. urens* petroleum ether leaf extracts

H/C	T1		T2	H/C	T3	
	^{1}H (*m*, J-value *Hz*)	^{13}C	^{1}H (*m*, J-value *Hz*)		^{1}H (*m*, J-value *Hz*)	^{13}C
1	1-1.8 (*m*)	38.42	1-1.8 (*m*)	1	…….….	……..….
2		27.25		2	…….….	75.08
3	3.65 (*m*)	79.05	3.25 (*m*)	3	1.8 (*m*)	31.1
4	…….….	38.78	…….….	4	2.65 (*t*, 13 *Hz*)	20.59
5	0.75-0.8 (*m*)	55.24	0.8-0.9 (*m*)	5	…….….	123.07
6	1.4-1.6 (*m*)	18.3	1.4-1.6 (*m*)	6	1.1-1.6 (*m*)	140.6
7	1.4-1.6 (*m*)	33.09	1.4-1.6 (*m*)	7		124.93
8		39.3		8		126.7
9	1.7-2 (*m*)	47.65	1.6-1.7 (*m*)	9		149.48
10	…….….	37.1	…….….	10		117.38
11	1.9 (*m*)	22.9	1.8-1.9 (*m*)	1'		39.93
12	5.12 (*t*, 5.8)	122.67	5.26 (*s*)	2'	0.93 (*s*)	21.1
13	…….….	143.6	…….….	3'		37.6
14	…….….	41.64	…….….	4'		32.76
15	1.5-2.1 (*m*)	27.7	1.5-2 (*m*)	5'		37.47
16	1.5-2.1 (*m*)	23.4	1.5-2 (*m*)	6'		24.52
17		46.5		7'		37.38
18	3.3 (*m*)	41.03	2.5-2.6 (*d*)	8'		32.85
19	1.2, 1.8 (*m*)	45.9	1.5-1.6 (*m*)	9'		37.54
20	…….….	30.7	1.3-1.5 (*m*)	10'		24.9
21	1.5-2 (*m*)	33.8	1.3-1.5 (*m*)	11'		39.45
22	1.5-2 (*m*)	32.46	2-2.3	12'		28.05
23	1.22 (*s*)	28.1	1.15 (*s*)	13'		22.7
24	1.16 (*s*)	15.57	0.92 (*s*)	2-CH_3	1.3 (*s*)	23.78
25	0.86 (*s*)	15.35	0.79 (*s*)	5-CH_3	2.04 (*s*)	11.9
26	1.02 (*s*)	17.1	0.93 (*s*)	7-CH_3	2.16 (*s*)	13
27	1.26 (*s*)	25.96	1 (*s*)	8-CH_3	2.09 (*s*)	12.1
28	…….….	180	…….….	4'-CH_3	0.93 (*s*)	19.76
29	0.92 (*s*)	33.09	0.94 (*d*, 2.35)	8'-CH_3	0.91 (*s*)	19.83
30	1.02 (*s*)	23.6	0.9 (*d*, 2.35)	12'-CH_3	0.94 (*s*)	22.8

1.12. In silico docking study of isolated compounds into KEAP-1 protein

Docking of the isolated compounds into KEAP-1 protein revealed that oleanolic (T1) and ursolic acids (T2) had the highest inhibitory affinity to the target receptor (**Table 4**) followed by α-tocopherol (T3), scoring -7.741, -7.967 and -7.05 kcal/mol, respectively, relative to the positive control 4'-bromoflavone (-8.282 kcal/mol), inferring their potential KEAP-1 inhibitory effect and, thus, cytoprotective action. Both T1 and T2 formed non-covalent bonding with the target protein at Gly 367 and Val 606 amino acids *via* their hydroxy and carboxy moieties (**Fig.7**). Results are in accordance with the reported studies on T1-T3 [51-53].

Conclusion

The study reported the chemopreventive effect of *C. urens* petroleum ether leaf extract through induction of NQO1 enzyme, in which folds of increase was recorded to be 4.79 times the control *via* DCPIP assay and further confirmed with increase of NQO1 protein expression. Phytochemical investigation of the active extract *via* GC/MS analysis enabled identification of 34 compounds in its unsaponifiable matter and 15 fatty acids as methyl esters. Isolation attempt *via* chromatographic techniques and spectral analyses revealed for 5 compounds, namely oleanolic acid (T1), ursolic acid (T2), α-tocopherol (T3), palmitic and stearic acids (T4 & T5), where T1 and T3 are reported for the first time in *C. urens* leaves. *In silico* molecular modelling study of the chemopreventive effect of the isolated compounds as KEAP-1 inhibitors showed that T1, T2 and T3 had the highest docking score compared to 4'-bromoflavone positive control. The study prompted to conduct *in vitro* and *in vivo* studies to assess the NQO1 inducing activity of *C. urens* petroleum ether leaf extract and further phytochemical investigation of its chemical content.

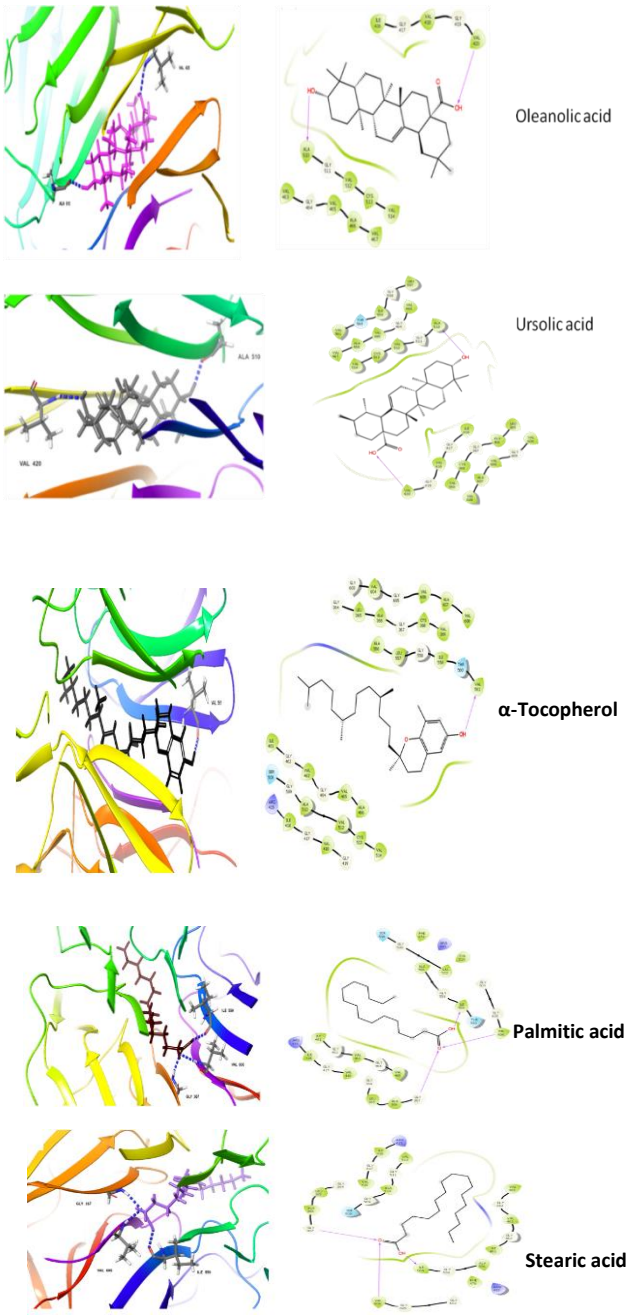

Fig. 7. 3D and 2D-molecular docking simulation of compounds (T1-T5) isolated from *C. urens* petroleum ether leaf extract showing hydrogen bond interactions with KEAP-1 protein (PDB ID: 4IQKD)

Table 4: Results of the in silico docking study of isolated compounds into Kelch-like ECH-associated protein 1 (KEAP1) (PDB: 4IQK)

Sample	Code	Ligand	Docking score kcal/mol	Hydrogen bond interactions
Positive control	…….	4'-Bromoflavone (4'-BF)	-8.282	Gly 367 and Val 606
***C. urens* isolated compounds**	T1	Oleanolic acid	-7.741	Val 420 and Ala 510
	T2	Ursolic acid	-7.967	Ala 510 and Val 420**.**
	T3	α-Tocopherol (Vitamin E)	-7.05	Val 561
	T4	Stearic acid	-1.968	Gly 367, Ile 559 and Val 606
	T5	Palmitic acid	-0.86	Gly 367, Ile 559 and Val 606

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Conflict of interests

Authors declare that there is no conflict of interests

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Arabic abstract الملخص العربي

تسلط الدراسة الضوء للمرة الأولى على الدور المحتمل لـ نباتين من العائلة النخيلية و هما *C. urens* و *C. mitis* في الوقاية من التسرطن كمحرضات لإنزيم NQO1 باستخدام طريقة DCPIP assay & western blot. من بين العينات التي تم اختبارها من مستخلصات الاوراق ذات اقطاب مختلفة ، أظهر مستخلص الاثير البترولي لاوراق *C. urens* يليه *C.mitis* الاكثر فعالية للتحريض في نشاط الإنزيم. تمّ عمل دراسة مقارنة لأول مرة بين المستخلصين باستخدام تحليل ال GC/MS حيث تم التعرف علي عدد 35 مقابل 34 مركب من الجزء الغير متصبن و علي عدد 29 مقابل 15 مركب من الاحماض الدهنية في *C. urens* و *C. mitis*, علي التوالي. و بمحاولة الفصل الكيميائي من المستخلص الفعال، تم فصل 5 مركبات و تعريفهم بواسطة الطرق الطيفية المختلفة ، و هم: oleanolic acid (T1) ، و ursolic acid (T2) ، و α-tocopherol (T3) ، و palmitic & stearic acids (T4 & T5) ؛ حيث يتم تعريف T1 و T3 لاول مرة في *C. urens*. تم إجراء تقييم النشاط الوقائي ضد التسرطن للمركبات المعزولة عبر النمذجة الجزيئية ضد مستقبلات KEAP-1 حيث أظهرت T1 و T2 و T3 درجة إرساء واعدة من -7.7 و -7.9 و -7.05 كيلو كالوري / مول ، على التوالي ، مقارنة بـ -8.2 كيلو كالوري. / مول للتحكم الايجابي بـ 4'-bromoflavone