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The Evaluation of *α*-amyrin from *Callistemon Citrinus*: A Study on Distribution and Cytotoxic Properties

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Abstract

Callistemon citrinus is an aromatic herb, its lemon-scented leaves and bioactive potential propose that it could find a niche as a flavoring spice, intensely in herbal teas and natural medicine formulations. The *C. citrinus* from ITERA Botanical Gardens is reported to contain *α*-amyrin, a triterpene with promising bioactivity, prevalent in various segments of *C. citrinus*. The *α*-amyrin has cytotoxic properties, which are of interest in both medicinal and food science topics. This study objects to isolate and measure the *α*-amyrin content from various parts of *C. citrinus*. The *α*-amyrin exhibited high concentrations in n-hexane bark extract by 39.27 ± 0.774 ppm and showed moderate-weak activity against A549, MCF7, and HeLa, with IC_{50} values of 54.14 ± 1.41, 82.79 ± 0.86, 69.35 ± 2.37 μM respectively. This research intends to support the Botanical Garden's greening campaign by investigating bioactive compounds against malignant human cancer cells.

Keywords: *C. citrinus*, Cytotoxic, *α*-amyrin, Screening content, Aromatic herb

Introduction

Callistemon citrinus (Myrtaceae), moreover familiar as "Sikat Botol" in Indonesia, is a aromatic plant originally from Australia Chauhan *et al.* [1] but is also frequent in various tropical zones such as Sumatera Island, Indonesia. in Sumatera, it primarily serves as an ornamental flowering shrub, *C. citrinus* has stunning red blossoms and luxuriant foliage Rios-Chavez *et al.* [2], this plant exhibits remarkable adaptability to various weather and environmental conditions. Accordingly, it demonstrates suitable for both ornamental and inclusion in reforestation purposes [3]. The *C. citrinus* emits a lemony fragrance as crushed, it's due to the presence of essential oils and terpenoids, making it potentially useful as a flavoring and herb agent. The lemony scent can be a base for herbal teas and flavorings in food preparations. Indigenous Australians using *C. citrinus* for food additive, traditional therapeutic practicing, utilizing it to alleviate pain, treating gastric disorders, and preventing infections [4,5]. The therapeutic attributes of *C. citrinus* are predominantly by its terpenoids and phenolic compounds [6]. The primary terpenoids constituents found in the essential oil of *C. citrinus* leaves include limonene, terpineol, and *α*-pinene [7,8]. Additionally, myrcene, linalool, and phytosterol have been identified in the several plant sections [9]. The prospective terpenoids from *C. citrinus* have been documented to exhibit potential anticancer properties *in vitro* investigations conducted on colon cancer cells [6,7].

The amyrins, classified as triterpenes [10], are reported to influence cytotoxic, gastric-protective, anti-implantation, and obesity properties [11-13]. Consequently, the occurrence of *α*-amyrin compounds explains their use in ethnobotanical approaches for

treating ulcers and wounds [14]. The triterpene *α*-amyrin has shown cytotoxic effects against various cancer cell lines such as leukemia (HL-60), glioblastoma (SF-295), colon cancer (Colo-205), and melanoma (MDAMB-435) [15-17]. Additionally, amyrins have been investigated against glioblastoma, breast and colorectal adenocarcinoma, liver and lung carcinoma, pancreatic adenocarcinoma, prostate, and kidney carcinoma cell lines [10,18]. However, there have been no reports of *α*-amyrin from *C. citrinus* and its cytotoxic activity, initiating from the ITERA Botanical Gardens in South Lampung, Sumatera, Indonesia. This study intentions to contribute to conservation efforts by investigating bioactive compounds from *C. citrinus* under reforestation program.

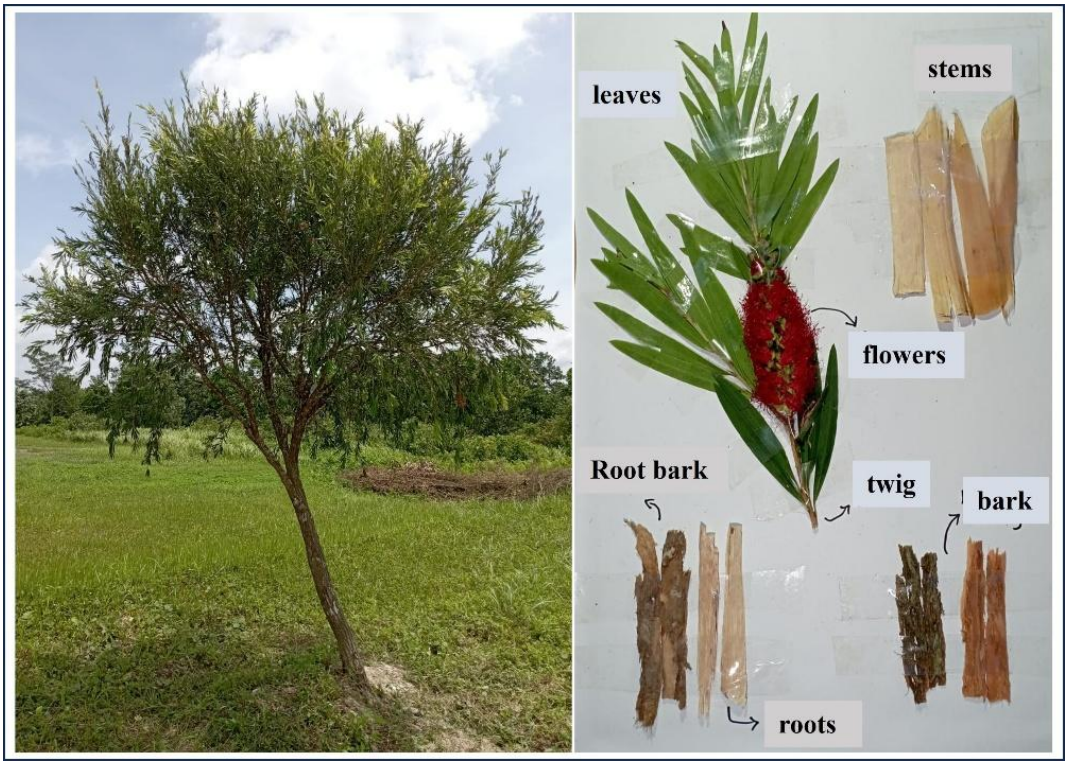

Figure 1 The ITERA Botanical Gardens greening campaign plant: The *C. citrinus*.

Materials and methods

Plant samples

The plant samples of *C. citrinus* were gathered in January 2023 from the ITERA Botanical Gardens (South Lampung, Sumatera Island, Indonesia), established at latitude 5°22'12.1"S (-5.368955132470356) and longitude 105°18'47.9"E (105.31392573578954). The specimen identified by the Bogor Herbarium, is stored under the reference code EH.TT.15527 at ITERA Botanical Garden.

General procedures

Several parts of *C. citrinus* were extracted through maceration method by MeOH. The subsequent steps involved separation and purification utilizing a chromatography column packed with silica gel 7734 (particle size: 0.063 - 0.2 mm) and Sephadex (Merck). Monitoring of the extract was performed using TLC on a PF254 plate, observed under UV light at wavelengths of 254 and 366 nm. The identification of *α*-amyrin structure was achieved through Brucker ^{1}H and ^{13}C NMR spectroscopy (solvent: Acetone-d6, frequency: 500 MHz, referencing TMS). Functional groups were examined using PerkinElmer FTIR spectroscopy. Mass spectrum analysis was conducted using the HP 5973 Mass Spectrometer with the JEOL JMS Ax-500 model. The concentration of *α*-amyrin was assessed using a Genesys 150 UV-Vis spectrophotometer by Thermo Scientific. The optical rotation was measured using a Krüss Optronic Polarimeter P 8000-T.

The *α*-amyrin isolation

Dried *C. citrinus* wood powder (1000 g) underwent extraction using 3 L of MeOH, resulting in a crude extract weighing 121.7 g. A portion of this crude extract (100 g) was diluted 3 times by 500 mL of acetone, yielding an acetone extract weighing 19 g. The acetone extract was subjected to column vacuum chromatography on silica gel, retaining a gradient of EtOAc-hexane (ranging from 10:90 to 0:100, in 100 mL increments per fraction) as the eluent, generating 32 fractions labeled A1 through A32.

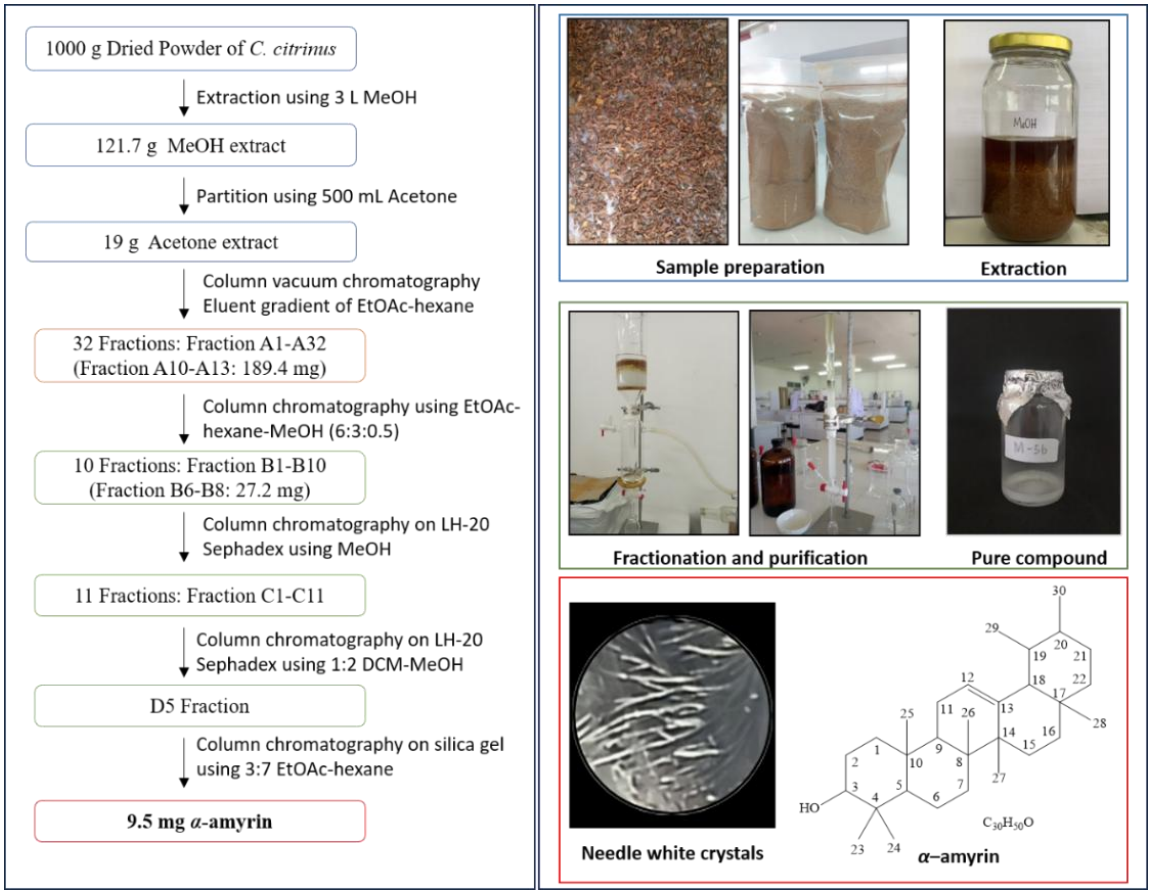

Figure 2 The *α*-amyrin isolation from *C. citrinus*.

Further column chromatography of fractions A10 through A13 (totaling 189.4 mg) on silica gel, using a mixture of EtOAc-hexane-MeOH (in a ratio of 6:3:0.5), produced 10 fractions labeled 1B through 10B. Fractions B6 through B8 (27.2 mm) underwent column chromatography on LH-20 Sephadex using MeOH as the eluent, resulting in 11 fractions labeled C1 through C11. These fractions were then subjected to additional column chromatography on LH-20 Sephadex, using a mixture of dichloromethane (DCM) and MeOH (in a ratio of 1:2) as the eluent, which provided the D5 fraction. The D5 fraction (17.53 mg) was purified by column chromatography on silica gel using an EtOAc-hexane eluent (3:7), resulting in the isolation of 4.4 mg of compound Y (identified as a mixture of amyrins) and 9.5 mg of compound X, identified as *α*-amyrin.

The *α*-amyrin determination content

The determination of the *α*-amyrin content, following the method outlined by Kurniawan *et al.* [19], was conducted using UV-Visble spectrophotometry. The analytical approach involved scanning at a rate of 1500 nm/min with data intervals of 1000 nm, maintaining a constant range at 2.0 nm. The *λ* max of the *α*-amyrin standard solution, measured within the UV range of 200 - 400 nm, was found to be 284 nm. To prepare the stock solution of *α*-amyrin (100 ppm), 5 mg of pure *α*-amyrin was dissolved in 50 mL of MeOH. The absorbance measurements exhibited linearity across the

range of 100 - 3.125 ppm through serial dilution, yielding a correlation coefficient of 0.997.

Cell lines culture process

Bioactivity tests were conducted on human cancer cells (HeLa, MCF7, and A549) using isolated *α*-amyrin. The cell culture followed the standard protocol of the European Collection of Authenticated Cell Cultures (ECACC), where cells were grown in Dulbecco Modified Eagle Media (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1x antimycotic solution (Sigma) under specific conditions (5 % CO2, 37 °C, 90 % humidity). Cell confluence was assessed using the trypan blue dye exclusion procedure, and cells were trypsinized and passaged when reaching 80 % confluence. Cultured cells were seeded in 96-well plates at a density of 1×104 cells/well in 100 *μ*L DMEM.

MTT assay procedure

The cytotoxicity assay was conducted using MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) approach. Paclitaxel was applied in varying concentrations onto the plate, then left to incubate for 48 h. Afterward, each well was washed with PBS, followed by the addition of MTT solution (100 *μ*L 5 mg/mL MTT, Themo), and further incubation for 4 h at 37 °C. Formazan crystals were then dissolved using DMSO (100 *μ*L/well), and absorbance was determined using a multiplate ELISA reader (Biobase) at a wavelength of 540 nm. Cell viability was calculated using the subsequent formula:

% Viability = OD of preserved cells/OD of unpreserved cells.

Results and discussion

The *C. citrinus* contains bioactive compounds, including *α*-amyrin, which have been reported for anti-inflammatory, antimicrobial, and cytotoxic properties. The *α*-amyrin exhibits needle-shaped white solid crystals by a melting point reaching around 184.2 to 185.5 °C. The molecular formula is determined as $C_{30}H_{50}O$, confirmed by EIMS with *m/z* values of 427 $[M+H]^+$ and 449 $[M+Na]^+$. The UV-Vis spectra demonstrate absorption at λ_{max} = 284 nm, suggesting electron excitation from the $\pi \rightarrow \pi^*$ orbital, a characteristic chromophore for unconjugated double bonds (C=C). The infrared spectrum of *α*-amyrin demonstrates a broad band at 3384 cm^{-1}, indicating the presence of a hydroxyl group, supported by the secondary alcohol band at 1051 cm^{-1}. Absorption bands at 2979 and 2863 cm^{-1}, as well as 1469 cm^{-1}, are indicative of aliphatic (C-H) groups. Additionally, absorption peaks at 1377 cm^{-1} suggest the presence of a geminal dimethyl group. The absorption band at 1656 cm^{-1} shows the presence of an alkene group. The optic rotation of *α*-amyrin $[\alpha]_D^{20}$ = +84.4 ° (*c* 0.5, $CHCl_3$) (lit: +84.8 ° (*c* 0.315, $CHCl_3$ Serbian and Csuk [26]) compared by *β*-amyrin $[\alpha]_D^{20}$ = +89 ° (*c* 0.315, $CHCl_3$) [26].

The NMR data of isolated *α*-amyrin, obtained using ^{1}H NMR in acetone-d_6 at 500 MHz (Brucker instrument), referenced by tetramethylsilane (TMS): δ_H 0.65 (1H, *d*), δ_H 0.71 (3H, *d*), δ_H 0.74 (3H, *s*), δ_H 0.85 (3H, *d*), δ_H 0.88 (3H, *s*), δ_H 0.94 (3H, *s*), δ_H 0.93 (3H, *s*), δ_H 0.99 (3H, *s*), δ_H 1.77 (2H, *t*), δ_H 1.85 (2H, *dt*), δ_H 1.96 (2H, *t*), δ_H 3.15 (1H, *dd, J* = 4.9; 11.4 Hz), δ_H 5.14 (1H, *t, J* = 3.5 Hz) C=C alkene representative. The ^{13}C NMR data: δ_C 38.8 (C1, *s*), δ_C 27.6 (C2, *s*), δ_C 79.6 (C3, *s*), δ_C 39.1 (C4, *s*), δ_C 55.1 (C5, *s*), δ_C 18.6 (C6, *s*), δ_C 32.2 (C7, *s*), δ_C 30.5 (C8, *s*), δ_C 47.4 (C9, *s*), δ_C 36.6 (C10, *s*), δ_C 23.5 (C11, *s*), δ_C 124.5 (C12/C13, *s*), δ_C 139.7 (C12/C13, *s*), δ_C 41.9 (C14, *s*), δ_C 27.0 (C15, *s*), δ_C 26.6 (C16, *s*), δ_C 33.5 (C17, *s*), δ_C 58.9 (C18, *s*), δ_C 39.8 (C19/C20, *s*), δ_C 31.0 (C21, *s*), δ_C 41.5 (C22, *s*), δ_C 28.2 (C23, *s*), δ_C 15.5 (C24/C25, *s*), δ_C 16.8 (C26, *s*), δ_C 23.2 (C27, *s*), δ_C 27.9 (C28, *s*), δ_C 17.2 (C29, *s*), δ_C 21.4 (C30, *s*). The ^{13}C NMR and ^{1}H NMR spectroscopic data shows the presence of 8 methyl groups, 1 olefenic proton at δ_H 5.14 (*t, J* = 3.5 Hz), and a Hidroxy proton at δ_H 3.15 (*dd, J* = 4.9; 11.4 Hz), the entire revealing of olealane pentacyclic type triterpenoid, both spectra are consistent by the previously reported data for *α*-amyrin [11].

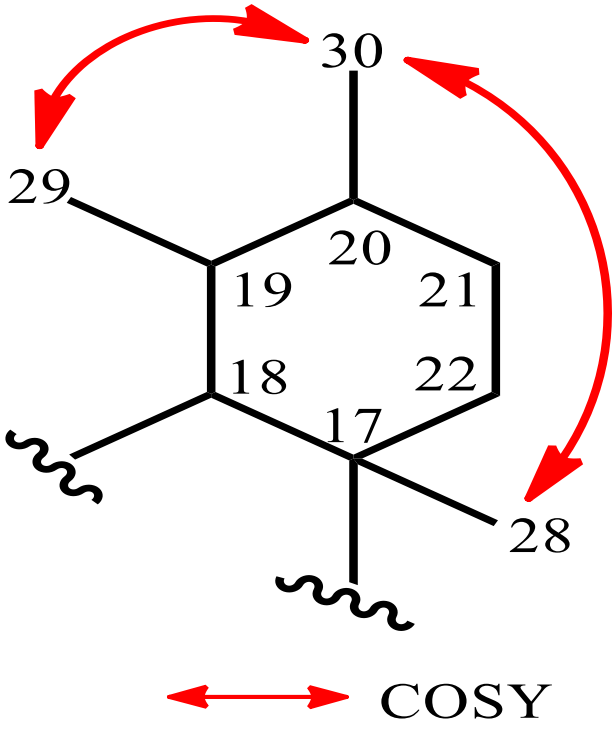

Figure 4 The $^{1}H^{1}H$COSY typical analysis of *α*-amyrin from *C. citrinus*.

The $^{1}H^{1}H$COSY analysis discovers correlations between proton at δ_H 0.93 (C23) and δ_H 0.74 (C24), the proton at δ_H 0.85 (C29) correlates with proton δ_H 0.74 (C30). Additionally, the proton at δ_H 0.94 (C28) correlates to the proton δ_H 0.74 (C30) [12,13]. The reciprocal proton correlation observed between C29 and C30 serves as a distinctive feature of *α*-amyrin, differentiating it by the *β*-amyrin, which are generally characterized by correlation uniquely with the methyl proton at C28 [14-17].

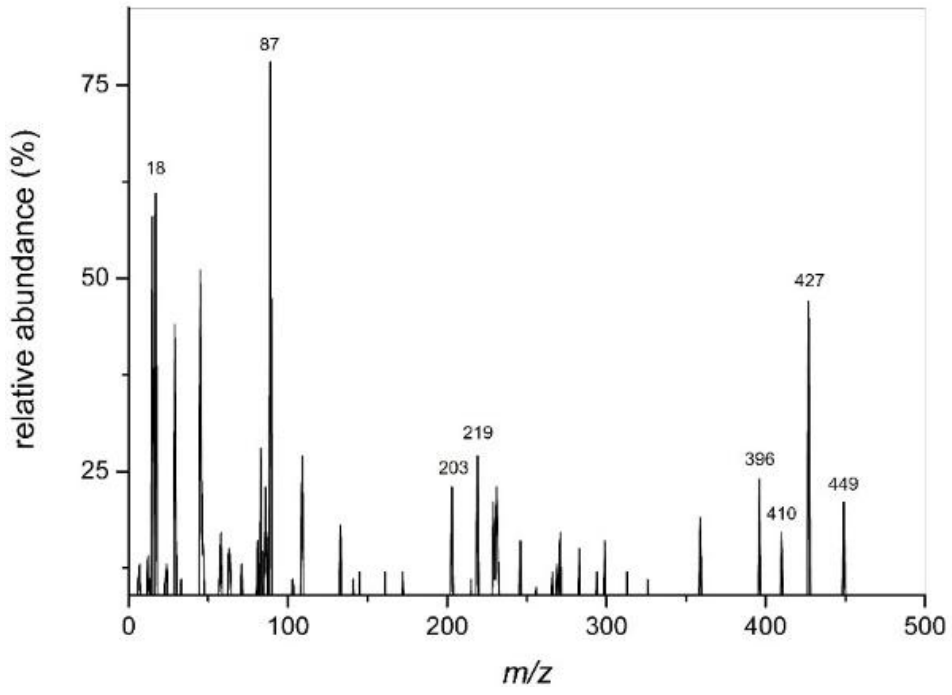

Figure 5 The EIMS fragmentation of *α*-amyrin from *C. citrinus*.

The mass fragmentation indicated an *m/z* of 427 $[M+H]^+$ corresponding to a molecular formula of $C_{30}H_{50}O$. The molecule displayed 6 double bond equivalents, with 5 arranged within a pentacyclic carbon framework and the remaining one as an alkene C=C bond. The fragments at *m/z* 219 and 203 resulting from a retro Diels-Alder fragmentation suggested the presence of a double bond at the C-12 position within the pentacyclic C-ring. The fragmentation observed at *m/z* 410 involved the removal of a hydroxy group (OH)•, corresponding to (M-17), *m/z* 396 (M-31), the loss of (CH_3OH)• was noted, additionally, *m/z* 86 (M-341) was attributed to the loss of $(C_{24}H_{36}O)$•. The spectroscopic data are related to the spectroscopic characterization studies of *α*-amyrin that have reported before [10,20].

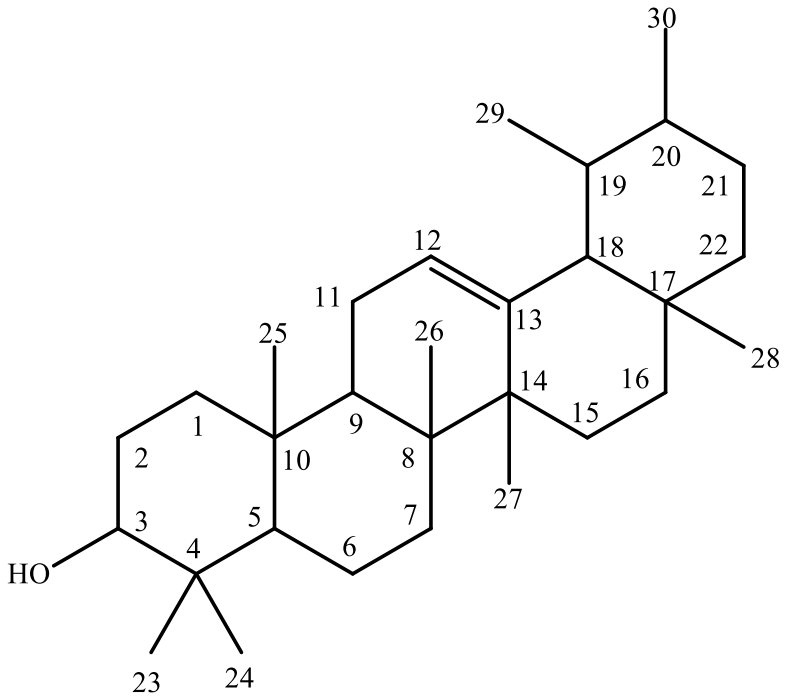

Figure 6 The isolated *α*-amyrin structure from *C. citrinus*.

The amyrins assist as precursors to ursolic and oleanolic acids. These secondary metabolic are derivated and divided into *α*-amyrin and *β*-amyrin [21]. The *α*-amyrin, which retains an ursane framework, is a pentacyclic triterpenol by the $C_{30}H_{50}O$ chemical formula of 3*β*-hydroxy-urs-12-en-3-ol [20].

Table 1 The *α*-amyrin concentration from several part extract of *C. citrinus.*

C. citrinus	***α*-amyrin content (ppm)**			
	***n*-hexane**	**H_2O**	**Acetone**	**MeOH**
Root	23.14 ± 1.005	5.17 ± 0.915	11.21 ± 0.792	6.81 ± 3.021
Wood	12.74 ± 1.870	3.89 ± 0.655	5.04 ± 0.838	4.27 ± 0.566
Leaf	6.054 ± 0.639	0.59 ± 0.198	1.68 ± 0.769	0.85 ± 0.448
Bark	39.27 ± 0.774	10.91 ± 0.231	16.66 ± 0.277	13.49 ± 0.209

The distribution and concentration of *α*-amyrin in the Mertaceae family, particularly in *C. citrinus*, have not been thoroughly established. The highest content of *α*-amyrin was discovered in bark of *C. citrinus* by 39.27 ± 0.774 ppm (*n*-hexane). The *α*-amyrin is a natural triterpene that can be isolated from a variety of plant resin sources [22]. The Burseraceae family, which includes *Bursera* and *Protium* genus, is known to

contain significant amounts of *α*-amyrin. Additional sources of *α*-amyrin have been identified, including Mexican copal with a concentration of 5 g/kg, *Cassia obtusifolia* which contains 140 mg/kg, and the resin of *Commiphora holtziana* with 200 mg/kg [9,10]. The *α*-amyrin, a plant resin as natural reservoir, is certainly present in entirety major parts of *C. citrinus* in varying concentrations (**Table 1**). Accordingly, the bark of *C. citrinus* is the most pointed source of *α*-amyrin. The *α*-amyrin has been isolated from several plant sources, including the resin of *Boswellia carterii*, stemwood and bark of *Populus euramericana*, the seed oil of *Amelanchier alnifolia* Nutt, the methanol extract of *Poncirus trifoliata* stem bark, the methanol extract of *Antiaris africana* Engler stem bark (1 mg/kg), the *n*-hexane extract of *Melastoma malabathricum L. leaves* (65 mg/kg), and the methanol extract of *Ficus pandurata* Hance stem bark and leaves (23 mg/kg) [8].

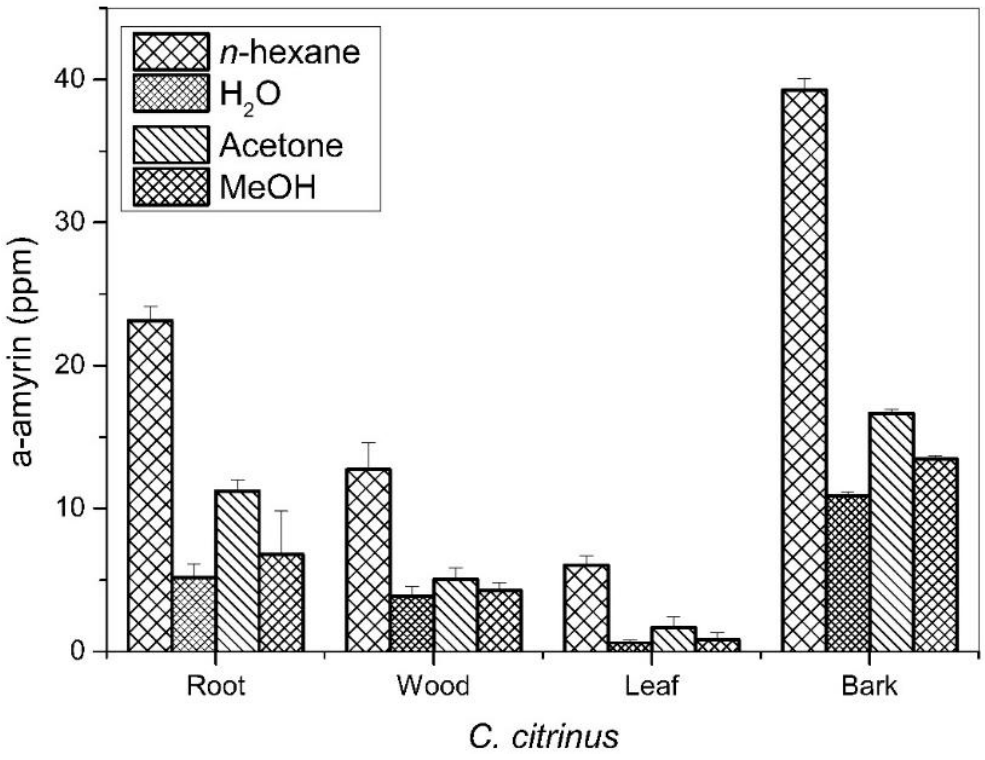

Figure 7 The *α*-amyrin content of *C. citrinus.*

The *α*-amyrin is present in numerous medicinal plants and in oleoresin extracted by carving the bark of different plant species. Studies suggest that *α*-amyrin also plays crucial biological roles [23]. The *α*-amyrin has demonstrated a broad spectrum of pharmacological activities both *in vitro* and *in vivo*, effectively targeting various health-related conditions.

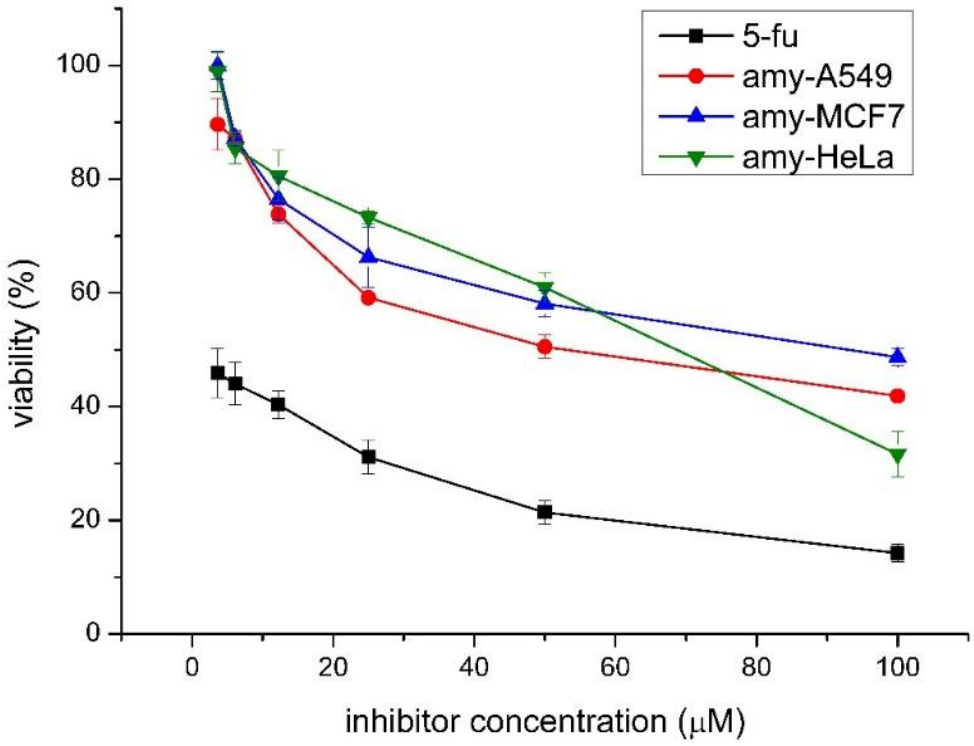

Figure 8 Cell viability of *α*-amyrin against human cancer cells.

These include inflammation, and infections caused by microbes, fungi, and viruses, as well as cancer cells [15,13,24,11]. The cytotoxic data for *α*-amyrin indicates varying levels of activity against different cancer cell lines, with IC_{50} values of 54.14 ± 1.41 *μ*M against A549 cells, 82.79 ± 0.86 *μ*M against MCF7 cells, and 69.35 ± 2.37 *μ*M against HeLa cells. The cytotoxic assay of *α*-amyrin examined against cervical adenocarcinoma (HeLa), breast adenocarcinoma (MCF7), and skin epidermoid carcinoma (A431) cells using the MTT assay, the results showed that *α*-amyrin exhibited weak to moderate activity, with IC_{50} values reaching from 30 - 80 *μ*M.

Table 2 The *α*-amyrin cytotoxic activity from *C. citrinus.*

Cancer cell	Sample (IC_{50}: *μ*M)		
	***α*-amyrin**	***n*-hexane**	**5-fu**
A549	54.14 ± 1.41	12.47 ± 1.63	6.68 ± 0.83
MCF7	82.79 ± 0.86	21.05 ± 3.71	5.13 ± 1.20
HeLa	69.35 ± 2.37	10.24 ± 0.93	3.26 ± 0.49

These results suggest that *α*-amyrin exhibits moderate cytotoxic activity against A549 cells, which are lung carcinoma cells, but shows weaker activity against MCF7 and HeLa cells (**Table 2**). Compared by the aliphatic triterpenoids revealing that the 24-ethylsterol chain has a stronger cytotoxic effect than the 24-methylsterol moiety. The alipahtic double bond between C-22 and C-23 plays an important role in its cytotoxic activity. In contrast, *α*-amyrin lacks an aliphatic chain, which causes its bioavailability in cells to reduction [25]. The *n*-hexane extract demonstrates significant cytotoxic activity, implying the presence of additional bioactive compounds within the extract that enhance its overall cytotoxic effect. It is important to note that specific IC_{50} values can vary depending on various factors such as experimental conditions, cell type, and other environmental variables. The *α*-amyrin alone shows varying degrees of efficacy across different cell lines, the synergistic effects of compounds in the *n*-hexane extract advantage to more pronounced cytotoxic

effects. These bioactivities properties associate it with other herbs that are valued for their health benefits and can complement their use in herbal remedies, teas, and tinctures.

Conclusions

This research intentions to support the Botanical Garden's greening program by investigating bioactive compounds from reforested plants. The bioactive compounds from *C. citrinus* contain essential oils and terpenoids, commonly found in herbs and spices used for their therapeutic properties. These compounds can be used in aromatherapy, holistic medicine, or as an additive to enhance the flavor and fragrance of certain dishes or herbal concoctions. The *α*-amyrin is distributed in various parts of the *C. citrinus* plant, the *n*-hexane extract from the bark of *C. citrinus* containing the highest *α*-amyrin content. The *α*-amyrin showed moderate to weak activity against A549, MCF7, and HeLa cells. The *α*-amyrin has ineffective in significantly suppressing cancer cell proliferation and inducing cell death. The significant cytotoxic activity observed in the *n*-hexane extract, which contains *α*-amyrin along with other bioactive compounds. The exploration of combined or synergistic treatments involving multiple compounds is a promising approach in future cancer therapy.

Acknowledgements

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