Egypt. J. Chem. **Vol. 64,** No. 12 pp. 7603 - 7610 (2021)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Apoptotic mechanism of lantadene A from *Lantana camara* leaves against prostatic cancer cells

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Abstract

Lantadene A (LA) which is one of the major pentacyclic triterpenoids in *Lantana camara* leaves was reported to exhibit anti-cancer property. However, the detail mechanism of LA inhibition against prostate cancer cells is still remained unknown. Hence, this study aimed to extract LA which was then used to treat LNCaP cells for the prediction of its apoptotic mechanism. A serial of separation techniques including maceration, solvent partition, crystallization and column chromatography was applied to recover LA. Approximately, 0.45%w/w LA was obtained from the plant leaves with 87.16% purity. The results found that the viability of LNCaP cells decreased with the increase of LA concentration with the IC50 of 208.4 µg/mL. High content screening showed the nucleuses of intact cells were started to collapse at the LA concentration more than 12.5 µg/mL, whereas the breakage of the mitochondrial membrane was observed together with the release cytochrome *C* into cytosols. The activities of caspases -3/7 and -9 were found to increase in a dose dependent manner. Cell cycle arrest was happened during the (G0/G1) phase at the concentration of LA more than 50 µg/mL. As a conclusion, LA was effective to inhibit the growth of LNCaP cells without any cytotoxic effects to RWPE-1 cells. The inhibitory action of LA followed the intrinsic pathway of mitochondria dependent mechanism.

Keywords: *Lantana camara*; lantadene A; prostate cancer; caspases; cell cycle arrest

1. Introduction

Prostate cancer is considered to be the second malignant tumor leading to mortality in males. The number of incidences is elevated with ages, especially over 60 years old males. The American Cancer Society announced that prostate cancer representing 19% of all other diagnosed cancer cases with about 26,730 of death in USA [1]. Although chemotherapy offers significant survival advantages in the treatment of prostate cancer, it is often related to the toxicity of normal tissues [2]. Hence, scientists and researchers

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Receive Date: 14 June 2021, Revise Date: 02 August 2021, Accept Date: 04 August 2021
DOI: 10.21608/EJCHEM.2021.80354.3997

continue to find alternative analogous which is lesser side effects, but more effective on cancer treatment.

The use of natural products for medication has been practicing since ancient times. The early use of plants as ethnomedicine can be traced back at least 60,000 years ago [3, 4]. *Lantana camara* is one of the traditional medicinal plants belonging to Verbenaceae family. It is also known as red sage, Spanish flag and Surinam tea plant and usually grown in tropical, subtropical and temperate areas [5]. *L. camara* has different flower colors like white, pink, red, violet and yellow. Its branches and stems are sometimes armed with twinge [6]. The leaves are broadly ovate, opposite, and have strong odor as shown in Fig. 1 [7].

Fig. 1. Plants of *Lantana camara*

Recent studies reported that the plant contained many phytochemicals with a wide range of pharmacological activities. The pharmacological properties included termiticidal [8], larvicidal [9], anti-bacterial [5], antioxidant [10], anti-inflammatory [6], anti-motility [11], anti-pyretic [12], anti-ulcerogenic [7], anti-hyperglycaemic [13], anti-mutagenic [14], and anti-cancer [15, 16]. Lantadene A (LA) which is a pentacyclic triterpenoid is the major phytochemical in the leaves of *L. camara* [17, 18]. The chemical structure of LA or 22β-angeloyloxy-3-oxoolean-12-en-28-oic acid is presented in Fig. 2. The phytochemical and its congeners were reported to exhibit anti-proliferation against different cancer cell lines [19-22], and possibly chemopreventive activity against carcinogenesis [23]. Specifically, LA was found to have antioxidant [24], hepatoprotective [25] and anti-inflammatory [26] properties. Sharma et al. [20] reported that the functional group of angeloyl, especially the electrophilic α,β-unsaturated C=O component in LA was the key player to inhibit different cancerous cell lines such as HL-60, HeLa, Colon 502713, and Lung A-549. They also mentioned methylation of carboxylic group may increase lipophilicity for better bioavailability in anticancer activities. Previous studies also highlighted the importance of betulinic acid, oleanolic acid and ursolic acid in inhibiting HeLa cells [27]. Those compounds are also triterpenoids having carboxylic acids as one of the functional groups in the basic pentacyclic structure.

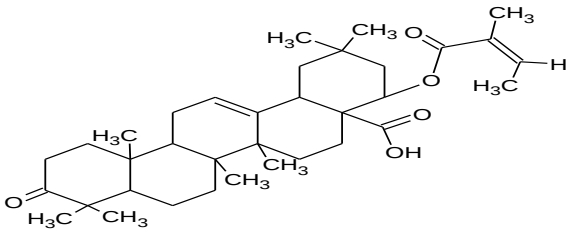


Fig. 2. Chemical structure of lantadene A (LA)

In the present study, LA was extracted and purified from the leaves of *L. camara* using a serial of separation processes from maceration, solvent partition, crystallization and consequently clean-up in column chromatography. The cytotoxicity of LA was evaluated in a dose dependent manner against the growth of prostate cancer cells (LNCaP) and prostate normal cells (RWPE-1). The plausible apoptotic pathway of LA in inhibiting LNCaP cells was examined based on the change of cell morphology in terms of nuclear intensity, cell membrane permeability and mitochondrial membrane potential, and caspases activities, as well as cell cycle arrest. The inhibitory action of LA against LNCaP cells is important to understand the programmed cell death for better drug design.

2. Materials and Methods

2.1 Chemicals and cell lines

Methanol, acetonitrile, acetic acid and chloroform were purchased from (Merck, Darmstadt, Germany). Dimethyl sulfoxide (DMSO), penicillin, streptomycin, fetal bovine serum (FBS) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Propidium iodide (PI) and RNase were obtained from BD Biosciences (San Jose, CA. USA). The cell lines of lymph node carcinoma from human prostate cancer (LNCaP), and human normal prostate epithelial cells (RWPE-1) were obtained from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA). The former cell line was

maintained in Roswell Park Memorial Institute Medium (RPMI 1640), where the latter was kept in keratinocyte.

2.2 Plant Material

The fresh leaves of *L. camara* were harvested from the Baghdad University Garden (Baghdad, Iraq). The plant was identified and authenticated by Dr. Zainab Abid Aun from the Department of Biology in the College of Sciences for Women (Baghdad University, Iraq). The specimen (registration number 1169) has been deposited in the National Herbarium of Iraq, Ministry of Agriculture, Baghdad, Iraq.

2.3 Extraction and purification of lantadene A

The collected leaves were dried and then ground into powder. The leaf powder (100 g) was mixed with 500 mL methanol and macerated for 24 hours with intermittent shaking. The extract was filtrated using muslin cloth and decolorized with 20 g activated charcoal to obtain yellowish solution. The methanol solution was concentrated by evaporation and reconstituted in a methanol-water (1:7) mixture (15 mL). The solution was extracted with chloroform (2 x 15 mL) by vigorous shaking in a separating funnel. The organic layer was collected and dehydrated by anhydrous sodium sulphate. The organic layer was then concentrated by a rotary evaporator. The concentrated residue was added with methanol (100 mL) and left to crystallize as white crystal (1.06 g). The white crystal was reconstituted in methanol and chromatographed through a silica gel packed column (30 g, 60–120 mesh) using the solvent system of chloroform–methanol (99.5:0.5). The eluted solution was concentrated in vacuo again and recrystallized in methanol to form pure lantadene A (0.45 g). The white crystal was dissolved in methanol and analyzed using HPLC (Shimadzu LC-2010A HT, Kyoto, Japan) at 240 nm. The separation was carried out using a Phenomenex C18 column (4 µm, 150×4.6 mm) at the isocratic gradient of mobile phase consisted of methanol: acetonitrile: water: acetic acid (68:20:12:0.1). The flow rate was 1.2 mL/min and injection volume was 20 µL.

2.4 Cell Line Culture

LNCaP cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. RWPE-1 cells were grown in K-SFM supplemented with recombinant epidermal growth factor and bovine pituitary extract. All cells were incubated in 5% CO2 at 37^{o} C for 3-5 days until reached 80-90 % confluency.

2.5 Cell viability assay

MTT assay was carried out to determine the cell viability of LNCaP and RWPE-1 after treated with different concentrations of LA ranged from 6.25 to 400 µg/mL according to the procedures described by Sharma et al. [19] with modification. DMSO was used as negative control. Briefly, 1×10^5 cells/well were seeded in a 96-well plate and incubated overnight at 37°C in 5% CO_2 to obtain 80-90 % confluency. The cells were then treated with LA and further incubated for 24 hours. A 50 µL of MTT was added to the treated cells to achieve the final concentration of 0.25 mg/mL and incubated for another 2 hours. After incubation, the formazan crystals were dissolved by DMSO. The viability of cells was determined at 570 nm using an ELISA microplate reader (Bio-Rad, Hercules, California, USA). The results are expressed in growth inhibition as calculated using Equation (1).

$$\text{Growth inhibition (\%)} = \frac{\text{OD treated sample}}{\text{OD control (DMSO)}} \text{ x } 100 \quad (1)$$

The IC50 is defined as the required concentration of LA to inhibit 50 % cell viability. The experiment was carried out in triplicate.

2.6 High content screening

Cellomics multiparameter cytotoxicity 3 kit (Thermo Scientific™, Pittsburgh, PA, USA) was used to evaluate cell nuclear intensity, cell membrane permeability, mitochondrial membrane potential (MMP) and cytochrome *C* release after treated with LA [28]. After 24 hours of treatment, cell permeability dye and MMP dye were added to the cells (1×10^4 cells/well) and incubated for 30 min at 37 °C. Blocking buffer (1×) was used in the fixation and permeabilization of cells. Cells were probed with primary cytochrome *C* antibody and secondary DyLight 649 conjugated with goat anti-mouse immunoglobulin G for 1 hour each. The staining solution was supplemented with Hoechst 33342 to stain the nucleus of cells. The analysis was performed

using the ArrayScan high content screening system (Cellomics, PA, USA).

2.7 Measurement of caspase-3/7, -8 and -9 activities

The activities of caspase-3/7, -8 and -9 were determined using Caspase-Glo-3/7, -8 and -9 kit (Promega, Madison, WI, USA), according to the manufacture's protocols. Cells (1×104 cells/well) were seeded and treated with LA overnight. DMSO was used as the negative control. The next day, 100 µL of caspase-Glo reagent was added to the each well and then incubated for 30 min. The activities of samples were measured using a Tecan Infinite® 200 Pro (Tecan, Männedorf, Switzerland) microplate reader. The experiment was repeated in triplicate.

2.8 Cell cycle arrest analysis

The cell cycle was evaluated using a flow cytometer (BD Biosciences, New Jersey, USA) according to the procedures described by Gao et al. [29] modification. Approximately, 1×10^5 cells/well were seeded and treated with LA for 24 hours. DMSO was used as negative control. The treated cells were then washed twice with PBS. The cells were fixed using 70% cold ethanol. The cells were stained with PI supplemented with RNase. The percentage of cells were measured in (G0/G1, S and G2/M) phases.

2.9 Statistical Analysis

The results were expressed as mean ± standard deviation. ANOVA was performed to determine the significance of results using GraphPad Prism 7 (La Jolla, CA, USA). The statistical significance is defined at $p \leq 0.05$.

3. Results and discussion

3.1 Cytotoxicity of lantadene A

In the present study, the technique of maceration could recover 1.06 %w/w of methanolic extract from *L. camara* leaves. The extract was further purified and cleaned using column chromatography to obtain 0.45%w/w LA. The presence of LA was detected using high performance liquid chromatography and its purity was found to be 87.16% (Fig. 3).

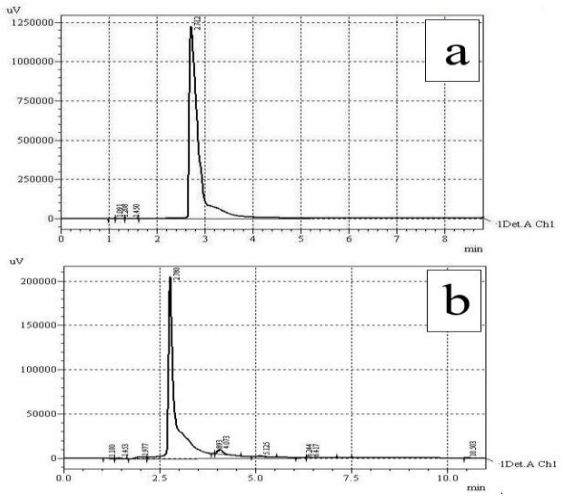


Fig. 3. HPLC analysis. (a) standard LA, largest peak appeared at retention time of (2.712). (b) purified LA, largest peak appeared at retention time of (2.780)

The obtained LA was used to treat both LNCaP and RWPE-1 cells in a dose dependent manner. The viability of LNCaP and RWPE-1 cells was decreased with the increase of LA concentration as illustrated in Fig. 4. The decrease was more significant for LNCaP cells than RWPE-1 cells after treated with LA. The IC50 was found to be 208.4 µg/mL for LNCaP cells and the value was about 4 times lower than that of 770.662 µg/mL for RWPE-1 cells. The difference explains that the inhibitory action of LA was selective enough against LNCaP cells, but non-cytotoxic to healthy cells. Anyhow, the performance of LA to inhibit the growth of LNCaP cells was approximately five times lower than the performance of docetaxel (IC50, 36.5 µg/mL) which is the standard drug of prostate cancer. The concentration of LA more than 100 µg/mL was cytotoxic to LNCaP cells. However, LA was non-cytotoxic to RWPE-1 cells because there were still more than 70% viable cells even treated with LA at the concentration up to 400 µg/mL.

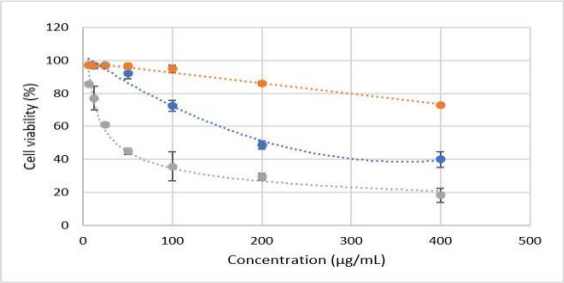
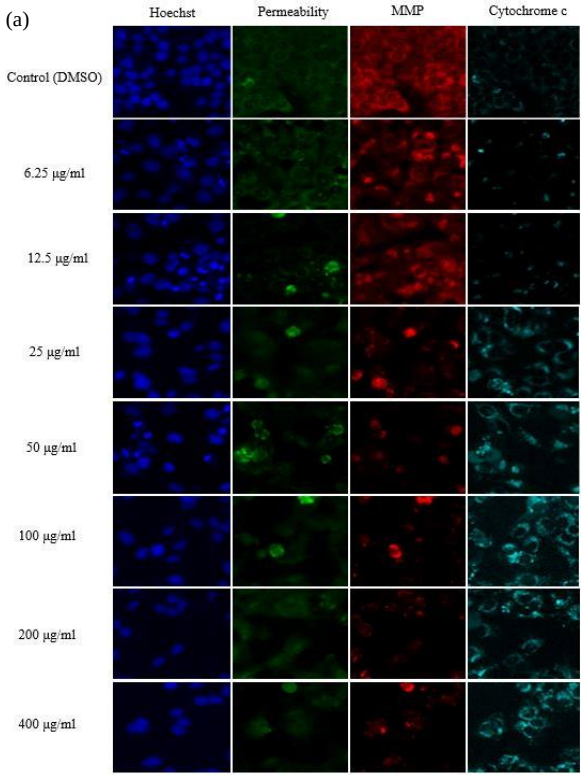


Fig. 4. Cell viability of lantadene A treated LNCaP (•) and RWPE-1 (•) cells compared to the performance of positive control, docetaxel (▲) in MTT assay. The results are expressed as mean ± SD from triplicate data

3.2 Collapse of LNCaP cells by lantadene A

The treatment of LA on LNCaP cells was analyzed by high content screening approach. The change of cell morphology explains the apoptotic pathway of LA.

In particular, cell mitochondria play a crucial role in intrinsic pathway of apoptosis for homeostasis [30]. Fig. 5a shows the occurrence of nuclear condensation for treated LNCaP cells after dyed with Hoechst 33342 (blue). The nucleuses of intact cells were fragmented at the concentration of LA starting from 12.5-400 µg/mL. The quantitative increment of nuclear intensity can be seen from the bar charts in Fig. 5b. The permeability of cell membrane was weakening as the YoYo dye (green) started to penetrate into cells. Hence, the intensity of green dye was getting obvious at higher LA concentration (Fig. 5c). On the other hand, the red intensity of cell images was getting reduced to explain the damage of mitochondria due to the breakage of mitochondrial membrane (50 – 400 µg/mL) (Fig. 5d). The reduction of mitochondrial membrane permeability was in line with the release of cytochrome *C* into cytosols (Fig. 5e). The collapse of mitochondrial membrane potential and the release of cytochrome *C* was not observed in control cells. Previous study also reported the collapse of mitochondrial membrane in rat cells treatment with LA [31].



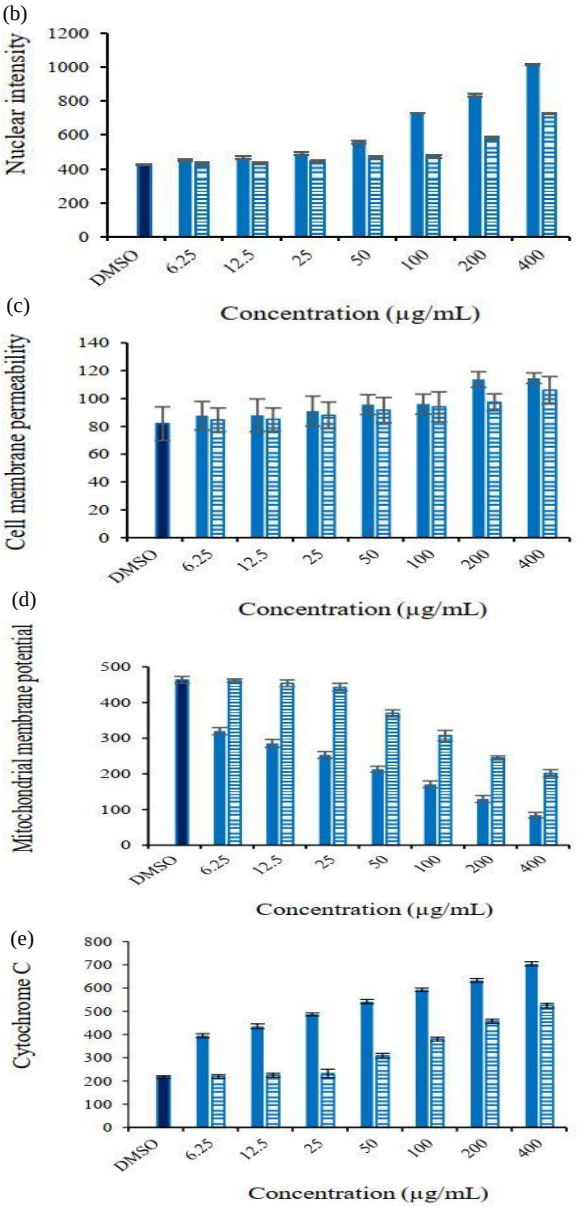


Fig. 5. (a). LNCaP cell morphological images treated with lantadene A (LA) from high content screening, and comparison of (b) nuclear intensity, (c) cell membrane permeability, (d) mitochondrial membrane potential and (e) release of cytochrome *C* from LNCaP cells treated with docetaxel (solid bar) and LA (line bar)

3.3 Activation of caspases for apoptosis

Caspases are activated to regulate apoptosis through a programmed cell death. The activities of executioner caspases 3/7 and initiator caspases 8 and 9 were analyzed after LNCaP cells were treated with LA as presented in Fig. 6. The figure shows exponential increment of caspases 3/7 and 9 in a dose dependent manner, especially at the concentration of LA higher than 50 µg/mL. There was only a slight elevation of caspase 8 starting from 50 to 400 µg/mL of LA. The disturbance of mitochondrial membrane

permeability caused by LA could release cytochrome *C* into cytosols. The phenomenon is considered to be the inducer factor leading to the activation of apoptosis. Cytochrome *C* would interact with WD40 domains of the apoptotic protease activating factor-1 (Apaf-1) in cytosols, and thus enabling the assembly of apoptosome [24, 25]. This platform initiated the activation cascade of caspase-9 and subsequently caspase-3 for apoptotic execution, while effector caspase-7 is required for apoptotic cell detachment. Therefore, LA most likely induced the intrinsic apoptosis through the damage of DNA and release of pro-apoptotic proteins from mitochondria into cytosols. Apoptosis was partly induced by death receptors of FAS ligand which interacted with the FAS receptor, and leading to the activation of caspase-8 through the extrinsic or death receptor-initiated pathway [26]. The observation of this study revealed that LA induced apoptosis of LNCaP cells mostly via the intrinsic pathway.

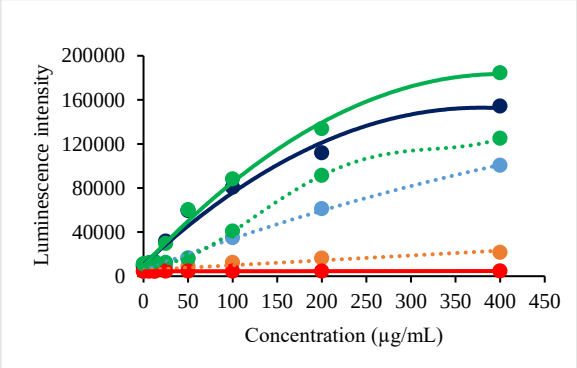


Fig. 6. Caspases -3/7 (blue), -8 (red) and -9 (green) activities of LNCaP cells treated with different concentrations of docetaxel (solid line) and lantadene A (dot line)

3.4 Lantadene A induced cell cycle arrest

Dysregulation of cell cycle was always associated with cancer development. The determination of cell cycle arrest is important in the development of anticancer drug. Therefore, the role of LA in the progression of LNCaP cell cycle was analyzed by flow cytometer. The cellular progression of apoptosis induced by LA was also compared to docetaxel. The results suggested that LA induced cell cycle arrest significantly in (G0/G1) phase at the concentration higher than 50 µg/mL (Fig. 7a). In contrast, there is no significant event of cell cycle arrest in synthesis (S) and mitosis (G2/M) phases (Fig. 7b and 7c). The results also indicated that the anti-proliferative action of LA was opposite to the inhibitory action of positive drug, docetaxel on cellular events. Docetaxel showed to exhibit cell cycle arrest in G2/M phase. LA was also found to cause cell cycle arrest in (G0/G1) phase of human leukemia (HL-60) cancer cells in previous studies [19]. Probably, LA inhibits the activity of growth-dependent cyclin-dependent kinase (CDK) which involves in DNA replication and initiates G1 to S phase transition [35].

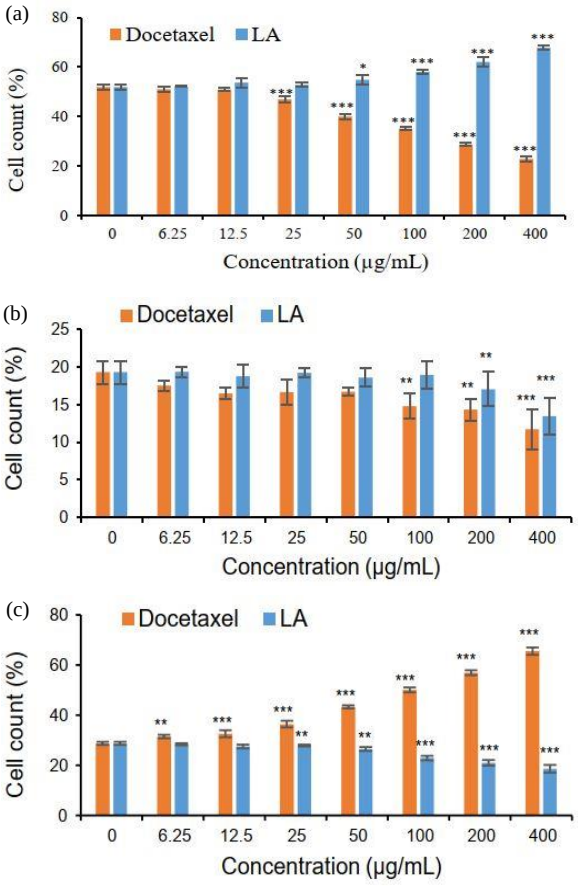


Fig. 7. LNCaP cell cycle arrest induced by docetaxel (orange bar) and lantadene A (blue bar) in a dose dependent manner observed in (a) G0/G1 phase, (b) synthesis phase and (c) G2/M phase. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

4. Conclusions

This study revealed that relatively pure LA (87.16%) could be extracted and isolated from the leaves of *L. camara* through a serial of separation processes including maceration, solvent partition, crystallization and column chromatography. LA was selective against the growth of LNCaP cells, but non-cytotoxic to RWPE-1 cells. The induction of apoptosis by LA followed the intrinsic pathway of mitochondria dependent mechanism. This was supported by the significant activation of caspases -3/7

and 9 in a dose dependent manner. Cell cycle arrest was obviously happened in the G0/G1 phase.

Conflicts of interest

There are no conflicts to declare.

Acknowledgment

This work was funded by University Technology Malaysia grant UTMHR (08G84).

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