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THE ANACARDIC ACID-LOADED NANOLIPOSOMES IMPROVED THE ANTICANCER ACTIVITY *IN VITRO*

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SUMMARY

Anacardic acid accounts for approximately 77% of the cashew nut shell liquid from *Anacardium occidentale*, which was reported to have anticancer potential. Due to its water insolubility, the compound is hard to absorb leading to less bioavailability. Currently, using nanolipsomes as drug carriers in general and for anacardic acid in particular could be an effective solution. In this research, anacardic acid was incorporated into nanoliposomes by employing the thin-film method. The results showed that the fabricated nanoliposomes with egg lecithin, cholesterol, and DSPE-PEG as components will have higher encapsulatted efficiency when compared with the fomula using soybean lecithin. The obtained anacardic acid nanoliposomes (AAL) present a size of 135.2 nm, a PDI of 0.095, and a negative zeta voltage of -27.9 mV. The AAL nanoparticles were also evaluated for their growth inhibitory capacity on several cancer cell lines by MTT assay. The GI_{50} (growth inhibition concentration at 50%) values were determined to range from 80.13 µM to 95.29 µM, of which AAL showed the strongest activity on NTERA-2 human cancer stem cells (80.13 µM). The NTERA-2 cell cycle was also significantly arrested at the S phase (19.43%) under treatment with anacardic acid nanoliposomes. This is the first time for AAL reported activity against cancer stem cells. The activity suggests the potential anti-cancer stem cells of anacardic acid nanolposomes.

Keywords: Anacardic acid, cell cycle, lecithin, nanoliposome, NTERA-2, S phase

INTRODUCTION

The cashew plant (*Anacardium occidentale* L.) is popularly distributed in many regions of Vietnam. As reported, approximately 77% of the cashew nut shells were removed during the primary processing of the nuts. Cashew nut shell liquid contains phenolic lipids (anacardic, cardanol, cardol, and 2-methyl-cardol), of which anacardic acid (AA) accounts for about 76.93% (Andayanie *et al*., 2019). AA suppressed the proliferation of several cancer cell lines, such as breast cancer (MCF-7 and MDA-MB

231), cervical, lung, liver, bladder, and other malignancies (Sung *et al*., 2008; Ten Cate *et al*., 2010). Zhao *et al*. (2018) reported that AA presented anti-proliferation activity on MDA-MB-231 cells with an IC_{50} value of 19.7 μM at 24 h of treatment. Anacardic acid had an effect on triple-negative breast cancer based on the adjustment of the key Hsp90-dependent tumor-related molecules or endoplasmic reticulum stress (ERS)-related molecules (Zhao *et al*., 2018). The anticancer activity of AA in PTEN-deficient colorectal cancer cells is related to inhibited histone acetyltransferase P300/CBP inhibitors, inhibitory subunits of nuclear factor-κBα-related cell survival, proliferation, invasion, and inflammation (Liu *et al*., 2020). In addition, Bogachek *et al*. (2016) reported that AA could exert an antitumor effect by inhibiting the SUMO pathway (Bogachek *et al*., 2016). Because of its anti-cancer potential, AA was considered a phytochemical agent for cancer therapy. Nevertheless, their bioactivities were limited due to their poor solubility in inorganic solvents (Araújo *et al*., 2020).

Nanoliposomes were spherical artificial vesicles that could be produced by natural phospholipids. They contained one or more lipid bilayers with discrete water spaces. Hydrophobic compounds exist between two membranes, while hydrophilic compounds reside in a hydrophilic core (Deshpande *et al*., 2013). Nanoliposomes play multiple functions in enhancing drug delivery, protecting the active compounds from environmental factors, improving product performance, reducing costs, and increasing therapeutic efficacy through reducing systemic toxicity, preventing the premature breakdown of encapsulated drugs (Deshpande *et al*., 2013; Aguilar-Pérez *et al*., 2020). Drug-carrying nanoliposomes could limit drug absorption in normal tissues, thus improving the therapeutic index of drugs. Nanoliposomes could preferentially target tumors with a passive pathway through a permeability-enhancing effect due to leaky tumor blood vessels, leading to an increase in tumor interstitial uptake capacity (Gogoi *et al*. al., 2016).

Due to AA's dipole character, it could be easily incorporated into nanoliposome bilayers. Therefore, nanoliposome technology is a feasible and effective solution to improve the bioavailability of AA. In this report, the AA-incorporated nanoliposomes were fabricated and evaluated for its *in vitro* improved anti-cancerous activity.

MATERIALS AND METHODS

Materials

Anacardic acid (AA) was isolated from cashew nutshells with a purity of 99% (provided by Prof. Tran Van Loc, Institute of Chemistry, Vietnam Academy of Science and Technology). Egg lecithin, soybean lecithin, cholesterol, DMEM medium with 2 mM L-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate, and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St.Louis, MO, USA). Antibiotic-Antimycotic and Trypsin-EDTA were originals from Gibco (ThermoFisher Scientific). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from AK Scientific (Union City, CA). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene gly-col)-2000] (DSPE-PEG-2000) from Avanti Polar Lipids Inc. (Alabama, USA).

Bangham Thin Film method for preparation of anacardic acid nanolipsomes (AAL)

AA was incorporated into nanoliposomal particles according to the thin film method (Bangham *et al.*, 1964), with minor modifications to be suitable for laboratory conditions. Accordingly, lipids including egg lecithin (EPC), soybean lecithin (SPC), cholesterol, DSPE-PEG-2000, and anacardic acid (AA) were dissolved in a dichloromethane (DCM) solvent. Anacardic acid was added to the lipid mixture fomulas before evaporating under vacuum to completely remove the solvent and create a thin lipid layer (thin film). In the next step, PBS (pH 7.2) was slowly added to hydrate the thin film layer. After the lipid layer was completely hydrated, the solution was sonicated at 2 atm with a 20-second sonication cycle and 10-second rest, repeated 10 times. Then, the solution was centrifuged at 12,000 rpm for 90 minutes to separate the supernatant and collect liposome residues.

Encapsulate efficiency detection method

The supernatant obtained from the above centrifugation has been used for the quantification of non-integrated AA by HPLC analysis and based on a standard curve. The efficient encapsulation was determined with the following formula:

$$\text{EE\%} = \frac{C_i - C_f}{C_i} \times 100$$

Where C_f is the unconjugated amount of **AA** into liposomes measured in the supernatant; C_i is the **AA** amount added into the lipid mixture.

Particle size distribution and zeta of the AA-nanoliposome analysis

The AAL re-suspended in PBS was used to detect physiochemical indicators such as particle size, particle distribution (PDI), and voltage. These features were accessed by using the dynamic light scattering (DLS) analysis on a Zetasizer Nano-Z instrument (Malvern Instruments, UK).

Cell culture

Human cancer cell lines, including A549 (human lung carcinoma, ATCC, CCL-185), HepG2 (human hepatocarcinoma, ATCC, HB-8065), Hela (human cervical carcinoma, ATCC, CRM-CCL-2), HT-29 (human colon carcinoma, ATCC, HTB-38), and NTERA-2 (human pluripotent human embryonal carcinoma, ATCC, CRL-1973), were grown in DMEM medium with 2 mM L-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate, supplemented with 10% Fetal Bovine Serum (FBS). Cells were sub-cultured after 2-3 days with a ratio of 1:3 and cultured in an incubator at 37 °C with 5% CO_2.

Anti-proliferative assay using the MTT method

The anti-proliferation of samples was evaluated using the MTT method, according to Mosmann *et al*. (1983). Briefly, cancer cells ($3x10^4$ cells/ml) were incubated with samples at different concentrations in a 96-well plate at 37°C with 5% CO_2. Ellipticine was used as a reference control, while the negative control contained cells treated with sample solvent (DMSO 1%). Wells containing only medium played as blank. After 72 hours of treatment, 20 µL of fresh MTT (5 mg/mL) was added to each well.

After 4 hours of incubation at 37ºC, the medium was removed. The formazan crystals were dissolved in 100 µL of 100% DMSO. The OD values were measured at 540 nm by using a spectrophotometer (BioTek Elx800). The percentage of cell growth inhibition was determined using the following formula:

$$\%\ \text{inhibition} = 100 - \frac{OD(sample) - OD(blank)}{OD(Negative) - OD(blank)} x100$$

Cell cycle analysis using flow cytometry

The cell cycle arrest activity of anacardic acid nanolipsomes was performed using the flow cytometry technique (Zhang *et al.*, 2018). Cells were placed in a 6-well plate at a density of 3 x 10^5 cells per well and cultured overnight in an incubator at 37°C with 5% CO_2. Cells were then treated with a range of concentrations of sample and incubated for 48 hours. The well with only DMSO 0.1% (final concentration) served as the negative control. After 48 hours of incubation, cells were detached using Trypsin-EDTA and collected in falcon tubes. After centrifugation and trypsin removal, cell pellets were washed with cold PBS and fixed with 70% ethanol for 2 hours. In the next steps, the ethanol was removed by centrifugation, and the cells were washed twice with PBS before adding RNase A. The RNase incubation was 15 minutes in a 37ºC bath, followed by the addition of propidium iodide (PI) solution (1 mg/mL) for 1 hour at RT. The effects of the samples on cell cycles were determined by using the flow cytometry Novocyte system (ACEA Bioscience Inc.) and NovoExpress software.

RESULTS AND DISSCUSSION

Determination of a suitable phospholipid ratio for the fabrication of nanoliposome complexes

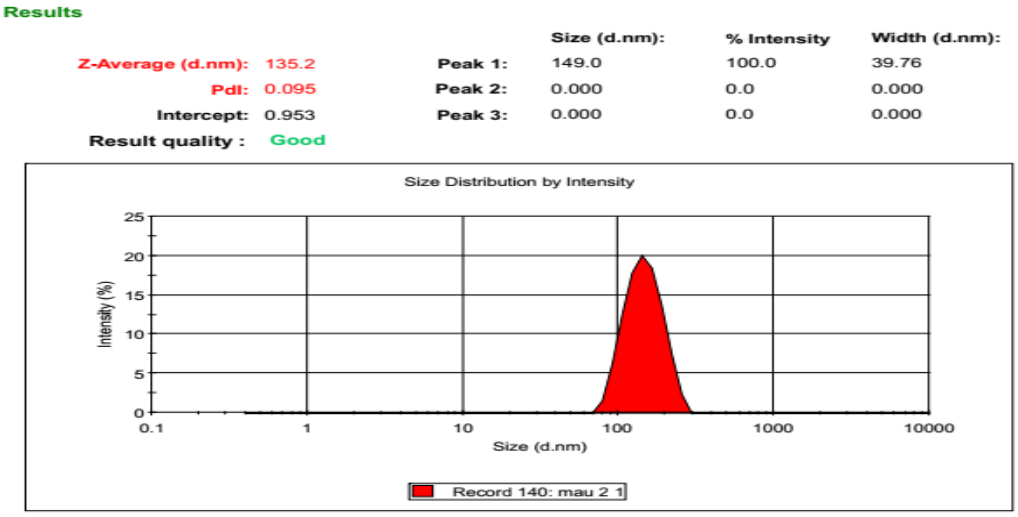
Lipid components building liposomes are necessary factors that have an effect on the morphology, stability, permeability, and efficiency of the assembly. Particularly, phospholipids play a central role in producing liposomes. The chemical structure of phospholipids and their natural origins, causing different physiological functions, could affect the characteristics of the liposome (Li *et al*., 2015). In this study, two formulas of liposomes in which some fixing components such as cholesterol, DSPE-PEG2000, and the drug anacardic acid (AA) in combination with either egg lecithin (EPC) or soybean lecithin (SPC) with an unchanged ratio were accessed. The loading efficiency of AA into nanoliposomes by using the two formulas with different proportions of SPC or EPC was examined. Results in Table 1 showed that the SPC-containing formulation had an AA-conjugated efficiency of up to 82.2%, while that of the EPC-containing liposomes was 97.5%. Thus, the liposomes using EPC as one ingredient allow AA integration with higher performance than the ones with the SPC component.

Determination of some properties of AA-nanoliposomal complexes

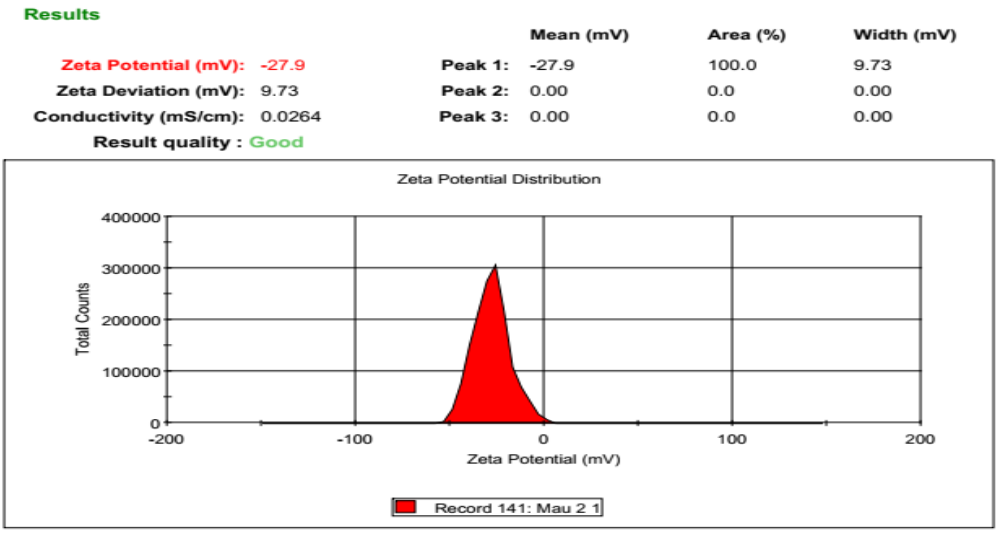
Some physiochemical features of the AA-nanoliposome, including size, zeta potential, and PDI index, were evaluated using the dynamic light diffraction (DLS) technique with a Zetasizer Nano-Z device (Malvern Instruments, UK).

Table 1. Determination of ratio and composition of phospholipids.

Element	Formula 1 (mol ratio)	Formula 2 (mol ratio)
Soybean Lecithin (SPC)	10	-
Egg lecithin (EPC)	-	10
Cholesterol	1	1
DSPE-PEG-2000	0.1	0.1
Anacardic acid (AA)	2	2
Efficient encapsulation (%)	82.2	97.5



(a)



(b)

Figure 1. The basic physiochemical characters of the nanoliposome-anacardic acid complex measured by the Zeta sizer system (Malvern, UK); (**a**) the size and PDI of the AAL; (**b**) the zeta potential of AAL.

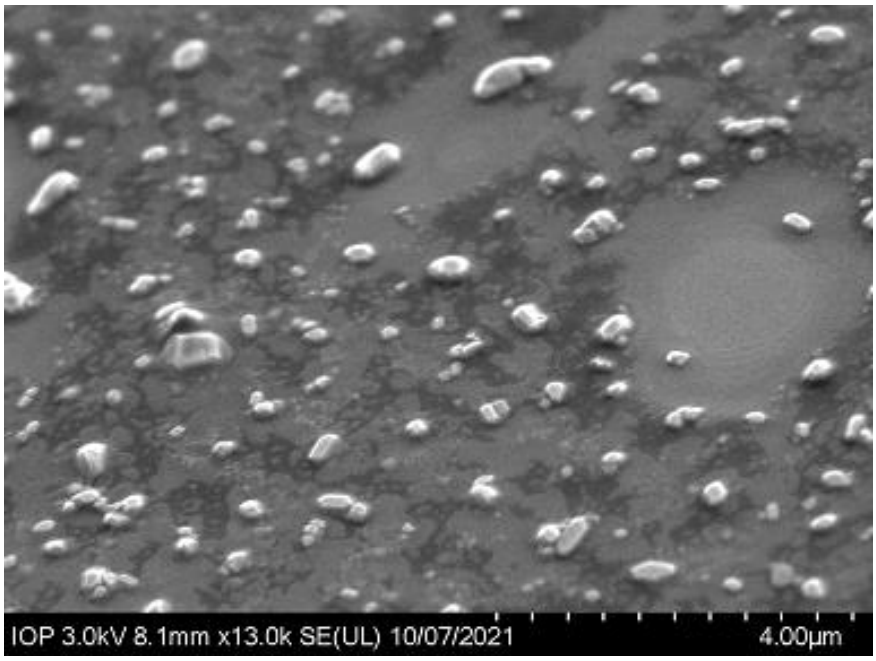


Figure 2. Anacardic acid–nanoliposome particle morphology under scanning electron microscope HITACHI 8100.

In Figure 1, the nanoliposomes presented a size of 135.2 nm. The PDI index of the liposome was 0.095. Normally, the PDI index spreads from 0 to 1 and the larger the DPI, the more uneven the structure of the system is in terms of size. The AA-nanoliposome has a small PDI, indicating that the AA-nanoliposome has a unique size. In addition, AA-nanoliposome presented a negative charge with a zeta potential in dex of -27.9 mV (Fig. 1). The zeta potential was known as a particle surface charge responsible for the repulsive energy barrier that controls the stability of dispersion and counteracts the closeness of particles and aggregation (Haeri *et al.*, 2014). Therefore, the high zeta potential in the liposome system was helpful for avoiding liposome agglomerates, thus maintaining the stability of the nanoliposome system. Figure 2 also exhibited that the morphology of the obtained AA-nanoliposome was almost in short rods and some in spherical shapes, as observed under a scanning electron microscope.

In vitro anti-cancer activity

The AA-nanoliposome (abbreviated as AAL) was evaluated for its anti-cancer activities against cervical cancer (Hela), liver cancer (HepG2), colon cancer (HT-29), and the human cancer stem cell line (NTERA-2). The results in Table 2 showed that the growth inhibitory activity of AAL depended on cancer cell lines and concentrations. The liposomal system expressed the effect on the different cell lines in the following order: NTERA2 > Hela > HepG2 > HT29, with IC_{50} of 80.13 ± 4.17 µM, 89.81 ± 4.41 µM, 93.94 ± 3.86 µM, and 95.29 ± 3.44 µM, respectively. Besides, AAL presented a weak effect against the growth of A549 human lung cancer cells, with IC_{50} value greater than 100 µM.

Table 2. The cell growth inhibitive capacity of AAL.

AA concentration (μM)	% Inhibition				
	Nanoliposome -blank				
	A549	**HepG2**	**Hela**	**HT-29**	**NTERA-2**
0	2.77±0.12	5.62±0.27	1.72±0.69	2.92±0.19	6.59±0.52
AA concentration (μM)	**AAL**				
	A549	**HepG2**	**Hela**	**HT-29**	**NTERA-2**
100	45.14±1.46	52.01±1.13	54.27±1.92	51.92±1.34	58.17±2.12
20	10.17±1.28	17.42±1.90	17.02±0.98	15.91±1.16	19.91±0.58
4	6.19±0.28	5.26±0.27	8.36±0.61	6.33±0.45	7.32±0.29
0.8	3.54±0.51	3.83±0.39	4.15±0.14	4.04±0.52	3.22±0.02
IC_{50}	>100	93.94 ± 3.86	89.81 ± 4.41	95.29 ± 3.44	80.13 ± 4.17

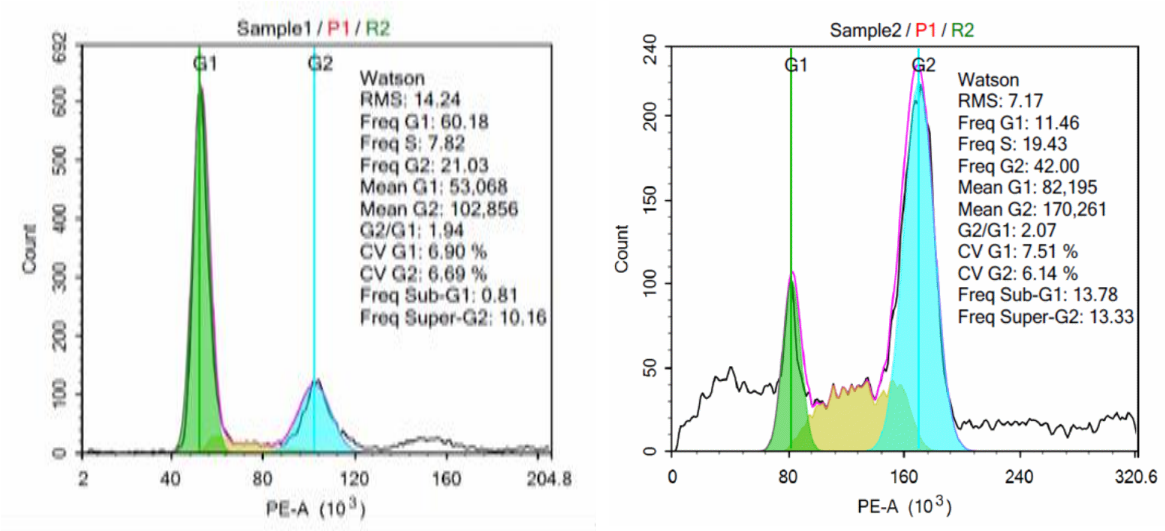
Effect of the nanoliposome-anacardic acid complex on the NTERA-2 cell cycle

Due to the fact that AAL exhibited the strongest anti-cancer activity on NTERA-2 cells, the nanocomplexes were further studied for their cell cycle arrest capacity on this cell line. Results from Table 3 showed that AAL significantly changed the phase proportions of the NTERA-2 cell cycle at both tested concentrations in comparison with the negative control. Specifically, the AAL harboring 100 μM of AA arrested cells in the G2/M and S phases had an increased percentage up to 42.00% and 19.43% compared to the negative control (21.03% and 7.82%), respectively. Meanwhile, the percentage of cells in G0/G1 was remarkably reduced to 11.46% compared to 60.18% of the negative control.

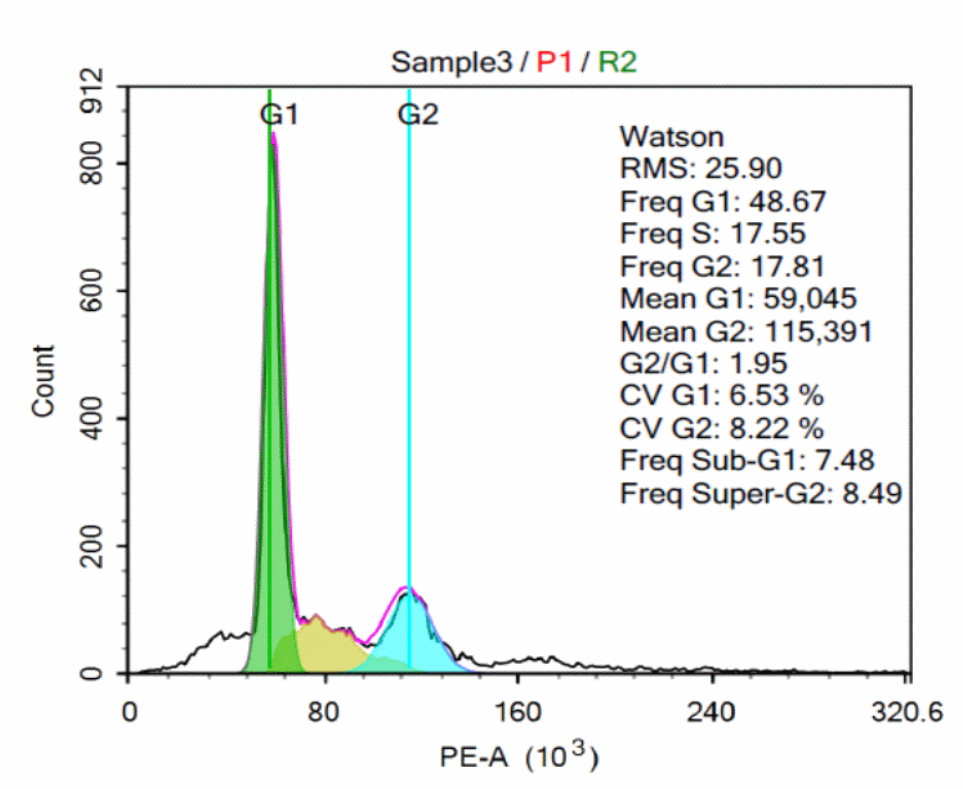
In contrast, cells that were incubated with AAL holding 20 μM of AA, had a slightly declining percentage of G0/G1, G2/M, and S proportions (Table 3). However, both tested concentrations of AAL increased the percentage of cells in the S phase, suggesting a potential apoptotic-inducing effect of the nanocomplexes.

Table 3. Percentage of NTERA-2 cells in different phases of the cell cycle.

Sample	Cells percentage (%)		
	G0/G1 phase	**S phase**	**G2/M phase**
Blank	60.18	7.82	21.03
AAL liposome (100 μM)	11.46	19.43	42.00
AAL liposomes (20 μM)	48.67	17.55	17.81



(a) **(b)**



(c)

Figure 3. The NTERA-2 cell cycle arrests under the treatment. (**a**) blank nanoliposome; (**b**) 100 µM of AAL; (**c**) 20 µM of AAL.

Natural compounds have been worldwide accepted as a potential medicinal source in drug development for the treatment of diseases in general and cancer in particular. However, only a small number of the natural compounds are used in clinical applications due to their poor bioavailability and undesirable side effects. By conjugating into nanoliposomal carriers, natural compounds could improve their solubility and bioavailability (Deshpande *et al*., 2013). In this study, anacardic acid was incorporated into nanoliposomes with high loaded efficiency, reaching 97.5%. The EPC as an ingredient in the fabricated formula presented a higher EE than using SPC as a component. According to Li *et al.* (2015), SPC and EPC are the two main sources of natural phospholipids. However, they do present some different characteristics, such as lecithin from egg yolks containing a higher amount of PC than in soybeans. In addition, egg yolk phospholipids, which are composed of long-chain polyunsaturated fatty acids of n-6 and n-3, mainly arachidonic acid (AA) and docosahexaenoic acid (DHA), that were absent from soybean lecithin, lead to different biochemical characteristics between them. Furthermore, the saturation level of egg yolk lecithin is higher than that of soybean lecithin, causing EPC oxidative stability to be better than that of soybean lecithin (Hager *et al*., 1993).

The obtained results in this study showed improved, promising anticancer activities of anacardic acid nanoliposome (AAL) against a category of cancer types, such as NTERA-2 cancer stem cells, hepatocarcinomas, cervical cancer cells, and colon cancer cells. AAL showed the highest activity against cancer stem cell lines (NTERA-2) with the lowest IC_{50} values of 80.13 ± 4.17 µM. AAL at both concentrations of 100 µM and 20 µM anacardic acid harboring also increased the percentage of S phase of the NTERA-2 cell cycle. All those results together suggest a potential anticancer capacity of the AAL complex in general and also target cancer stem cells as well. The compound might regulate the expression of several genes involved in cell cycle, apoptosis, and signaling (Hsieh *et al*., 2011).

CONCLUSIONS

In this study, anacardic acid was incorporated into the nanoliposomal complex by the thin-film method with the highest encapsulated efficiency of 97.5% with egg lecithin in the fabricated components. The formulated AAL had a short rod form with a size of 135.2 nm, a PDI index of 0.095, and a zeta potential of -27.9 mV. The anacardic acid nanoliposomes showed growth inhibitory activity against several cancer cells, with IC_{50} values ranging from 80.13 to 95.29 µM of anacardic acid. The strongest activity was presented on the NTERA-2 cancer stem cell line. The nanocomplex also exhibited a better effect on the NTERA-2 cell cycle proportion, suggesting potential apoptotic induction-activity.

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REFERENCES

Aguilar-Pérez KM, Avilés-Castrillo JI, Medina

DI, Parra-Saldivar R, Iqbal HMN (2020) Insight into Nanoliposomes as Smart Nanocarriers for Greening the Twenty-First Century Biomedical Settings. *Front Bioeng Biotechnol* 8: 579536.

Andayanie WR, Nuriana W, Ermawaty N (2019) Bioactive compounds and their antifeedant activity in the cashew nut (*Anacardium occidentale* L.) shell extract against *Bemisia tabaci*, (Gennadius, 1889) (Hemiptera: Aleyrodidae). *Acta Agric Slov* 113: 281–288.

Araújo JTC, Lima LA, Vale EP, Martin-Pastor M, Lima RA, Silva PGB, Sousa FFO (2020) Toxicological and genotoxic evaluation of anacardic acid loaded-zein nanoparticles in mice. *Toxicol Rep* 7: 1207–1215.

Bogachek MV, Park JM, De Andrade JP, Lorenzen AW, Kulak MV, White JR et al. (2016) Inhibiting the SUMO pathway represses the cancer stem cell population in breast and colorectal carcinomas. *Stem Cell Rep* 7(6): 1140–1151.

Deshpande PP, Biswas S, Torchilin VP (2013) Current trends in the use of liposomes for tumor targeting. *Nanomedicine* 8: 1509–1528.

Gogoi M, Kumar N, Patra S (2016) Multifunctional magnetic liposomes for cancer imaging and therapeutic applications. In: Holban AM, Grumezescu G, editors. Nanoarchitectonics Smart Delivery Drug Targeting. Elsevier; Amsterdam, The Netherlands. pp. 743–782.58.

Haeri A, Alinaghian B, Daeihamed M, Dadashzadeh S (2014) Preparation and characterization of stable nanoliposomal formulation of fluoxetine as a potential adjuvant therapy for drug-resistant tumors. *Iran J Pharm Res* 13(Suppl): 3–14.

Hager AA, De Paoli T, Ihlo JE, Farach HA, Poole Jr CP (1993) Stability study of lecithin liposomes during storage using ESR. *Spectrochim Acta - A: Mol Biomol Spectrosc* 49: 1999–2005.

Hsieh CC, Hernández-Ledesma B, de Lumen BO (2011) Cell proliferation inhibitory and apoptosis-inducing properties of anacardic acid and lunasin in human breast cancer MDA-MB-231 cells. *Food Chem* 125(2): 630–636.

Li J, Wang X, Zhang T, Wang C, Huang Z, Luo X, Deng Y (2015) A review on phospholipids and their main applications in drug delivery systems. *Asian J Pharm Sci* 10(2): 81–98.

Liu Y, Yang EJ, Shi C, Mou PK, Zhang B, Wu C, Lyu J, Shim JS (2020) Histone Acetyltransferase (HAT) P300/CBP inhibitors induce synthetic lethality in PTEN-deficient colorectal cancer cells through destabilizing AKT. *Int J Biol Sci* 16(11): 1774–1784.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55–63.

Sung B, Pandey MK, Ahn KS, Yi T, Chaturvedi MM, Liu M, Aggarwal BB (2008) Anacardic acid (6-nonadecyl salicylic acid), an inhibitor of histone acetyltransferase, suppresses expression of nuclear factor-kappa B-regulated gene products involved in cell survival, proliferation, invasion, and inflammation through inhibition of the inhibitory subunit of nuclear factor-kappaB alpha kinase, leading to potentiation of apoptosis. *Blood* 111(10): 4880–4891.

Ten Cate R, Krawczyk P, Stap J, Aten J A, & Franken N A (2010) Radiosensitizing effect of the histone acetyltransferase inhibitor anacardic acid on various mammalian cell lines. *Oncol Lett* 1(4): 765–769.

Zhang B, Lai Y, Li Y, Shu N, Wang Z, Wang Y et al. (2018) Antineoplastic activity of isoliquiritigenin, a chalcone compound, in

androgen-independent human prostate cancer cells linked to G2/M cell cycle arrest and cell apoptosis. *Eur J Pharmacol* 821: 57–67.

Zhao Q, Zhang X, Cai H, Zhang P, Kong D, Ge X, Du M, Liang R, Dong W (2018) Anticancer effects of plant derived Anacardic acid on human breast cancer MDA-MB-231 cells. *Am J Transl Res* 10(8): 2424–2434.