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GRAVIOLA (ANNONA MURICATA) EXTRACT LOADED ON CHITOSAN NANOPARTICLES MODULATES HDACS IN TONGUE SQUAMOUS CELL CARCINOMA

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ABSTRACT

Background: Oral cancer is considered the 6th most frequent cancer type worldwide. Different approaches were proposed to tackle the disease, though the traditional therapies failed to successfully treat the disease. Hence, natural medicines became under focus for their potential anticancer properties utilizing emerging concept of HADCs inhibition that offered potential strategy for cancer therapy.

Methods: In the present study, the efficacy of Graviola extract (*Annona muricata*) loaded on chitosan nanoparticles (CNPs) was assessed for its potential role in modulating HDACs in tongue squamous cell carcinoma (SCC-25). Cells were treated with *A. muricata* loaded on CNPS (A-CNPs) and emetine (as a reference drug) loaded on CNPS (E-CNPs), and HDACs 1, 2, 3, 6, and 8 proteins were quantified using Simple Step ELISA™ and The intensity was measured by ROBONIK P2000 ELISA READER at 450 nm.

Results: A-CNPs revealed a significant decrease ($p<0.000$) in the expression of HDAC1, 2, 6, and 8 in SCC-25 cells at values (7, 2.35, 1.23, 82.5 & 3 ng/mL, respectively) compared to control untreated cells (12.81, 4.03, 2.19, 86.99 & 10.453 ng/ml). Meanwhile, E-CNPs downregulated all HDACs more than A-CNPs.

Conclusion : Annona muricata loaded on CNPs was capable of downregulation of HDAC 1 and 2, 3, 6 & 8 in SCC-25 cell line which might implicate its potential role as anti-cancer agents through modifying HADCs expression.

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Clinical relevance : Understanding the role of HDACs in oral cancer might open the gate in front of novel natural drugs that have less side effects and high accuracy. The present study sheds light on the performance of A. muricata in controlling the propagation of OSCC in vitro, and this might be useful after extensive in vivo studies.

KEYWORDS: Annona muricate, Graviola, Emetine, Histone deacetylases, oral cancer

INTRODUCTION

Oral cancer (OC) is the sixth most frequent cancer worldwide [1], and it is one of the most prevalent tumors in high-risk regions of the globe. The majority of the recognized risk factors, including cigarette smoking, alcohol consumption, and betel nut chewing, are habits that raise the likelihood of contracting the disease. Due to its high mortality rate, early detection is of the outmost importance [2-4]. Traditional chemotherapies are not adequate in many cases, given the adverse effects and off-targeting, thus, the search for natural-based novel therapies is becoming a mandate.

The tropical evergreen fruit tree Graviola (*Annona muricata*) is cultivated worldwide. Aborigines in tropical Africa and South America employ graviola's bark, fruits, leaves, pericarp, seeds, and roots to treat a variety of illnesses, including cancer, with decoctions. In vitro and preclinical animal models are used to examine Graviola's capacity to target disease while preserving the survival of healthy cells. Graviola consists of about 212 phytochemicals. Graviola contains bioactive compounds such as annonaceous acetogenins, alkaloids, flavonoids, sterols, and others [5-7]. Anti-cancer activity of Graviola was ascribed to the presence of Acetogenin (ACG), a fatty acid that modifies numerous physiological processes, including suppression of epidermal growth factor receptor (EGFR), and downregulation of PI3K/Akt, RAS, NF-B, and JAK/STAT [8, 9]. This activity is significantly enhanced when the plant extract was formulated in nanoscale

Histone acetylation is a major process involved in gene expression[10]. histone deacetylase enzymes (HDACs) undo chromatin acetylation and modify expression and activity of several genes and proteins involved in both cancer initiation and progression [11] . There are 18 human HDACs grouped according to their sequence similarities, some of them were evidenced to be implicated in regulation of cancer cell proliferation, migration, angiogenesis, immunological evasion, and treatment resistance, making them important clinical targets[12]. HDAC inhibitors could be potential strategy to treat cancer. So far, some HDACi have gained the FDA's permission like Vorinostat, romidepsin and others for the treatment of many cancers [13-14].

Nanomedicine is a comprehensive sector of science and technology that integrate various zones of medical practice such as disease diagnosis, treatment, prevention providing innovative solutions for human health medicinal enhancement [15]. implementing the nanoscale technology insighted scientists for advanced an interdisciplinary approach to adopt the applications of biotechnology, nanomaterials, biomedical robustness, and genetic engineering incorporated below the broad heading of nanomedicine [16].

Chitosan is a natural nanostructured polymers that could act as scaffold that provide a favorable environment for tissue regeneration and temporary controlled release of signaling molecules [17]. It was previously used in different aspects in dentistry. It showed both a high biocompatibility and germicide character against oral biofilms. It has also been used

as a coating for poly(lactic-*co*-glycolic) acid for the delivery of ciprofloxacin for treating root canal infections. Chitosan nanoparticles recorded a lower cytotoxicity and DNA fragmentation compared to propolis [18].

Moreover, Kim et al. reported unique-structured nanohybrids through self-assembly of biomedical inorganic/organic nano-units, comprised of bioactive inorganic nanoparticle core (hydroxyapatite or bioactive glass) and chitosan cartridge . in general, the nanohybrids self-assembled with their distinctive features (ultrahigh inorganic content, nano-topography, high flexibility, numerous-medicinal delivery, and cellular triggering, can be considered as possible 3D tissue regenerative platforms[19].

Up to the authors knowledge there is limited literatures regarding the use of chitosan-based nanomaterials for encapsulation of bioactive compounds like graviola extracts relying on their high biocompatibility for many applications as multifunctional smart materials. As the nanoencapsulation with natural polymers has been documented as a encouraging approach to producing nanodrugs with sustainable stability and bioactivity [20].

Hence, the aim of the present study was to examine Graviola extract loaded on chitosan nanoparticles as potential inhibitor for different types of HDACs in oral tongue squamous cell carcinoma cell line.

MATERIALS AND METHODS

Cell culture

The human tongue squamous cell carcinoma cell line (SCC-25) was obtained from the American Type Culture Collection (ATCC). Cells were cultured using DMEM (Invitrogen/Life Technologies) supplemented with 10% FBS (Hyclone), 10 μg mL^{-1} of insulin (Sigma), and 1% penicillin-streptomycin. Cells were plated 24 h prior to treatment to reach at least 75% confluence.

Preparation of the loaded drug and treatments

In the present study graviola extract and emetine was loaded on nano-chitosan since it offers superlative chemical properties and protection of the loaded materials. The polycationic nature of CS makes it water-soluble and a bio-adhesive that easily couples to the negatively charged surfaces of cell membranes, hence, it amplifies the connection to surfaces promoting the time of union for perforation of drug particles to ensure drug perception inside culture cells according to the calculated concentration. Moreover, the biocompatibility and anti-microbial effect of chitosan made it very attractive candidate as drug carrier [21].

Loading *A. muricata* and emetine on chitosan NPs (CNPs) was performed according to the normal laboratory procedures and assessed by TEM. IC50 for both treatments was calculated. Chemicals and reactants were from Sigma and Invitrogen. SCC was cultured at 1.6 x 10^4 cells/well in a 6-well TC plate, then the cells were treated with either chitosan-loaded Annonaceae or chitosan-loaded emetine at different concentrations (0.39, 1.00, 1.56, 6.25, 25, and 100 μg mL^{-1}) for 48 h.

Cytotoxicity assay

The viability of SCC cells was assessed using 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT). After treatment, the old media were replaced with 150 mL fresh media and 50 μL MTT solution (5 mg mL^{-1} in PBS), and the plates were stored in a humidified atmosphere with 5% CO_2 for 4 h at 37°C. The developed formazan crystals were dissolved by adding DMSO (200μL/well), and then the plates were incubated for 30 min. at 37°C. The OD was read at 550 nm using a spectrophotometric microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

The tested compounds

A Annonaceae muricata leaves extract was purchased for the local market in Its pure formula (Graviola leaves extract/Soursop Leaves Extract (2oz)- by Purelife Herbs company, Egypt.

Emetine hydrochloride sterile solution was obtained from Sigma-Aldrich (Formula: C29H40N2O4· 2HCl, Molecular weight: 553.6 g/mol, CAS-No.: 316-42-7).

The main source of commercial chitosan is manufactured by deacetylation of its main component polymer chitin. Chitin comes from remnants of crustacean shells (shrimp, prawn, crab and lobster). The bioactive chitosan was bought from Sigma-Aldrich (Catalogue no. 448877, St. Louis, MO, USA). The level of deacetylation is 75–85% based on viscosity 200–800 cP at 1% acetic acid for medium-molecular- weight and 190–310 kDa [22].

Preparation of encapsulated NPs

Chitosan NPs were prepared by ionotropic gelation method which revealed good efficacy for targeted drug-delivery applications. *A. muricata* plant and emetine NPs were formulated as Nano-suspension with poly acrylic acid (PAA) and calcium chloride. PAA solution was prepared at a concentration of 0.05% (in water) with NaOH to pH 8 and 0.1% calcium chloride.

Chitosan mixture (1% (w/v) was prepared by mixing chitosan powder in distilled water (DW) containing 0.05 M NaCl with 1% acetic acid (w/v), then stirred overnight using a magnetic stirrer (300 rpm for 24 h) at room temperature. $CaCl_2$ solution (0.25% w/v) was prepared by dissolving $CaCl_2$ in DW. The pH of the chitosan mixture was modified to 5.5 using a 2 M NaOH solution. Afterward, it was purified by a 0.45-μm cellulose acetate filter to remove any unsolved chitosan. TPP (0.25 %) was liquefied in double-distilled water at a concentration of 0.25% by weight and purified through a 0.25-μm cellulose acetate filter.

The chitosan-CaCl2 mixture solution was added dropwise to the tested materials at serial concentrations. Chitosan/*A. muricata* and chitosan/emetine were stirred at 550 rpm for 30 min. The prepared NPs were rapidly centrifuged at 10,000 rpm for 15 min and then washed with distilled water. Finally, the prepared NPs were freeze-dried at -65 °C for 72 h. The NPs were stored in a desiccator to maintain their physiological stability until further analysis. NP suspensions were sonicated and sanitized with UV exposure prior to application.

Morphology of Chitosan Nanoparticles

The size and shape of nanoparticles was investigated by using Transmission electron microscopy (TEM) JEM-2100 high resolution at an accelerating voltage of 200 kV. Samples for TEM were developed by inserting a droplet of colloid suspension in the solvent on a Formvar carbon-coated, 300-mesh copper grid (Ted Pella) and permitting them to evaborize in air. Dispersal and average size were assessed using an image analysis software. Transmission electron microscopy (TEM) of micro@ CSNPs showing the spheroidal shape with size less than 100 nm particles [23].

Zeta potential analysis.

The encapsulated chitosan NPs were examined to ascertain mean average particle size & distributions and physical stability by Zetasizer ZS90 instruments (Malvern, UK) by suspending the nanoparticles in deionized water. The zeta potential was assessed by calculating the electrophoretic motility. All the procedures were done at 25°C [24] .

Encapsulation Efficiency (%)

The amount of graviola extract and emetine encapsulated in the NP was investigated utelizing a UV-Vis spectrophotometer (Cary series UV-Vis-NIR, Australia). After the dissociation of NPs from the commixture, the saturation of the supernatant was registered at 272 nm and the concentration of

free graviola extract was evaluated based on the standard curve of A[25]. Then EE % and LC were calculated according to the following equations:

Entrapment efficiency % =

$$\frac{intialConc.-freeconcentration}{intialConc} \times 100$$

Drug loading (%) = sum of drug in CSNP/Total weight of CSNP×100

Quantification of HDAC

Quantitative measurement of Histone deacetylase (HADC1, 2, 3, 6 & 8) in treated and untreated SSC cells was performed using Simple Step ELISA™, which implements affinity tag labeled capture antibody and a reporter conjugated detector antibody that immune capture the analyte in suspension. The generated signal was proportional to the amount of bound analyte. The intensity was measured by ROBONIK P2000 ELISA READER at 450 nm.

Cell cycle analysis

To identify the variables in the cell cycle upon treatments, cells were harvested by centrifugation at 600 rpm for 5 min in cold 70% ethanol, and then centrifuged again at 600 rpm for 5min and left at 4 °C for 2h. Cells were then handled with 50 µg mL^{-1} PI, 0.1% Triton X-100 and 50 µg mL^{-1} RNAse for 25 min and incubated at room temperature in a dark place. The PI fluorescence was calculated utilizing a FACScan flow cytometer (BD FACSCaliburTM). Information was studied to show the cell cycle distribution in the SCC-treated and untreated cells.

Apoptosis detection

To capture cell death, treated and untreated SCC cells were gathered after treatment and resuspended in 100 µL Annexin V binding buffer and 5 µL Annexin V Alexa Fluor 488. After 15 min incubation in the dark, 4 µL PI diluted in 1x Annexin V binding buffer (1: 10) was put on and incubated for 15 min in the dark. After incubation, 500 µL of Annexin V binding buffer was added on to wash the Annexin/PI-stained cells. Annexin/PI staining was monitored on flow cytometer (BD FACSCaliburTM). Annexin V-FITC binding was examined at Ex 1⁄4 488nm; Em 1⁄4 530 nm using the FITC signal detector (FL1) and PI staining by the phycoerythrin emission signal detector (FL2).

Statistical analysis

Variables presented as mean ± standard deviation (± SD). Analysis of variance and Tukey's HSD post-hoc multiple 2-group comparisons were employed. Two-sided *p-values* less than 0.05 was regarded as statistically significant. Statistical computations were done by IBM SPSS (IBM Corp, Armonk, NY, USA) release 22, Microsoft Windows. All experiments were performed in triplicate.

RESULTS

Characterization of the chitosan-loaded NPs

In the present study, CNPs were loaded by either *A. muricata* (A-CNPs) or emetine (E-CNPs) to enhance their efficacy in controlling SCC cells. Transmission electron microscopy (**Fig. 1**) revealed the expected sizes of the formulated A-CNPs (50-100 nm).

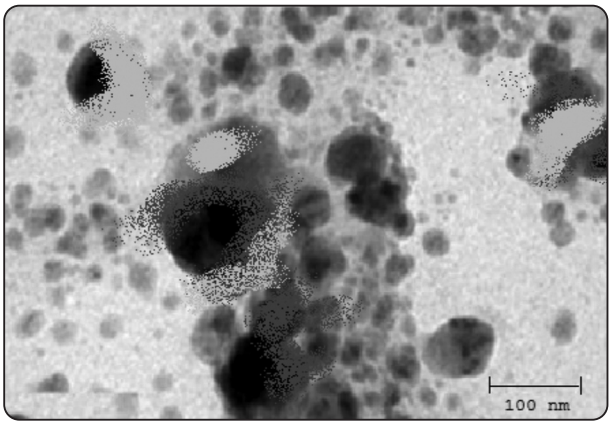

Fig. (1) TEM of A-CNPs shows the formulated compound at the expected size.

Cytotoxicity assay

Tongue SCC cells were treated with either Annonaceae or emetine loaded on CNPs. The IC-50 was 7.64 μg mL^{-1} for A-CNPs and 4.57 μg mL^{-1} for E-CNPs. (**Fig. 2**).

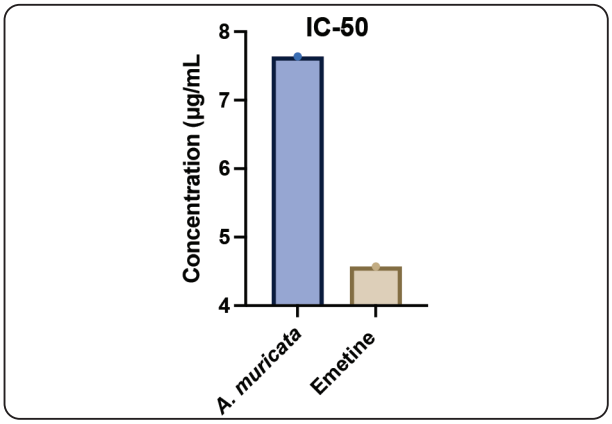

Fig. (2) IC-50 as calculated by MTT assay.

HDACs expression level

In the present study, tongue cancer cells (SCC-25) were treated with either A-CNPs or E-CNPs. HDACs 1,2,3,6 & 8 were assessed upon treatment, and the data showed a significant decrease in the expression of all tested HDACs (p = 0.000) at values for A-CNPs group ***(7.080, 2.35, 1.23, 82.54 & 3.0188 ng/ml repectively)*** Emetine, as a reference drug, has also decreased the levels of all HDACs ***(5.48, 2.06, 124, 69.66 & 3.63 ng/ml)*** compared to control untreated cells (**Table 1**). HADCs 1, 2, 6, and 8 were expressed at significantly lower levels in the loaded emetine treatment compared to the loaded Annonaceae treatment (**Fig. 3**). The untreated cells exhibited higher expression levels of HDAC6 (86.99 ng mL^{-1}) compared to the other HDACs 1, 2, 3 and 8 (12.8, 4.03, 2.19, and 10.45 ng mL^{-1}, respectively). Moreover, there was a significant reduction in all tested HDACs between Annonaceae and emetine except for HDAC3, which exhibited no significant variation between these two treatments (p = 0.429).

Cell cycle analysis

The distribution of cell cycle phases was affected by treating SCC cells with A-CNPs or E-CNPs. Data revealed that A-CNPs treatment has resulted in arresting cells early at the G1 phase (67.76%) compared to the positive control (E-CNPs; 41.33%) and negative control (54.15%). Additionally, a large population of cells (23.89%) has been detected in the pre-G1 phase (**Fig. 4**).

TABLE (1) Multiple comparisons for HDAC expression within groups (Tukey's test).

HDACs	Groups	Mean ± SD	*P*-Value (CI 95%)
HDAC1	Control	12.8167±0.17	0.000 all groups
	A-CNPs	7.0820±2.53	0.000 (*A. muricata* to Emetine)
	E-CNPs	5.4863±2.44	
HDAC2	Control	4.03033±0.0633	0.000 all groups
	A-CNPs	2.35750±0.5	0.015 (*A. muricata* to Emetine)
	E-CNPs	2.06800±0.6	
HDAC3	Control	2.19533±0.142	0.000 all groups
	A-CNPs	1.23100±0.38	0.429 (*A. muricata* to Emetine)
	E-CNPs	1.12425±0.35	
HDAC6	Control	86.9900±0.836	0.000 all groups
	A-CNPs	82.5425±5.23	0.000 (*A. muricata* to Emetine)
	E-CNPs	69.6600±4.36	
HDAC8	Control	10.4533±0.155	0.000 all groups
	A-CNPs	3.0188±2.03	0.000 (*A. muricata* to Emetine)
	E-CNPs	3.6350±1.85	

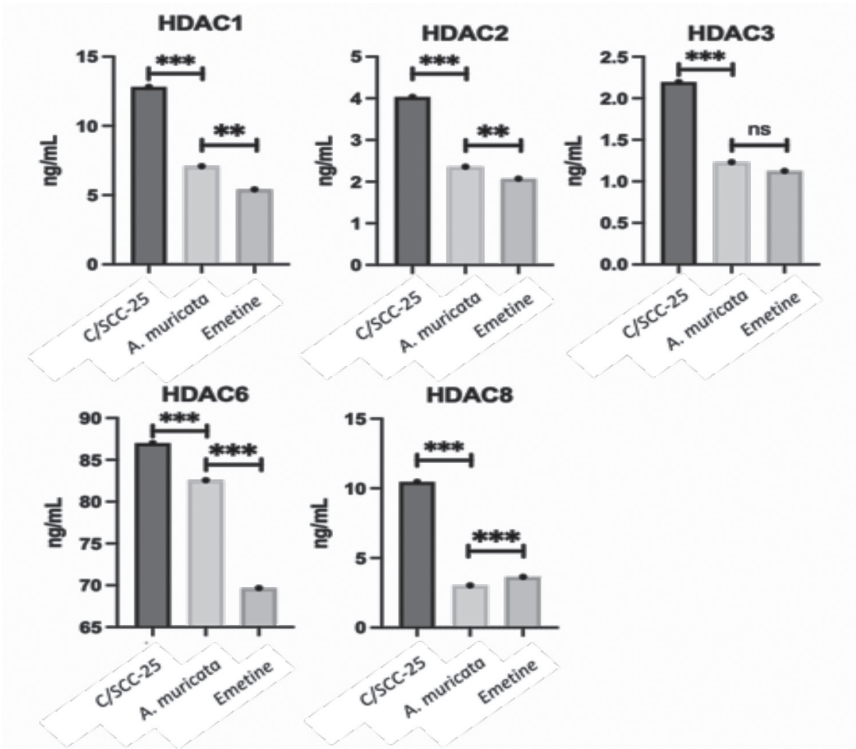

Fig. (3) The mean values of the expression profile of HDACs in different treatments and control.

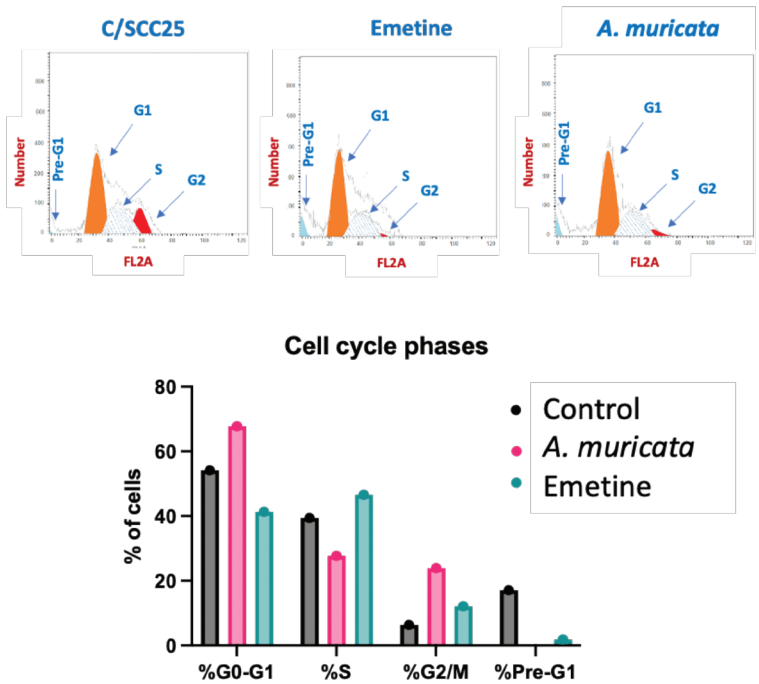

Fig. (4) Cell cycle analysis. DNA content in treated and untreated SCC-25 cells was quantified using flow cytometry.

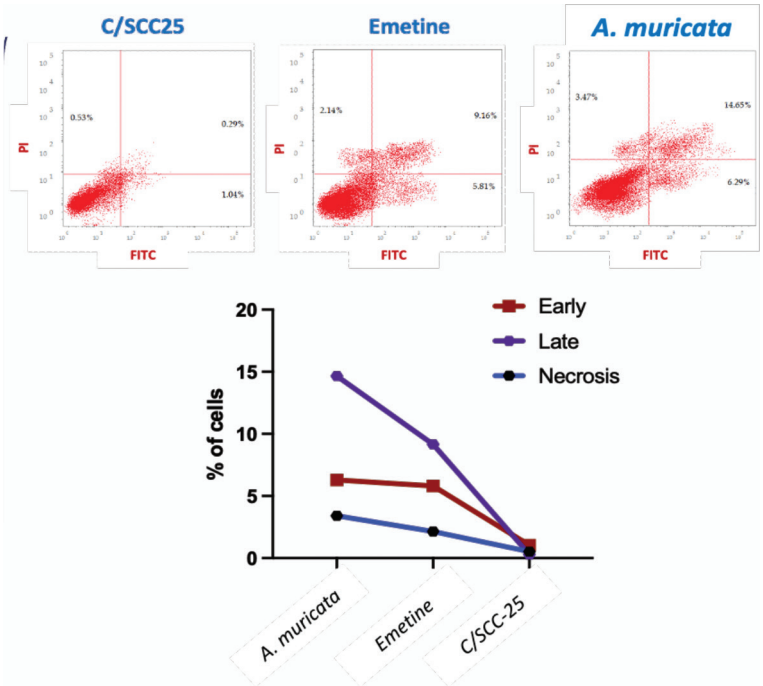

Fig. (5) Apoptosis detection. Total apoptosis of the treated and untreated SCC-25 cells was assessed using PI staining. Data showed that A. muricata treatment has resulted in higher late apoptosis percentages compared to emetine and control cells.

Apoptosis detection

Annexin-V/PI staining data (**Fig. 5**) showed that A-CNPs at 7 μg mL^{-1} promoted apoptosis as the percentage of cells that committed late apoptosis was 14.6%. Meanwhile, E-CNPs treatment has resulted in 9.16% late apoptosis, compared to untreated cells, which yielded 0.29% of the cells undergoing late apoptosis.

DISCUSSION

The most prevalent clinical therapies for oral cancer include surgery, radiation, and chemotherapy but still of reduced efficacy [26]. Histone posttranslational changes, which affect gene transcription, chromatin remodeling, and nuclear architecture, may contribute to cancer development. HDAC inhibitors (HDACi) represent an attractive rising target future drugs for therapy of many chronic diseases, especially cancer [27]

It has been suggested that medicinal plants offer alternative treatment options for various types of cancers, including oral cancers. *A. muricata* contains potential cancer-fighting chemicals, including alkaloids, phenols, and acetogenins [28]. In the present study, we evaluate the role of *A. muricata* in suppressing HDACs in SCC. The Data revealed that *A. muricata* has decreased the concentration of all HDACs ***(7.080, 2.35, 1.23, 82.54 & 3.0188 ng/ml)*** compared to contro***l (12.81, 4.03, 2.19, 86.99 & 10.453 ng/ml) at*** ($p = 0.000$) (**Table 1**). Emetine, as a reference drug, has also decreased the levels of all HDACs ***(5.48, 2.06, 124, 69.66 & 3.63 ng/ml)*** compared to control at ($p = 0.000$).

Best of our knowledge, no study was done assessing the modulation of graviola extract on HDACs but other studies in literature reported ability of natural compound like Resveratrol (RVT), a, bioactive polyphenolic compound used on hepatoma cell lines HepG2, Hep3B, and HuH7, resulted in block of the proliferation of all cell lines with significant impediment of HDACs, and hyperacetylation of the histones in HepG2 cells. [29]

HDACs regulate the work of genes for cell survival, proliferation, apoptosis, and metabolism[30], and downregulate several genes, including those involved in suppressing tumor formation[31], thus treatments that regulate HDACs might regulate cell proliferation and apoptosis. Our data indicated the percentages of cells undergoing late apoptosis were 14.65%, 9.16%, and 0.29% in *A. muricata*, emetine, and untreated SCC cells, respectively. This indicates that the antiproliferative effect of *A. muricata* was higher than emetine (the reference chemotherapeutic drug). The majority of HDACs are highly upregulated in different types of cancers[30], thus, the repressive effect of *A. muricata* on all HDACs studied nominates it to be a potent antiproliferative agent. Taking into consideration the role HDACs as regulators of the genes managing cell growth, cell death, and metabolism. Thus, *A. muricata* could then be regarded as a potential HDACi, which might be used in combinations to synergize the approved chemotherapeutic drugs [32].

In the line of our result, it was reported that reduction of HDAC 1 in tumor cells disrupted G_2/M shift and hindered cell division as revealed by suppression of dividing cells and raised number of apoptotic cells [33].

$p21^{WAF1/CIP1}$ is cyclin depedant kinase inhibitor which inhibit cell cycle progression . It was reported that when HDAC1 and 2 bind to the $p21^{WAF1/CIP1}$ genes, this bonding induces hypoacetylation and down regulation of $p21^{WAF1/CIP1}$expression, which permits cell cycle progression. Treatment by HADC inhibitors promoted acetylation of cyclin dependent kinase inhibitors resulting in cell-cycle block in G1 and hindrance of tumor expansion [34].

Different studies revealed that suppression of HDAC1, 2, and 3, was essential for FLIP inhibition and caspase-8 function and stimulation of apoptosis in NSCLC. They concluded that HDAC inhibitors promoted sensitization of malignant cells for TRAIL-dependent death mechanism [35].

Another study reported that HDAC 3 straight links with cyclin A and modulate its acetylation state which allow cell cycle S phase progression and mitosis. Disability of HDAC3 at metaphase change cyclin A acetylation by PCAF/GCN5, which promote its degradation resulting in inhibition of mitosis. The authors concluded that reducing HDAC3 leads to cell block in S and G_2/M phases due to loss of cyclin A stability [36]. Noteworthy, in the present study both annonaceae and Emetine NPs have the same reduction value on HDAC3 expression in scc-25 cells.

It was found that HDAC6 is upregulated in squamous cell carcinoma particularly at advanced stage where aberrant HDAC 6 overexpression fostering cell cycle propagation G1-S phase, angiogenesis and epithelial -mesenchymal transition. Moreover, the authors reported that the expression of HDAC6 was oppositely linked with survival ($P \leq 0.001$) [37].

Other species of Annonaceae such as *A. squamosa* also exhibited anticancer activity against breast cancer cells (MCF-7), colon cancer cells (CaCo-2), liver cancer cells (HepG2), and prostate cancer cells (PC3) [38, 39]. This may open the gate for revisiting natural medicine as a safe, cost-effective alternative to chemotherapy.

CONCLUSION

Annona muricata loaded on CNPs was capable of downregulation of HDAC 1 and 2, 3, 6 & 8 in SCC-25 cell line which might implicate its potential role as anti-cancer agents through modifying HADCs expression.

However, the limitation of the present study requires more investigations on different types of cell lines and solid tumors to confirm the anti-cancer effect of Graviola extracts and analyze its effect on epigenetic cellular response.

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Author contribution

SO, SA-G, UE-D, JA-H, GA-A: Conducted the practical work, SO: Wrote the final raft. SI and HS: Revised and approved the final draft.

Conflict of interest

The authors declare no conflict of interest.

Informed consent

Not applicable

Ethics Approval

This study was approved by the research ethical committee faculty of dentistry, Cairo university (24- 4- 23)

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