Synthesis, Characterization and Cytotoxicity Evaluation of *Syzygium Aromaticum* L. Bud (Clove) Essential Oil Nanoemulsion

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Research

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Abstract

Background: The present study performs to improve the medicinal properties of *Syzygium Aromaticum* L. by processing *S. Aromaticum* L. Bud essential oil (SABE) to the Nano Emulsion drug delivery system (SABE-NE) and investigate its anti-tumor and apoptotic impacts on human HT-29 colon cancer cells and to evaluate the toxicity of SABE-NE. Applying the ultra-sonication method and characterization by DLS and FESEM analysis facilitates the nano-emulsification procedure. Then both human cancer (HT-29) and normal (HFF) cell lines were evaluated based on the SABE-NE apoptotic and cytotoxic impacts. In an in-vitro section, flow cytometry method, Cas3 gene profile, AO/PI cell staining, and MTT assays apply to analyze the apoptotic and cytotoxic activities, and in further analysis measuring liver lipid peroxidation and antioxidant genes expression (SOD, CAT, and GPx) investigate in mice organs alterations.

Result: As a result, produced 131.2 nm SABE-NE induces apoptosis response and cellular death (Cas3 up-regulation and enhanced SubG1 peaks), and subsequently, the HT-29 cells' viability can reduce significantly, while HFF cells indicate confined cytotoxic impacts. Moreover, in-vivo test results on mice liver demonstrate the cytoprotective properties of SABE-NE (reduced lipid peroxidation and increased antioxidant enzymes gene expression and non-detectable cytotoxic impacts).

Conclusion: We produced a novel nature nanoemulsion drug delivery system called SABE-NE, which is cell-specific apoptotic inducer and thus can be utilized as an efficient anti-cancer compound for human colon cancer treatment. However, to verify and approve its cell-specific anti-tumor activity, further supplementary studies are required.

Background

Colon cancer is a third common malignancy and counts as the fourth cancer-associated death cause worldwide. It seems to have, 700,000 colon cancer death among about 1,400,000 patients, globally [1]. Previous studies have used several types of therapy strategies such as radiotherapy, surgery, and chemotherapy for colon cancer treatment.

Despite the numerous undesirable side effects of chemotherapy in liver and kidney organs, unfortunately, the method is widely applied as the only tumor-suppressive tool in colon cancer therapy. Based on researches, chemotherapy affects the liver by inducing lipid peroxidation and reduces renal functionality in the kidney [2]. Different kinds of bio-compatible phytochemicals have been used considerably as an alternative remedy instead of harmful chemical drugs in cancer therapy [3]. Due to the ample content of phenolic structures in *Syzygium Aromaticum* L. (clove) essential oil, it can be used as an edible antioxidant and antibacterial compound [4]. Previous approaches have been reported its medicinal role against bacterial and fungal disease; likewise, lung and breast cancers [5]. Researchers have applied a variety of anticancer compounds and drug delivery systems successfully in the field of cancer therapy by strategies that increase drugs' efficiency and specificity such as nanoemulsionalization techniques.

Nanoemulsion systems improve the anticancer compound's safety and efficiency by encapsulating toxic or therapeutic agents which makes them highly bio-compatible.

These bio-compatible nano-droplets have been utilized for enhancing Phyto-compounds (plants' essential oil substances) solubility and bioavailability, which increase the efficiency and safety of therapeutic compounds [6]. Nanoemulsions are a 20-200 nm transparent/opaque nano-droplets consisting of polar/nonpolar bioactive compounds and emulsifiers [7]. Regarding the nanoemulsion formation principles, ultrasonic-based emulsification is a common high energy-consuming strategy ($\sim 108 - 1010$ Watt per kilogram (W.kg^{-1})). However, high-pressure homogenization and spontaneous emulsification are two distinct methods applying in nano emulsification [8]. Ultrasonication is a simple, fast, clean, and cost-effective procedure for nano emulsification [9]. The emulsions disintegration usually performs in two methods: 1- Erupting oil into the water which makes interfacial waves in an acoustic field; 2- Suspension agitation by low-energy waves. A generalized principle of nanoemulsion systems formulation is Hydrophobic encapsulation in two stages of suspension liquid Nonpolar-Dispersed Phase (DNP) and Polar-Continuous Phase (PCP) [10]. These two widespread steps are triggered in the pharmaceutical industry as well as the bio-compatible efficient delivery systems [11]. Augmentable stability and quickly disruption are nanoemulsions properties which can be programmed to release their constituents surrounding the cells [12]. Therefore, they have been used prevalently for delivering therapeutics into cancer cells and inducing apoptotic death in lung and breast cancers [13]. Apoptotic Cas-3 gene is a well-known marker of apoptosis and down-regulated in cancer cells. Moreover, several kinds of research support its main role in inducing apoptotic death. In this regard, *Syzygium Aromaticum* L. (Rutaceae) essential oil nanoemulsion (SABE-NE) is produced to investigate their anticancer and cytotoxic impacts on human colon cancer cells (HT-29) and compare their histopathologic impacts in the mice Liver, Kidney, and Jejunum tissues.

Results

In-vitro section

SABE oil properties

The SABE oil extraction analysis shows 5.1 ± 0.26 percent oil content among the bud part. According to the GC-MS analysis, SABE oil has various phytochemical contents including Eugenol (69.8 %), Caryophyllene (9.1%), Eugenyl acetate (5.2%), and Humulene (7.3%), which have been shown to have anti-cancer activity (31-34) (Table 1A, B).

Table 1

The *Syzygium aromaticum* L. bud essential oil properties and processing data. A) The *Syzygium aromaticum* L. bud essential oil content; B) The phytochemical composition of *Syzygium aromaticum* L. bud essential oil; C) The nano-emulsion characteristic of *Syzygium aromaticum* L. bud essential oil. SABE: *Syzygium aromaticum* L. bud essential oil

A The SABE content		
Plant material		Essential Oil Content (%)
Syzygium aromaticum L. bud		5.1±0.26
B The phytochemical composition of SABE		
Peak	Compound	% of total
1	Eugenol	69.8
2	Caryophyllene	9.1
3	Humulene	7.3
4	Eugenyl acetate	5.2
C The nano-emulsion characteristic of SABE		
Particl size (nm)	Poly dispersity Index	Zeta potential (mV)
131.2	0.248	-42.1±0.68

SABE-NE characterization

Figure 1A indicates the SABE-NE size dispersion by distributions intensity. The DLS indices (DPI and Z-average), stability index (Zeta potential), and morphology features have been shown in Table.2C and Fig. 1B. According to the results, 30 minutes of ultrasonication with 750 W power makes 131.2 nm emulsions with sufficient 0.248 DPI value (Table 1C) (35). The emulsions zeta potential was measured at -42.1±0.68 mV, which means an appropriate good degree of stability for SABE-NE regarding B. Salopek et al recommends (36). Also, the morphology results of FESEM microscopy indicate the pseudo-spherical shape for SABE-NE nanoemulsions (Fig. 1B).

Cytotoxic impacts of SABE-NE on HT-29 colon cancer cells

A meaningful dose-dependent correlation among cancer HT-29 cell line and SABE-NE treatment concentrations is observable (Fig. 2) (p-value = < 0.001), while there is no notable relationship between normal HFF cells and SABE-NE. The 48-hours-IC₅₀ value of SABE-NE has been calculated at 74.8 µg/mL. It can be explained that the SABE-NE has a cell-specific cytotoxic impact on HT-29 colon cancer cells.

The Cas-3 gene expression in HT-29 colon cancer cells

A significant Cas-3 up-regulation following increasing doses of SABE-NE in treated cells shows a dose-dependent impact of it on HT-29 cells (Fig. 3 ($p < 0.001$)). The results indicate a notable apoptotic activity of SABE-NE in human HT-29 colon cancer cells.

Flow cytometry data and AO/PI–fluorescent imaging of HT-29 cells

According to the results (Fig. 4), the cellular death in HT-29 cells seems to be an apoptotic type. There is a significant relationship between the increasing SABE-NE treatment concentrations and Sub G1 peaks ($p < 0.001$).

In-vivo section

The histopathology study in treated mice

Both Fig. 5 and pathologist report indicates no major cellular death in mice liver, kidney, and jejunum tissues. However, a meaningful jejunum villus, crypts, and goblet cell enlargement are observable in Table 2. Therefore, it can be described that SABE-NE has no cytotoxic influence on normal jejunum cells.

Table 2 Morphocharacteristic of mice received different concentrations of nanoemulsion from Syzygium aromaticum L. bud essential oil				
Jejunum morphocharacteristic	0 mg/kg body weight	10 mg/kg body weight	20 mg/kg body weight	SEM
Villus Height(μm)	284.4 ^c	316 ^b	346 ^a	3.23
Villus Width(μm)	72 ^c	80 ^b	87 ^a	2.16
Crypt Depth(μm)	75 ^c	99 ^b	110 ^a	4.81
Goblet Cells(μm)	2.1 ^c	2.8 ^b	3.4 ^a	0.21
SEM: standard error of the mean				
Means with different superscript letters differ significantly ($p<0.05$)				

Liver peroxidation and antioxidant gene expression profile in treated mice

The SABE-NE meaningfully reduces the peroxidation of lipids in the mice liver tissue (p -value= < 0.001) (Fig. 6). Therefore, it can be interpreted that it has cytoprotective effects on mice liver cells, which is appropriate in suppressing the side effects of chemical drugs.

The expression of antioxidant genes (SOD, CAT, and GPx) in mice liver

The genes expression profile of mice liver cells indicates a significant overexpression in antioxidant enzymes (SOD, CAT, and GPx) ($p < 0.001$) (Table 3).

Table 3
Antioxidant genes expression analysis in the liver of mice received different concentrations of nanoemulsion from *Syzygium aromaticum* L. bud essential oil.

Genes	0 mg/kg body weight	10 mg/kg body weight	20 mg/kg body weight	SEM
SOD	1 ^c	1.6 ^b	3.5 ^a	0.26
CAT	1 ^c	1.1 ^b	2.4 ^a	0.17
GPx	1 ^c	1.2 ^b	1.8 ^a	0.10
SEM: standard error of the mean				
Means with different superscript letters differ significantly (p<0.05)				

Discussion

To the best of our knowledge, this is the first study producing the SABE-NE as a novel optimized therapeutic system and studying its apoptotic, antioxidant, and cytotoxic, impacts on HT-29 human colon cancer cells comparing with mice liver, kidney, and jejunum cells. We analyzed the phytochemical contents of extracted SABE oil and synthesized the stable 131.2 nm pseudo spherical SABE-NE (Table 1,2 - Fig. 1) and investigate their meaningful cell-specific cytotoxic impacts on HT-29 human colon cancer cells compared with normal HFF cells ($p \leq 0.001$) (Fig. 2). Moreover, we detected a meaningful Cas-3 up-regulation and increased -Sub G1 peaks by enhancing SABE-NE treatment concentration in HT-29 cells ($p \leq 0.001$) (Fig. 3 and 4), which verify the apoptotic type of death.

Finally, the SABE-NE dietary treatment concentration impacts on mice liver, kidney, and jejunum cells were studied. The results indicate it has no remarkable negative impacts on the mentioned mice cells. According to results, SABE-NE not only improves the antioxidant enzymes (SOD, CAT, and GPx) mission in normal liver cells but also decreases the levels of lipid peroxidation. Moreover, it can enlarge the jejunum villus, crypt, and goblet cell size (Table 3 and 4) (Fig. 5 and 6). Therefore, we suggest that it could be used as an efficient natural cell-dependent anti-cancer compound.

Nanoemulsions as a safe bio-compatible efficient drug delivery systems have revealed a high degree of safety and amazingly modified the cancer therapy strategies [14]. Nanoemulsions as the drug carriers are used for safe-transporting therapeutics and phytochemical agents towards the cells. They improve their contents' efficiency and optimize their biological properties such as antibacterial, antioxidant, and anti-cancer activities [15, 16]. There are several studies utilizing the essential oils of various types of plants as therapeutic nanoemulsions such as thyme, eucalyptus, neem, lemongrass, clove oil, and citronella [15, 16].

Antioxidant and apoptotic activities are considered as the most two important properties of anti-cancer compounds, due to their potential of affecting on reactive oxygen specious (ROS) cellular levels. It is well known that whenever ROS levels increase and do not refine, a normal to cancer transformation reveals

and alters the gene expression profile in oxidative stress conditions [17]. In this regard, the most two main cellular response systems such as antioxidant defense systems and pathways of apoptosis response are programmed to be activated in stressed cell.

In other words, cancer cells try to neutralize reactive radicals by improving their antioxidant defense systems including up-regulation of CAT, SOD, and GPx. Moreover, they can suppress apoptotic pathways and thus apoptotic death by decreasing the expression of apoptotic genes. The accuracy of cell proliferation and growth is reliable because of the apoptosis pathways' responsibility, which is weakened in cancer cells [18].

Cas-3, as the effector of apoptosis response, is responsible for inducing death in response to increased amounts of ROS in stressed-cells. Its activation leads to revealing apoptotic morphology in cells including fragmentation of DNA and vesiculation of cells. Fortunately, the cytotoxic properties of various plant essential oils such as *Satureja khuzistanica* Jamzad, *S. zenkeri*, *Solanium spirale* Roxb, *Casearia sylvestris*, *A. lepidophyllus*, and *Cedrelopsis grevei*, have been approved to have an apoptotic impact on breast cancer cells by up-regulating Cas3 gene [19, 20].

Syzygium aromaticum L. (clove) due to its components including tannins, flavonol glucosides, and phenolic volatile oils (eugenol, acetyl eugenol) has the high degree of antioxidant activity among the other plants. The clove phytochemical compounds are known to act as a strong antioxidant, antiproliferative, antibacterial, antiseptic, and anti-inflammatory agent, which make it appropriate cancer chemopreventive compound [21], which are improvable by nanoemulsion drug delivery systems [11]. Nanoemulsions due to their individual capability in improving the solubility and efficiency of therapeutic compounds such as dicumarol, tetanus toxoid, insulin, and anti-cancer agents in the drug delivery process, have widely attracted researchers' attention in pharmaceutical industries and the field cancer therapy [22, 23].

Eugenol (4-allyl (-2-methoxy phenol)) as a natural phenolic compound in honey and *Syzygium aromaticu*, *Cinnamomum verum*, and *Pimenta racemosa* has been approved to be served as pin reliever, antiseptic, antibacterial, antiviral, antioxidant, and anti-inflammatory compound. Also, eugenol has apoptotic activity on diverse cancer cell lines including mast cells, melanoma cells, and HL-60 leukemia cells [24, 25].

The antioxidant enzymes (SOD, CAT, and GPx) overexpression in mice liver tissue after treatment doses of SABLE-NE approves its cytoprotective role in normal liver cells. Saravana Kumar Jaganathan et al demonstrated that eugenol induces apoptotic death in human HT-29 colon cancer cells by up-regulating the apoptotic genes such as caspase 3 and P53. They suggested eugenol as a natural chemopreventive compound in colon cancer treatment.[26] Also, Kim et al investigated the apoptotic activity of eugenol on G361 melanoma cells by detecting Caspase 3 protein after the cells' treatment with eugenol [27]. In this regard, we investigated that SABLE-NE can reveal apoptotic activity and thus induce cellular death in HT-29 colon cancer cells. Up-regulation of Cas3, SubG1 peaks enhancement, and fluorescent AO/PI imaging indicate apoptotic death in HT-29 cells. Moreover, its impacts on antioxidant enzymes (SOD, CAT, and

GPx) status in the treated mice liver approve its cytoprotective effects on normal mice liver cells, which support the cause of lipid peroxidation reduction in such cells.

Conclusion

According to results, the novel safe natural SABE-NE significantly induced cell-dependent apoptotic death by up-regulating caspase 3 in the HT-29 colon cancer cell line. We suggest SABE-NE as a novel efficient drug delivery system containing eugenol and additional bioactive agents existing in clove essential oil to be used in colon cancer treatment approaches. However, further cancerous and normal cell lines are required to verify the nanoemulsions specificity.

Material And Methods

Chemical material

The nano emulsification and cell culture material including the polysorbate 80 and 20, Fetal Bovine Serum (FBS), MTT [3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide], trypsinized- DMEM culture medium, polyethylene glycol (PEG), antibiotic, and acridine orange dye were purchased from Merck (Germany). Cancer (HT-29) and normal (HFF) cell lines were prepared from the Pasteur Institute of Iran (Tehran, Iran). All reagents and solvents not mentioned in this paragraph were bought from Sigma (Germany).

SABE oil extraction and purification

The *Syzygium Aromaticum* L. Bud (SAB) was dried and powdered. 100 g of SAB was added in 500 ml double distilled water. The solution was boiled 120 minutes to collect the volatile oil using the Clevenger apparatus. Following that, CaCl₂ was used to dry the extracted oil [28]. SAB essential oil (SABE) was collected and weighted. Finally, the oil phytochemical contents were analyzed by using gas chromatography-mass spectroscopy (GC-MS).

The preparation of SABE-NE

To prepare CABE-NE, we mixed water (97 g) with CAE oil (3 g). The ratio of essential oil to non-ionic surfactant was 1:3 (v/v). Then, the ultrasonication was applied to homogenate emulsions at 20kHz ultrasonic frequency and 750 watts for 30 minutes sonication [29].

Nanoemulsions characterization

The polydispersity index (PDI) and the size of nanodroplets were estimated applying dynamic light scattering method (DLS) with a (Malvern Instruments Ltd., UK). The mean droplet diameter (Z-average) value was calculated based on the distributions' intensity. A Field Emission Scanning Electron Microscopy (FESEM) was used to define the morphology of SABE-NE. Moreover, the 1/100 diluted

samples were prepared for analyzing the nanoemulsion Zeta potentials, which were analyzed by a Zetasizer (nanoparticle SZ-100) at 25°C. All measurements were performed in triplicates.

Cell culture

The cancerous (HT-29) and normal (HFF) cell lines were cultured at 37°C in DMEM cell culture media supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 mg/mL).

MTT assay

The cytotoxic potential of SABE-NE on both cancer (HT-29) and normal (HFF) cells was checked in triplicate culture plates for 48 hours at different concentrations of SABE-NE (125, 62.5, 32, and 15 µg/ml). Then, the previous medium was removed and renewed with MTT (0.5 mg/mL)-supplemented media. The mediums passed 3 hours incubation at 37°C and renewed by media containing dimethylsulfoxide (DMSO). The last medium passed 10 minutes stirring. In the end, the absorbance at 570 nm was read for all samples utilizing plate reader spectrophotometer [29]. The cells viability was calculated by the following equation: Cell viability (%) = (OD sample/OD control) × 100.

Gene expression analyzing

Both cancerous and normal cell lines passed 10 hours of treatment at four different concentrations of SABE-NE (125, 62.5, 32, and 15 µg/ml) in triplet samples. Then, the RNeasy Mini kit (Qiagen, Hilden, Germany) was utilized to extract their transcriptome and their specific cDNA library was synthesized applying the Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany). To amplify cDNA, the primer sets of Cas-3 as the most known apoptotic gene and GAPDH as a famous control housekeeping gene were designed (Table 4). Finally, we applied an SYBR green PCR Master Mix (Qiagen, Hilden, Germany) to amplify cDNA and performed a comparative real-time PCR by applying a Stratagene Mx-3000P real-time thermocycler (Stratagene, La Jolla, CA). Moreover, we performed standard curves to verify the gene amplification process [29].

Table 4

The primer sets characteristics used in this study. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; Cas-3: Caspase 3; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase.

	Gene	Forward (5' → 3')	Reverse (5' → 3')
A549 cell lines	Cas-3	gctggatgccgtctagagtc	atgtgtggatgatgctgccca
	GAPDH	gaaggtgaaggtcggagtc	gaagatggtgatgggatttc
Mice tissues	SOD	gagacctgggcaatgtgact	gtttactgcgcaatcccaat
	CAT	acatggtctgggacttctgg	caagttttgatgccctggt
	GPx	caagttttgatgccctggt	tcggacgtacttgagggaat
	GAPDH	gactcaacagcaactccac	tccaccacctgttgctgta

Flow cytometry measurement and fluorescent staining (AO/PI)

The cells which were cultured for 48 hours were treated using different concentrations of SABE-NE (15, 32, 62.5, and 125 µg/ml). Then, after washing and mixing the cells with triton X100 (0.2%), Propidium iodide (PI), and sodium citrate (0.1%), the cells were incubated for 5 minutes at 37°C in dark condition. Finally, the cell cycle status was defined by a FACScan laser flow cytometer (FACSCalibur, Becton Dickinson, USA). The fluorescent staining was performed by mixing the fluorescent dyes (AO/PI) with washed-harvested cells. The cell status comparison was evaluated by a fluorescence microscope.

In vivo Study

The *in-vivo* study were designed by employing 15 female Balb C mice (20 ± 4 g), which were purchased from the BuAli research center of Mashhad, Iran. The animals' maintenance condition was as following instruments: 1- Animals were kept in cages; 2- the maintenance temperature and humidity were at 24 ± 1°C and 50 ± 10%, respectively; 3- Animals had 12-hour dark period intervals and standard Ad libitum feeding pellet diet and water. They were divided into 3 groups (n=5). To adopt mice with lab environment, seven days gap was considered before mice dietary treatment plane. A group receiving free- SABE-NE enriched-diet was defined as the control group, and the other 2 groups were received with two different dietary treatment doses of SABE-NE (10 and 20 mg/kg body weight). The experiment lasts for 30 days. All the research protocols used in this experiment were approved by the ethical committee of Azad university of Mashhad and the laws, norms, and regulations dealing with international animal ethics were followed (22nd September 2019).

Histopathological study, biopsy preparation, and samples staining

In order to study the histopathological impacts of SABE-NE, a portion of the mice liver, kidney, and Jejunum were sliced and washed twice by NaCl (0.9%) serum. After weighting tissue biopsies, the

samples were fixed by formalin solution (10%) and settled into the tubes. Then, the paraffinized- samples were sliced into 5 µm thin layer. Hematoxylin and eosin were utilized to stain the prepared samples as Cardiff et al have described [30]. Finally, an inverted microscope was used to study the morphology of the cells. An expert pathologist interpreted the morphology of the samples.

Antioxidant genes expression pattern of mice liver samples

The expression pattern of liver cell antioxidant genes (SOD, CAT, and GPx) was studied in the mice liver feeding with three different concentrations of SABA-NE (20, 10, and 0 mg/Kg body weight). The mentioned genes were analyzed by designing their specific forward and reverse primer (Table 4) according to the described protocol in the previous section.

Lipid peroxidation measurements

Lipid peroxidation measurements were performed as beyrami et al described [31]. Malondialdehyde (MDA) as a lipid peroxidation marker reacted with thiobarbituric acid (TBA) and generated reddish color observable at 532 nm. The intensity of color shows the concentration of MDA.

Statistics

All statistical measurements were calculated by SPSS 21 statistical software. ANOVA test analyzing was carried out and less than p-values less than 0.001 were defined as meaningful results.

Declarations

Ethics approval and consent to participate

All protocols to use the *in vivo* study were reviewed and confirmed by Ethics committee of Islamic Azad University of Mashhad, IRAN. All animal handling method was accomplished as per the regulations of the Islamic Azad University of Mashhad, IRAN.

Consent to participate

NA/Not applicable

Consent to Publication

Not applicable

Availability of data and materials

The datasets applied during the current study are available on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AVM, GRK, NS: Study design, experimental work, formal analysis and writing original draft; EK and EO: analysis, methodology, project administration, supervision, review, and editing of the original draft; All authors read and approved the final manuscript.

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Figures

Figure 1

Particle size properties. A) refer to the size dispersion of *Syzygium aromaticum* L. bud essential oil nano-emulsion analyzed by DLS (Size dispersion by intensity and volume); B) Morpho characteristic of

Syzygium aromaticum L. bud essential oil nanoemulsion analysed using FESEM. FESEM: Field Emission Scanning Electron Microscopy.

Figure 2

MTT assay data. A and B show the HFF and HT-29 cell lines viability, respectively. The cells were under different SABE-NE treatment doses. SABE-NE: Syzygium aromaticum L. essential oil nanoemulsion. “***” indicates the $p < 0.001$.

Figure 3

The expression analysis of caspase-3 gene in HT-29 cell line during 10 hours incubation with different concentrations of SABE-NE; SABE-NE: Syzygium aromaticum L. essential oil nanoemulsion; “ ***” indicates the p-value at less than < 0.001 levels.

Figure 4

The HT-29 colon cancer cells death type and cell cycle status after treatment with different doses of SABE-NE. A) The AO/PI staining of HT-29 cell line treated using different concentration of SABE-NE (125, 62.5, and 31.2 µg/mg) using fluorescence microscopy. B) Flow cytometry data indicating the subG1 values in A549 cell line treated with different concentrations of SABE-NE; SABE-NE: Syzygium aromaticum L.essential oil nanoemulsion

Figure 5

The histopathological analysis of liver, kidney and jejunum of the mice feed by different concentrations of SABE-NE (20, 10, and 0 mg/Kg). A, B, and C indicating the mice liver, kidney, and jejunum tissues alterations; SABE-NE: *Syzygium aromaticum* L.essential oil nanoemulsion.

Figure 6

The lipid peroxidation percentage in the mice liver feeding with different concentrations of SABE-NE; SABE-NE: *Syzygium aromaticum* L.essential oil nanoemulsion; Charts bearing different letters differ significantly ($p < 0.05$).