Biocatalysis and Agricultural Biotechnology 20 (2019) 101227

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Development, characterization and evaluation of cinnamon oil and usnic acid blended nanoemulsion to attenuate skin carcinogenicity in swiss albino mice

Alok Mukerjee, Himanshu Pandey, Abhishek K. Tripathi, Sunil K. Singh*

Department of Pharmaceutical Sciences, Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad, 211007, India

ARTICLE INFO

Keywords:
Cancer
Nanoemulsion
DMBA
Croton oil
Cinnamon oil

ABSTRACT

The objective of the present study was to formulate and characterize cinnamon oil and usnic acid blended nanoemulsion (CUN) and evaluate its efficacy against chemical induced skin carcinogenesis. CUN was prepared using ultrasonic emulsification method and prepared nanoformulations (CN1–CN9) were characterized for particle size, surface morphology, zeta potential, polydispersity index and transmittance. Anticarcinogenic effect of topical CUN was determined in DMBA/croton oil-induced skin carcinogenic swiss albino mice. Biochemical study was performed on isolated skin tissues homogenate of mice at the end of experiment (16th week). Histopathological analysis was performed to observe the effect of CUN on animals skin. Results exhibited that all the formulations were spherical and of nanosized range. Batch CN7 was considered as optimized formulation due to lowest particle size (96.39 nm), optimum zeta potential (-27.13 mV) and highest percentage transmittance (99.76%). CUN significantly reduce tumors in chemical induced carcinogenic mice. It restored all antioxidant enzymes in skin tissues. Histopathological study showed a minor disturbance in the skin architecture, reduced number and size of keratinized pearls, acanthosis and tumor in comparison to negative control group. It can be concluded from the study CUN may be used as an effective topical formulation to attenuate skin carcinogenesis.

1. Introduction

Cancer is a complex disease affecting people worldwide, influenced by an interplay between non-modifiable (genetic) and modifiable (environmental) risk factors (Wong et al., 2018). Exposure to ultraviolet radiation (UV) is the most common modifiable risk factor for skin cancer (Watson et al., 2016). Skin cancer is a very common forms of cancer occur due to excess exposure to ultraviolet (UV) light. Skin cancer can be malignant and non-melanoma (basal cell and squamous cell arcinomas) both are associated with intense sun exposure (Chummun; McLean, 2017). UV radiation triggers epithelial skin tumorigenesis processes that may cause DNA damage and oxidative stress. It results in alteration of cell structure and functions due to inflammatory reactions (Awad et al., 2018). Oxidative stress activate inflammasomes (intracellular multiprotein platforms) that nucleat NLR (nucleotide-binding domain and leucine-rich repeat) family and secrete pro-inflammatory mediators (interleukin 1β and 18). It has reported that mutation in NLRP1 underlying the etiology of dermatological disorder and enhance susceptibility to cancer (Tang; Wang, 2016).

Medicinal plants constitute a common alternative for cancer prevention and treatment in many countries around the world (Mehta et al., 2010; Desai et al., 2008). Currently, there are numerous drugs are available in the market for cancer chemotherapy that exhibit cell toxicity, induces genotoxic, carcinogenic, and teratogenic effects in non-tumor cells (Philip, 2005). These side effects limit the use of chemotherapeutic agents despite of their high efficacy in treating target malignancy. Therefore, the search for novel drugs that are both effective and non-toxic bioactive plant products has been increased (Kinghorn et al., 2004). Cinnamon is obtained from the inner bark of genus Cinnamomum with most popular species of Cinnamomum zeylanicum of the Lauraceae family, it is native to Sri Lanka and Asian countries. It is commonly used as spice worldwide (Vasconcelos et al., 2018). Cinnamon oil has diverse biological and pharmaceutical applications such as antimicrobial, antidiabetic, antipyretic and antioxidant capacity (Lee and Balick, 2005; Ghosh et al., 2013). Usnic acid (UA), a low-molecular weight dibenzofuran derivative [2,6-diacetyl- 7,9-dihydroxy-8,9b-dimethyl-1,3[2H,9bH]-dibenzo-furandione] isolated by certain species of lichen. Usnic acid is widely distributed in lichen species of Usnea, Lecanora, Cladonia, Ramalina, Evernia and Parmotrema. Usnic acid possesses many biological and pharmacological

* Corresponding author.
E-mail address: meetsunilsingh@gmail.com (S.K. Singh).

https://doi.org/10.1016/j.bcab.2019.101227
Received 12 May 2019; Received in revised form 21 June 2019; Accepted 1 July 2019
Available online 02 July 2019
1878-8181/

applications such as antiproliferative, antimicrobial activity against human and plant pathogens, antiviral, antiprotozoal, anti-inflammatory, analgesic activity as well as cytotoxicity and antitumor activity (Campanella et al., 2002; Ribeiro-Costa et al., 2004). Nanoemulsions (NEs) are most efficient dispersed nanosystems of droplet size ranging to submicron size. Nanoemulsions play an important role in various biomedical applications like targeted drug delivery system, as cosmetics, and cell culture based technology (Hu et al., 2016; Jonkman et al., 2014). Thus, in present study nanoencapsulation of usnic acid and cinnamon oil was designed, therefore supply of alternative dosage form to improve the bioavailability of drugs allowing its usage in therapeutics. With this concept in mind, the main aim of the current study was to investigate the skin anti-carcinogenicity potential of cinnamon oil and usnic acid blended nanoemulsion.

2. Materials and method

2.1. Drugs, chemicals and reagents

Cinnamon oil, usnic acid, linseed oil, oleic acid, isopropyl palmitate, arachis oil, light liquid paraffin, isopropyl myristate, ethyl oleate, tween 80, span 20, tween 20, span 80, ethanol, isopropyl alcohol, propylene glycol and poly ethylene glycol 400 were purchased from Sigma-Aldrich, St. Louis, MO, USA. 7, 12-dimethylbenz (a) anthracene (DMBA) and croton oil were procured from Sigma-Aldrich, St. Louis, MO, USA. Chemicals and solvents were of analytical grade and distilled water was used throughout the study.

2.2. Preparation of CUN

The cinnamon oil and usnic acid blend (CUB) was prepared by mixing in 4:1 ratio in a beaker at room temperature. Cinnamon oil was itself used as an oil for nanoemulsion preparation, the solubility of CUB was determined in surfactants such as Span 20, Span 80, Tween 20, Tween 80 and co-surfactants like ethanol, propylene glycol, alcohol and polyethylene glycol 400. Nanoemulsion was prepared by the ultrasonic emulsification method. Based on solubility of CUB in surfactant and co-surfactant, Tween 80 and ethanol, respectively were selected for nanoemulsion preparation. In this method, accurately weighed quantity of CUB was dissolved in premeasured quantity of ethanol followed by addition of Tween 80. Ethanol and Tween 80 was mixed in different concentrations (S_{mix}), the mixture was homogenized using magnetic stirrer followed by addition of distilled water sufficiently to get a uniform, homogenized emulsion. Cinnamon oil and usnic acid blended nanoemulsion (CUN) was obtained by sonication of prepared emulsion for 30 min. Nine different batches of CUN (CN1–CN9) was prepared by varying concentration of S_{mix} as shown in Table 1.

2.3. Characterization of CUN

2.3.1. Globule size and morphology studies

Optical microscopy was used to determine globule size of CUN. Particle size analyzer (Hydro 2000 SM, Malvern Instruments, UK) was used for analysis of particle size distribution. Sample (1–2 ml) was placed in a disposable zeta cell. It was run to obtain the zeta size value at 25 °C. The analysis was performed in triplicate and then average value was calculated (Patel et al., 2013). Transmission electron microscopy (TEM) Hitachi (H-7500) was used to evaluate microstructure of CUN at room temperature. CUN photo-micrographs was obtained by drying on a microscopic carbon coated grid then viewed under microscope.

2.3.2. Zeta potential

Zeta sizer version 6.2 (Malvern Instruments, UK) was used to determine zeta potential of CUN. Briefly, sample (1 ml) was placed in a disposable zeta cell and analyzed by inserting palladium electrode in distilled water in the cuvette at 25 °C. The value of zeta potential was based on measurement of distribution of the electrophoretic mobility of particles using the laser Doppler velocity technique (Zainol et al., 2015).

2.3.3. In vitro release study

Franz diffusion cell (3.14 cm^2 diffusion area and 15.5 ml cell volume) was used to determine in vitro release profile of CUN. Franz diffusion cell is an artificial cellulose acetate membrane, clamped between the donor and the receptor chamber. This cell is filled with freshly prepared phosphate buffer solution of pH 5.5 and maintained at 37 °C ± 1 °C. The membrane was equilibrated to 10 mg of drug onto the donor side before application of the nanoemulsion. Aliquots (1.0 ml) were removed after definite time interval and analyzed for drug content after appropriate dilutions using UV visible spectrophotometer (Zainol et al., 2015).

2.3.4. Stability studies

Stability of prepared CUN was determined during storage for a period of 6 months at room temperature. CUN was studied for physical stability such as phase separation, accelerated centrifugation cycle, creaming and flocculation; and chemical stability such as drug content, particle size and zeta potential determinations after 6 months of storage.

2.4. In vivo skin anticarcinogenic effect of CUN

2.4.1. Preparation of gels for application on animal skin

CUB and CUN gels were prepared by soaking with carbopol 980 (0.5%, w/w) and glycerol (5%, w/w) in water for 2 h individually and the solutions were dispersed in distilled water using magnetic stirrer. The preparation of gel formulations of CUB and CUN were performed by centrifuging both the formulations individually at 2000 rpm for

Table 1
Preparation and characterization of Cinnamon oil and usnic acid blended nanoemulsion (CUN).

Batch	S_{mix} (%wt/wt)	Water (%wt/wt)	Globule size (nm)	PDI (%)	Zeta potential (mV)	T (%)
CN1	25	70	316.00 ± 3.12	0.315 ± 0.015	−25.69 ± 2.13	95.14
CN2	27	68	296.12 ± 2.11	0.251 ± 0.121	−31.49 ± 3.13	96.66
CN3	29	66	187.47 ± 3.27	0.221 ± 0.023	−26.42 ± 3.25	97.42
CN4	31	64	178.53 ± 2.64	0.215 ± 0.021	−31.05 ± 1.32	98.24
CN5	33	62	161.38 ± 3.22	0.196 ± 0.017	−29.13 ± 0.25	99.68
CN6	35	60	142.58 ± 3.54	0.171 ± 0.014	−29.34 ± 0.22	99.32
CN7	37	58	96.39 ± 2.38	0.250 ± 0.022	−27.13 ± 1.21	99.76
CN8	39	56	120.17 ± 2.73	0.271 ± 0.021	−28.21 ± 1.32	99.59
CN9	41	54	142.41 ± 3.45	0.294 ± 0.011	−29.30 ± 1.29	99.68

Data are expressed mean ± SD (n = 3), Cinnamon oil and usnic acid was used in the ratio of 4:1 (%, w/w); PDI, Polydispersity index; T, Transmittance.

20 min (Hussain et al., 2016).

2.4.2. Rheological evaluation of prepared CUN gel

The viscosity of prepared gel formulations were determined by using Brookfield DV-E viscometer. The viscosity of CUN gel was measured at 920 cP at 100 rpm. The spindle (TF S-64) was rotated at 10 rpm and pH was adjusted using triethyl amine at pH 7.

2.4.3. Experimental animals

Swiss albino mice weighing 20–30 g, 6–8 weeks old were selected for the study. Animals were procured from central animal house of United Institute of Pharmacy, Naini, Allahabad, India. The protocol was approved by Institutional Animal Ethical Committee (Approval no: UIP/IAEC/2014/April/22) and care was provided to the animals according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.4.4. Induction of tumor

Tumor was induced by applying 7, 12-dimethylbenz (a) anthracene (DMBA) and croton seed oil topically on dorsal side of animals. Hairs on caudal portion of animals were removed using electric clipper 2 days before starting of the treatment. DMBA was applied by dissolving at concentration of 100 µg/100 µl in acetone. Tumor initiation by DMBA was promoted by application of croton seed oil (100 µl) after 2 weeks. Croton seed oil was mixed in acetone to give a solution of 1% dilution and applied three times in a week, for the next 14 weeks (Sharma and Goyal, 2015).

All animals were observed daily and weighted weekly during the experimentation period of 16 weeks. Appearance of tumors of skin were examined and recorded at weekly intervals. Final evaluation was performed by taking into consideration only those tumors that persists for 2 weeks or more than that with a diameter more than 2 mm.

2.4.5. Experimental design

Animals were divided into five groups to observe the effect of CUN on DMBA/croton oil-induced oxidative and inflammatory responses. Each group containing 10 animals. Different grouping of animals were as follows:

Group – 1 Negative control; Acetone (100 µl) was applied topically to the mice of this group.

Group – II Positive control; DMBA (100 µg/100 µl of acetone) was applied once topically then after two weeks croton oil (100 µl, 1%, v/v) in 0.2 ml acetone was applied topically three times per week until the end of the experiment (up to 16 weeks).

Group – III CUB treated experimental group (peri-post initiation) Animals of this group treated with application of CUB (20µmol/mice) topically. Starting at 7th day prior to DMBA application and continuing throughout the experimental duration (i.e. 16 weeks).

Group – IV CUN treated experimental group (post initiation); The treatment pattern of DMBA and croton oil was same as that in Group – II. CUN (20 µmol/mice) by applied topically. Starting from the time of croton oil application until the end of the experiment.

Group – V CUN treated experimental group (peri-post initiation); Animal of this group treated with application of CUN (20 µmol/mice) topically. Treatment pattern was same as that in Group – III.

2.4.6. Morphology study

The morphology of tumors after treatment was determined on following criteria:

Cumulative number of tumors were estimated by observing the appearance of tumors till the completion of experiment. Percentage tumor incidence was determined by counting the number of tumors on each mice. Tumor yield was calculated by average number of tumors per mouse. Tumor burden was estimated by average number of tumors divided by each tumor-bearing mouse. Diameter and weight of each tumor was estimated at the end to the experiment. Body weight of each animals was measured weekly.

2.4.7. Average latent period

It is the time lag between application of drugs and appearance of 50% tumors. It was determined by multiplying developed tumors per week (F) by the number of weeks (X) and divided by the total number of tumors produced (N).

$$\text{Average latent period} = \frac{FX}{N}$$

2.5. Biochemical study

Animals were sacrificed after 16 weeks followed by excision of dorsal skin. The skin was washed thoroughly with NaCl (0.9%, pH 7.4). The excised skin tissues were weighted accordingly and kept dry. A tissue homogenate (10%) was prepared from the excised skin tissue sample in 0.15 M Tris-KCl (pH 7.4) to determine levels of different antioxidant enzymes.

2.5.1. Reduced glutathione (GSH)

GSH level in skin tissues was estimated as total non-protein sulphydryl group by the method as described by Moron et al. (1979). GSH level in tissues was expressed as µmol/g of tissue.

2.5.2. Lipid peroxidation (LPO)

LPO level in skin tissues was estimated spectrophotometrically using thiobarbituric acid reactive substance according to the method of Ohkhawa et al., 1979, with slight modification. LPO level in mice skin tissues was expressed as nmol/mg of tissue.

2.5.3. Catalase

The method of Aebi (1984), was used for estimation of catalase in skin tissues of mice. It was expressed as U/mg of tissue, where U was µmol of H_2O_2 disappearance/min.

2.5.4. Superoxide dismutase (SOD)

SOD level in excised skin tissues was estimated as method described by Marklund and Marklund (1974). SOD level was expressed as unit/mg protein.

2.5.5. Total proteins

Total protein in skin was measured by the method of Lowry et al. (1951). The level of total proteins in skin tissues was expressed by mg/g.

2.5.6. Glutathione peroxidase

The level of glutathione peroxidase in skin tissues was estimated by the method Lawrence and Burk (1976).

2.6. Histopathological study

The skin was excised out after 16 weeks (the commencement of treatment) for histopathological study and fixed in freshly prepared 10% formalin fixative for 24 h at 4 °C. Then, tissue was embedded in paraffin wax. A vertical section of skin (4 µm thick) was cut and stained using haematoxylin and eosin. Histological changes studied using a light microscope.

2.7. Statistical analysis

All values are represented as mean ± SD. The significant values were determined using an analysis of variance followed by Bonferroni test using Graph Pad Prism version 8.1. Data were significantly different at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in comparison to control group.

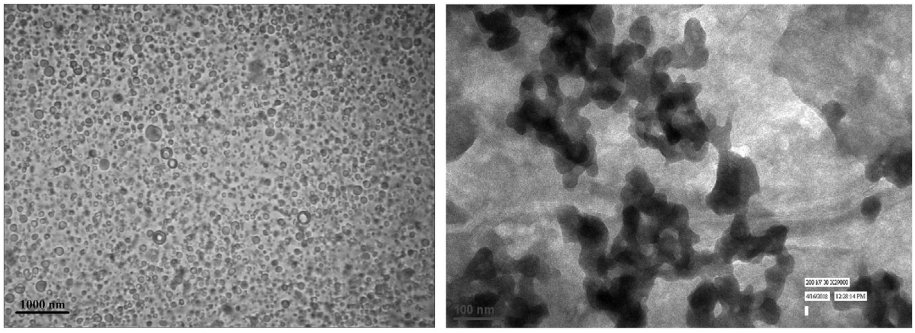

Fig. 1. Images of CUN globule. (A) Optical microscopy (B) TEM image.

3. Result

3.1. Preparation and characterization of CUN

Fig. 1A shows the optical microscopy of CUN. Results exhibited that the globules of CUN were spherical in shape and size of the globules were decreasing with increase in concentration of S_{mix} in the formulations (Table 1). Batch CN1 containing 25% of S_{mix} that showed globule size of 316 nm; however, least globule size was obtained with batch CN7 (96.39 nm) containing 37% of the S_{mix}. CUN globules were of nanosized range. Fig. 1B shows the TEM image of CUN. All nine batches exhibited nano size ranged particles that was well evident with low PDI values. PDI indicates uniformity of droplet size in the formulation. Batch CN7 showed optimum zeta potential values (−27.13 mV) with highest percentage transmittance (99.76%) with lowest droplet size. Thus, batch CN7 was selected as optimized batch containing 4% cinnamon oil, 1% usnic acid, 37% Smix and 58% deionized water as continuous phase. It was further selected for evaluation of anticancer studies. The process for the preparation of NE was optimized by varying the stirring speed at different time interval i.e. 10 min, 20 min and 30 min followed by the globule size determination. Based on the minimum globule size, a stirring speed of 800 rpm and stirring time of 30 min were selected as the optimized process parameters to obtain drug-loaded NE.

3.2. In vitro release study of prepared CUN

The maximum drug release (93.19%) from CUN was achieved within 8 h; however, 94% drug was release up to 24 h. The small globule size was one of the factors that contributed to the increased penetration of skin. The presence of surfactant i.e. Tween 80 also contributed to release higher percentage of drug from NE. Fig. 2 shows the graph of drug release from dialysis bag with respect to time.

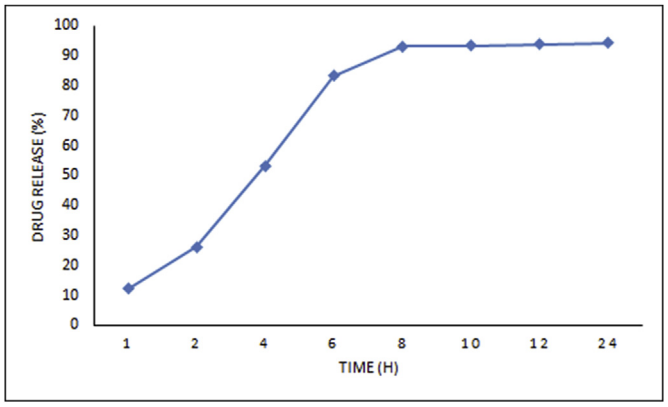

Fig. 2. Percentage drug release profile of CUN.

Table 2
Stability study of CUN nanoemulsion on storage.

Time (days)	Globule size (nm)	Polydispersity index (%)	Zeta potential (mV)
0	96.39 ± 3.34	0.250 ± 0.31	−27.13 ± 2.41
30	97.43 ± 2.21	0.251 ± 0.16	−27.01 ± 3.23
60	99.35 ± 2.55	0.254 ± 0.22	−26.12 ± 3.15
90	99.79 ± 3.75	0.254 ± 0.15	−26.01 ± 2.32
120	101.44 ± 3.02	0.256 ± 0.42	−24.14 ± 1.26
150	101.97 ± 2.32	0.256 ± 0.13	−24.08 ± 2.66
180	103.39 ± 3.77	0.257 ± 0.28	−24.05 ± 3.33

Data are represented as mean ± SD (n = 3).

3.3. Stability studies

Results showed that CUN was stable after 180 days regarding globule size (103.39 nm), zeta potential (−24.05 mV) and polydispersity index (0.257%) (Table 2).

3.4. Morphological study

Results shown that CUN was found effective in reducing skin carcinogenesis in mice (Table 3). Body weight of animals was reduced after application of DMBA/croton oil in negative control animals. Treatment with CUN resulted in increase of animals weight (32.43 g) week by week in comparison to control group animals (34.21 g). Fig. 3 shows the appearance of 100% tumors in chemical induced mice, however, no tumor was found in negative control animals (Fig. 3A). Animals of positive control group (Fig. 3B) showed highest number of tumors (39.36) that get reduced to 7 peri-post treatment with CUN (Fig. 3E). CUB (23.65) and CUN post (14.35) also showed reduction in number of tumors in mice (Figure – 3C and 3D, respectively). Tumor yield was restored (1.16%) and tumor multiplicity was reduced to 3.51 week after application of CUN peri-post. The average latent period in CUN treated mice was increased to 13.52 week after application of CUN peri-post.

3.5. Biochemical study

Topical application of CUN was significantly restored all the enzymatic level in the body (Table 4). Application of toxic chemicals for 16 weeks caused increase in the level of TBARS (131.25 m mol/100 g Tissue) that was reduced to 100.02 in CUN peri-post mice in comparison to control animals (98.75). However, the level of other antioxidant enzymes such as SOD (54.22 IU/mg Protein), CAT (42.44 µmol H_2O_2 consumed/min/mg Protein), GSH (1.45 µmol/mg Tissue), GPx (14.21) and Total protein (54.87 mg/g Tissue) was also decreased due to application of DMBA/croton oil in positive control animals; these antioxidant enzymes level i.e. SOD (61.89 IU/mg Protein), CAT (55.49 µmol H_2O_2 consumed/min/mg Protein), GSH (4.15 µmol/mg Tissue), GPx (32.67 nmol NADPH oxidized/min/mg Protein) and Total

Table 3
Chemopreventive potential of CUN on chemical induced skin carcinogenesis in mice.

Treatment groups	Body weight (g)		No. of tumor	Tumor yield	Avg. latent period (week)	Tumor multiplicity (week)	Tumor incidence (%)
	Initial	Final					
Negative control	25.27 ± 3.23	34.21 ± 3.43	——	NA	NA	NA	NA
Positive control	26.43 ± 2.14	23.84 ± 3.65	39.36 ± 4.58	6.56 ± 0.46	8.05 ± 0.02	6.58	100
CUB	25.23 ± 2.19	28.22 ± 3.87	23.65 ± 4.73***	3.83 ± 0.47***	10.95 ± 0.015***	5.75	66.6
CUN post	27.22 ± 2.43	30.37 ± 2.43	14.35 ± 4.04***	2.32 ± 0.40***	11.94 ± 0.034***	4.65	50
CUN peri post	26.21 ± 3.54	32.43 ± 3.28	07 ± 2.52***	1.16 ± 0.25***	13.52 ± 0.04***	3.51	33

Data are expressed mean ± SD (n = 3), significantly different at *p < 0.05, **p < 0.01, ***p < 0.001 in comparison to control group. CUB- Cinnamon oil and usnic acid blend, CUN- Cinnamon oil and usnic acid blended nanoemulsion.

protein (107.08 mg/g Tissue) were restored by topical application of CUN peri post. Likewise, CUB and CUN post groups also showing restoration of enzymatic levels in comparison to negative control animals.

3.6. Histopathological study

Histopathological investigations showed normal histology in the skin of negative control group (Fig. 4A) that is starting from the outer side keratin layer, to the epidermis, dermis and basal layer. Carcinogen treated positive control group indicated the occurrence of squamous cell carcinoma with dysplastic epithelium. Histopathological study showed disarrangement of skin layers in the form of hyperkeratosis, thickening of the epidermis (epidermal hyperplasia), erosion of the epidermis and dermal invasion. The sebaceous glands and hair follicles were also damaged. The tumors of positive control mice (Fig. 4B) showed large keratinized pearls, thickening of the epidermis (acanthosis), high infiltration of lymphocytes and invasion of epidermal cells in the dermis. Experimental Group CUB and CUN-post showed less damage to the skin, reduced hyperkeratosis and epidermal hyperplasia as shown in Fig. 4C–D, respectively. CUN-peri post treated mice restored disturbance in the skin architecture, reduced number and size of keratinized pearls, acanthosis and tumor (Fig. 4E).

4. Discussion

In the present study we have prepared cinnamon oil and usnic acid blended nanoemulsion for topical application against skin carcinogenicity. Nanoemulsions (NEs) are mixture of two immiscible liquids to form a single phase containing oil, water, surfactant, and co-surfactant systems as well as much larger surface-area-to-volume ratio (Teo et al., 2017). In our preparation, cinnamon oil was itself acting as oily phase; Tween 80 was surfactant and ethanol was selected as co-surfactant. NEs

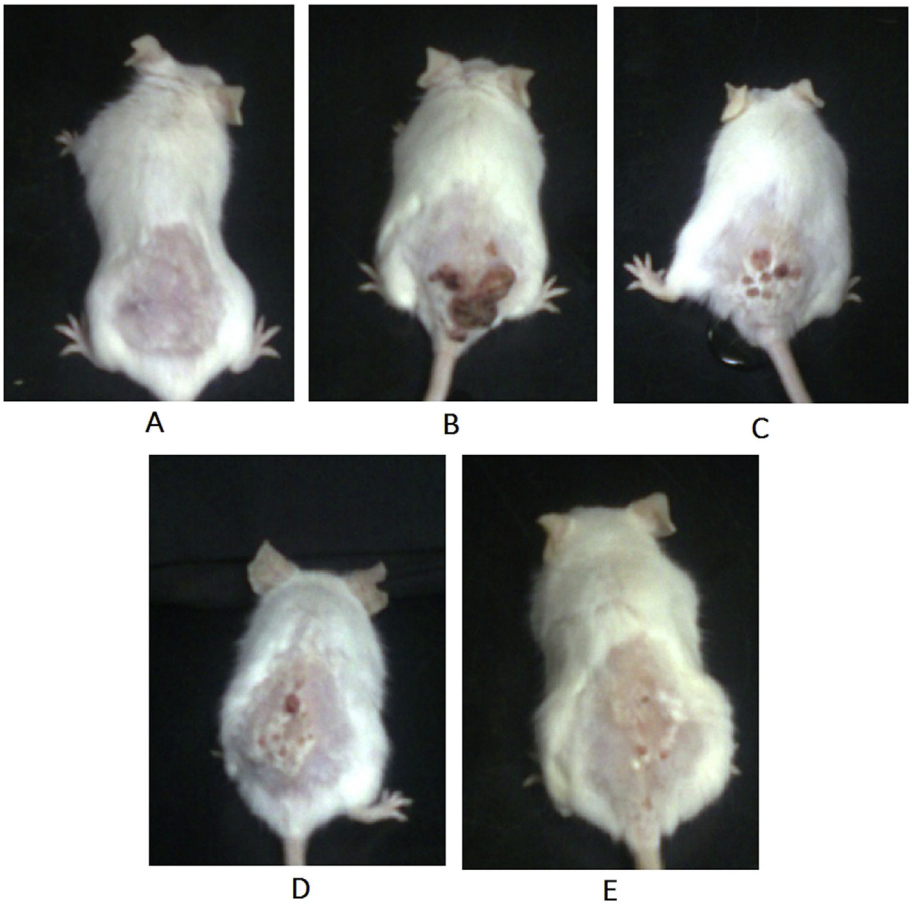

Fig. 3. Morphological changes in tumor appearance due to application of CUN. A = Negative control, B = Positive control, C = CUB, D = CUN post, E = CUN peri post.

Table 4
Biochemical parameters of cancer induced mice treated with CUN.

Treatment groups	TBARS	SOD	CAT	GSH	GPx	TP
Negative control	98.75 ± 5.36	67.64 ± 0.55	55.55 ± 4.17	5.09 ± 1.22	33.75 ± 3.32	103.71 ± 7.19
Positive control	131.25 ± 5.77***	54.22 ± 0.36***	42.44 ± 3.04***	1.45 ± 1.73***	14.21 ± 3.9***	54.87 ± 6.95***
CUB	121.05 ± 4.24*	55.28 ± 0.45*	47.83 ± 3.45**	2.77 ± 0.91***	22.14 ± 3.24***	70.16 ± 9.54**
CUN post	106.25 ± 2.79***	56.48 ± 1.13**	50.61 ± 3.17***	3.31 ± 0.81***	28.81 ± 8.06***	72.73 ± 12.77***
CUN peri post	100.02 ± 5.59***	61.89 ± 0.69***	55.49 ± 4.14***	4.15 ± 0.70***	32.67 ± 1.67***	107.08 ± 13.58***

Data are expressed mean ± SD (n = 3), significantly different at *p < 0.05, **p < 0.01, ***p < 0.001 in comparison to control group. CUB, Cinnamon oil and usnic acid blend; CUN, Cinnamon oil and usnic acid blended nanoemulsion; TBARS, Thiobarbituric acid reactive substances (m mol/100 g Tissue); SOD, Superoxide dismutase (IU/mg Protein); CAT, Catalase (μmol H_2O_2 consumed/min/mg Protein); GSH, Glutathione (μmol/mg Tissue); GPx, Glutathione peroxidase (nmol NADPH oxidized/min/mg protein); TP, Total Protein (mg/g Tissue).

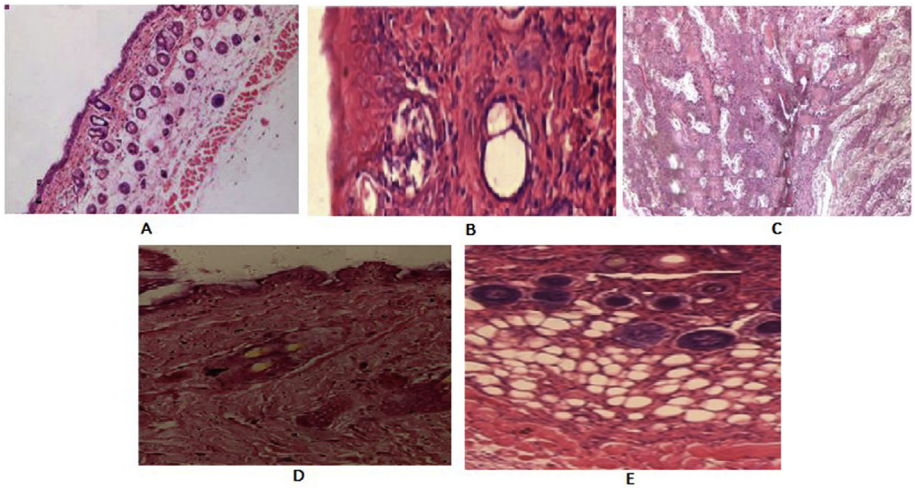

Fig. 4. Histopathology of cancer cells treated with CUN. A = Negative control, B = Positive control, C = CUB, D = CUN post, E = CUN peri post.

having the unique characteristics that include clear, very small droplet diameter (20–200 nm), higher optical clarity, thermodynamically stable, increased bioavailability, isotropic liquid mixtures (Tadros et al., 2004). However, visible transparency or translucency to the naked eye, good stability against aggregation of droplet (Kaur et al., 2017), and small the particle size (Khalid et al., 2017; Shanmugapriya et al., 2018). The particles size of CUN was 96.39 nm and other batches were also nano size ranged. CUN exhibited uniform spherical globules, highest percentage transmittance and lowest droplet size. The small globule size of CUN contributed to the increased penetration of skin as observed during in vitro release for 24 h. The presence of surfactant i.e. Tween 80 also contributed to the release higher percentage of drug from NEs. CUN was stable over six months observation; slight change in globule size (103.39 nm), zeta potential (−24.05 mV) and polydispersity index (0.257%) were noted.

CUN significantly reduced skin carcinogenesis in mice after 16 weeks. The topical application of CUN gel caused increase in animals body weight, decreased tumor diameters and numbers. Tumor yield was restored (1.16%) and tumor multiplicity was reduced to 3.51 week after application of CUN peri-post. Cinnamon oil possess antitumor and cytotoxicity activities through inhibiting cell proliferation as well as inducing apoptosis has been already reported (Ghosh et al., 2013; Meghani et al., 2018). The volatile oils of cinnamon are commonly collected from C. camphora, C. burmannii, C. cassia, C. verum, C. osmophloeum, and C. zeylanicum (Jayaprakasha and Rao et al., 2011). The common chemical constituents found in cinnamon oils are cinnamaldehyde, cinnamic acid, cinnamate, cinnamyl acetate, trans-cinnamaldehyde, camphor, eugenol, caryophyllene oxide, L-borneol, b-caryophyllene, α-terpineol, terpinolene, α-cubebene, and α-thujene (Senanayake et al., 1978; Vangalapati et al., 2012). CUN was containing blend of cinnamon oil and usnic acid incorporated into nanoemulsion formulation for topical delivery. Usnic acid is a lichen, generally found in the species of Usnea, Lecanora, Cladonia, Ramalina, Evernia and Parmotrema. It possesses several biological activities including antiproliferative and antimicrobial activities (Campanella et al., 2002). The synergistic effects of plant metabolites and their interaction at multiple points within the cell offer higher efficacy during chemoprevention process. Cinnamon oil has partially soluble in water and strong odor therefore bioavailability of drug is less (Donsi et al., 2012; Chuesiang et al., 2019). For overcoming this problem, colloidal nanoemulsions delivery systems have been developed to encapsulate cinnamon oils in small droplets however, it can be more easily utilized or accumulated in biological system (Komaiko and McClements, 2016; Ryu et al., 2018). Thus, incorporation into nanoemulsion formulation was an effective step to get the more bioavailability of oil over skin and deeper tissues.

Cancer is the major cause of death around the globe (Singh et al., 2018), the occurrence of skin cancer is much higher than other kinds of cancers (Linos et al., 2016). Malignant cells shows intense oxidative stress especially in the tumor microenvironment in comparison to normal cells (Bernardes et al., 2015). Antioxidants inhibits cell apoptosis occurs due to oxidative stress by removing oxidative free radicals, maintaining the homeostasis of the host immune system, circumvent ROS-mediated tumor cell damage and hence finding its way in cancer treatment (Block et al., 2008; Thyagarajan and Sahu, 2018). They neutralize electrical charge and inhibiting free radicals, taking extra electrons from other compounds (Bjelakovic et al., 2007). CUN significantly restored oxidative stress markers in animals tissues. The enzymatic levels of SOD, CAT, GSH, GPx and Total protein were restored by topical application of CUN peri post. Natural compounds acting as antioxidants are important in the chemoprevention of diseases and protect against UV-radiation (Kohlhardt-Floehr et al., 2010; Nadeem et al., 2018). It has reported that cinnamon oil is an effective antioxidant used for reducing oxidative stress (Iqbal et al., 2005) and usnic acid possess oxidative neutralizing capacity (Suwalsky et al., 2015).

Thus, CUN may be an effective topical formulation against skin cancer.

5. Conclusion

It can be concluded from the study that CUN possess significant anticancer and antioxidant properties in the pathogenesis of DMBA/croton oil-induced skin carcinogenesis while applying topically. It was demonstrated that prevention of tumor production by naturally occurring antioxidant compounds like cinnamon oil and usnic acid in nanoemulsion vesicles could be effective strategy for prophylaxis of DMBA/croton oil-induced skin carcinogenesis. Therefore, CUN can be a suitable candidate for clinical studies for development of drug skin carcinogenicity.

Declaration

The authors declare no conflict of interest.

Acknowledgement

The authors are thankful to the institute for providing all facilities required during the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101227.

References

Aebi, H., 1984. Catalase: in vitro. In: Colowick, S.P., Kaplan, N.O. (Eds.), Method in Enzymology105. Academic Press, New York, pp. 121–126.
Awad, F., Assrawi, E., Louvrier, C., et al., 2018. Photoaging and skin cancer: is the inflammasome the missing link? Mech. Ageing Dev. 172, 131–137.
Bernardes, S.S., de Souza-Neto, F.P., Ramalho, L.N., et al., 2015. Systemic oxidative profile after tumor removal and the tumor microenvironment in melanoma patients. Cancer Lett. 361, 226–232.
Bjelakovic, G., Nikolova, D., Gluud, L.L., Simonetti, R.G., Gluud, C., 2007. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. J. Am. Med. Assoc. 297, 842–857.
Block, K.I., Koch, A.C., Mead, M.N., Tothy, P.K., Newman, R.A., Gyllenhaal, C., 2008. Impact of antioxidant supplementation on chemotherapeutic toxicity: a systematic review of the evidence from randomized controlled trials. Int. J. Cancer 123, 1227–1239.
Campanella, L., Delfini, M., Ercole, P., Iacoangeli, A., Risuleo, G., 2002. Molecular characterization and action of usnic acid: a drug that inhibits proliferations of mouse polyomavirus in vitro and whose main target is RNA transcription. Biochimie 84, 329–334.
Chuesiang, P., Siripatrawan, U., Sanguandeekul, R., Julian, D., Mclandsborough, L., 2019. Antimicrobial activity of PIT-fabricated cinnamon oil nanoemulsions: effect of surfactant concentration on morphology of foodborne pathogens. Food Control 98, 405–411.
Chummun, S., McLean, N.R., 2017. The management of malignant skin cancers. Surgeon 35 (9), 519–524.
Desai, A.G., Qazi, G.N., Ganju, R.K., 2008. Medicinal plants and cancer chemoprevention. Curr. Drug Metabol. 9, 581–591.
Donsi, F., Annunziata, M., Vincensi, M., Ferrari, G., 2012. Design of nanoemulsion based delivery systems of natural antimicrobials: effect of the emulsifier. J. Biotechnol. 159, 342–350.
Ghosh, V., Saranya, S., Mukherjee, A., Chandrasekaran, N., 2013. Cinnamon oil nanoemulsion formulation by ultrasonic emulsification: investigation of its bactericidal activity. J. Nanosci. Nanotechnol. 13, 114–122.
Hu, Q., Gerhard, H., Upadhyaya, I., Venkitanarayanan, K., Luo, Y., 2016. Antimicrobial eugenol nanoemulsion prepared by gum Arabic and lecithin and evaluation of drying technologies. Int. J. Biol. Macromol. 87, 130–140.
Hussain, A., Samad, A., Singh, S.K., Ahsan, M.N., Haque, M.W., Faruk, A., Ahmed, F.J., 2016. Nanoemulsion gel-based topical delivery of an antifungal drug: in-vitro activity and in-vivo evaluation. Drug Deliv. 23 (2), 642–657.
Iqbal, S., Bhanger, M.I., Farooq, A., 2005. Antioxidant properties and components of some commercially available varieties of rice bran in Pakistan. Food Chem. 93, 265–272.
Jayaprakasha, G.K., Rao, L.J., 2011. Chemistry, biogenesis, and biological activities of cinnamomum zeylanicum. Crit. Rev. Food Sci. Nutr. 51, 547–562.
Jonkman, J.E., Cathcart, J.A., Xu, F., Bartolini, M.E., Amon, J.E., Stevens, K.M., Colarusso, P., 2014. An introduction to the wound healing assay using live-cell microscopy. Cell Adhes. Migrat. 8, 440–451.
Kaur, A., Saxena, Y., Bansal, R., Gupta, S., Tyagi, A., Sharma, R.K., Ali, J., Panda, A.K., Gabrani, R., Dang, S., 2017. Intravaginal delivery of polyphenon 60 and curcumin nanoemulsion gel. AAPS Pharm. Sci. Technol. 18, 2188–2202.
Khalid, N., Shu, G., Holland, B.J., Kobayashi, I., Nakajima, M., Barrow, C.J., 2017. Formulation and characterization of O/W nanoemulsions encapsulating high concentration of astaxanthin. Food Res. Int. 102, 364–371.
Kinghorn, A.D., Su, B.N., Jang, D.S., Chang, L.C., Lee, D., Gu, J.Q., 2004. Natural inhibitors of carcinogenesis. Planta Med. 70, 691–705.
Kohlhardt-Floehr, C., Boehm, F., Troppens, S., Lademann, J., Truscott, T.G., 2010. Prooxidant and antioxidant behaviour of usnic acid from lichens under UVB-light irradiation–studies on human cells. J. Photochem. Photobiol., B 101 (1), 97–102.
Komaiko, J.S., McClements, D.J., 2016. Formation of food-grade nanoemulsions using low-energy preparation methods: a review of available methods. Compr. Rev. Food Sci. Food Saf. 15, 331–352.
Lawrence, R., Burk, R.F., 1976. Glutathione peroxidase activity in selenium-deficient rat liver. Biochem. Biophys. Res. Commun. 71 (4), 952–958.
Lee, R.A., Balick, M.J., 2005. Rx: Chocolate. Expl.: J. Sci. Heal. 1, 136.
Linos, E., Katz, K.A., Colditz, G.A., 2016. Skin cancer—the importance of prevention. JAMA Intern Med 176 (10), 1435–1436.
Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Landall, A.J., 1951. Protein estimation with Folin phenol reagent. J. Biol. Chem. 193e265.
Marklund, S., Marklund, G., 1974. Involvement of the superoxide anion radical in auto oxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem. 47, 469–474.
Meghani, N., Patel, P., Kansara, K., Ranjan, S., Dasgupta, N., Ramalingam, C., 2018. Colloids and Surfaces B: biointerfaces Formulation of vitamin D encapsulated cinnamon oil nanoemulsion : its potential anti-cancerous activity in human alveolar carcinoma cells. Colloids Surfaces B Biointerfaces 166, 349–357.
Mehta, R.G., Murillo, G., Naithani, R., Peng, X., 2010. Cancer chemoprevention by natural products. Pharm. Res. (N. Y.) 27, 950–961.
Moron, M.A., Depierre, J.W., Mannervick, B., 1979. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Acta Biochim. Biophys. Sin. 582, 67–78.
Nadeem, M.T., Yasmin, A., Saima, H., Imran, M., Shahid, M.Z., Afzaal, M., 2018. Antioxidant capacity of cinnamon extract for palm oil stability. Lipids Health Dis. 17 (1), 1–8.
Ohkhawa, H., Ohishi, N., Yogi, K., 1979. Assay for lipid peroxidation in animal tissue by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.
Patel, H.C., Parmar, G., Seth, A.K., Patel, J.D., Patel, S.R., 2013. Formulation and evaluation of o/w nanoemulsion of ketoconazole. Pharma Science Monitor 4 (1), 338–351.
Philip, P.A., 2005. Experience with docetaxel in the treatment of gastric cancer. Semin. Oncol. 32, S24–S38.
Ribeiro-Costa, R.M., Alves, A.J., Santos, N.P., Nascimento, S.C., Gonçalves, E.C., Silva, N.H., Honda, N.K., Santos-Magalhaes, N.S., 2004. In vitro and in vivo properties of usnic acid encapsulated into PLGA-microspheres. J. Microencapsul. 21, 371–384.
Ryu, V., McClements, D.J., Corradini, M.G., McLandsborough, L., 2018. Effect of ripening inhibitor type on formation, stability, and antimicrobial activity of thyme oil nanoemulsion. Food Chem. 245, 104–111.
Senanayake, U.M., Lee, T.H., Wills, R.B., 1978. Volatile constituents of cinnamon (Cinnamomum zeylanicum) oils. J. Agric. Food Chem. 26 (4), 822–824.
Shanmugapriya, K., Kim, H., Sivagnanam, P., 2018. Astaxanthin-alpha tocopherol nanoemulsion formulation by emulsification methods: investigation on anticancer, wound healing, and antibacterial effects. Colloids Surfaces B Biointerfaces 172, 170–179.
Sharma, J., Goyal, P.K., 2015. Chemoprevention of chemical-induced skin cancer by Panax ginseng root extract. J Ginseng Res. 39 (3), 265–273.
Singh, K., Bhori, M., Kasu, Y.A., Bhat, G., Marar, T., 2018. Antioxidants as precision weapons in war against cancer chemotherapy induced toxicity – exploring the armoury of obscurity. Saudi Pharmaceut. J. 26 (2), 177–190.
Suwalsky, M., Jemiola-Rzeminska, M., Astudillo, C., Gallardo, M.J., Staforelli, J.P., Villena, F., Strzalka, K., 2015. An in vitro study on the antioxidant capacity of usnic acid on human erythrocytes and molecular models of its membrane. Biochim. Biophys. Acta 1848 (11), 2829–2838.
Tadros, T., Izquierdo, P., Esquena, J., Solans, C., 2004. Formation and stability of nanoemulsions. Adv. Colloid Interface Sci. 303–318.
Tang, L., Wang, K., 2016. Chronic inflammation in skin malignancies. J. Mol. Signal. 11, 1–13.
Teo, S.Y., Yew, M.Y., Lee, S.Y., Rathbone, M.J., Gan, S.N., Coombes, A.G., 2017. In vitro evaluation of novel phenytoin-loaded alkyd nanoemulsions designed for application in topical wound healing. J. Pharm. Sci. 106, 377–384.
Thyagarajan, A., Sahu, R.P., 2018. Potential contributions of antioxidants to cancer therapy: immunomodulation and radiosensitization. Integr. Cancer Ther. 17 (2), 210–216.
Vangalapati, M., Satya, N.S., Prakash, D.S., Avanigadda, S., 2012. A review on pharmacological activities and clinical effects of cinnamon species. Res. J. Pharmaceut. Biol. Chem. Sci. 3 (1), 653–663.
Vasconcelos, N.G., Croda, J., Simionatto, S., 2018. Antibacterial mechanisms of cinnamon and its constituents : a review. Microb. Pathog. 120, 198–203.
Watson, M., Holman, D.M., Maguire-Eisen, M., 2016. Ultraviolet radiation exposure and its impact on skin cancer risk. Semin. Oncol. Nurs. 32 (3), 241–254.
Wong, S.H., Kwong, T.N., Wu, C.Y., Yu, J., 2018. Clinical applications of gut microbiota in cancer biology. Semin. Canc. Biol. 55, 28–36.
Zainol, N.A., Ming, T.S., Darwis, Y., 2015. Development and characterization of cinnamon leaf oil nanocream for topical application. Indian J Pharm Sci 77 (4), 422–433.