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Nanoformulation of cinnamon oil/usnic acid blend: - An attempt to improve chemopreventive effect and reduce hepatotoxicity

Sunil K Singh, Alok Mukerjee, Himanshu Pandey and Abhishek Tripathi

Abstract
Introduction: Natural originated substance are a common alternative for prevention and treatment of various type of cancer. Usnic acid also have a good potential as chemopreventive agent. But its low water solubility and hepatotoxicity, restrict use of this as chemopreventive agent. So here attempt has been focused to formulate cinnamon oil and Usnic acid blend nanoemulsion (CUN), for improvement of chemopreventive effect and reduce hepatotoxicity of Usnic acid.
Material and method: A series of Nanoemulsion (NE) were prepared using ethanol (Co-surfactant) and Tween-80 (Surfactant) by using ultrasonic emulsification method. CUN antitumor activity was evaluated on an BL16-F10 melanoma tumor cell implanted in C57BL/6 mice and estimated by percentage tumor inhibition. CUN sub chronic toxicity were evaluated by Biochemical estimation of blood and histopathological study of liver.
Result: CUN improved the activity of usnic acid by 51.81% in contrast with its cinnamon oil/ usnic acid blend form (CUB). Hepatocytes vacuolization and lymphocytes infiltration in portal area was observed in animal treated with CUB. However, this hepatotoxicity was significantly reduced when animal were treated with CUN.
Conclusion: Result of the present study suggested that CUN had potent antitumor activity with reducing hepatotoxicity of usnic acid.

Keywords: Cinnamon oil, usnic acid, nanoemulsion, antitumor

1. Introduction
Cancer is the most dreaded disease of mankind causing mortality and morbidity globally [1]. Now days it accounts for nearly more than 15% of all death recorded annually, (WHO Statistics, 2013) [2]. Skin cancer is one of the most prevalent type of cancer, which arises due to exposure of environmental carcinogenic chemical and excessive chronic exposure of UVB radiation. Skin cancer mainly two types, Non-melanoma skin cancer (NMSC) and cutaneous malignant melanoma. (CMM) [3]. Malignant melanoma is an metastatic tumor of the melanocyte in the epidermis layer of skin. Despite of tumor types, most tumor have some common features, like acquired the same set of functional activity during their growth and development. Generally, tumor cells are grow under abnormal regulated cell growth signals can efficiently avoid normal apoptosis and have the ability to invade to surrounding tissue and metastasize. Phenotypic heterogeneity of cancer cell is responsible for their resistance to currently used chemotherapeutic drugs and account for tumor relapses and treatment failures [4]. Most of chemotherapeutic affect only limited cell within the tumor while the growth of chemo- resistant tumor cells remain unaffected by these agent. So, needed a new potential anticancer agent that would multitargeted but selectively inhibit growth of tumor cell is necessary. For attempt to achieved this goal could be pursued by isolating efficient natural chemopreventive compound, those potentially leading to inhibition of tumor progression at its terminal stage.
Usnic acid has become the most extensively studied lichen metabolite [5]. It is mainly found in certain lichen species (Alectoria, cladonia, usnea, lecanora, ramalina and evemia). Usnic acid has been show to exhibit antiviral, antiproliferative, antitumor and anti-inflammatory properties [6]. Usnic acid is a non-genotoxic antineoplastic agent that works in a p53 gene regulated apoptosis in a independent manner and makes it a potential candidate for novel chemopreventive agent. but application of usnic acid have somewhat restricted due to high hepatotoxicity and lower water solubility [7, 8].

Nano- technology is valuable for formulating new therapeutic dosage forms. It can protect drugs from degradation in biological fluid and improve their penetration into cells. Nanosystem may be able to improve the efficacy of existing drug [9].

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. Caspase-3 and Caspase-9 colorimetric assay kit were purchased from Bio vision research products, USA. The rest of the chemicals utilized were obtained from local firms and were of analytical grade.

2.2 Preparation and characterization of cinnamon oil/ Usnic acid bland nanoemulsion

Nanoemulsion was prepared according to the method of Mukherjee *et al*., 2019. Cinnamon oil (4 parts) and usnic acid blend (1 part) were mixed in a beaker at room temperature to prepare cinnamon oil and usnic acid blend (CUB). Cinnamon oil and usnic acid blended nanoemulsion (CUN) were prepared by dissolving CUB in the pre-measured quantity of ethanol (co-surfactant) and Tween 80 (surfactant) and then mixed (S_{mix}). The mixture was homogenized using magnetic stirrer and continuing addition of distilled water to a certain extent till a uniform and homogenized emulsion was obtained. The emulsion was sonicated for 30 min to get Cinnamon oil and usnic acid blended nanoemulsion (CUN), which was further processed to evaluate its anticarcinogenic property.(9)
The particle size and surface charge potential of CUN were determined using a Zetasizer (Nano-ZS90, Malvern, United Kingdom). The Globule size and polydispersity of prepared nanoemulsion formulations were determined by photon correlation spectroscopy through Malvern analyzer. All results are based on the average of at least three measurement of different samples for same batch of prepared nanoemulsion [10, 11].

2.3 Antitumor activity of cinnamon oil/ Usnic acid blend nanoemulsion

Experimental Animals- The animals selected for the study were C57BL/6 mice 4-6 weeks old, weighing 25-30 g. The animals were divided into four groups containing six animals in each group. The animal was acclimatized in the laboratory environment before the experiment and handled carefully according to CPCSEA guidelines. Institutional Animal Ethical Committee approved the above protocol with approval no. UIP/IAEC/2014/April/22. The laboratory conditions were 24 ± 1ºC temperature with 50 ± 5% relative humidity in 12 hr light/dark cycle. The animals were giving a pelleted diet and water *ad libitum.*

2.4 Experimental design

The experiments were undertaken after IAEC approval. In the present investigation four groups of six animals each were randomised into positive and negative control, CUB and CUN treated groups. Mice of group I served as positive control, in this group healthy animal was added without any treatment. For induction of tumor, Group II, III and IV animals were injected subcutaneously with B 16 melanoma tumor cell (5 x 10^6 cell/ml/animal) as described by N.P.da Silva Santos *et al*. Treatment of the animals of group III was started 24 hour after tumor cell inoculation with daily i.p injection of CUB (0.5% tween 80) and group IV with CUN at a dose of 15 mg/kg/day for 7 days. The positive and negative control groups of animal were treated daily with phosphate buffered saline (pH 7.4) for 7 days. After a week of treatment, the animals were anaesthetised and sacrificed. Blood sample were collected in CBC bottles for haematological study. Tumor and liver were excised for weight measurement. The antitumor activity was evaluated in terms of percentage tumor inhibition.

$$\% \text{ Tumor inhibition} = (C-T) / C \times 100$$

[Where C = Avg. tumor weight of the control group, T= Avg. tumor weight of the treated group] [10].

2.5 Biochemical assays

Blood sample from mice were collected into CBC bottles for red blood cells (RBC), White blood cells (WBC), and haematocrite (Ht) determination. Caspase-3 and Caspase-9 activity in tumor cell were measured using caspase-3 colorimetric assay kit and caspase-9 colorimetric assay kit respectively according to manufacturer's instructions (Biovision Research products, 980 Linda vista Avenue, Mountain View, USA.)

2.6 Survival of tumor bearing mice

Male swiss albino mice, 4-6 weeks old, weighing 25-30g were acclimatized for approximately one week and randomly divided into three groups of six animals in each. B 16 melanoma tumor cells (5 x 106 cell/ml/animal) were injected subcutaneously in the inguinal area of the animals. After 24 hours tumor inoculation, animals of group II and group III received daily i.p injections of CUB and CUN at a dose of 15 mg/ kg/day for 7 days. Group I received phosphate buffered saline (pH 7.4) daily for 7 days. All group animals behaviour was observed during experimental protocol and recorded time of survival.

2.7 Subchronic toxicity of usnic acid

Male swiss albino mice, 4-6 weeks old, weighing 25-30g were acclimatized for approximately one week and randomly divided into three groups of six animals in each. The two groups II^{nd} and III^{rd} of animals received daily i.p injections of CUB (0.5% Tween 80) or CUN (15mg/kg/day) for 15 days. The control group I^{st} was only received phosphate -buffered saline. Throughout the experimental study period, the animal behaviour was observed and body weights recorded on daily basis. Clinical toxicity of drug was monitored such as tremor, rigidity, ataxia and morbidity of animals. After treatment, the animals were anaesthetised and sacrificed by cervical dislocation. Blood was collected and performed biochemical analysis. Estimate blood urea nitrogen (BUN), Alanine aminotransferase (AST), creatinine (CRT), were determined by help of biochemistry analysis kit. After the animal sacrificed, liver were removed, measured weight of liver and performed histopathology.

2.8 Statistics

The data were analysed using one –way analysis of variance (ANOVA) and matched –pair comparisons were also performed using the Tukey test. The value of $p<0.05$ were considered statically significantly significant.

3. Result

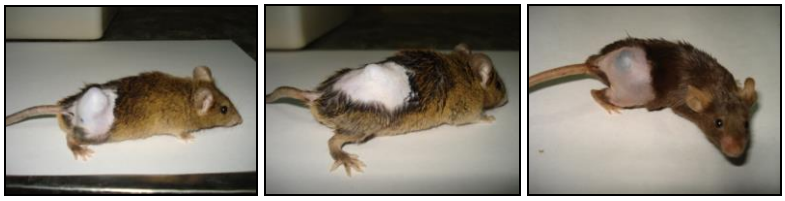
3.1 Preparation and characterization of cinnamon oil/ Usnic acid bland nanoemulsion

Nanoemulsion of cinnamon oil/ usnic acid blend (CUN),

result exhibited that the globule size of CUN were spherical in shape and mean size of the globules 96.39 ± 2.38 mm with a low polydispersity index (PDI= 0.250±0.022). PDI indicate uniformity of droplets size in the formulation is present. Moreover, the result also showed the CUN was stable after 180 days regarding globule size (103.39± 3.77nm), Zeta potential (-24.05mv) and polydispersity index (0.257±0.28). Selected formulation batch showed optimum zeta potential (-27.13) with highest percentage transmittance (99.76%) with lowest droplet size. Selected batch formulation contain 4% cinnamon oil, 1% usnic acid, 37% Smix and 58% deionized water as continuous phase.

3.2 Antitumor activity of cinnamon oil/ usnic acid blend nanoemulsion (CUN)

Body weight and liver weight significantly decreased in sarcoma 180 treated animals compared to negative control animals. (Table-2).In sarcoma- 180 bearing mice, tumor volume (192.6±53.72mm^3) and tumor weight (1.33±0.04g) was observed. CUB and CUN groups animal showed reduction in tumor weight and tumor volume as compared to positive control. In contrast, treatment with CUN produced a significant reduction in tumor weight (0.281±0.013g) and tumor volume (69.8±7.14mm^3) as compared with the CUB treated animals (tumor weight- 0.97±0.06 and tumor volume-134.5±15.14 mm^3). CUN treated animal was showed 78.87% tumor inhibition as compared to positive control group. CUN improved the activity of usnic acid by 51.81%. in contrast with its cinnamon oil/ usnic acid solution (CUB) form which showed antitumor activity of 27.06% as compared with the positive control group animals.

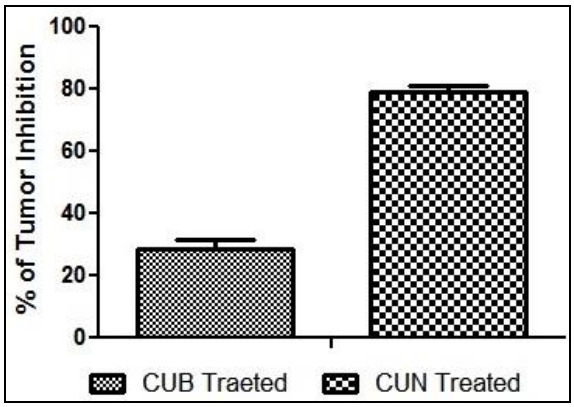


(A) (B) (C)

Fig 1: The Gross Appearance of skin melanoma tumours in B-16 Cancerous cell induce mice (A) Controlled Animal (B) CUB treated (C) CUN Treated

Table 1: Effect of CUB and CUN on body weight, tumor volume, tumor weight, % tumor inhibition and lever weight of experimental animal in each group. (Value are means±SD)

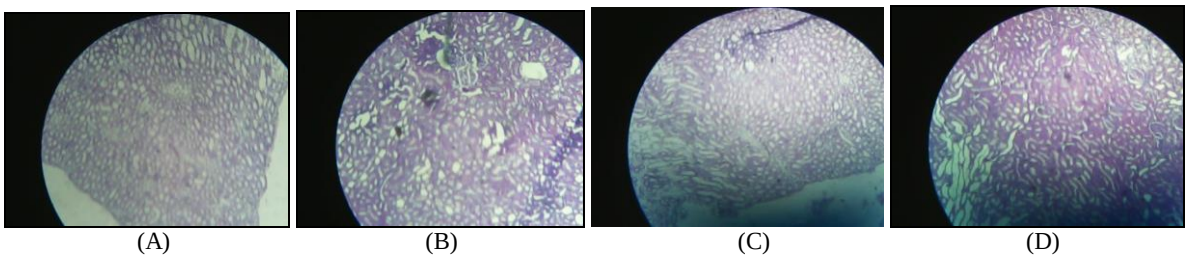
Group	Body Weight (Gram)		Tumor Volume	Tumor Weight	% tumor inhibition	Liver Wt.
	Initial	Final				
Negative Control	25.56±1.73	32.67±0.45	-	-	-	1.07±0.05
Positive Control	25.63±1.95	21.67±1.23	192.6±53.72	1.33±0.04	-	0.79±0.07
CUB Treated	27.48±1.87	26.53±1.32	134.5±15.14	0.97±0.06	27.06	0.98±0.06
CUN Treated	26.91±1.44	34.83±2.04	34.83±7.14	0.28±0.01	78.87	1.09±0.06



Graph 1: Evaluation of anti-tumor activity of CUB and CUN against BL-16 melanoma tumours bearing mice (CUB- Cinnamon oil/Usnic acid Blend, CUN- Cinnamon oil/Usnic acid blend nanoemulsion)

3.3 Histopathological evaluavtion

The histopathological evaluation of liver showed morphological alteration in the liver of the animals treated with either solution or emulsion of cinnamon oil/usnic acid blend. Hepatocytic vacuole and an intensive lymphocytes infiltration in portal space can be observed in the liver of animals treated with CUB (Fig-3a) in comparison with the positive controls. On the other hand the liver of animals treated with CUN showed less vacuolization of hepatocytes and a mild lymphocytes and a mild lymphocytes infiltration in portal spaces. Liver histopathological analysis indicates that the CUN was able to reduce hepatotoxicity when compared with CUB treated animals.



(A) (B) (C) (D)

Fig 2: Histopathological evaluation of lever in BL-16 Tumor bearing mice (H&E, 50x). (A)- Negative Control (B) Positive Control (C) CUB Treated (D) CUN Treated

3.4 Evaluation of caspase-3 and caspase-9 activity

Caspase-3 and Caspase-9 activity in tumor tissue were measured using caspase calorimetric assay kit according to manufacturer's instructions (Biovision Research Products, 980 Linda Vista, Avenue, Mountain view, USA). Activity of caspase-3 and caspase-9 in tumor tissue of control and experimental animals in each group are shown in Table-4. Significantly caspase-3 and caspase-9 level was decreased in tumor tissue compared to negative control group animals. CUN treated tumor bearing animals showed significantly restored the activity of caspase-3 and caspase-9 activity.

Table 2: Levels of Caspase 3 and Caspase 9 activities in tumor tissue of controlled and experimental animal in each group. Value are mean±SD of 6 animals.

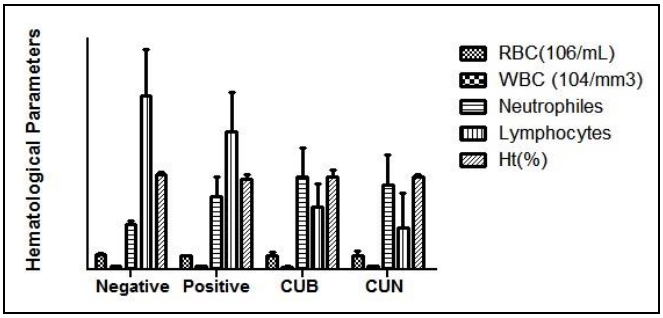
Groups	Caspase 3	Caspase 9
Negative Control	0.157±0.014	0.108±0.012
Positive Control	0.085±0.018	0.072±0.016
CUB Treated	0.134±0.012	0.093±0.014
CUN Treated	0.159±0.13	0.110±0.018

3.5 Haematological evaluation

Haematological analysis did not showed any significant change in RBC level in treated and untreated B 16 melanoma tumor bearing mice as compared with negative control group of animals (from 6.12±2.01 to 6.4±0.6 x 10^{6} cells/ ml), Table-5. No significant reduction in wbc level, lymphocytes and neutrophils were observed between the treated groups and the untreated positive group.

Table 3: Haematological finding in mice treated with CUB and CUN at a dose of 15mg/kg for 7 days. Values are presented as means±SD. (RBC- Red blood cells count, Ht- Heamatocrit WBC- White blood cells count, Negative control- Untreated healthy mice, Positive control- Untreated BL-16 tumor bearing mice.

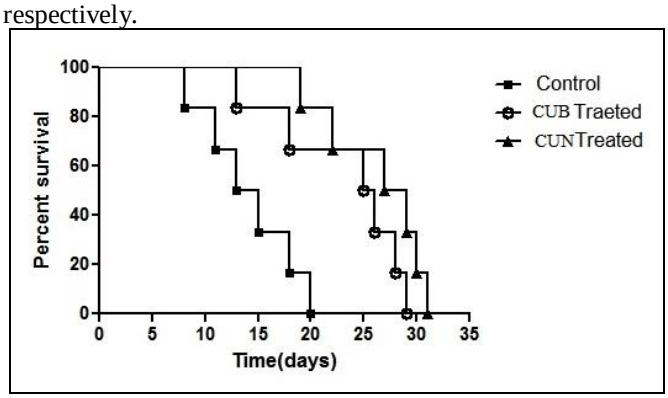
Group	RBC(10^6/mL)	Ht(%)	WBC (10^4/mm^3)	Lymphocytes	Neutrophiles
Negative Control	6.4±0.6	41±1	1.5±0.03	74.9±20	19.5±15
Positive Control	5.9±0.4	39±2	1.6±0.03	59.8±0.7	31.5±8.5
CUB Treated	6.2±1.16	40±3	1.3±0.09	49.8±10	40.0±12.5
CUN Treated	6.12±2.01	40±1	1.5±0.03	53.0±15	36.6±13



Graph 2: Haematological evaluation in groups of animal treated with CUB and CUN or without treatment during BL-16 melanoma cell induce tumor.

3.6 Survival of tumor bearing mice

Survival of BL-16 melanoma tumor bearing mice was evaluated on the basis of behaviour and clinical features of different group of animals. In the first week of all treated group of animals preserved normal behaviour and clinical features. From the second week onwards animals presented bristling hair, reduced weight and reflex and irregular respiration and consequent death. In the positive control group the death was recorded 20 days after the tumor inoculation. 50% of the animals that treated with CUB had died at 25 days and all animals died 29 days after the tumor inoculation. Moreover in the group treated with CUN, a 50% death was recorded on the 27 days and remaining of the animals survived for 34 days. These results show that the CUN was increase 26.47% and 41.17% survival rate as compared with CUB treated and Positive Control respectively.



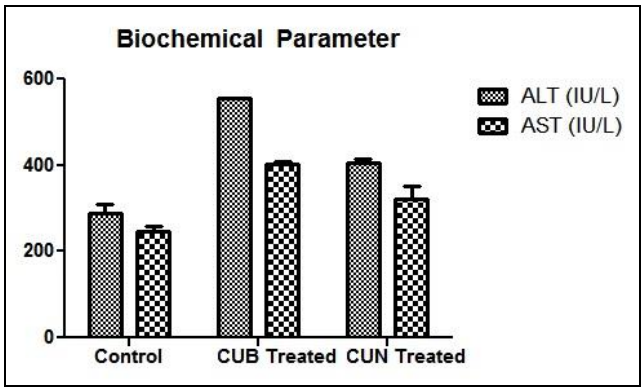
Graph 3: Survival evaluation of BL-16 tumor bearing mice treated with CUB and CUN as compared with untreated tumor bearing mice.

3.7 Subchronic toxicity of CUN

In this present investigation subchronic toxicity of CUN was evaluated by estimation of Blood urea nitrogen (BUN), creatinine (CRT), alanine amino transferase (ALT) and aspartate aminotranferase (AST) in serum. All estimated value of BUN, CRT, ALT and AST in serum is shown in table-6. No significant alteration in serum levels of BUN and CRT were observed with the CUB or CUN treated animals. But AST and ALT level significantly higher than negative control group. CUN treated group animals showed slightly reduce level of ALT and AST as compared with CUB treated animals.

Table 4: Serum biochemical evaluation in C57 BL/6 mice treated with CUB and CUN at a daily dose of 50mg/kg for 15 days. (BUN- Blood urea nitrogen, CRT- Creatinine ALT- Alanine amino transferase, AST- Aspartate amino transferase)

Group	BUN(mg/dl)	CRT (mg/ml)	ALT (IU/L)	AST (IU/L)
Control	141±10.5	1.09±0.08	287.44±21.30	245.51±12.80
CUB Treated	154±5.58	0.84±0.10	554±0.82	403.10±6.76
CUN Treated	140±8.18	0.81±0.10	406±9.42	320.45±30.36



Graph 4: Evaluation of AST and ALT level in serum of untreated and treated animal with CUB and CUN

4. Discussion

Usnic acid possesses antitumor and antiprolifiretive activity, but in fact due to high hepatotoxicity and low water solubility of usnic acid has restricted its clinical use in anti cancer therapy [6]. However, the side effects of usnic acid only occur when applied at high doses. Experimental animals are reported to remain healthy when administered in nanoemulsion form with some other adjuvant [12]. Antitumor activity of CUN were improved upto 26.47% in comparison with CUB on B 16 melanoma tumor bearing mice. These finding could be explained by the increase bioavailability of usnic acid in the tumor tissue and more effectively inhibit the abnormal cell proliferation B 16 melanoma tumor bearing mice. Usnic acid adverse effects were investigated in cultured murine hepatocytes. It suggested that hepatotoxicity induced by usnic acid was related to the inhibition of the mitochondrial function by inhibiting of the electron transport chain [13]. Usnic acid also increases production of free radical and increase oxidative stress which cause cellular death. This hepatotoxicity was significantly reduced as animal were treated with CUN. A recent study suggested that the anti-proliferative activity of usnic acid against MCF-7 and Breast cancer cells is related with P^{53} gene activity. Usnic acid non-genotoxic anticancer agent which works in a P^{53} gene related activity in independent manner [14, 15]

The haematological result suggested that usnic acid either in CUB or CUN form caused no significant change in haematological parameters. Immunological parametres (Lymphocytes and Neutrophils) also unaltered. It support that the usnic acid has no effect on the immune response of host [16, 17]. Caspase-3 is a caspase protein that interacts with caspase-8 and caspase-9 in process of apoptosis. During programmed cell death caspase-3 and caspase-9 are activated [18]. Level of caspase-3 and caspase-9 significantly decreased in tumor bearing mice [19]. Results of the study showed that the normal process of apoptosis is disrupted in tumor bearing untreated mice. CUN treated animal significantly restored caspase-3 and caspase-9 activity, and help in stimulation of the apoptosis in tumor tissue without much induced cell necrosis. No significant alteration found in serum level of BUN and CRT in CUN and CUB treated animals as compared with negative control healthy animals. It suggested that usnic acid do not have nephrotoxicity, when administered as a long term treatment. Moreover the hepatotoxicity present in considerable amount in CUB treated animals as compared with CUN treated animals. High serum level of transaminase observed in CUB treated animals. It suggested a chronic hepatic dysfunction caused by usnic acid. Taransaminase level slightly recovered in CUN treated animal. It indicate CUN was able to reduced hepatotoxicity.

5. Conclusion

The present study thus demonstrated the antitumor activity of nanoemulsion of cinnamon oil/usnic acid blend in tumor bearing mice. The finding showed that the CUN was able to improve antitumor activity and significantly reduced the hepatotoxicity of usnic acid. This investigation also underlying mechanism might be associated with inducing apoptosis on tumor cells, modulating activity of caspase-3 and caspase-9 with P^{53} gene regulated activity. Hence, CUN might have potential to be promising effective chemotherapeutic agent.

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