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Original paper

Antitrichomonal activity of nanoemulsion of carvacrol on *Trichomonas galline*: formulation development and i*n vitro* characterization

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ABSTRACT. In recent years, increasing attention has been paid in veterinary medicine to find novel natural resources to reduce the use of synthetic drugs, avoid side effects, and for better compliance of the animals' owners. Metronidazole has been used for many years in the treatment of birds' trichomonosis. Carvacrol is a terpenoid and several biologic activities was attributed to it. The present study developed and characterized a carvacrol nanoemulsion (NanoCAV) and investigated its antitrichomonal activity on *Trichomonas gallinae*, the causative agent of pigeon trichomonosis, under *in vitro* condition and compared it with carvacrol (CAV) and the standard antitrichomonal dug, metronidazole (MTZ). Additionally, cytotoxicity of the developed formulation to the fibroblast cell line was evaluated. The NanoCAV mean size and surface charge were 80.5 nm and -31.2 mv, respectively. No significant cytotoxicity was observed for the NanoCAV. Incorporation efficiency of NanoCAV was measured as 75%. Results of antitrichomonal activity assay showed 12 h fifty percent lethal concentrations of 0.39 and 0.27 µg/ml for CAV and NanoCAV, respectively. The NanoCAV based on *in vitro* activity and low cytotoxicity, can be further studied for its efficacy and safety profile in the pigeons.

Keywords: terpenoid, metronidazole, cytotoxicity, trichomonosis, pigeons

Introduction

Avian trichomonosis is a parasitic disease caused by the protozoan *Trichomonas gallinae*. These parasites live mainly in the bird's upper digestive tract, where they can cause granulomatous lesions that obstruct the oesophagic lumen, leading to the death of birds as a result of severe starvation [1]. However, it is well documented that virulence varies between the strains and some may reach parenchymatous organs, generate necrotic foci in them and result in more severe complications [2]. Columbidae are known as the parasite's main host, particularly the domestic pigeon (*Columba livia*), which has been considered responsible for the worldwide spread of *T. gallinae* [3].

Trichomonas gallinae has significant health and economic impacts on the poultry industry, especially pigeons and game birds [4]. Outbreaks of trichomonosis have resulted in wide-ranging mortality, mainly in breeding populations [5,6]. Nitroimidazoles are the drugs of choice for the treatment of trichomonosis. However, sub-therapeutic dosing and prophylactic use of these drugs against trichomonosis led to the emergence of resistant strains [7].

Carvacrol [2-methyl-5-(1-methylethyl)-phenol] is a monoterpenoid compound that is found in essential oils of aromatic plants including oregano, pepperwort, thyme and wild bergamot. It has a characteristic odor of oregano and exhibits numerous biological and pharmacological activities [8].

Several studies have reported activity of carvacrol and carvacrol bearing essential oils against several protozoan parasite species. The effect of essential oils obtained from *Origanum vulgare* L. (oregano) and *Thymus vulgaris* L. (thyme) on growth and ultrastructure forms of *Trypanosoma cruzi* have been investigated, and promising trypanocidal activity of oregano and thyme essential oils have been reported [9]. It has been shown that among some terpenoids, which are the common constituents of the essential oils, carvacrol was the most potent and safest for the treatment of visceral and cutaneous leishmaniosis both *in vitro* and *in vivo* [10,11].

In recent years, there has been significant progress in the area of developing nanoparticle-based drug delivery systems. Nano-drug delivery systems have the advantages of improving drugs bioavailability and distribution, consequently increasing their efficacies [12]. A recent study developed a nanostructured lipid carrier as a strategy to enable the use of carvacrol in the leishmaniosis clinical therapy. It has been demonstrated that encapsulation of carvacrol in nanolipid carriers provided a lower cytotoxicity relative to free carvacrol and increased its *in vitro* toxicity on the amastigote forms of *Leishmania*. Moreover, some pharmacokinetic parameters including mean residence time and volume of distribution were higher for nanocarvacrol in comparison to the free carvacrol [13].

Based on the above, the present study aimed to develop and characterize a carvacrol nanoemulsion formulation (NanoCAV) and examine its *in vitro* antitriconal activity against *T. gallinae*. Besides, the antitrichomonal efficacy and cytotoxicity of the nanoemulsion were compared with carvacrol conventional emulsion (CAV).

Materials and Methods

Preparation of NanoCAV

A nanoemulsion of carvacrol was formed spontaneously in an oil phase of glycerol monooleate (GMO), Cremophor RH-40® and poly ethylene glycol (PEG) 400 (1:8:1 ratio). Various amounts of carvacrol (10, 25, 50, 100, and 200 mg) were added to 10 gram of oil phase. Carvacrol, oil, surfactant and co-surfactant were stirred at 100 rpm for 2 h. To complete the mixing process, further sonication for 1 h by using a bath sonicator was done. Deionized water was added to the oil phase at a ratio of 5:1 to obtain nanoemulsion during stirring [14]. For comparison between nanocarvacrol and carvacrol, a conventional emulsion was prepared using 16% GMO and CremophorRH-40® (4%). carvacrol (0.25% w/v) was added under stirring to GMO and Cremophor RH-40®. The mixture was then dispersed in water under stirring for 30 min to form an emulsion.

Particle size, zeta potential and polydispersity index analysis

The particle size, the surface charge (zeta potential), and polydispersity index (PDI) for the NanoCAV were determined by a Nano-ZS ZEN 3600 particle size analyzer (Malvern Instruments, UK). The scattering intensity was assessed at an angle of 90° and at 25°C.

Incorporation efficiency of carvacrol in NanoCAV

Calculation of the incorporation efficiency of carvacrol in NanoCAV was performed by using high performance liquid chromatography (HPLC) method [15]. The freshly prepared NanoCAV was centrifuged at 13000 rpm for 20 min, and the supernatant was recovered. The carvacrol concentration in the obtained supernatant was determined using a HPLC system consisted of a guard column (ODS 4 mm 3.0 mm I.D, security guard, Phenomenex, Torrance, CA, USA) and a C18 column (Prodigy ODS 250 4.6 mm, 5 m, Phenomenex, Torrance, CA, USA). The mobile phase composed of ACN: H2O (50:50) and 10 µl of the sample was injected to the HPLC system. The flow rate of the mobile phase was 1.0 ml/min and the UV wavelength was set at 274 nm. The limit of detection (LOD) was 0.02 µg/ml based on a signal-to-noise ratio of 3:1, whereas the limit of quantitation (LOQ) was 0.04 µg/ml based on a signal-to-noise ratio of 6:1. The incorporation efficiency was calculated based on the following equation:

Incorporation Efficiency % = (A – B)/A × 100

Where A = Total amount of carvacrol added for preparation of the nanoemulsion and B = Total amount of carvacrol in supernatant after centrifugation.

Parasite

Samples were taken using microbiology swabs from membranous lesions in oropharyngeal area of trichomonosis suspicious pigeons. Prepared wet smears were examined under a light microscope at

×100 and ×400 magnifications to confirm the existence of *T. gallinae* as the causative agent. Parasite were cultured in tryptone/yeast extract/maltose (TYM) medium supplemented with 10% fetal calf serum (Sigma, Germany) and incubated at 37°C [16]. Cultures were observed on consecutive days to check the growth of *T. gallinae*. Sub-cultures were done when the parasites showed more than 95% mobility and normal morphology [17]. Design of the study was approved by the IAU institutional ethics committee based on the animal welfare law with code number of IR.IAU. BABOL.REC.1400.068.

In vitro antitrichomonal activity

The method used for the *in vitro* assay was that described by Tabari et al. [1] with some slight modifications. To examine the susceptibility of *T. gallinae* to CAV and NanoCAV, sterile multi well plates were used to incubate the trophozoites with the corresponding concentrations of CAV and NanoCAV. A volume of 100 µl of culture medium containing 1×10^4 parasites counted by using a neobar slide and pipetted into each well, as well as NanoCAV and CAV to reach final concentrations of 5, 2.5, 1.25, 0.625 and 0.312 µg/ml. Metronidazole (MTZ) was used as the standard antitrichomonal dug at the same concentrations to other tested formulations. Control wells did not receive any treatment. Subsequently, to generate anaerobic conditions a layer of 50 µl of vaseline was added to wells. All assays were in triplicate. The wells were examined with an inverted microscope every 24 h for 3 consecutive days.

Cytotoxicity assay

To assess the cytotoxicity of NanoCAV, an MTT assay was used. In brief, 1×10^4 primary fibroblast cells were obtained from Pasteur Institute of Iran, North Research Center (Amol), seeded on a 96-well plate and cultivated for 24 h at 37°C (5% CO_2). Afterwards, the cells were exposed to NanoCAV and CAV at different concentrations ranging 0 to 50 µg/ml, and incubated for 1 day. After 24 h exposure, 5 µl of MTT reagent was added. Then, isopropanol 50% and 10% sodium dodecyl sulphate (SDS) were added and incubated at 37°C for 5 h. Finally, all samples were measured spectrophotometrically at 540 nm. All experiments were performed in triplicate.

Statistical analysis

In the present study, statistical analysis was performed using SPSS software version 22 (Chicago, Illinois, USA). Statistical analysis was performed by comparing the means in different groups by one-way analysis of variance (ANOVA) and Tukey post hoc test. The lethal concentrations 50 and 90 (LC_{50} and LC_{90}) were calculated using Probit regression analysis. Values of $P<0.05$ were considered significant.

Results

Particle size, and zeta potential of NanoCAV

Mean size of NanoCAV is to 80.5 nm, and poly dispercity index is equal to 0.22 (Fig. 1A). The zeta potential of the nano-MTZ was -31.2 mv (Fig. 1B). These results indicate that the NanoCAV has been formulated on the nanometric size and the particles are homogenous in the dispersion.

Incorporation efficiency in NanoCAV formulation

Obtained HPLC results showed that in the developed formulation incorporation efficiency of carvacrol in the NanoCAV was 75%.

Cytotoxicity assay

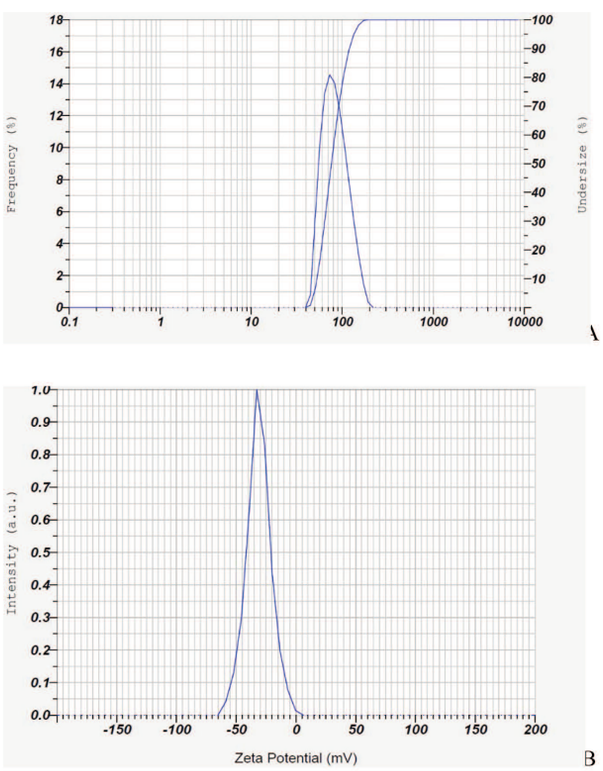

Figure 1. Particle size and polydispersity index distribution (A) and zeta potential (B) for NanoCAV

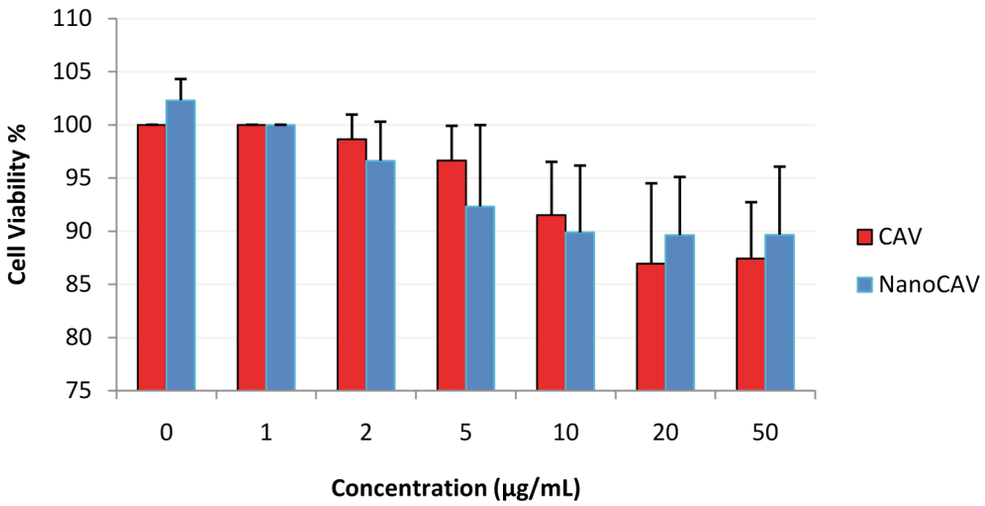

Figure 2. Mean viability of fibroblast cells exposed to different concentration of carvacrol (CAV), and nanoemulsion of carvacrol (NanoCAV) for 24 h. Data are presented as mean± standard error

Table 1. I*n vitro* fifty and ninety percent lethal concentrations (LC_{50} and LC_{90}) of carvacrol (CAV) and nanocarvacrol (NanoCAV) and the standard antitrichomonal, metronidazole (MTZ), on *Trichomonas galline*

Components	Concentration (µg/ml)	12 h mortality (%)±SD[a]	LC_{50} (µg/ml)(LCL-UCL)	LC_{90} (µg/ml) (LCL-UCL)	χ^2 (df)[b]
Carvacrol (µg/ml)	0.156	39.33±1.15	0.39(0.19–0.59)	1.86(1.56–2.34)	0.81(3) ns
	0.312	50.66±3.05			
	0.625	56.66±3.05			
	1.25	60.66±3.05			
	2.50	96.66±1.15			
	5.00	100.00±0.00			
Nanocarvacrol (µg/ml)	0.156	39.33±1.15	0.27(0.20–0.42)	1.71(1.06–3.82)	1.88(4) ns
	0.312	58.66±3.15			
	0.625	70.00±4.60			
	1.25	80.00±1.15			
	2.50	95.33±1.15			
	5.00	100.00±0.00			
Metronidazole (µg/ml)	0.156	13.44±1.15	2.17(1.30–2.90)	9.72(8.42–11.65)	3.86(5) ns
	0.312	24.66±1.02			
	0.625	33.66±3.05			
	1.25	42.66±7.02			
	2.50	59.66±3.05			
	5.00	70.66±1.50			

Explanations: SE: standard error, LCL 95%: lower confidence limit, UCL 95%: upper confidence limit, ns: not significant (P>0.05)

[a]values are mean±SE of three replicates, [b]Chi-square, df: degrees of freedom

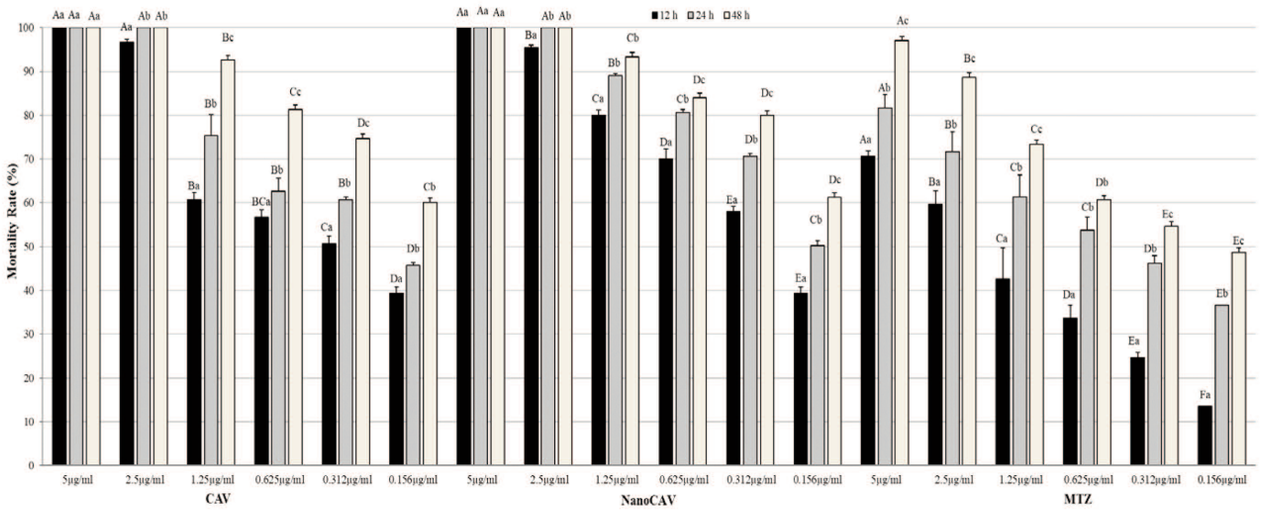

Figure 3. Antitrichomonal activity of different concentrations of CAV (carvacrol) and NanoCAV (nano emulsion of carvacrol) in comparison to MTZ (metronidazole) on trophozoites of *Trichomonas gallinae* at 12, 24, and 48 h time points.
T-bars represent standard errors. Within each concentration of tested compounds, columns marked with different letters (lowercase) are significantly different between times of exposure (Repeated measures ANOVA, Bonferroni test, $P<0.05$).
Within each tested compound, columns marked with different letters (uppercase) in each time of exposure (12, 24, and 48 h, separately) are significantly different between concentrations (ANOVA, Tukey's HSD test, $P<0.05$)

Viability of primary fibroblast cells incubated with different concentrations of NanoCAV (0–50 µg/ml) was determined after 24 h (Fig. 2). NanoCAV and CAV especially at higher concentrations induced a slight cytotoxicity but the cytotoxic effect was not significant.

Antitrichomonal effect

Antitrichomonal activity of different concentrations of CAV and NanoCAV on trophozoites of *T. gallinae* at different time points is shown in figure 3. All tested concentrations of CAV and NanoCAV except 0.156 µg/ml at 12 h resulted in higher than 50% mortality rates in *T. gallinae*. At 12 h time period, the highest tested concentration, 5 µg/ml, in both CAV and NanoCAV caused 100% mortality rates; however, for MTZ at the same concentration and time point, 70.66% mortality was recorded. The mean mortality rate of trophozoites after 24 h for the concentration of 1.25 µg/ml of nanocarvacrol was 89%, while the same concentrations of carvacrol and MTZ were less toxic and caused 75, and 61.33% mortality, respectively. At 48 h, MTZ at the lowest tested (0.156 µg/ml) concentration resulted in 54.66% mortality; however, CAV and NanoCAV caused 74.66 and 80% mortality. A significant effect of time was observed for NanoCAV and CAV at concentrations of 1.25, 0.625, and 0.312 µg/ml ($P<0.05$). For MTZ, at all tested concentrations and time points, significant interaction of time and concentration were noted ($P<0.05$).

The 12 h fifty and ninety percent lethal concentrations (LC_{50} and LC_{90}) of CAV, NanoCAV, and MTZ are shown in table 1. Based on the calculated values, the most potent tested compound was NanoCAV with LC_{50} and LC_{90} values of 0.27 and 1.71 µg/ml fallowed by CAV with LC_{50} and LC_{90} values of 0.39 and o.89 µg/ml. The LC_{50} value obtained for the positive control drug MTZ was 2.17 µg/ml. Since there is an overlap between carvacrol and nanocarvacrol in the 95% confidence limits of these two compounds, the difference between the LC_{50} and LC_{90} values of these two compounds is not significant ($P>0.05$). However, both of these compounds showed a significant difference compared to MTZ ($P<0.05$).

Discussion

Emerging resistance to metronidazole and nitroimidazole in *T. gallinae* is an important health concern in avian medicine. Resistant strains of *T. gallinae* are a threat for wild birds along with pigeons which can cause high range mortality rates in bird's populations [5]. Many studies have done on

medicinal plants as alternative sources of antitrichmonal compounds for treatment of birds' trichomonosis [7,17–19]. Youssefi et al. [17] evaluated the antitrichomonal efficacy of *Artemisia sieberi* essential oil on *T. gallinae* under both *in vitro* and *in vivo* conditions. Essential oil of *A. sieberi* at the dose of 50 mg/kg after 7 days of treatment resulted in improvement of pigeons' trichomonosis and total eradication of trophozoites in crop lavage. In comparison to MTZ, the standard antitrichomonal drug, no significant difference was observed between the essential oil treatment protocol and the standard drug (P<0.05). More important no side effect or complication was observed in pigeons treated with *A. sieberi*.

In spite of numerous researches on essential oils and extracts of plants for their antitrichomonal activity, plant-based products had poor market penetrations probably due to variations in the chemical constituents of taxonomically similar plants according to the used parts and the time or geographical area of collection [20]. To solve this problematic issue, isolation of plant bioactive materials and using them as leading compounds for development of novel antitrichomonals is an efficient approach. Carvacrol is one of the most studied bioactive terpenoid compounds; its antiprotozoal, antiparasitic, acaricide, and insecticide properties has been previously described [10,21,22]. Nano formulation of carvacrol possesses the advantages of increasing its physicochemical stability and water solubility; also enhances its biological activities. Several studies demonstrated higher efficacy of nano formulations of carvacrol against pathogenic microorganisms [23–25]. In the present study, NanoCAV showed promising antitrichomonal activity against trophozoites of *T. gallinae*, with 24 h LC_{50} value of 0.27 µg/ml and low cytotoxicity. In line with this finding, Galvão et al. [13] encapsulated carvacrol in nanolipid carriers and demonstrated its lower cytotoxicity and higher antileishmanial activity in comparison to carvacrol. However, in our study, based on the 12 h LC_{50} values no significant difference was noted for *in vitro* antitrichomonal activity of NanoCAV relative to CAV. On the other hand, it was reported that nanoencapsulation of carvacrol resulted in higher antileishmanial activity of formulation in the infected mice [13]. Since no *in vivo* study was done for NanoCAV, predicting antitrichomonal activity of this formulation in the infected pigeons is speculative and further *in vivo* studies are needed to warrant efficacy and safety of this formulation in infected birds.

In conclusion, the developed formulation of NanoCAV based on *in vitro* activity and low cytotoxicity, can be further studied for its efficacy and safety profile in the trichomonosis infected birds, and may serve as a leading approach in the treatment of bird trichomonosis.

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