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In vitro study of curcumin calcium carbonate phosphate nanoparticles (Curcumin-NPs) impacts on the meriz goat's coccidian oocysts

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Abstract

Nanoparticles biosynthesis has an essential and increased role in delivering medical compounds. Calcium carbonate phosphate nanoparticles ($CaCO_3$-NPs) were prepared as a stabilized amorphous and incorporated with herbal curcumin extract as an anticoccidial agent *in vitro*. $CaCO_3$ - NPs were tested against local meriz goat coccidian oocysts. Concentrations were used 2, 4, 8, 16, 30 and 50 mg/ml shows oocysticidal effects and sporocystidal effects at concentration of 100, 200, 400, 800 and 1000 µg. Sporulation inhibition assay was used for 24 and 48 hours. Results of significant oocysticidal effect were seen to inhibit in the concentration of 30 - 50 mg/ml and able to inhibit the sporulation of meriz coccidian parasite oocysts at a rate of 92.54±3.51%. The sporocysticidal effect was also significant with a curcumin nanoparticles concentration of 400-1000 µg/ml with a rate of 98.1±2.11%. The stability of prepared curcumin nanoparticles was examined against various pH levels 4.01, 7, and 9.21 at multiple temperatures 4, 25, 60, and 100°C. Investigation after 1, 6, 12, and 24 hours of treatment occurs according to various treatments. Stability was assessed by spectrophotometric indicated significant reductions for pH 4 and 9 after one hour of treatment and at the temperature of 60°C and 100°C after 12-24 hours of treatment. These results reflect promising hopes of exploiting $CaCO_3$ curcumin nanoparticles to eradicate coccidiosis as they are composed of and prepared from natural substances.

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Introduction

Nano-biotechnology is a division of science that is exploited to enhance the efficacies of nanoparticles (NPs) for various applications of therapeutic drug categories by using numerous techniques and methods to study, design, and fabricate substances at atomic and molecular levels. The original concept of investigating ingredients and biological systems at the nanoscale went backs to more than 40 years ago when Richard Feynman presented a lecture in 1959 at the annual meeting of the American Physical Society at the California Institute of Technology (1). Recently, nanotechnology can include research, and advanced work on materials and species at length scales 1 to 100 nm. Nanotechnology is vital to biology since several biological parameters have molecular structures at the nanoscale levels. These parameters cover a wide variety of basic structures, such as proteins, polymers, carbohydrates (sugars), and lipids, which have a countless variety of physical, chemical, and functional properties (2,3). Nanoparticles are classified into diverse classes, for example, inorganic nanoparticles, organic nanoparticles, ceramic nanoparticles, and carbon-based nanoparticles. The inorganic nanoparticles are more sub classified into metal

and oxide nanoparticles, such as oxide. Correspondingly carbon base nanoparticles are classified into fullerene, carbon nanotubes, graphene, carbon nanofiber, and carbon black nanoparticles, which are furtherly classified based on dimensions, such as one-dimension nanoparticles, two-dimension nanoparticles, and three-dimension nanoparticles (4,5).

Recently, tissue-specific criteria have been utilized to characterize homing peptides and prepare nanoparticles to target drug delivery toward specific body organs. This strategy decreased adverse treatment complications on unintended tissues (6). Scientists have also prepared nanoparticles to increase the effectiveness of applied medications and minimize off-target effects (7,8). In addition, drug-loaded nanoparticles, for instance, liposomes, were synthesized and designed to bind to tissue-specific epitopes to release incorporated medication at the intended organs, such as the placenta (8,9). However, these nanoparticles may exert certain complications and inhibit specific physiological functions (10). $CaCO_3$ has to be significantly considered in research due to its rewards, including affordability, low toxicity, biocompatibility, cytocompatibility, pH sensitivity, sedate bio-degradability, and environmentally responsive materials (11,12). Additionally, modification of NPs for therapeutic purposes has concerned considerable attention by researchers to improve the solubility, stability, circulation half-life, and biodistribution of the encapsulated agent (13,14).

Turmeric Curcumin (*C. longa*) as an old-style medicinal herb was castoff for various determinations for refining of overall health and also as a medication in many cases (15,16). Curcumin was intermittently used in animal health due to its growth increment, antimicrobial, anti-inflammatory, and neuroprotective effects (13,17). The dried turmeric rhizome consists of 3-6% terpenes and terpenoids, 6-8% proteins, 6-10% fats, 60-70% carbohydrates, and 3-6% fibers (18). Curcumin or diferuloylmethane [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], diferuloylmethane, diferulylmethane, natural yellow 3 with known structure. It is a hydrophobic and polyphenolic complex extracted from *Curcuma longa* (19). Curcumin is also used to reduce parasitic coccidiosis disease's pathological manifestations in animal and poultry industries (20). Many studies on curcumin were done either *in vivo* or *in vitro* (20,21) using the direct raw or extract materials of the compound (13,22) as well as in *ex vivo* (23) to ensure medicinal application of these effects.

Previously (24), local meriz goats (*Capra ibex*) showed a high infection rate 37.67% with six coccidias of genus *Eimeria* of various species *E. alijevi, E. christenseni, E. caprovina, E. minasensis, E. megaembryonica, E. ninakohlyakimovae,* and *E. megaembryonica*, this infection rate necessitated some suggestions and trials to work on the prevention, treatment, and reduction of infection rates via using nanoparticles as a novel approach to improving the health and welfare of meriz goats, which are considered species of the most economically significant animals.

In this study, CaCo3 - curcumin NPs were used to investigate their effects on the oocysts (as Oocysticidal) and the sporocysts (as sporocysticidal) of coccidian parasite of genus *Eimeria* that infected local meriz goats *in vitro*. These effects of CaCo3 - curcumin NPs will be examined at various levels of concentration, time, pH, and temperature.

Materials and methods

Herbal extract of curcumin

Curcumin of 99% purity was purchased from (Sigma-Aldrich), which contain Curcumin, ≥94% (curcuminoid content), ≥80% (Curcumin) according to product comparison guide of the company.

Curcumin nanoparticles preparation

$CaCO_3$ nanoparticles were provided from a local chemical store, and the amorphous phase was prepared according to Rao *et al*. (12,25). Briefly, the aqueous solutions of $CaCl_2$, $NaCO_3$, $Na_2HPO_4.12H_2O$) with deionized distilled water 100 ml were used for each. $CaCl_2$ (10 mM), 0.07419X2 g of $NaCO_3$, $Na2CO_3$ (0.107419X2g) and 0.1074X2g of $Na_2HPO_4.12H_2O$ were dissolved in 20 ml of deionized distilled water. Sodium Phosphate hydrate solution was added as a drop by drop to $CaCl_2$. All substances in the solution were allowed to mix for 15 minutes at 30°C. Curcumin solution (w/v) preparations in proper concentrations at 2, 4, 8, 16, 30 and 50 mg per ml for oocysticidal and 100,200,400, 800 and 1000 µg for sporocysticidal effects, were prepared with sterilized deionized distilled water. These solutions were added simultaneously into the $CaCO_3$ solution.

Oocysticidal and sporocysticidal effects

Oocysts of a coccidia parasite were isolated from local meriz goats as previously described (24). Five grams of fresh fecal sample were collected in sporadic containers, then transferred to the lab and mixed with distilled water v/v (1X10). The sample was sieved with four layers of sterilized cotton gauze and washed three times with PBS. After that, 0.15 ml was placed on a clean glass slide, the coverslip was adapted, and total coccidia oocysts were counted. One hundred oocysts were used for each treatment to estimate the oocysticidal effect by preventing further development and sporocysticidal effect by preventing the formation of sporocysts and sporozoites inside it, and followed by a simple compound microscope (X40). This procedure was previously performed in the poultry type of coccidia (22,26). Then, the proper concentration of curcumin- NPs was used according to the planned time and pH described above.

Stability of curcumin-NPs

Preparation was tested against various pH in different standard buffer solutions at pH 9.21, pH 7, and pH 4.01. Stability was tested after 1, 6, 12, and 24 hrs. by screening change in the spectrum of a spectrophotometer at 424 nm. This is the first study that examines the stability of curcumin-NPs using this method. Thus, it is considered a novel method for this type of NPs.

Thermal stability of prepared curcumin - NPs

It is examined at 4, 25, 45, 65, and 100°C for 1, 6, 12, and 24 hrs. (For boiling, just 1, 2, and 3 hrs. were used). The screening was done as described above for both pH and temperature with the aid of a spectrophotometer according to protocols used in previous studies (12,25).

Statistical analysis

All tests were done f to minimize possible reading errors. Statistics of Chi-square for both, oocysticidal and sporocysticidal effects was adapted, and ANOVA was used for pH and thermal stability with the aid of Duncan's multiple range test by using PROC GLM procedure of SAS (27).

Results

According to previously published scientific research, the $CaCo_3$ - curcumin NPs were prepared successfully. It shows well and observed effects according to the following sequence of current treatments against the local meriz *Eimeria* coccidia parasite (Figure 1).

Table 1 explains the results of the In-vitro oocysticidal effects of curcumin-NPs against local meriz (*Capra ibex)* coccidian parasites (temp/time/concentration). The data showed significant variations according to used *p* values (P=0.05 and P=0.01). In 2 mg/ml concentration, it is clear that sporulation inhibition rate arranged from 25.7 to 37.5% for various temperatures and times. In a concentration of 4 mg/ml$^-$ and above, the results exhibited a significance from 12 hours at 39°C and up. These results reflected that prepared curcumin-NPs effectively inhibited the sporulation process of coccidia oocysts *in vitro* according to various concentrations at different temperatures and rates of 25.7-88.3%.

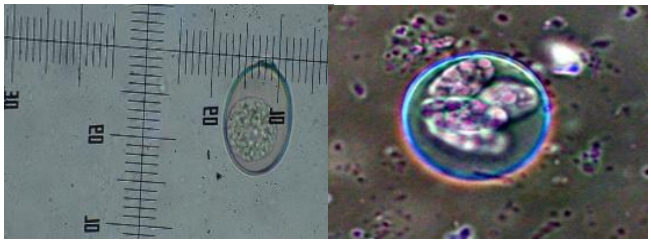

Figure 1: Oocysts of Meriz coccidian (left is unsporulated, right is sporulated with four sporocysts each of them contains two sporozoites) X40 wet preparations.

Table 2 showed that the prepared NPs started their effect at the lower concentration (100mg. ml$^-$) at about 55.4 - 66.9% at various times (after 1 and 3 h) and different temperatures 25°C and 37°C. The sporocysticidal effects exhibited significant variations in relationship with the concentration, time, and temperature from 55.3% to 66.9% for 100 mg /ml to be significantly different (P=0.001) from the 200 mg/ml concentration above. The total sporocysticidal effect was seen to start at a concentration of 400 mg/ml after three h of treatment at 39°C, whereas for lower used temperature 25°C and time 1 h, the total sporocysticidal effect started at a concentration of 1000 mg/ml. These results indicate that prepared were very effective in their sporocysticidal activity of coccidia oocysts *in vitro* at various concentrations and different temperatures at a rate of 55.3-100%. Also, the stability of curcumin-NPs preparation was proved against various pH and thermal stability of prepared curcumin-NPs.

Table 1: Curcumin-NPs *in vitro* oocysticidal effects against local meriz coccidian parasites

Curcumin- NPs (mg/ml)[1]	Temperature 25°C (hr.)				Temperature 39°C (hr.)			
	12	24	48	72	12	24	48	72
2 (mg/ml)[1]	25.7^{a}	30.1^{a}	31.6^{a}	31.5^{a}	30.2^{a}	32.4^{a}	37.5^{a}	39.9^{b}
4 (mg/ml)[1]	26.3^{a}	29.1^{a}	30.5^{a}	31.7^{a}	50.6^{b}	53.8^{b}	52.5^{b}	55.5^{b}
8 (mg/ml)[1]	41.2^{b}	45.8^{b}	51.9^{b}	59.3^{c}	49.1^{b}	53.7^{b}	56.3^{c}	66.9^{c}
16 (mg/ml)[1]	50.1^{b}	55.4^{c}	59.2^{c}	60.1^{c}	69.9^{c}	72.2^{c}	79.1^{d+}	80.1^{d+}
30 (mg/ml)[1]	53.2^{b}	59.2^{c}	60.1^{c}	65.3^{c}	62.1^{c}	69.2^{c}	70.3^{c}	80.4^{d+}
50 (mg/ml)[1]	55.8^{c}	60.3^{c}	66.6^{c}	70.9^{c}	60.7^{c}	64.1^{c}	72.1^{c}	88.3^{d+}
Control negative (2% Pot Dichromate w/v)	21^{a}	1^{++}	0.5^{++}	0.5^{++}	12^{++}	1^{++}	0.5^{++}	0.5^{++}
Control positive (0.5 mg Amprolium /ml)	95^{+}	99^{+}	100^{+}	100^{+}	99^{+}	100^{+}	100^{+}	100^{+}

1= Vs. 100 oocystes / ml // Normal rectal goat temp is 39// By Chi square. a=Is significance in lower than others (c, b and d). b= Is significance in P=0.05 to others (a and b). c= Is significance in P=0.05 to b, and in P= 0.01 to a. d= Is significance in P=0.01 to all others (a, b and c). += Is none significance among all these treatment vs. control positive. ++=Is significance (P=0.05 and P=0.01) for all treatments vs. control negative.

Table 2: Curcumin-NPs *in vitro* sporocysticidal effects against local meriz coccidian parasites

Curcumin- NPs (µg/ml)[1]	Temperature 25°C		Temperature 39°C	
	1 hs	3 hs	1 hs	3 hs
100 (µg/ml)[1]	55.3[a]	59.7[a]	61.2[a]	66.9[a]
200 (µg/ml)[1]	70.4[b]	89.5[c]	90.2[c]	99.1[c]
400 (µg/ml)[1]	85.2[c]	95.4[c]	98.1[c]	100[c]
800 (µg/ml)[1]	90.1[c]	100[c]	98.2[c]	100[c]
1000 (µg/ml)[1]	100[c]	100[c]	100[c]	100[c]
Negative control (PBS)	0	2.1	0	20.9[?]
Positive Control (Amprolium 50 µg/ml)	80.5[b]	100[c]	95.6[c]	100[c]

1= Vs. 100 sporocysts / ml // Normal rectal goat temp is 39^0C// Chi square is used. a= is significance (P=0.05) vs. b. b= is significance (P=0.05) vs. a and c. c= is significance (P=0.01) vs. a, and (P=0.05) vs. b. ?= Coccidia sporocysts are vital (in about 20.9/100) vs. this time and temperature.

Table 3 shows the curcumin - NPs stability ratio versus various pH and temperatures by using absorbance at 424 nm through the release method. The NPs were stable, and the data has revealed a lower releasing ratio 13%, 5%, and 10% from curcumin at 4°C at various pH 9.21, 7, and 4.01 with no significant variations correlated to the prepared concentration. Releasing ratio started to elevate with an increasing temperature 25°C and above. The pH played a major role in releasing curcumin as the temperature raised. Significant (P= 0.001) variation was observed from 4°C to 100°C. The releasing ratio was seen to be stable and acceptable 13-45% until 60°C for various pH readings. The data from this study shows that the curcumin - NPs preparations were stable at the range of releasing time in about 13-94% according to various temperatures and pH.

Table 3: Curcumin - NPs stability ratio vs. various pH and temperatures

Temperatures*	pH 9.21	pH 7	pH4.01	Concentration
4°C	13[a] %	5[a] %	10[a] %	100 mg / ml Curcumin-NPs in PBS
25°C	33[b] %	27[b] %	39[b] %	100 mg / ml Curcumin-NPs in PBS
60°C	38[b] %	35[b] %	45[b] %	100 mg / ml Curcumin-NPs in PBS
100°C	94[c] %	90[c] %	85[c] %	100 mg / ml Curcumin-NPs in PBS
Blank	0 %	0 %	0 %	PBS solution
Standard	97 %	95 %	98 %	100 mg/ml of Pure Curcumin in PBS

* = Average of 1, 2, and 3 h for each tested preparation was counted and ANOVA was used. a= none significance among them. b= significance (P=0.01) vs. a and c. c= significance (P=0.01) vs. a and b.

Discussion

Coccidiosis is a significant protozoa disease of many animals such as sheep, goats (28), camels (29) as well as poultry of various species (30,31). Herbal pharmacological medicine shows an attractive category in control of it by inhibiting its life cycle. Effectivity of inhibiting sporulation process of coccidia oocysts *in vitro* was observed by Manafi (32), who is showed that herbal medicines in chicken (*in vivo*) leads to a reduction in the clinical signs of coccidiosis by degeneration of schizonts of Oocysts, which indicates a decrease in the sporulation of the parasite. In addition, Kheirabadi *et al.* (33) have used herbal medicines in chicken to reduce sporulation and oocysts per gram of coccidia. Both studies were conducted *in vivo*, however, in chicken, the herbs are not specified, and they are unrelated to Curcumin- NPs.

Furthermore, Yadav *et al.* (21) have discussed the influence of different doses of curcumin herbs on many aspects of chicken performance, including their effects against coccidia. The researchers used an *in vivo* challenge test with food supplemented with extract of curcumin, which was obtained from the herbal plant *Curcuma longa.* In their study, they have observed similar activities. Similar effects were seen in previous studies on poultry (22,34), but not for *in vitro* work, animal species such as meriz goats, using different doses, times, and temperatures.

These bioactivities of curcumin could be attributed to the components that have been derived from a rhizome of turmeric (*Curcuma longa*), which may interact with several cellulars or molecular targets (such as many cellular communication molecules as NF-KB, JAKs/STATs, MAPKs, TNF-γ, IL-6, PPARγ, and TRPV1). Moreover, the antitumor, antimicrobial, and wound-healing effects are due to curcumin bioactivity (35). The suppression of gluconeogenic gene expression could be the cornerstone of this action for all eukaryotic cells (36). Also, the ability of curcumin to modulate the functions of multiple signal

transductions is linked with the attenuation of acute and chronic diseases (37).

The preparation of nanoparticles from natural resources has shown specific medicinal effects that can be useful in treating specific kinds of pathological disorders (38,39). Many studies show an agreement that curcuminoids as bioactive compounds of *C. longa* L. can remain stable while exposing to heating treatment during the preparation process (40). Furthermore, Oshi *et al.* (41) found that the release rate of curcumin from the core-shell nanoparticles was low at a pH mimicking the stomach and small intestine. In contrast, it was higher at a pH mimicking the colon and not dissolved in the upper GIT, resulting

in the high availability of the drug in the colon for therapeutic activity. In addition, Sun *et al.* (42) clarified that $CaCO_3$ nanoprobe resists low pH effect *in vitro*. Previous investigation (10) on other preparations of nanoparticles displayed that they can remain stable for 28 days, but these studies have used 4°C as the only storage temperature. Researchers have also demonstrated that nanoparticle preparations show efficient releasing properties as in this study regarding the incorporated materials at the site of action (8,9) or different NPs compounds used (12,37).

Conclusion

Curcumin- NPs prepared in the current study showed influential oocysticidal and sporocysticidal effects on coccidia oocysts In -vitro. These findings are considered promising strategies to be applied in the field as the compounds are derived from natural sources, and they are reliable to be exploited for the prevention and control of coccidia and to be prepared as a pharmaceutical formulation. Regardless of complex techniques used in the preparation and investigation of nanoparticles, the current study revealed that spectrophotometric methods are applied easily in the study stability of nanoparticles, which may be essential for further research when field application may be investigated lately.

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Conflict of interest

The authors declares that there are no any of conflicts of interest with regards to the manuscript

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دراسة في الزجاج لتأثيرات الأجسام النانوية لكاربونات الكالسيوم الكركمية على أكياس بيوض أوكريات معز المرعز

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الخلاصة

يعد التخليق الحيوي للجسيمات النانوية مهم جدا في إيصال المركبات الطبية المستخدمة لأغراض علاجية في بعض الأمراض. وبعد تحضير الجسيمات النانوية من كاربونات الكالسيوم والمحملة بمستخلص الكركمين والمستقرة امفوتيريا استخدمت في الزجاج لعلاج الكوكسيديا الاكرية في المختبر. استخدمت تراكيز مختلفة من الجسيمات النانوية الكركمية ٢,٤، ٨، ١٦، ٣٠ و ٥٠ ملغم/مل كمبيدة لأكياس بيوض كوكسيديا معز المرعز (مثبطة للتبوغ) ومبيدة لأبواغ أكياس البيوض بتراكيز مختلفة ١٠٠ ،٢٠٠، ٤٠٠، ٨٠٠، ١٠٠٠ ميكروغرام/مل. تمت متابعة وقت تثبيط الأبواغ بعد ٢٤ و ٤٨ ساعة وكانت النتائج بمعنوية عالية للتراكيز ٣٠ -٥٠ ملغم / مل وبنسبة ٩٢,٥٤±٣,٥١ %. وكان التأثير المبيد لأبواغ أكياس البيوض عالي المعنوية أيضاً بتراكيز ٤٠٠ - ١٠٠٠ مايكرو غرام وبنسبة ٩٨,١±٢,١١ %. وعند دراسة استقرارية الأجسام النانوية الكركمية المحضرة تجاه الحامضية ٤,٠١، ٧ و ٩,٢١ وبدرجات حرارة مختلفة ٤، ٢٥، ٦٠ و ١٠٠ وبأوقات مختلفة ١، ٦، ١٢ و ٢٤ ساعة بمختلف المعاملات المستخدمة باستخدام المطياف الضوئي لم يلاحظ تأثير معنوي بالاستقرارية إلا بعد ساعة فاكثر بدرجات الحامضية المتطرفة عند ٤,٠١ و ٩,٢١ وكذلك بدرجات الحرارة ٦٠ و ١٠٠ درجة مئوية بعد ١٢ - ٢٤ ساعة. هذه النتائج تعكس أمال واعدة باستخدام الجسيمات النانوية الكركمية في مكافحة الكوكسيديا الاكرية كونها مركبات طبيعية.