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Original Article

Synthesis, Characterization, and Anti-parasitic Activity Evaluation of the Synthesized Chalcone against *Toxoplasma Gondii* Isolated from Cases of Abortion in Al-Najaf City

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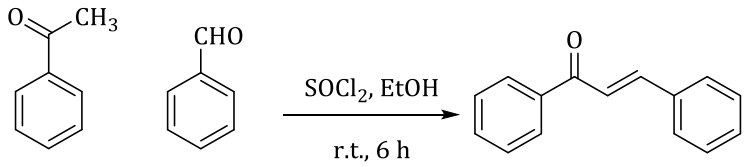
ABSTRACT

Toxoplasmosis is a well-known and widespread parasite infection. Although diagnosis has come a long way, therapeutic approaches have not changed since the 1940s. Using $SOCl_2$/EtOH as a catalyst, the Claisen-Schmidt condensation method has been used in this study to effectively synthesize chalcone. The anti-parasitic effect of the synthesized chalcone was evaluated at doses of 1, 10, 50, 100, 250, 500, and 1000 µg/ml against isolated *Toxoplasma Gondii* from aborted cases compared with spiramycin. The isolated *T. Gondii* in this therapeutic investigation was used as strong positive specimens for inoculum on Vero cells for parasite propagation. A significant reduced the fold expression values (2^-ΔΔCT), which are a measure of how well chalcone inhibits the proliferation of parasites. Compared with spiramycin, which demonstrated an IC_{50} of 281.19 g/mL, chalcone has an inhibitory concentration (IC_{50}) of 142.9 µg/mL. The results of this study reveal that chalcone can be used as a medication to treat toxoplasmosis because it had an antiparasitic impact on *T. Gondii*. To assess the therapeutic index of chalcone, molecular, synergistic, and *in vivo* monitoring investigations are essential.

GRAPHICAL ABSTRACT



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Introduction

Toxoplasma Gondii, an intracellularly spreading parasitic protozoan, is the major cause of the important worldwide disease toxoplasmosis. It is a common disease that can affect any warm-blooded mammal, including about one-third of people, wherever in the world [1,2]. Ninety percent of pregnant *T. gondii*-infected women don't have any symptoms. After a five to eighteen-day incubation period, pregnant women with symptoms were compared with non-pregnant women with an infection such as influenza [3-5]. During the oocyst development stage, Felidae family members such as cats may behave as the final hosts. This stage is a component of the faeces life cycle of cats that is resistant to environmental factors and spreads infection through oral intake. One of the main ways that warm-blooded species, such as humans or other animals, acquire the illness is through ingesting oocysts. *T. gondii* bradyzoites reside, surrounded by a glycan-rich cyst wall, predominantly in brain and muscle tissue and which are transitional hosts for oocysts. Trans-placental transfer, blood transfusion, organ transplantation, lab-acquired inoculations, and mechanical transmission by flies, cockroaches, and earthworms are other infection methods [6-9].

In the past, there were two antibiotic-based anti-parasitic treatments. For the prevention and treatment of toxoplasmosis, these antibiotics have an inhibitory effect on the protein synthesis of parasites [10]. To avoid fetal infection, spiramycin was typically used as a prophylactic. Another way to treat the disease was with a combination of sulfonamide and pyrimethamine, which should be stopped 14 weeks before pregnancy [11].

The prevalence of spiramycin resistance is highly correlated with the use of medications in a particular area and may be increased in various ways. Due to the failure of spiramycin and the increasing incidence of fetal infection with each trimester, sulfadiazine and pyrimethamine were later used during pregnancy. Since the encysted bradyzoite parasite, which can be reactivated later in life, was not affected by any of the treatments indicated above. Therefore, toxoplasmosis was not completely eradicated [12].

Many fruits and vegetables contain phytochemicals called flavonoids, which have a range of biological effects including anti-inflammatory, anti-cancer, antiviral, and cardio- and neuroprotective characteristics. These biological functions are influenced by the type of flavonoid and its therapeutic mechanism of action to treat diseases. Numerous flavonoids, which are found in abundance throughout the plant kingdom, are biosynthesized utilizing precursors known as chalcones, which have biological effects compared with those of flavonoids [13-19].

There is a significant effort being made to find and combine innovative therapies for the latent and acute *T. gondii* infection forms, even as human disease therapy advances [10]. Therefore, it is quite interesting to investigate anti-parasitic effectiveness of chalcones against *T. gondii.* The main goal of this study was to evaluate anti-parasitic properties of chalcone with those of the commonly used drug spiramycin.

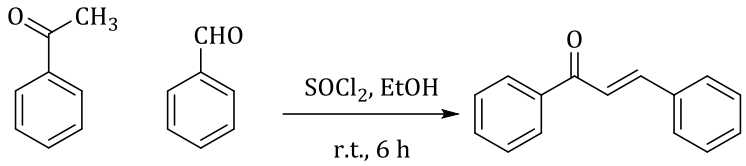
Materials and Methods

Acetophenone and benzaldehyde were both obtained from British Drug Houses in the United Kingdom (UK) and Lobachemie in India, respectively, for utilization as reactants at University of Kufa/College of Pharmacy/Laboratory of Pharmaceutical Chemistry/Najaf, Iraq. By using Shimadzu Fourier-transform infrared (FTIR) 8400 spectrophotometer and Potassium bromide disks, the Infrared (IR) spectrum of the synthesized chalcone was recorded. Via the electrothermal melting point equipment from Cole-Parmer Ltd (UK), the melting point of the product was obtained by using the open capillary technique [18]. Through the ascending thin layer chromatography technique and a mobile phase consisting of ethyl acetate: petroleum ether (30:70), the progress of the reaction and the purity of the product were examined. The spot of the synthesized chalcone was made visible either through Ultraviolet 254 nm light irradiation, derivatization, or reactivity to iodine vapor.

Chemical synthesis of chalcone

The mixture of benzaldehyde (1.01 mL, 10 mmol) and acetophenone (1.16 mL, 10 mmol) was stirred

for 6 hours in an ice bath before adding thionyl chloride (0.75 mL, 8 mmol). After being stirred for 20 and 30 minutes, the solution started to congeal. The mixture was precipitated by adding 10 mL of cold, distilled water before filtering after standing for a whole night. The resultant solid was then rinsed twice with cold water (50 mL), once with diethyl ether (20 mL), and once with ethanol (20 mL). The previously washed solid was dried to produce chalcone. This procedure was carried out in line with the following plan scheme [18].



Scheme 1: The synthetic diagram of chalcone

Spiramycin and chalcone solutions

The spiramycin and produced chalcone, each weighing 0.01 gm, were first dissolved individually in a minimal amount of dimethyl sulfoxide (DMSO). The final volume of these two solutions was then brought up to 10 ml by diluting them with Dulbecco's Modified Eagle Medium (DMEM). The DMSO percent was never more than 0.1% [18]. Next, these solutions were heated to 37 °C and agitated for roughly an hour over a magnetic stirrer. These solutions were used as stock solutions, from which maintenance media were used to produce (1, 10, 50, 100, 250, 500, and 1000 µg/ml) concentrations.

Vero cell lines

African green monkey kidney (Vero) cells provide a suitable host cell system for proliferation and maintaining the *T. gondii* role in cytopathic effects. These cells were donated kindly by Faculty of Pharmacy, Kufa University for this study.

Vero cell culture inoculation

A 25 cm^3 flask was used to create Vero cells. The pH at 6.8 to 7.1 was maintained for media of growth and maintenance. Fetal calf serum (FCS) was used to generate growth and maintenance media at 10 % and 2% concentrations, pretreated for cell culture with penicillin and streptomycin at concentrations (100 IU/mL) and (100 µg/mL), respectively. Until a full monolayer had formed, the cells were incubated and monitored at 37 °C every day, and then the inoculation of the cells was done with half milliliter of parasite inoculum for every flask of cell culture. The flasks were incubated at 37°C for one hour, and then washed three times with a maintenance medium before being checked every day for three to seven days to see the occurrence of any parasite growth, observation of cytopathic effect (CPE), and the emergence of the amastigote phase.

Detection by real-time quantitative polymerase chain reaction technique

For amplifying a 71 base pairs DNA fragment of the *T. gondii* B1 gene, quantitative real-time PCR was used to detect the conserved section of the *T. gondii* gene that was acquired from women (20-40 years old) who lost a pregnancy due to toxoplasmosis disease [20,21]. Subsequently, for amplifying 112 base pairs of RE sequence, nested PCR technique was employed and by using one microliter of the PCR products (extended 10:1) for *T. gondii* after the previous amplification as a second-row adjunct and displacing it with the internal group of the primers. The proper product size was confirmed via a melting curve study. The B1 gene was amplified in two steps by PCR that was tuned for the specific beginning DNA concentration of fifty micrograms per milliliter. For the primary PCR step, denaturation was carried out by subjecting samples to heat at ninety-four degrees Celsius for three minutes, tracked by (forty to fifty cycles) at ninety-seven degrees Celsius heating for thirty seconds. For annealing and extension, heating at sixty-two degrees Celsius for forty seconds and seventy-two" degrees Celsius for fifty seconds were needed, respectively. The final extension was done by heating (seventy-two degrees Celsius) for five minutes by using a smart cycler real-time PCR

system (Smart cycler, U.S.A., version 2.0d). For the secondary PCR, an initial denaturation was performed by samples heating at ninety-five degrees Celsius for thirty seconds, tracked by (fifty to sixty cycles) at ninety-seven degrees Celsius for fifteen seconds, forty-five degrees Celsius for thirty seconds, and seventy-two degrees Celsius for forty-five seconds. The last extension was accomplished by heating at seventy-two degrees Celsius for ten minutes [22]. Oligonucleotide primers were used with a fluorescence-labeled TaqMan probe to amplify the *T. gondii* B1 gene and RE sequence were including primer B1 gene, forward "5`-"GAAAGCCATGAGGCACTCCA-3`, reverse 5`-TTCACCCGGACCGTTTAGC-3`, and probe 5`(6FAM) CGGGCGAGTAGCACCTGAGGAGATACA (TAMRA)-3`", "RE sequence forward 5`-AGAGACACCGGAATGCGATCT-3`", RE sequence reverse 5`-TTCGTCCAAGCCTCCGACT-3`" and RE sequence probe 5`(6FAM) "TCGTGGTGATGGCGGAGAGAATTGA (TAMRA)-3`, β-actin forward 5`-AGAGCTACGAGCTGCCTGAC-3` and β-actin reverse 5`-AGCACTGTGTTGGCGTACAG-3` primers were also used as an internal control gene"[23], all these primers were manufactured by Alpha DNA Company, Canada.

RNA isolation and real-time quantitative polymerase chain reaction technique

Total RNA was isolated from groups of Vero cell lines that had been microtitrated with chalcone by using YTzol pure RNA reagent to evaluate the expression of mRNA of the *T. gondii* B1 gene by qRT-PCR. Imbeaud S. *et al.* stated that concentration and purity of the nucleic acid were determined by using a Biodrop spectrophotometer (bio drop, UK) [24]. Subsequently, by using a cDNA synthesis kit, samples of equivalent amounts were supplied for reversing transcriptase to be converted into (cDNA). Twenty-five microliters of the volume of reaction agents at the mentioned conditions of real-time quantitative polymerase chain reaction technique was down-regulated or over-expressed for detecting whether the B1 gene after treatment with chalcone as compared with spiramycin by real-time quantitative polymerase chain reaction technique ($2^{-\Delta\Delta Ct}$ method). The expression levels of the RE gene from the cDNA were determined. In gene expression, the fold-change was compared with a calibrator sample and standardized to a "housekeeping gene β-actin" [25-27].

Statistical Analysis

Utilizing Microsoft Excel 2010, graphic statistical processing (Mean and R^2) was carried out.

The blotting of the concentration log of any substance tested vs. ΔC_T (delta cycle threshold) average was used to fit the half inhibitory concentration, with the $P<0.05$ value considered in all tests as significant statistically. The fold expression values of the RE gene gained on the real-time quantitative polymerase chain reaction technique were estimated comparative to a "housekeeping gene β-actin" by using the relative quantification of the Livak method.

Results

Synthesis of chalcone

Table 1 indicates that when chalcone was eluted on a TLC plate by using an ethyl acetate: petroleum ether (30:70) mobile phase, it produced with a good yield (75%) and a single spot with an R_f value of 0.58. Numerous characteristic absorption bands were visible in the IR spectrum of the synthesized chalcone at *v* values of 1665, 1604, and 1560 cm^{-1} (Table 2).

Table 1: physicochemical data for the synthezed chalcone

Chemical name	Molecular formula	M.w. (g/mol)	Physical appearance	Yield (%)	M.P. (°C)	R_f
(E)-3-(phenyl)1-(phenyl)prop-2-en-1-one	$C_{15}H_{12}O$	208	White crystalline powder	75	56-57	0.58

Table 2: IR spectral data and elemental analysis of the synthesized chalcone

IR band [KBr] υcm^{-1}	Interpretation
3028	Aromatic C-H stertching vibration
1665	C=O stertching vibration of ketone conjugated to both benzene and alkene
1604	C=C trans stertching vibration of alkene conjugated to both benzene and carbonyl group
1560	stertching vibration of C=C skeleton
1222	C-O asymmetric stertching vibration of ketone
823	Aromatic C-H out of plan-bending viberation 1,4-disubstituted benzene

Anti-parasitic effect of chalcone and Vero cell line

To investigate the effect of the prepared chalcone agent anti-parasitic against *T. gondii* tachyzoites compared with that of spiramycin. Vero culture has been used for *T. gondii* propagation to obtain a highly qualified *T. gondii* source with less possible host cell contamination (Figure 1-A). Figure 1-B depicts the formation of a rosette of intracellular tachyzoites in Vero culture within 4-7 days following *T. gondii* post-infection.

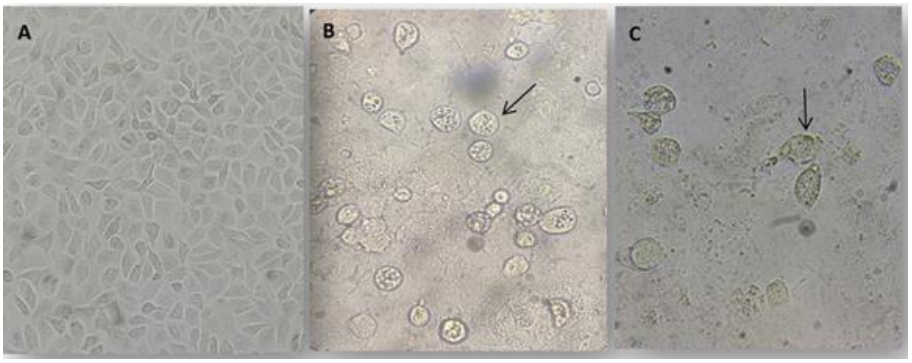


Figure 1: A-Vero culture before infection with *T.gondii*; B & C-Vero culture after infection with *T.gondii* and appearance of Rosette of intracellular tachyzoites

The synthesized chalcone microtitration at the concentrations (1 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml, and 1000 g/ml) indicated a positive correlation between the concentration of the chalcone and CT cycle number (R^2=0.8301) and considerable multiplication inhibition of fold expression values ($2^{-\Delta\Delta Ct}$). IC_{50} (the half inhibitory concentration) of the synthesized chalcone equivalent to 142.9 µg/ml in comparison to spiramycin, which demonstrated (IC_{50}=281.19 µg/ml) (Figures 3, 4, and 5). Given that the RE gene CT of the chosen original *T. gondii* strain was equivalent to 12.69 "(where, ΔC_T= -6.24 & 75.4091 copy number) and amplified throughout real-time quantitative polymerase chain reaction assay, it is obvious that chalcone functions to inhibit the growth of that strain (Figure 2-A).

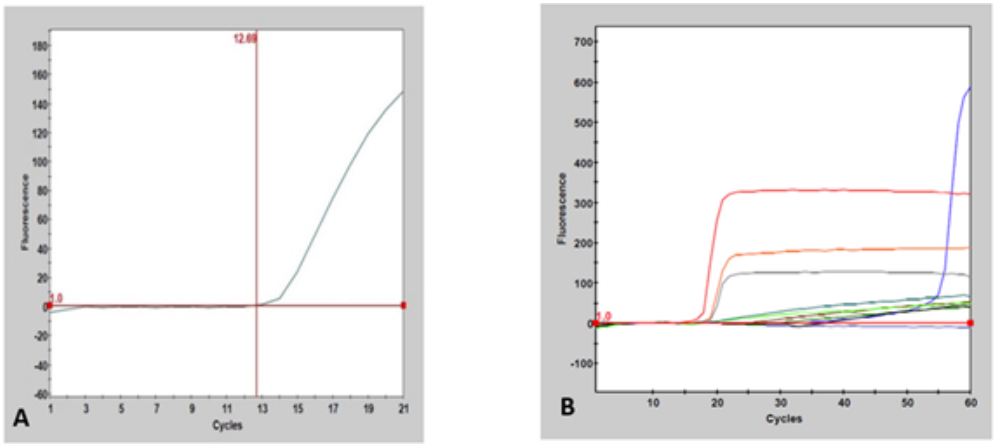


Figure 2: *T.gondii* RE gene expression before (A) and after treatment with different concentrations of chalcone (B) presented by nested qRT-PCR

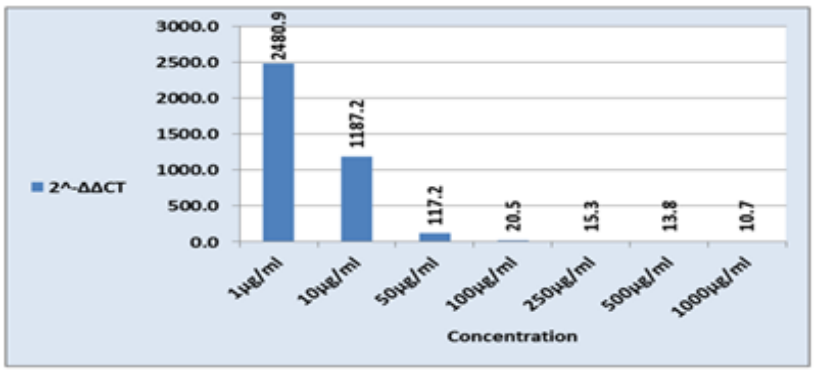


Figure 3: chalcone anti-parasitic effect presented by plotting fold experssion values (2^-ΔΔCT) versus concentration

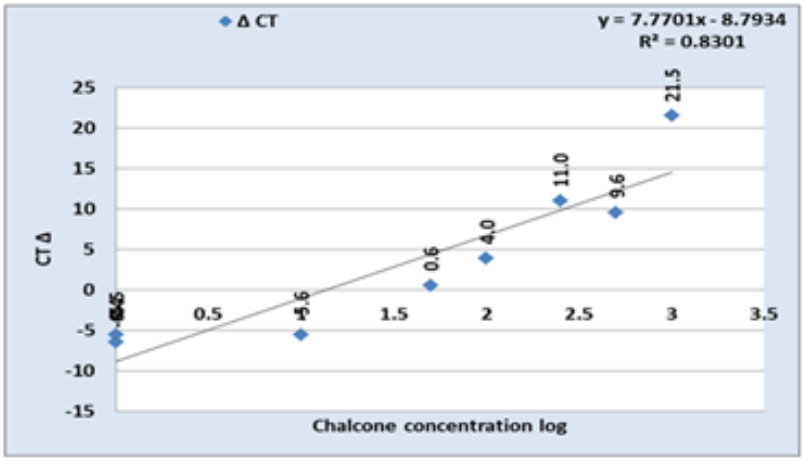


Figure 4: chalcone anti-parasitic effect presented by plotting concentration log versus the average of ΔCT

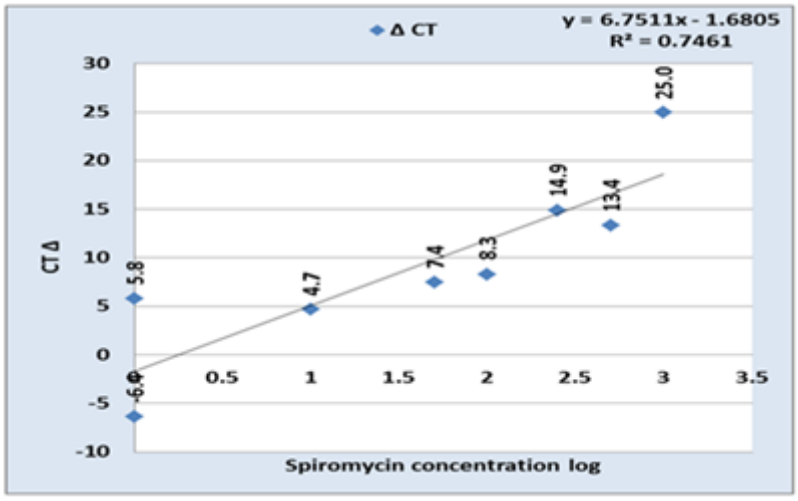


Figure 5: Spiramycin anti-parasitic effect presented by plotting concentration log versus the average of ΔCT

Discussion

T. gondii tachyzoites were successfully propagated in Vero cells by using an improved approach, resulting in high yields and viability. The SABP1 of the zoonotic parasite *T. gondii* (sialic acid binding protein-1), which is found on its outer membrane, is able to quickly bind to surface sialic acid of the host cell. Therefore, the SABP1 presence in *T. gondii* is of great sgnificance in invasiveness of parasite. So, sialic acid binding protein-1 appeared to facilitate *T. gondii* attachment and improve parasite invasion via sialic acid receptors [28].

Any nucleated cell can become infected by *T. gondii*. However, the primary cell lines chosen as the host cells of infection have a considerable impact on the *T. gondii* rate *in vitro* growth. According to the reports, Vero cells significantly ($P<0.05$) enhance the formation of *T. gondii* tachyzoites. Furthermore, earlier research has shown that different genotype strains have dramatically varied *in vitro* growth rates. This work indicated that *T. gondii* could be propagated and maintained by using Vero cells, since the amplification characteristic showed that such copy number was multiplied many times by culturing on Vero cells.

Chalcones are naturally occurring substances initially isolated from Chinese licorice (*Glycyrrhizae inflate*). It was unable to extract the naturally stable chalcones obtained from plants since chalcone synthase; the enzyme that converts chalcones into flavonoids exists. It is an ideal starting material for creating numerous heterocycles used in drug design, such as flavones, indols, isoxazole, quinolinone, and benzofuranone [29].

Chalcones can be made in various ways, but they are all essentially made by condensing aromatic ketones with aromatic aldehydes by using the right condensing agents [29,30]. In this study, chalcone was produced by a Claisen-Schmidt reaction between equimolar amounts of phenylethanone and phenylmethanal in an acidic environment (HCl), tracked by dehydration. "HCl was produced *in situ* by reaction of $SOCl_2$ with ethanol. This" procedure produced a good yield of chalcone quickly and without the creation of any unwanted byproducts. Using this synthetic method, a variety of chalcones with enhanced pharmacological potency and reduced side effects can be prepared and used as novel pharmaceutical agents in a broader range of effective therapeutic fields [18].

The results indicate that we would soon be able to utilize chalcone as a substitute drug because they suggested that it may have anti-parasitic potency comparable to or perhaps greater than spiramycin. This is in line with the observations of other researchers that chalcone compounds may have an intriguing antibacterial activity profile [16,19]. Hofei Si *et al.* emphasized in 2018 that Licochalcone A has a significant anti-*T. gondii* activity *in vitro* and *in vivo* and could be employed as a potential anti-*Gondii* treatment medicine. Licochalcone A may also carry out similar functions by impeding the lipid metabolism of parasite [31]. Therefore, it seemed fair to predict that our synthetic chalcone would exhibit the same behavior.

Despite this, chalcone derivatives continue to captivate researchers in the medical profession due to their uncomplicated synthesis, straightforward chemistry, and a variety of encouraging biological actions. However, chalcones have not yet received the attention they deserve, particularly given their high potential as a chemical source for creating and designing novel effective medications [29]. The strengths of this study are the value of the chalcone as a treatment for toxoplasmosis, its relatively easy and fast synthesis, and the successful propagation of the *T. gondii* tachyzoites in Vero cells without limitations. This followed in the same line as prior studies that noted the value of chalcones, predominantly as target proteins inhibitors linked to the *T. gondii* pathogenesis. In the future, it will be crucial to use molecular docking to investigate potential chalcone substitution patterns to develop highly effective inhibitors or modulators that are selective for a particular parasite receptor. According to the findings of this study, chalcone may be used as a treatment for toxoplasmosis because it had an anti-parasitic impact on *T. gondii*. *T. gondii* was successfully maintained and multiplied by Vero cells. In addition, it is necessary and advised to conduct *in vivo*, molecular, and synergistic impact monitoring studies to keep track of the therapeutic index of chalcone.

Conclusion

We have successfully synthesized chalcone efficiently by using $SOCl_2$/EtOH as a catalyst and characterized by IR spectroscopy. The synthesized compound was subjected to anti-parasitic evaluation against *T. gondii*. Compared with spiramycin, which demonstrated an inhibitory concentration (IC_{50}) of 281.19 µg/ml, chalcone has an IC_{50} of 142.9 µg/ml. Hence, the synthesized

chalcone shows strong anti-parasitic activity against *T. gondii* than that of spiramycin.

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Authors' contributions
All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

Conflict of Interest
All participating authors declare no conflict of interest and approve the final article.

Ethical issues
In this study, except for maintaining the secrets of the patient by the Helsinki Treaty, it is assured to patients that their information will be confidential and will be used only for research. In addition, no additional costs were imposed on patients. The proposal is approved by the Ethics Committee of the University of Kufa/College of Pharmacy.

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