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Evaluation of mono and combined nitrofurantoin therapy for toxoplasmosis in vivo using murine model

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ABSTRACT
Toxoplasmosis is a frequent disease with an estimated prevalence of more than one billion human cases worldwide and over one million new infections each year. It is classified as a neglected tropical disease by the CDC since 2019. The disease may pass unnoticed in healthy individuals but could be fatal in the immunocompromised. Moreover, no effective treatment is available against the chronic form of the disease. Available anti-Toxoplasma drugs are associated with many side effects. Therefore, search for new more reliable, more efficient, and less toxic therapeutic agents is a continuous endeavor. This study assesses the potential use of nitrofurantoin, a compound with well-established antimicrobial properties, as a potential anti-Toxoplasma drug in vivo. It compares its efficacy to the commonly used anti-Toxoplasma agent spiramycin by molecular and histopathological methods in acute and chronic infection. The results demonstrate a significant ability to eliminate the parasite (P < 0.001) whether used as mono- or combined therapy with spiramycin in the acute and chronic stages. When compared to the anti-Toxoplasma drug spiramycin, nitrofurantoin achieved similar efficacy in the acute and chronic infection (P = 0.65 and P = 0.096, respectively). However, better results were obtained when using a combination of both drugs (P < 0.001). Additionally, nitrofurantoin showed good inhibitory effects on the inflammatory process in the liver, kidney, and uterus of the experimentally infected animals. In conclusion, nitrofurantoin can be considered as a potential anti-Toxoplasma agent. Nevertheless, further studies are recommended before consideration for clinical trials.

1. Introduction
Toxoplasma gondii is one of the most successful parasites worldwide; it is an intracellular apicomplexan protozoon with an assembly of intermediate hosts including man. The parasite is the etiological agent of toxoplasmosis, a disease that has an enormous impact on the animal husbandry industry as well as human health and has contributed to severe economic losses [1]. Toxoplasmosis is classified as the second most common cause of deaths and the fourth leading cause of hospitalizations attributed to foodborne diseases [2]. T. gondii has a ubiquitous nature, it overcomes the immune system by using the host’s machinery factors to survive and replicate and thus can persist for the host’s entire life [3].

In humans, the parasite can be transmitted by various means but predominantly via the oral and congenital routes. Entering the body, the sporozoites are released from the oocyst, invade the intestinal epithelium and multiply intracellularly. The host cell dies and the tachyzoites are released, they invade adjacent cells and disseminate all over the body [4]. In immunocompetent individuals, the tachyzoites are transformed into bradyzoites and form tissue cysts in different organs, and the disease may pass unnoticed. However, acute toxoplasmosis presents as a mild or asymptomatic disease that evolves, under the host immune response, into a persistent chronic disease in healthy individuals. Chronic toxoplasmosis establishes as latent tissue cysts in the brain and skeletal muscles. In immunocompromised patients, chronic toxoplasmosis may reactivate, leading to a potentially life-threatening condition [5]. The degree of severity of congenital toxoplasmosis is inversely related to the gestational trimester at which the infection is acquired. Infection of the fetus during the first trimester often leads to abortion, stillbirth, or a child born with severe abnormalities of the brain and eyes, such as hydrocephalus, intracranial calcifications, deafness, mental retardation, seizures, retinochoroiditis, and even blindness [6]. T. gondii can affect many organs such as the lymph nodes, the eyes, the central nervous system, the kidneys, the uterus, and the liver. Previous studies have
proposed that hepatic involvement in toxoplasmosis occurs early in the course of infection and in many cases; it may go unnoticed as it may not produce any laboratory or clinical changes despite producing pathological changes [7]. McAuley [4] also suggested that an initial infection occurs in the uterus, leading to extra villous trophoblast infection and consequently causing fetal infection.

The gold standard method of diagnosis remains the serological detection of antibodies [8] detection of the parasite DNA by polymerase chain reaction assays (PCR) which has the ability to detect the parasite even at low concentrations [9]. Whereas treatment of toxoplasmosis relies on using a combination of anti-Toxoplasma drugs that interfere with the folate pathway [10], there are many concerns with the currently available medications. For instance, many chemotherapeutic agents have been reportedly linked to severe side effects such as life-threatening allergies, bone marrow suppression, and renal and hepatic problems [11]. Besides, many of the currently used drugs are not suitable for pregnant women, and parasites in AIDS patients show resistance against these drugs [12]. All these factors have urged the search for new more reliable, more efficient, and less toxic therapeutic agents.

Several medicinal plants such as Myrrh [13], Piper nigrum, Capsicum frutescens, Curcuma longa [14], Nigella sativa [15], Azadirachta indica (neem), Melia azedarach [16], and Zingiber officinale [17] have been tried, and some proved to have promising effects. In spite of that, each one had its own limitation. Recently, the emergence of nitro-containing compounds – originally used for the treatment of tuberculosis – as potent anti- Trypanosoma agents has ignited the investigations of their roles as alternative anti-parasitic drugs [18]. The lethal potential of these drugs is attributed to the production of free radical species, reactive oxygen species, and reactive nitrogen species which react with the pathogen cell wall enzymes [19]. One of these compounds, nitrofurantoin, originally used to treat urinary tract infections is active through inhibition of the citric acid cycle, DNA synthesis, and protein synthesis. This compound is known to be effective against a myriad of microorganisms, including gram-positive and gram-negative bacteria and various pathogens [18]. Nitrofurantoin is a hypoxic agent with activity against a myriad of anaerobic pathogens. It has also been used as an animal food additive due to its anti-bacterial and anti protozoal effects [20].

It is usually well tolerated and can be used safely during pregnancy. The potential use of nitrofurantoin as an anti-Toxoplasma drug has not been widely explored till now; only few studies have assessed its effect on the brain of murine models without any reference to other commonly affected organs like the liver, uterus, and kidneys [20]. Therefore, this study aims to explore the effect of nitrofurantoin alone or combined with spiramycin compared to spiramycin alone by studying the pathological changes in liver, kidney, and uterus of experimentally infected mice with T. gondii as well as the parasite load in blood samples.

2. Materials and methods

2.1. Parasite strain

Me49 non-virulent strain of T. gondii (obtained from the zoonotic diseases department at the National Research Center, Giza, Egypt) was regularly maintained by repeated oral inoculation of Swiss albino mice via gastric tube with 0.1 ml of brain homogenate of previously infected mice containing, approximately 1 × 10^5 tissue cysts/ml every 8 weeks to establish chronic toxoplasmosis. The mice brains were ground using sterile pestle and mortars, then diluted with saline to a concentration of 1 × 10^5 cysts/ml obtaining brain cyst suspension by using a hemocytometer [21]. Brains were then homogenized in a tissue homogenizer with 1 ml saline each, and one drop of brain suspension was added onto a microscopic glass slide, and the number of cysts was counted under a high power lens (x40) [22].

2.2. Experimental animals

A total of 90 laboratory-bred female Swiss albino mice 6 weeks-old weighing approximately 20–25 g each were included in the study. The mice were housed in plastic cages (six mice/cage) with white wood chips for bedding, fed by commercial complete food mixture and tap water for drinking provided ad libitum, and maintained under controlled conditions of lighting (12 h light/12 h dark cycle) and temperature (25 ± 2°C).

2.3. Drugs materials and administration

Spiramycin: Spiramycin was available as a film coated tablet produced by Medical Union Pharmaceuticals. Tablets (704 mg) were grinded then dissolved in distilled water (9 ml for each tablet) to make suspension (18 ml, two tablets were used). The homogenized suspensions were administrated orally (0.05 ml containing 4 mg for each mouse) [23].

Uvamine Retard: Uvamine Retard was available as capsules (100 mg) form. The active ingredient is nitrofurantoin, produced by Medical Union Pharmaceuticals. Capsules were evacuated then dissolved in distilled water (2.5 ml for each capsule) to make suspension, and the homogenized suspensions (10 ml, four tablets were used) were administrated orally to mice (0.05 ml containing 2 mg for each mouse) [20].
2.4. Experimental design
Mice were inoculated with 0.1 ml of the brain cyst suspension orally equal to 10 of avirulent Me49 strain of *T. gondii*/mouse.

2.4.1. Experimental groups
Animals were classified into three main groups:

- Group I: Non-infected, non-treated (normal control): included six mice.
- Group II: Infected non-treated mice (infected control). Mice were inoculated orally by 0.1 ml of brain cyst suspension: included 12 mice.
- Group III: Infected treated mice (experimental group). This group was further divided into six subgroups, each included 12 mice:
  a. Group III a: Infected mice treated with spiramycin 200 mg/kg for 2 weeks starting on the fourth day post infection
  b. Group III b: Infected mice treated with nitrofurantoin 100 mg/kg for 2 weeks starting on the fourth day post infection
  c. Group III c: Infected mice treated with combination of 100 mg/kg nitrofurantoin and 200 mg/kg spiramycin for 2 weeks 4 days post infection.
  d. Group III d: Infected mice treated with spiramycin 200 mg/kg for 2 weeks starting 6 weeks post infection (drug control for chronic phase of infection).
  e. Group III e: Infected mice treated with nitrofurantoin 100 mg/kg for 2 weeks starting 6 weeks post infection (drug control for chronic phase of infection).
  f. Group III f: Infected mice treated with combination of nitrofurantoin and spiramycin for 2 weeks starting 6 weeks post infection (drug control for chronic phase of infection).

2.4.2. End of experiment
At the end of the experiment (8 weeks), all mice were sacrificed, their liver, kidneys, and uterus were removed and sent for histopathological examination.

2.5. Pathological examination
The internal organs of the mice (liver, kidney, and uterus) were collected after culling and fixed in neutral buffered formalin 10%. The fixed samples were washed, dehydrated, and embedded in paraffin melted wax. The tissues were sectioned at 4–5 microns thickness and stained with hematoxylin and eosin (H&E) as routine stain for histopathological examination [24].

2.6. Molecular test
A peripheral venous blood sample (about 1 mL) was collected into EDTA tube. Each sample was mixed and divided into two Eppendorf tubes and then stored at −80°C for further processing. QIAamp DNA blood mini kit (Qiagen, Germany) was utilized for DNA extraction following the manufacturer’s directions. Extracted DNA concentration was assessed by NanoDrop 2000c Spectrophotometer (Thermo Scientific, U.S.A.). Readings were assessed at wavelengths of 260 and 280 nm.

Then, amplification and quantification of DNA were done by real-time PCR in ABI7900 (Applied Biosystems, CA, U.S.A) using SuperReal Premix Plus QuantiTect. Kit, SYBR Green (Tianjin, Shanghai) according to the manufacturer’s instructions and using the specific primers: TOXO 4: 5’CGCTGCAGGGAGGACGAAAGTTG3’ and TOXO 5: 5’CGCTGCAGACACAGTGATCTGATT3’. Real-time cycler conditions were 95°C for 15 min for initial denaturation, followed by 40 cycles of 95°C within 30 secs for denaturation, 55°C for 1 min for annealing, and 72°C for 1 min for extension step.

Due to the relative nature of quantification method, an adjustment was required for each sample. Briefly, DNA was diluted $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$ fold prior to amplification by real-time PCR and a standard curve was derived to obtain optimal amplification conditions [25].

2.7. Statistical analysis
Software (SPSS, Version 26.0 for Windows) was used. Qualitative data were summarized as frequency and percentage, while quantitative data as Mean and standard deviation, median, and inter-quartile range (IQR) after establishing their non-normality by K-S test (One-Sample Kolmogorov–Smimov Test) of normality for some variables, ANOVA test (F value): used to compare mean of more than two groups of quantitative data with using LSD as post hoc test. Kruskal–Wallis (KW) test and Mann–Whitney (MW) U test were used to analyze non-parametric quantitative variables. Differences were considered significant at $P \leq 0.05$.

3. Results
3.1. Histopathological findings
Histopathological examination of liver, kidney, and uterus of non-treated mice infected (Group II) showed severe granular degeneration and cell necrosis with the presence of tissue cyst. Liver examination revealed granular degeneration and necrosis of some hepatic cells (Figure 1a). Kidneys showed signs of acute nephritis in the form of severe vacuolar degeneration and sloughing of lining epithelial cells of renal tubules in the lumen associated with focal mononuclear inflammatory cell
infiltration mainly lymphocytes (Figure 2a); while the uterus showed severe degeneration and focal desquamation of lining epithelial cells of endometrium associated with decrease in number of uterine glands in the field and diffuse leukocytic inflammatory cell infiltration (Figure 3a).

Groups treated with nitrofurantoin monotherapy (Group III b & III e) revealed a better response to treatment in chronic stage of infection than in acute stage especially in the uterus represented by mild-to-moderate degenerative changes and inflammatory cell infiltration (Figures 3b & 6a). On the other hand,
groups treated with spiramycin (Group III a & III d) showed no significant difference in response to treatment between acute (Figures 1, 2 & 3) and chronic infection (Figures 4, 5 & 6).

The best results in histopathology were achieved in combination groups (Group III c & III f) whether in acute or chronic infection. It was superior to other group treatment with each drug alone; studied organs (liver, kidney, and uterus) showed mild degenerative changes, perivascular edema, and mild focal inflammatory cell infiltration. In addition, combination therapy showed relatively better results in chronic infection.

Figure 3. Uterus of mouse infected with T. gondii. (a) Non-treated: showing severe degeneration and focal desquamation of lining epithelial cells of endometrium associated with decrease in number of uterine glands in the field and diffuse leukocytic inflammatory cell infiltration (H&E, X100). (b) Treated with nitrofurantoin for 2 weeks 4 days P.I.: showing severe degeneration, necrosis and sloughing of lining epithelial cells of endometrium with some diffuse inflammatory cell infiltration (H&E, X100). (c) Treated with combination of spiramycin and nitrofurantoin for 2 weeks 4 days P.I.: showing mild degenerative changes of lining epithelial cells of endometrium associated with mild focal inflammatory cell infiltration (H&E, X100). (d) Treated with spiramycin for 2 weeks 4 days P.I.: showing partial degeneration and sloughing of lining epithelial cells of endometrium with some inflammatory cell infiltration (H&E, X200).

Figure 4. Liver of mouse infected with T. gondii. (a) Treated with Nitrofurantoin 6 weeks P.I.: showing granular degeneration of hepatic cells associated with focal area of inflammatory cell infiltration (arrow) and some individual necrosis of some hepatic cells (arrowhead) (H&E, X200). (b) Treated with combination of Spiramycin and Nitrofurantoin 6 weeks P.I.: showing mild vacuolar degeneration associated with mild focal perivascular inflammatory cell infiltration (H&E, X100). (c) Treated with Spiramycin for 6 weeks P.I.: showing vacuolar degeneration of hepatic cells with focal area of inflammatory cell infiltration in portal area (H&E, X200).
3.2. Molecular results

There was a significant reduction of the parasite load in different groups receiving mono and combined therapy compared to the infected non-treated group ($P < 0.001$) with superiority of the combined therapy groups (Group III c and III f). Comparison between different groups was performed and their statistical significance is presented in Table 1.

As depicted from Table 1, nitrofurantoin was as effective as spiramycin in terms of decreasing the parasite load both in the acute and chronic phases and the
### Table 1

Mean *T. gondii* reduction rate in blood samples from experimentally infected non-treated mice (Gp II) and after treatment with spiramycin 4 days P.I. (Gp IIIa), nitrofurantoin 4 days P.I. (Gp IIIb), combined therapy 4 days P.I. (Gp IIIc), spiramycin 6 weeks P.I. (Gp IIId), nitrofurantoin 6 weeks P.I. (Gp IIIe) and combined therapy 6 weeks P.I. (Gp IIIf).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Median (IQR)</th>
<th>Marker</th>
<th>Mean ±SD</th>
<th>KW, P</th>
<th>MW1, P</th>
<th>MW2, P</th>
<th>MW3, P</th>
<th>MW4, P</th>
<th>MW5, P</th>
<th>MW6, P</th>
<th>MW7, P</th>
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</thead>
<tbody>
<tr>
<td>Gp II</td>
<td>3845000 (3772500–3912500)</td>
<td>3857000 ± 121293.95</td>
<td>47.36, &lt;0.001</td>
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<tr>
<td>Gp IIIa</td>
<td>1125000 (922500–1483750)</td>
<td>1174500 ± 326679</td>
<td>3.78, &lt;0.001</td>
<td>0.47, 0.001</td>
<td>0.45, 0.65</td>
<td>2.8, 0.005</td>
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<tr>
<td>Gp IIIb</td>
<td>1202500 (801250–2212500)</td>
<td>1455500 ± 744876.76</td>
<td>3.78, &lt;0.001</td>
<td>0.47, 0.001</td>
<td>0.44, 0.1</td>
<td>2.8, 0.005</td>
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<tr>
<td>Gp IIIc</td>
<td>652500 (496500–800000)</td>
<td>632600 ± 185960.69</td>
<td>3.78, &lt;0.001</td>
<td>0.47, 0.001</td>
<td>0.48, 0.001</td>
<td>2.8, 0.005</td>
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<tr>
<td>Gp IIId</td>
<td>1275000 (926750–1632500)</td>
<td>1279000 ± 396874.12</td>
<td>3.78, &lt;0.001</td>
<td>0.47, 0.001</td>
<td>0.49, 0.62</td>
<td>2.8, 0.005</td>
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<tr>
<td>Gp IIIe</td>
<td>1605000 (1173750–2120000)</td>
<td>1678000 ± 55983.67</td>
<td>3.78, &lt;0.001</td>
<td>0.47, 0.001</td>
<td>0.48, 0.001</td>
<td>2.8, 0.005</td>
<td></td>
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<tr>
<td>Gp IIIf</td>
<td>800000 (752500–846500)</td>
<td>799700 ± 172003.91</td>
<td>3.78, &lt;0.001</td>
<td>0.47, 0.001</td>
<td>0.48, 0.001</td>
<td>2.8, 0.005</td>
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P1 = P value comparing group II against other groups. P2 = P value comparing each group against group II. P3 = P value comparing each group against group IIIa. P4 = P value comparing each group against group IIIb. P5 = P value comparing each group against group IIIc. P6 = P value comparing each group against group IIId. P7 = P value comparing each group against group IIIe.
4. Discussion

Toxoplasmosis affects more than one billion people worldwide with an estimate of 1.1 million new infections each year. While the disease may pass unnoticed in immunocompetent subjects, it could be life threatening in those with weakened immune system [13]. Unfortunately, side effects and drug resistance are associated with chemotherapeutic agents commonly used to treat toxoplasmosis and discovering alternative therapy remains an ongoing challenging endeavor [26]. An ideal anti-Toxoplasma drug has to be able to penetrate the tissue cyst and not only effectively against the proliferative stage [27]. One of the recently sought alternatives is nitrofurantoin. For many years, this compound has been used to treat urinary tract infections. It also has anti-depressant, anti-convulsive, and anti-arrhythmic properties [18]. Besides, many of its derivatives have well-known anti-microbial activities. In their study, Sharma and Anand [28] stated that nitrofurantoin derivatives are potential schistomicidal agents as they proved to have prophylactic potency against Schistosoma japonicum in vivo. In addition, nitrofurantoin derivatives proved effective against filariasis and trypanosomiasis [29].

A more recent study suggested that nitrofurantoin has been used with success as an anti-Toxoplasma agent both in vivo and in vitro [20]. This study reported inhibition of T. gondii growth in the peritoneal cavity by 44.7% compared to the negative control group following treatment with 100 mg/kg of nitrofurantoin for 4 days. The same study also assessed the effect of lower concentrations of nitrofurantoin but found it ineffective against T. gondii and reported that 100 mg/kg is the optimally effective and safe dose. In our study, we went further and explored its effect on different organs, whether used alone or in combination with the well-known anti-Toxoplasma drug spiramycin.

It is worth noting that we used the less-virulent strain of T. gondii (Me49) so we could assess the drugs’ efficacy in the acute and chronic stages. Different strains of T. gondii exert different degrees of virulence with type I RH strain being the most virulent and associated with 100% mortality, while type II and III are considered less virulent and commonly associated with chronic infection [30]. Besides, we used PCR to detect the reduction in the parasitic load in blood. A different study reported that more than 50% of patients treated with spiramycin retained T. gondii DNA in blood and remained infected [31]; consequently, assessing the parasite DNA is of utmost importance to judge the efficacy of a drug.

Our experiment was extended to 8 weeks to ensure complete maturation and make certain that cyst tissue affects most organs (chronic phase of toxoplasmosis). In this line, Martínez-Gómez et al. [32], used the Me49 strain and found that the ideal time for brain isolation is 8 weeks post-infection when infecting the mice orally. This disagrees with the previous report of Clemente et al. [33] who found it better to isolate brain cysts 4 weeks post-infection. This might be attributed to different mice and parasite strains.

Our results proved that nitrofurantoin can be used as a potential anti-Toxoplasma drug as it demonstrated a significant ability to eliminate the infection compared to the infected control group (P < 0.001). This was proved by decreased parasitic load demonstrated by PCR, absence of tissue cysts, and better histopathological features of the studied organs. This agrees with Yeo et al. [20] who studied the effect of nitrofurantoin against T. gondii by measuring T. gondii induced serum ALT and AST levels as well as malondialdehyde and glutathione levels and found it significantly effective in reducing all markers and therefore recommended it as a promising drug. In the same line, Maddison, Page, and Church [34] suggested that nitrofurantoin can be lethal to parasites as it produces reactive oxygen species that react with cellular macromolecules causing destruction to the parasite. Similarly, Gerpe et al. [35] proposed that nitrofurantoin can be used as an anti-parasitic agent as it interacts with some parasite proteins such as prosta-glandin F2α synthase and cytochrome P450 reductases. Therefore, we can conclude that nitrofurantoin can achieve the intended function against T. gondii via a myriad of mechanisms.

On the other hand, Remington [36] argued that nitrofurantoin compounds have no ability in eliminating the tissue form of trypanosomes and stated that they can only be used against blood parasites. However, our results showed that no tissue cyst was found in the examined organs when using nitrofurantoin whether alone or in combination with spiramycin as. This difference could be attributed to the different response of parasites to different drugs or the use of compound derivatives. This has been said, a recent study concluded that nitrofurantoin demonstrated partial efficacy against trypanosomiasis compared to its analogs that showed no efficacy against the parasite [37]. Besides, drug actions may differ in vivo and in vitro. Payne et al. [38] reported that some drugs offered poor protection in vivo despite showing excellent results in vitro.

In the present study, the combination of nitrofurantoin and spiramycin achieved the best therapeutic efficacy in treating infections in both acute and chronic
phases. This was demonstrated by the significant reduction of the parasitic load as well as improvement in histopathological changes. This could be attributed to the synergistic action of these drugs explained by the antioxidant effect of nitrofurantoin and the inhibitory effect of spiramycin on protein synthesis and its good bioavailability [39].

In conclusion, nitrofurantoin demonstrated potential usefulness as a therapeutic agent for treating toxoplasmosis whether used as mono- or combined therapy with spiramycin as it succeeded in eliminating the infection. It also proved to have a similar protective effect on different organs as the commonly used anti-Toxoplasma drug spiramycin. This was achieved by reducing damage and inflammation in the studied organs. This study represents a good starting point for further discovery of its full range of action against different T. gondii strains and other parasites as well as the survival rates of experimental animals. It is recommended to further assess the effect of different doses of the drug when used as a combined therapy and use a combination of molecular and serological diagnostic methods.

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Author contributions
All authors contributed to the study conception and design. Material preparation was made by Dr Asmaa Elkholy and Dr Shereen Kishik. Molecular tests were performed by Dr Omnia Alsaid. Pathological examination was performed by Dr Ashraf Barakat and Dr Ashraf Sorour. Data collection and analysis were made by Dr Mona Elawady and Dr Rita Wassef. The first draft of the manuscript was written by Dr Rita Wassef, who is also the corresponding author. All authors revised and approved the final manuscript and agreed on submission to the chosen journal.

Ethics approval
This study was conducted at the National Research Center (NRC), Giza, Egypt, and the experiment was carried out according to the institutional guidelines and in line with the principles of the Declaration of Helsinki after approval of the Research Ethics Committee.

Data availability statement
The data that support the findings of this study are available from the corresponding author, [RW], upon reasonable request.

References


