Preparation and Evaluation of Azithromycin as Rectal Suppository to Treat Bacterial Infection of COVID-19

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Abstract

In this study, azithromycin was prepared for the first time as solid dispersed system with curcumin then formulated as suppository dosage form. The goal of these steps to achieve the maximum activity and to avoid side effect that might appear with oral route which is associated with this drug especially during the treatment of Coronavirus secondary bacterial infection. In this study, the solid dispersion system was prepared then characterized by Differential Scanning Calorimetry (DSC), Powder X-ray Diffractometry (PXRD) and Fourier Transform Infrared Spectroscopy (FTIR). The antibacterial activity was measured by the agar well-diffusion method. Eight different suppository formulas were prepared. These formulas were subjected to the official suppository test such as dissolution test (Apparatus I) and content uniformity test. The DSC and XPDR results revealed the formation of solid dispersed system. The solid dispense system that was formulated using glycerin in the suppository base showed the best release for both azithromycin and curcumin nearly 98% and 100% respectively. The antibacterial activity results showed that the physical mixture and the solid dispersion system showed double value of zone of inhibition rather than the pure drugs. This study will be interested in Drugs Company to find alternative route of drug administration for serious infection that associated with gastrointestinal tract (GIT) symptoms.

Keywords: Azithromycin, Curcumin, Covid-19, Co-amorphous, Solid dispersion

Introduction

Coronavirus disease 2019 (COVID-19) was brought on by a newly discovered severe acute respiratory syndrome coronavirus (SARS-CoV-2) (1-3). COVID-19 infections are characterized by fever and respiratory symptoms such as dry cough and dyspnea. Diarrhea, nausea, vomiting, and stomach discomfort are some of the gastrointestinal symptoms that may occur together with respiratory symptoms (4). More severe complications such as pneumonia, acute respiratory distress syndrome (ARDS), sepsis, thrombosis, shock, and acute renal and/or heart failure may develop in immunocompromised patients (5).

Oral route of drug administration becomes problematic as patients begin to lose the ability to swallow medication. Studies have shown that patients at the end of life have a greater need for alternative medicine delivery methods with up to 70% of patients needing an opioid delivery method other than oral (6-7). Rectal drug delivery is a reliable alternative to oral and parenteral routes of administration for medication delivery of protein peptides.
and for avoiding first pass metabolism in part (8). The rectal route is the most effective and cost-effective treatment option for people who have trouble swallowing, particularly children and certain adults. In some cases, such as those involving nausea and vomiting, this method is advantageous (9). The suppository is a solid medication dose type that is typically used in the urethra, vagina, and rectum. They dissolve or melt inside the bodily cavity and then disappear. The rectal route offers various benefits, including the ability to avoid the unfavorable effects of meals on medication absorption and the hepatic first pass impact (10). Suppositories are preferable to alternative dosage forms for administering medications because they reduce adverse effects, particularly gastrointestinal irritation, and eliminate disagreeable taste (6). In the treatment of prescribed case of covid-19 there is a lot of medication used, in early line treatment such as dexamethasone, hydroxychloroquine, azithromycin, and favipiravir are all recommended due to their efficiency (11).

A second-generation synthetic macrolide antibiotic with a broad spectrum of activity is azithromycin (AZ) often used to treat a variety of bacterial and mycobacterial infections (12). AZ is a class-II (low solubility/high permeability) active pharmaceutical ingredient according to the biopharmaceutical classification system (BCS) (13). Polymeric amorphous solid dispersion (PASD) has been favored as a means to obtain the fundamental advantage afforded by an amorphous form, namely enhanced apparent solubility and dissolution, which often translates to an improvement in bioavailability. As an alternative, the term "co-amorphous" was created (14). One of the most difficulties emerging during the therapeutic agents’ development that contains an antibiotic is their low water solubility (15). The main properties of both materials are seen in Table 1.

Table 1. Properties of the selected ingredients

<table>
<thead>
<tr>
<th>Properties</th>
<th>Azithromycin dehydrates</th>
<th>Curcumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Macrolides (12)</td>
<td>Text anti-oxidant, anti-inflammatory, anti-cancer, anti-rheumatic, antidiabetic (16), antibacterial, antiprotozoal, antiviral, hypoglycemic, anticoagulant (9).</td>
</tr>
<tr>
<td>Mechanism of action</td>
<td>Azithromycin prevents bacteria from growing by interfering with their protein (6).</td>
<td>Curcumin's antioxidant capacity was assessed by its ability to prevent the controlled initiation of styrene oxidation (5).</td>
</tr>
<tr>
<td>Chemical structure</td>
<td></td>
<td><img src="image" alt="Chemical structure" /></td>
</tr>
<tr>
<td>Melting point</td>
<td>126 ºC (17)</td>
<td>183.0 ºC (13)</td>
</tr>
<tr>
<td>Solubility</td>
<td>Practically insoluble in water, freely soluble in ethanol and in methylene chloride (6)</td>
<td>Curcumin is a liposoluble compound, organic solvents such as hexane, acetone, methanol, isopropanol, ethyl acetate and ethanol (15-18)</td>
</tr>
<tr>
<td>BCS class</td>
<td>Class II (low water solubility and high permeability) (19)</td>
<td>(BCS) class IV (poor solubility and permeability) (20)</td>
</tr>
</tbody>
</table>
Materials and Methods

Materials

Azithromycin dihydrate from SDI Samarra/Iraq, turmeric curcumin from Xi’an Sonwu Biotech Co., Ltd., soluplus from BASF pharmaceutical industries, Germany, sodium lauryl sulphate (SLS) Thomas Baker (Chemicals) Pvt. Ltd., India, ethanol from Thomas Baker (Chemicals) Pvt. Ltd., India, PEG (1500, 4000), tween 80 from Merck-Germany and glycerin from Nacalai Tesque Co. (Kyoto, Japan).

Methods

Preparation of physical mixture

Azithromycin Dihydrate and curcumin (carrier) were precisely weighed and then thoroughly mixed for five minutes in a glass mortar in a 1:2 drug-to-carrier weight ratio. The samples were kept for further research (21).

Preparation of solid dispersion formulas

The solvent evaporation (S.E) method was used to create solid dispersions (SDS). In a 1:2 weight ratio, AZ and curcumin are combined respectively. After that, ethanol was used to dissolve the combination. A rotary vacuum evaporator was used to remove the solvent under reduced pressure for 20 min at 60 °C. For additional characterizations, the resultant solid dispersions were kept in a desiccator (22).

Fourier transform infrared spectroscopy (FTIR)

On a Shimadzu 8300 Fourier transform infrared system (Shimadzu, Japan) instrument, the FT-IR of the drug and curcumin as received was captured between 4000 and 400 nm. After that, the resultant spectrum was interpreted (23).

Powder x-ray diffractometry (PXRD)

The X-ray diffractometer (XRDP-6000 Shimadzu - Japan) was used to take the PXRD readings, with the following settings used: voltage 40 kV, current 30 mA, and scanning speed 1/min over a range of 10–90° (24).

Differential scanning calorimetry (DSC)

DSC 60 (Shimadzu, Japan) was used to analyze the drug and curcumin in their raw state, physical mixture, and solid dispersion. The samples (5 to 6 mg powder sealed in an aluminum pan) were heated at a rate of 10 °C/min with an argon atmosphere between 35 and 400 °C (17).

Determination of saturated solubility

To evaluate the increase in drug and curcumin solubility as received physical mixtures before S.E and combination after S.E, saturation solubility test was carried out. Excess amounts (approximately 100 mg) of the drug were added to 100 mL of phosphate buffer (pH = 7.4). Samples were stirred at 200 RPM at 25 °C for 48 h, filtered by a 0.45μm syringe filter, suitably diluted and analyzed by a UV spectrophotometer at 208 nm for AZ and 425 nm for curcumin (25).

Suppository preparation

The formulations were improved in terms of drug release characteristics, excipient compatibility, and pharmaceutical qualities (26). Table 2 summarizes final formulations considered. PEGs (a PEG mixture of 80% w/w PEG 1500 and 20% w/w PEG 4000) (27) were melted at 65 °C in a water bath and then AZ and excipients were dispersed under mechanical stirring at 150 rpm to produce suspended AZ and excipient suppositories (as received physical mixing and solid dispersion). For co-melted AZ suppositories, PEGs were pre-melted in an oven at a high temperature (130 °C) above the melting temperatures of all constituents before the addition of the medication and excipients. The mixture was heated in the oven until the ingredients melted and then it was mixed to create a homogeneous mixture. Before being put into 2 g suppository molds, the mixture was cooled to a temperature of 55–60 °C for all suppositories. Before further examination or usage, they were stored in their molds in the refrigerator and allowed to harden at room temperature in a desiccator for at least 24 hours (28).

Table 2. Composition of different formulas of suppository contains different percent of additives.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose</td>
<td>2g</td>
<td>2g</td>
<td>2g</td>
<td>2g</td>
<td>2g</td>
<td>2g</td>
<td>2g</td>
<td>2g</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>250 mg</td>
<td>___</td>
<td>250 mg</td>
<td>___</td>
<td>250 mg</td>
<td>250 mg</td>
<td>250 mg</td>
<td>250 mg</td>
</tr>
<tr>
<td>Azithromycin S.E</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>250 mg</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>Curcumin</td>
<td>___</td>
<td>500 mg</td>
<td>500 mg</td>
<td>___</td>
<td>250 mg</td>
<td>250 mg</td>
<td>250 mg</td>
<td>250 mg</td>
</tr>
<tr>
<td>Curcumin S.E</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>500 mg</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
</tr>
<tr>
<td>Soluplus</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>250 mg</td>
<td>250 mg</td>
<td>250 mg</td>
<td>250 mg</td>
</tr>
<tr>
<td>PEG 1500 w/w</td>
<td>80%</td>
<td>80%</td>
<td>80%</td>
<td>80%</td>
<td>80%</td>
<td>80%</td>
<td>80%</td>
<td>80%</td>
</tr>
</tbody>
</table>
In vitro dissolution study

Ph. Eur. Apparatus I (basket apparatus) was used for the dissolution tests. Each vessel contained 250 mL of phosphate buffer (pH 7.4 ± 0.2) as the dissolution medium. In the dissolution medium, baskets containing suppositories (n = 3/batch) were positioned and rotated at a speed of 75 rpm. A temperature of 37 ± 0.2 °C was chosen. At 15-30-45-60-120 minutes, 2 mL samples were manually taken from each basket and replaced with fresh medium under the same pH and temperature conditions. The selected wavenumber (λmax) for each of the withdrawn samples was obtained using a UV-Vis spectrophotometer after filtering with a 0.45 µm syringe filter.

Determination of content uniformity

A spectrophotometric approach was used to determine the content uniformity test. In 100 ml of phosphate buffer pH (7.4), the suppositories were individually melted and dissolved. Following dilution, UV-Vis spectrometry was used to detect the absorbance at a wavelength of 208 nm for azithromycin and for curcumin (425 nm). Antibacterial activity (zone of inhibition determination)

In-vitro antibacterial activity of drug as received, physical mixture, and solid dispersion was investigated through the agar well-diffusion method against isolates of *Pseudomonas aeruginosa* (*P. aeroginoga*). The bacterial isolates of *P. aeroginoga* were cultured in nutrient broth and incubated for 24 hours at 37 °C. Muller-Hinton agar was prepared according to manufacturer instruction to evaluate the antibacterial activity of drug samples. Then 100µL of 1.5 x 10^8 cell/ml obtained from bacterial suspension of *P. aeroginoga* and spread on each petri dish by using a sterile cotton swab. Aseptically and by using cork borer a well (7 mm in size) was done in each dish.

Seventy μl of distilled water was added to 8 μg/ml of drug samples (received, physical mixture, and solid dispersion) then vortexing and centrifuge at 13000 RPM for 5 min. The supernatant of each sample (drug as received, physical mixture, and solid dispersion) was transferred into the Muller-Hinton agar wells. Plates were incubated at 37 °C for 24 h and the inhibition zone around the wells was measured using a ruler. Ceftazidime (1 gm) was used as a standard drug for comparison of its antibacterial activity on *P. aeroginoga* with the effect of drug samples.

**Statistical analysis**

Unless otherwise specified in the experimental section, all results were provided as the mean of triplicate standard deviation. One-way ANOVA and the post hoc test (LSD) were used for the statistical analysis. There was a statistically significant difference when (p<0.05). However, the difference was deemed statistically insignificant when (p>0.05). Statistical calculation was done using Statistical Package for the Social Sciences (SPSS) 16.0 software.

**Results and Discussion**

**Fourier transform infrared spectroscopy (FTIR)**

Table 3 and Fig 1 shows the FT-IR curves of pure AZ. S.E: physical mixture and combination with curcumin. There are variances in the C-H stretching vibration between various samples. The possibility of creation of hydrogen bonds in AZ before and after S.E was suggested by the broad band's change from (2978 cm⁻¹ to 2966 cm⁻¹). A notable variation was also seen in the carbonyl group's (C=O) stretching vibration peak. Before S.E, the stretching vibration peak was sharp and intense and the wavenumber was 1716 cm⁻¹. After S.E, the wavenumber of the peak was 1724 cm⁻¹. The shift from 1716 cm⁻¹ to 1724 cm⁻¹ in the C=O region that might indicates the formation of hydrogen bonding might be due to amorphous formation.
Table 3. FTIR spectra characteristic peaks of azithromycin dihydrate as received, solvent evaporated, physical mixture of drug with curcumin and combination of drug and curcumin.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>As received</th>
<th>S.E</th>
<th>Physical mixture</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-H stretching</td>
<td>2978</td>
<td>2966</td>
<td>2970</td>
<td>2935</td>
</tr>
<tr>
<td>C=O ketone</td>
<td>1716</td>
<td>1724</td>
<td>1716</td>
<td>1720</td>
</tr>
<tr>
<td>C-H in alkane</td>
<td>1377</td>
<td>1377</td>
<td>1373</td>
<td>1373</td>
</tr>
<tr>
<td>C-O-C ether stretching</td>
<td>1157</td>
<td>1156</td>
<td>1153</td>
<td>1157</td>
</tr>
</tbody>
</table>

Figure 1. FTIR spectra of pure and solvent evaporated azithromycin dihydrate and curcumin, physical mixture and combination of azithromycin and curcumin.

Table 4 and Fig 1 shows the FT-IR of pure curcumin, S.E, physical mixture and combination with drug. A sharp peak at 3502 cm⁻¹ for pure curcumin demonstrates the existence of the hydroxyl functional group (–OH) was shifted to 3495 that might indicate amorphous formation due to the possibility of H-bond formation (13-38).

Table 4. FTIR spectra characteristic peaks of curcumin as received, solvent evaporated, physical mixture with drug and combination of drug and curcumin.

<table>
<thead>
<tr>
<th>FORMULATION</th>
<th>As received</th>
<th>S.E</th>
<th>Physical mixture</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>–OH hydroxyl group</td>
<td>3502</td>
<td>3502</td>
<td>3502</td>
<td>3495</td>
</tr>
<tr>
<td>C=C</td>
<td>1624</td>
<td>1624</td>
<td>1624</td>
<td>1624</td>
</tr>
<tr>
<td>C=O carbonyl group</td>
<td>1504</td>
<td>1504</td>
<td>1504</td>
<td>1508</td>
</tr>
<tr>
<td>C-O enol group</td>
<td>1273</td>
<td>1269</td>
<td>1273</td>
<td>1273</td>
</tr>
</tbody>
</table>

**Powder x-ray diffractometry (PXRD)**

Figure (2) shows the XRPD patterns of pure azithromycin, curcumin, and physical mixture of as received material and after solvent evaporation. Received azithromycin displays multiple diffraction Braggs peaks that were reported in study done by Aucamp et al., which might indicate that the drug is crystalline in form (39).

Curcumin exhibits several distinct Braggs peaks. In the case of a physical mixture of azithromycin and curcumin Braggs peaks still visible as a sign of crystalline nature. After the solvent evaporation of azithromycin, there is a reduction in intensity and number of Braggs peaks. Additionally, the Braggs peak strength is reduced in the solvent evaporated curcumin suggesting that both the drug and the carrier may lose some of their crystallinity and return to highly amorphous form (21).
Figure 2. XRPD patterns of pure azithromycin and curcumin- physical mixture and solid dispersion of drug and curcumin.

**Differential scanning calorimetry (DSC)**

Figure (3) shows the DSC thermogram of pure azithromycin- curcumin- and physical mixture of as received material and after solvent evaporation. Pure azithromycin exhibits a single strong endothermic fusion peak at 135 °C which corresponds to their melting point (126°C) (40). One prominent endothermic fusion peak at 179°C, which corresponds to their melting temperature of 183 °C, is also visible in the DSC of curcumin (41). The physical mixture showed one endothermic peak at 147°C. The sharp endothermic peak of azithromycin after solvent evaporation became broader and revealed a decrease in melting peak which was previously described by Li- X.- et al. (42). This could be an indication that azithromycin's crystallinity has decreased. This result is consistent with XRPD data in a reduction of the crystallinity of azithromycin after solvent evaporation. For solvent evaporated azithromycin-curcumin mixture (SD) shows broad endothermic peaks at (174°C). This is consistent with the creation of partial amorphization according to XRPD data.

Figure 3. DSC thermograms of pure azithromycin and curcumin- physical mixture and solid dispersion of drug and curcumin.
Saturated solubility

Table 5 displays the findings of the saturated solubility analysis of AZ and curcumin in the as received, S.E., physical mixture, and combination. The findings demonstrated that the S.E. formula is more soluble than the drug as received and that the drug and curcumin combination is more soluble than as received and the physical mixture form. This increase can be attributed to the partial amorphization (17).

Table 5. Saturated solubility study of pure drug and curcumin: physical mixture and combination

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Saturation solubility (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ as received</td>
<td>6.4 ± 0.51</td>
</tr>
<tr>
<td>AZ S.E.</td>
<td>11 ± 0.13</td>
</tr>
<tr>
<td>Curcumin as received</td>
<td>0.135 ± 0.22</td>
</tr>
<tr>
<td>Curcumin S.E.</td>
<td>0.147 ± 0.19</td>
</tr>
<tr>
<td>Azithromycin in physical mixture at λmax 208</td>
<td>7.2 ± 0.68</td>
</tr>
<tr>
<td>Curcumin in physical mixture at λmax 425</td>
<td>0.141 ± 0.30</td>
</tr>
<tr>
<td>Azithromycin in combination at λmax 208</td>
<td>7.4 ± 0.71</td>
</tr>
<tr>
<td>Curcumin in combination at λmax 425</td>
<td>0.221 ± 0.20</td>
</tr>
</tbody>
</table>

In-vitro dissolution studies

Figures 4 illustrate the results of dissolution tests for all formulations. Within two hours, pure AZ (formula A) dissolve at a rate of about 4.5%. After physical mixing (formula C) and physical mixing with soluplus (formula E) dissolution rates non significantly increased to about 15% and 21.8% respectively. When compared to pure azithromycin, the dissolving profile of SD (formula D) was significantly (p < 0.05) increase (approximately 17.7% of the medication dissolved in 15 min.) and had a cumulative drug release of about 36.5% after 120 min. The percentage of drug release in the case of (formula H) physical mixture, soluplus and tween 80 after 120 min is 87%, while in the case of (formula G), the cumulative drug release is 90.6%. The rate of dissolution profile for (formula F) is about 97.8%. In comparison with pure drug and physical mixture there was a significant (p < 0.05) increase in formula F, G and H.

For curcumin in Figure 4 pure curcumin and physical mixture showed the least release rates about 11.4% and 12.9% respectively which is non-significant. Combination of drug and curcumin (formula D) and physical mixture with soluplus (formula E) showed non-significant increase in the rate of drug release about 23% and 31% respectively. The significant (p < 0.05) increasing in the dissolution rate (102.4%) was obtained with the use of soluplus and glycerin (formula F) and 95.2% with the use of tween 80 (formula H) (43). The addition of soluplus (an amphiphilic polymeric solubilizer with a high hydroxyl group) useful for dissolving poorly water soluble drugs (44), glycerin (water-soluble base (45) and tween 80 (By reducing the interfacial tension between the product and the dissolving fluid) non-ionic surfactants can produce higher dissolve rates with very little mucosal irritation. This increment in both dissolution rate of azithromycin and curcumin might be attributed to the partial amorphous formation for both materials in binary and ternary mixture as validated by DSC and XRPD data.

Content uniformity

Using spectrophotometry, the drug content in various formulations was calculated at the λ max of both drug and curcumin as shown in Table 6.
Tabla 6. Percentage of drug content in different formulas

<table>
<thead>
<tr>
<th>Formulations</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azithromycin content (mean ± SD; n = 3)</td>
<td>100 ± 1</td>
<td>100.5 ± 1.3</td>
<td>99.98 ± 2</td>
<td>100.7 ± 1.5</td>
<td>100 ± 1.8</td>
<td>100.2 ± 0.7</td>
<td>100.4 ± 0.9</td>
<td>99.6 ± 1</td>
</tr>
</tbody>
</table>

Antibacterial activity (zone of inhibition determination)

In agar well diffusion assay, the results showed that the drug samples (received, physical mixture, and solid dispersion) showed an antibacterial activity against the bacterial isolates of *P. aeruginosa*. For the azithromycin, curcumin, and solid dispersion of azithromycin and curcumin combination samples, the highest inhibition zone was shown in solid dispersion of azithromycin and curcumin in compare with the standard drug, ceftazidime (Figure 5). There was no difference in the inhibition zone between curcumin and the combination of azithromycin and curcumin as received and after solvent evaporation, while the inhibition zone for the solvent evaporator of azithromycin was higher than the received (Figure 6 and 7). Previous in vitro and in vivo proved that curcumin has antioxidant and anti-inflammatory activity against bacterial infection. The current in vitro study revealed that curcumin may have a strong or moderate impact on one of the most Gram-negative bacteria, *P. aeruginosa* that diagnosed as a more important bacterial species in superinfecting bacteria with infection of COVID-19. Curcumin interrupted several cellular processes in bacterial growth that results in alteration of bacterial cell membrane permeability that will lead to generate excess reactive oxygen species (ROS) in bacteria. The results of the current study came in accordance with the results of a previous study done by Bahari et al., who showed that the curcumin has a synergistic effect when it is used with antibiotic. And this will enhance the action of azithromycin against bacterial infection especially the secondary bacterial infection after viral infection such as infection with COVID-19.

Figure 5. Antibacterial activity against *P. aeruginosa*. AZM (azithromycin), Cur(curcumin), AZM-C(solid dispersion), SD(standard drug).

Figure 6. Zone of inhibition for drug as received and after solvent evaporation

Figure 7. (A) Zone of inhibition for Com (physical mixture of AZ and curcumin) and com-s (combination after S.E), (B) Zone of inhibition for azithromycin as received and after solvent evaporation and (C) Zone of inhibition for curcumin as received and after solvent evaporation.
Conclusion

In this study, the preparation of antibiotics (azithromycin) was prepared with the best nearly 100% of the drug was released within two hours. The preparation of co-amorphous system ensures both good dissolution and antibacterial activity with using curcumin as coformer and for its antibacterial effect which is potentiate azithromycin action by this combination.

Acknowledgment

The authors express acknowledge to the Department of Pharmacetics- College of Pharmacy/ Mustansiriyah University (Baghdad-Iraq) for providing the support and facilities to carry out the investigation.

Conflicts of Interest

The authors have no conflicts of interest regarding this investigation.

Funding

The author declare that they have no funding support for this study.

Ethics Statements

This research did not use in vivo study.

Author Contribution

Study conception and design: Mena Raid Khalil• Ghaidaa S. Hameed• Dalya Basil Hanna; data collection: Mena Raid Khalil; analysis and interpretation of results: Mena Raid Khalil• Ghaidaa S. Hameed• Dalya Basil Hanna; draft manuscript preparation: Mena Raid Khalil. All authors reviewed the results and approved the final version of the manuscript.

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