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Lawsone attenuates acute low dose ethanol-induced skin inflammation in A431 epidermoid carcinoma skin cells and its possible mechanisms in targeting interleukin (IL)-1α *in silico*

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ABSTRACT

Standard treatment for acute skin inflammatory conditions comes with unwanted side effects. Traditionally, *Lawsonia inermis* has been used to treat skin-related ailments, with lawsone as the main active phytochemical is predicted to inhibit a skin pro inflammatory cytokine, IL-1α. This study is aimed to evaluate the anti- inflammatory effect of lawsone in acute ethanol-induced skin inflammatory condition *in vitro*, by targeting IL-1α *in silico*. Basically, the IC_{50} of lawsone on human epidermoid carcinoma cell line (A431) was obtained by using MTT proliferation assay, followed by acute low dose ethanol-induced inflammatory skin condition on A431 cells to determine cell viability. Next, molecular docking study was done by utilizing Autodock Vina software, and further analyzed by ProteinsPlus and PyMol softwares. Meaningful interaction that exist in the binding of lawsone to IL-1α was determined by molecular docking results comparison with two other compounds (kirenol and curcumin diglucoside). *In vitro* study showed the IC_{50} of lawsone in low cytotoxicity level at 150 µM. Skin anti-inflammatory assay indicated that lawsone has highly significant ($p<0.05$) anti-inflammatory activity at 9.375 µM at low concentration, but not effective at higher concentrations (more than 18.75 µM). *In silico* studies indicated binding of lawsone to IL-1α with top binding affinity of -5.2 kcal/mol, at binding residues of Asp65 and Ile68. Docking comparison analysis to determine meaningful interaction comparison indicated three key IL-1α binding residues common to most tested compounds, such as Ile68 and Asp65 thus supported the predicted mechanistic pathway. This highlighted lawsone potential as a future skin anti-inflammatory agent with minimal toxicity.

Keywords: *Acute inflammatory skin condition; lawsone; IL-1α and meaningful interaction comparison*

INTRODUCTION

Skin inflammation is one of the conditions that are susceptible to everybody regardless of their ages (Tabassum et al., 2010). Several examples of inflammatory skin diseases are atopic dermatitis, rashes, psoriasis, eczema, bacterial and fungal infections. These occurrences commonly arise due to the impairment of the skin integrity caused by either pathogens

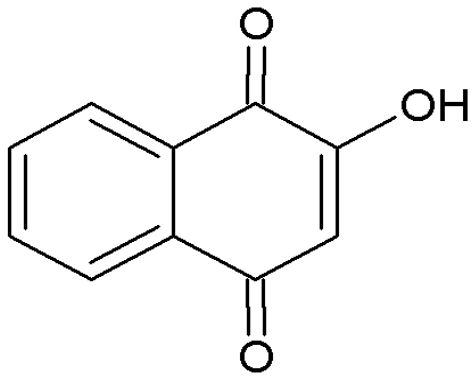
infection or tissue injury and activation of the host immune cells as a defence and protection mechanism (Robert & Kupper, 1999; Medzhitov, 2010). Similar to general inflammation, skin inflammation is mediated by a myriad of events, including inflammatory cytokines, to commence the cascade of an inflammatory process (Feghali & Wright, 1997; Medhitov, 2010). For example, IL-1α is a pro-inflammatory cytokine that plays a significant role in regulating both acute and chronic inflammation with additional factors of being site-specific which predominates mainly in the epidermis layer of the skin (Feghali & Wright, 1997; Feldmeyer et al., 2010).

Along with other several studies (O'Shaughnessy et al., 2010; Fenini et al., 2017; Lee et al., 2018), it has been indicated that IL-1α is an attractive potential therapeutic target for inflammatory skin conditions by blocking its signalling and activation pathway. As reviewed by Bou-Darhgam et al. (2017), overexpression of pro-inflammatory cytokine IL-1α has a positive correlation with intensification and advancement of skin diseases such as psoriasis, atopic dermatitis, neutrophilic dermatoses, skin phototoxicity, and skin cancer. Thus, IL-1α is predicted as a potential drug target for the prevention and treatment of various skin diseases (Jensen, 2010).

Since Sulzerberger and Witten first introduced it in 1952, topical corticosteroid has been prominently used for a wide range of dermatological conditions associated with immunological and inflammatory reactions due to its high efficiency (Coondoo et al., 2014). Nowadays, it can be considered as the standard treatment for many cutaneous inflammatory conditions (Devaraj et al., 2019). However, these benefits come with various unwanted side effects. Side effects of topical corticosteroids can be divided into two categories which are local side effects and systemic side effects. Localised side effects are more prevalent compared to systemic (Coondoo et al., 2014; Devaraj et al., 2019). Hence, it is vital to venture into other alternatives treatment with considerably less undesirable effects, preferably those compounds from natural products due to their potential healing properties.

Figure 1

Structure of lawsone (2-hydroxy-1,4-naphthoquinone)



Lawsonia inermis or henna is notoriously known for its valuable benefits in cosmetic and traditional medicine, and many studies have been done to investigate its therapeutic properties (Chaudhary et al., 2010; Hsouna et al., 2011; Raja et al., 2013; Singh & Luqman, 2014). This plant has several pharmacological activities such as antioxidant, anti-inflammatory, antipyretic, analgesic and antifungal properties (Ali et al., 1995; Chengajah et al., 2010; Borade et al., 2011; Suleiman & Mohamed, 2014). Lawsone (2-hydroxy-1,4-naphthoquinone), also known as hennatonnic acid, is a naphthoquinone and it is the main compound that is responsible for the dyeing properties of *L. inermis* (Kirkland & Marzin, 2003). The small structure of lawsone is made up of naphthoquinone ring and hydroxyl group where the biological activity of the lawsone is rendered by the presence and position of the hydroxyl group. As one of the simplest form of naphthoquinones in nature, lawsone serves as an essential scaffold for the synthesis of various interesting bioactive compounds (Jordão et al., 2015).

Throughout the years, numerous reports on the pharmacological activities of lawsone such as wound healing, anti-inflammatory, analgesic and antipyretic effect have been reported (Ali et al., 1995; Lopez et al., 2014; Adelii-Sardau et al., 2019). Although a few toxicity studies have reported the toxic effect of henna and lawsone upon oral ingestion (Nohynek et al., 2004), topical application of henna extracts on epidermal layer of the skin was found to be safe, as most of the henna extracts will remain on the skin with only low absorption levels by the fluids (Kraeling et al., 2007). Nevertheless, more recent studies have proven that the controversy on the safety of henna as a potential medicinal agent was found to be unreliable and negligible, where henna and lawsone was documented to be safe and do not produce toxic effects both *in vitro* and *in vivo* (Ali et al., 1995; Marzin & Kirkland, 2004; Norwegian Scientific Committee for Food Safety, 2005; Klaus et al., 2010). This showed the applicability of henna, and thus its main phytochemical constituent lawsone, for potential skin application, supported with a long history of the use of henna in folk medicine and cosmetic for skin and hair applications. Therefore, this study aims to evaluate the potency of lawsone that may possess possible skin therapeutic effect by its ability to exhibit skin anti-inflammatory property *in vivo*, followed by determination of its mechanistic study via inhibition of IL-1α *in silico*. Determination of meaningful interaction was used as a validation step for *in silico* study to complement the overall results and thus provide a more reliable data to this study.

METHODOLOGY

In vitro study

Lawsone (2-hydroxy-1,4-naphthoquinone)

Pure lawsone used was in powdered form and was purchased from Sigma Aldrich (St. Louis, MO, USA). Lawsone stock solution was prepared at a concentration of 1 M by dissolving in DMSO. The stock was further diluted using serial dilution to get final treatment concentrations ranging between 1000 to 7.8125 µM.

Cell culture

High glucose Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin streptomycin and phosphate buffer saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell line

Human epidermoid cell carcinoma A431 was a generous gift from Professor Dr Tan Wen Siang from Faculty of Biotechnology, Universiti Putra Malaysia, which was initially purchased from ATCC (American Type Culture Collection). A431 was maintained in DMEM supplemented with 10 % fetal bovine serum and 1 % penicillin streptomycin (antibiotic), in a humidified CO_2 incubator with 5 % CO_2 at 37 ˚C.

MTT cell viability assay

The cell viability and the IC_{50} of the lawsone on A431 cell lines were determined by using MTT assay (Zakaria et al., 2011). Cells were seeded into 96-well microtiter plate at a cell density of $1x10^5$ µg/ml with 100 µl volume per well. The seeded cells were incubated for 24 h at 37 ˚C in a humidified 5% CO_2 incubator. Next, the cells were treated for 24 h with several concentrations of lawsone ranging from 1000, 500, 250, 125, 62.5, 31.25 and 15.625 and 7.8125 µM, at 100 µl volume per well. Cells maintain with media only without any treatment was used as control. Then, the media treated with lawsone in each well was removed and replaced with new media before the addition of 10 µl MTT solutions (5 mg/ml) before further incubated for 3-4 h. After incubation, medium in each well was removed before 100 µl of DMSO was added to dissolve the formation of formazan product. The 96-well plate was read at 570 nm against a reference wavelength of 630 nm.

$$\text{Cell viability (\%)} = (OD_{sample} / OD_{negative.\ control}) \times 100$$

Acute skin inflammatory assay

The acute skin inflammatory assay and experiment model was carried out by referring to the method explained by Neuman et al. (2010), with slight modifications. The technique was a modification of the MTT assay method with the addition of inflammation induction. The viability of the cell was measured upon lawsone treatment, and induction of inflammation condition was done by ethanol at 50 mM concentration.

The A431 cells were seeded in the 96-well plates at a cell density of $1x10^5$ µg/ml with 100 µl volume per well. The next day, the cells were treated with 100 µl of lawsone at several lawsone concentrations ranging from 1000 to 7.8125 µM before incubated for another 24 h. Cells maintained in media and induced with ethanol but without treatment of lawsone were used as the negative control. Then, the cells were incubated for 24 h at 37 ˚C in a humidified 5 % CO_2 incubator. After treatment, the cells in each well were exposed with 50 mM of ethanol to induce acute inflammation conditions and further incubated for 24 h. Next, the media and treatment in each well were removed and replaced with new media before 10 µl MTT solution was added into each well and further incubated for 3-4 h at 37 ˚C. At the end of the period, the media in each well was removed and followed by the addition of 100 µl of DMSO. The plate was read at 570 nm against a reference wavelength of 630 nm to measure the viability of the A431 cells.

In silico study

Molecular docking was carried out for *in silico* study to further understand and characterise the behaviour and binding mode as well as chemical interaction between lawsone (ligand) and IL-1α (protein macromolecule) at the molecular level (Meng et al., 2011). This study was carried out as a way to determine further the possible mechanistic pathway underlying the inflammation inhibition by lawsone. In this study, the IL-1α was chosen as a protein target. The A431 cell line that was used in this study was originated from the human epidermal skin layer, where IL-1α is a pro-inflammatory cytokine that predominates this layer and plays a significant role in skin inflammation (Feldmeyer et al., 2010). The IL-1α protein was obtained from RSCB Protein databank in '.pdb' format file with PDB ID: 5UC6, which consist of a combination of 1L-1α and other foreign components at a resolution of 2.1 Å.

Protein preparation

The structure was prepared by using AutodockTools to create and isolate structure consisting only IL-1α (Trott & Olson, 2010). Both lawsone and 1L-1α were developed into a PDB, and eventually PDBQT formats which were the required format for AutoDock Vina to compute and run the molecular docking job.

Setting up of docking grid box and configuration

Grid box was set up to direct and specifies where the ligand has to dock and bind on the IL-1α. The grid box encompassed all specific amino acids sequence that previously been found to inhibit the activity of IL-1a. The parameters of the grid box used are outlined in Table 1 below:

Table 1

Docking grid box parameters

Protein	Size		Coordinate	
IL-1α	x-dimension	24	x-dimension	97.207
	y-dimension	18	y-dimension	53.86
	z-dimension	20	z-dimension	109.61

Running docking

Ren et al. (2017) reported that ten amino acids were responsible for being the inhibitory binding sites of IL-1α which were Met15, Arg16, Ile 18, Lys60, Ser61, Asp 65, Ala66, Lys67, Ile68 and Trp113. As recommended by Trott and Olson (2010), molecular docking was run by using AutoDock Vina for its speed and accuracy in docking. Autodock Vina software was computed by using the command prompt. The result was further analysed by using PyMol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC) and ProteinsPlus [Zentrum für Bioinformatik: Universität Hamburg - Proteins Plus Server (Fahrrolfes et al., 2017)] to view the configurations of each binding pose and to elucidate in more detail the interactions of the binding mode respectively.

Molecular docking comparison: Determination of meaningful interaction to IL-1α

Molecular docking comparison was carried out as a way to determine meaningful interaction with IL-1a. The contrast between lawsone was carried out with another two different compounds, which are curcumin diglucoside (Chem ID: 46173989) and kirenol (Chem ID: 1573672). All three compounds were docked on the same IL-1α structure (PDB ID: 5UC6) with the same binding site as well as same grid box parameters used in the molecular docking of lawsone. Structures of both curcumin diglucoside and kirenol were obtained from PubChem. Both curcumin diglucoside and kirenol underwent the same molecular docking protocol as lawsone, which includes the same binding site and grid box parameter for the molecular docking to the IL-1α. All three compounds followed similar steps of molecular docking, and the final results were analysed for comparison. Curcumin diglucoside and kirenol were chosen as the comparison in this study due to the previous studies that mentioned the ability of curcumin diglucoside (Xu et al., 2018) and kirenol (Wu et al., 2017) to bind and inhibit IL-1α *in silico*. Binding interaction analysis of the three compounds was later combined and compared to previously reported *in vitro* and *in silico* studies as a way to determine meaningful interactions possessed by lawsone on IL-1α binding inhibition.

Statistical analysis

All the *in vitro* data obtained were analysed by one way- ANOVA followed by Dunnet test as post hoc test. The statistical analysis was analysed by using IBM SPSS Statistic version 24.

RESULTS

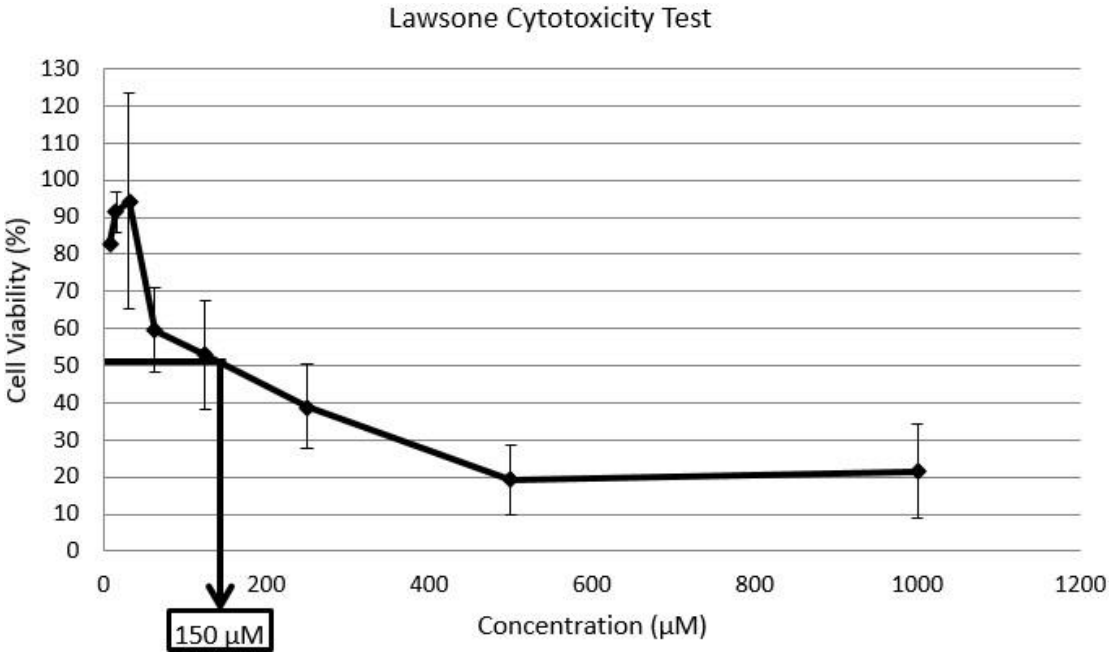
***In vitro* anti-inflammatory skin assay**

IC_{50} of lawsone

The determination of IC_{50} of lawsone on A431 cell viability was determined by using MTT assay. The result shown in Figure 2 showed the IC_{50} of lawsone on the percentage of A431 cell viability at various treatment groups was determined at 150 μM by using the graph as reference. The IC_{50} value was used as a reference dose for the subsequent skin anti-inflammatory assay.

Figure 2

Cytotoxic effects of different concentration of lawsone on A431 cells



Notes: A431 cells were treated with various concentrations of lawsone (1000 µM, 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15.625 µM and 7.8125 µM) in 24 hours. At the end of the treatment period, cell viability was measured by using the MTT assay. IC_{50} of lawsone was determined at 150 µM. The value represented as mean ± standard error of mean of three determinations. The data was analysed by using one way ANOVA.

Acute anti-inflammatory activity of lawsone

In this study, the anti-inflammatory effect of lawsone on A431 cell line was carried out by using MTT assay, with some modifications to include the addition of inflammatory inducers. The A431 cells were treated with the decreasing concentrations of lawsone from 150 (IC_{50}), 75 (1/2 IC_{50}), 37.5 (1/4 IC_{50}), 18.75 (1/8 IC_{50}), 9.375 (1/16 IC_{50}) and 0 µM and incubated for 24 h. This was followed by the induction of inflammation by ethanol at 50 mM for another 24 hours incubation period to mimic the acute inflammatory condition. The result shown in Figure 3 showed that all the treatment groups were not significantly different except for the treatment group at a concentration of 9.375 µM when compared to a negative control group (0 µM concentration of lawsone). Thus, the result indicated that the 9.375 µM of lawsone had a significant effect on acute ethanol-induced skin cells. The mean of cell viability of this treated group was 79%, which showed that lawsone demonstrated anti-inflammatory effect towards A431 cells at low concentration only, but not significant at high lawsone concentrations.

Determination of the inhibitory mechanism of lawsone on IL-1α *in silico*

Crystal structure and hydrophobic pocket residues of Il-1α.

Crystal structure of Il-1α obtained from PDB (PDB ID: 5UC6) consists of 2 chains where chain A is the Il-1α and chain B is an aptamer binding to the hydrophobic pocket that act as an inhibitory site of Il-1α (Figure 4). IL-1α is made up of entirely β-strands with one α-helix. The core structure is a six-stranded β-barrel, and another six β-strands form three hairpins that serve as the bottom of the barrel. Since only IL-1α structure is needed, chain B was deleted therefore docking work was carried out by using Chain A.

According to Ren et al. (2017), the hydrophobic pocket which served as the inhibitory site of IL-1α was composed of ten residues of amino acid. The ten amino acids were Met15, Arg16, Ile18, Lys60, Ser61, Asp65, Ala66, Lys67, Ile68, and Trp113 (Figure 5).

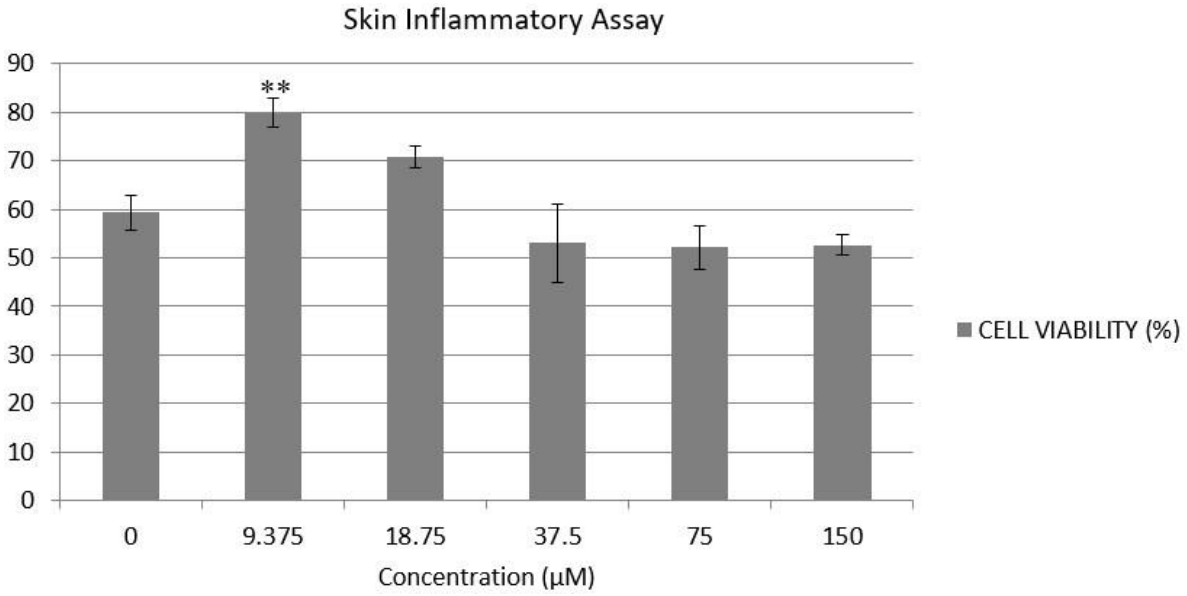
Auto dock result

The result of the molecular docking between lawsone to IL-1α is shown in Table 2. AutoDock Vina predicted nine best binding modes of orientation between lawsone and IL-1α that were ranked from the highest binding affinity to the lowest binding affinity. The most negative measurement indicated the best binding affinity. The best-docked configuration with the highest binding affinity was selected for further in detail analysis.

The best predicted binding result computed by AutoDock Vina was explained in Figure 6. Lawsone can bind to the active site of the binding pocket of IL-1α with best biding affinity of -5.2 kcal/mol. The aromatic ring of the lawsone resides in the hydrophobic pocket of the IL-1α at Isoleucine 68 (Ile68) residue. Simultaneously, one moderate hydrogen bond was formed between the hydroxyl group of the lawsone and the carbonyl group of the IL-1α at Aspartate 65 (Asp 65) residue. The distance of the hydrogen bonding was measured and determined at 2.9 Å.

Figure 3

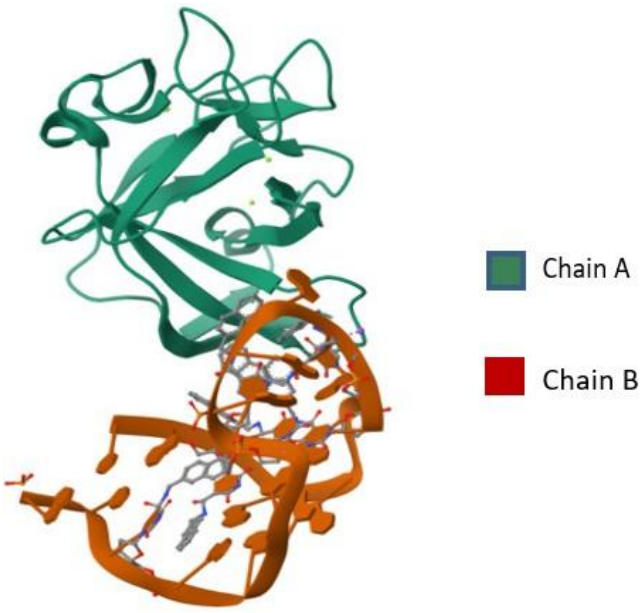
Percentage of cell viability of different concentrations of lawsone treatment upon acute ethanol induction in A431 cells



*Notes: A431 cells were treated with five different concentrations of lawsone (150 µM, 75 µM, 37.5 µM, 18.75 µM, 9.375 µM) for 24 hours followed with induction by ethanol for another 24 hours. A431 cells with 0 µM concentration of lawsone were used as negative control. At the end of treatment and induction by ethanol period, the cell viability was assessed by using MTT viability assay. The values are presented as mean ± standard error of mean of three determinations and were indicated by ** showed significant difference (p<0.01) relative to the untreated cell.*

Figure 4

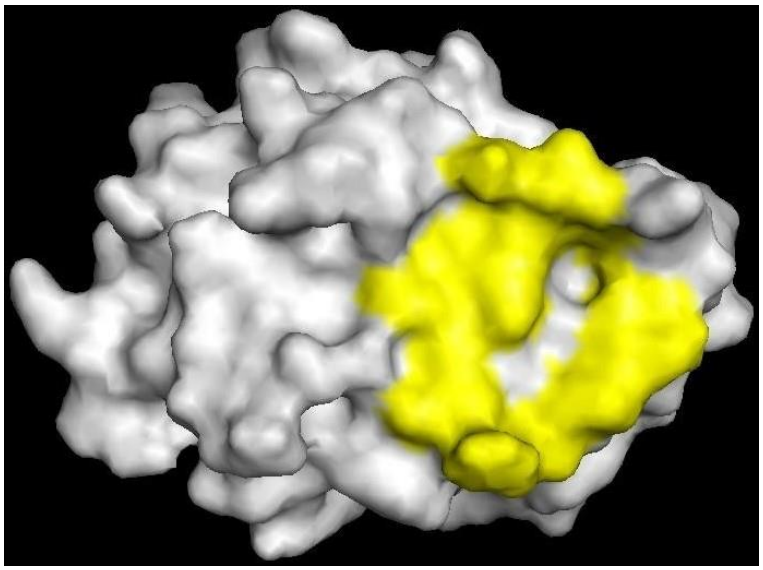
Crystal structure of IL-1α obtained from PDB (PDB ID: 5UC6 - reprinted with permission)



Notes: Chain A is the Il-1α and Chain B is an aptamer binding to the hydrophobic pocket (inhibitory site) of Il-1α.

Figure 5

Crystal structure of IL-1α visualized using PyMol



Notes: The ten amino binding residues on IL-1α is highlighted in yellow colour revealing a hydrophobic groove suitable for binding of the small molecule.

Table 2

Predicted binding poses of lawsone to IL-1α

Pose	***Binding affinity (kcal/mol)***
1	*-5.2*
2	*-5.0*
3	*-5.0*
4	*-4.9*
5	*-4.9*
6	*-4.8*
7	*-4.8*
8	*-4.7*
9	*-4.6*

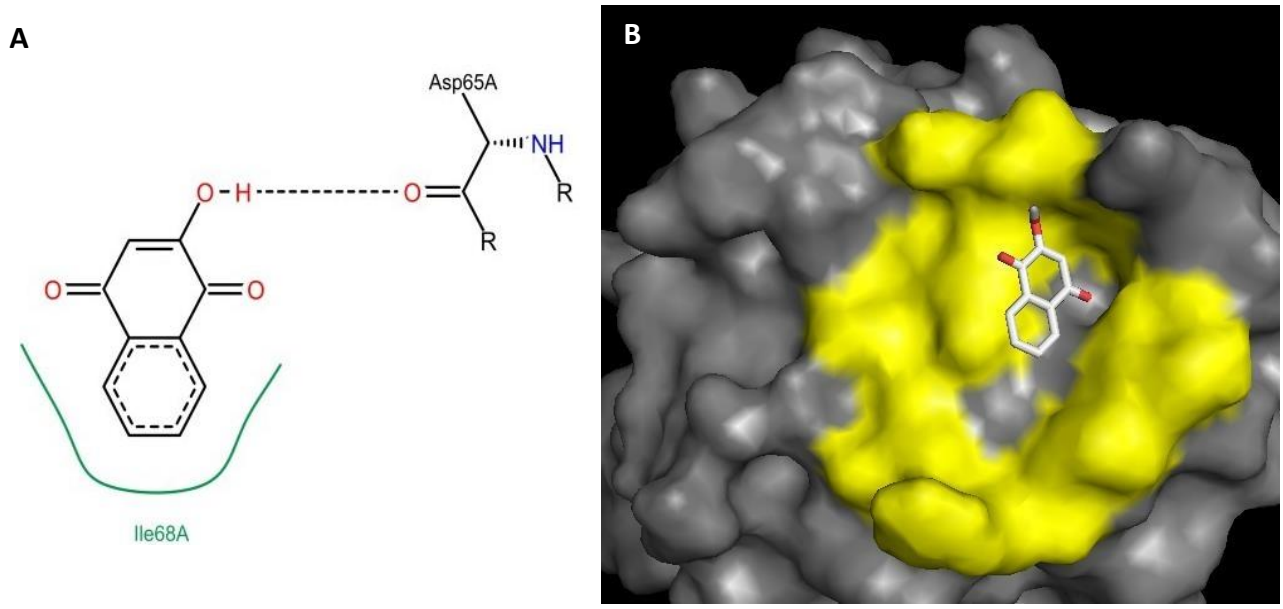
Notes: Auto Dock Vina predicted nine best binding modes of lawsone to Il-1α that are ranked from the highest binding affinity to the lowest.

Molecular docking comparison: Determination of meaningful interaction to IL-1α

The result on the comparison of binding ability to IL-1α between lawsone, curcumin diglucoside (Chem ID: 46173989) and kirenol (Chem ID: 1573672) between previously reported molecular docking studies (Xu et al., 2018) for curcumin diglucoside and (Wu et al., 2017) for kirenol and the current study is shown in Table 3. Comparison between these three compounds was performed on IL-1α (PDB ID: 5UC6) using the same binding site and same grid box parameters as the current study. In previous studies, different crystal structures of IL-1α were used for molecular docking analysis; where kirenol was docked on IL-1α (PDB ID: 2ILA) and curcumin diglucoside was docked on IL-1α (PDB ID: 2L5X) using different binding software. The aim was to compare the binding ability of different compounds to the active site of IL-1α assigned in this study and to relate the result with the previous biological study as a way to predict meaningful interaction of the compounds.

Figure 6

The best binding mode of lawsone to IL-1α



Notes: Lawsone was embedded to IL-1α with a binding affinity of -5.2 kcal/mol. (A) Lawsone interacted with Aspartate65 and Isoleucine68, as shown in the diagram above. The result was obtained from ProteinsPlus. (B) The best binding pose of lawsone embedded into IL-1α viewed using PyMol.

From the docking comparison in Table 3, no similarity was found on the interacting amino acid residues between the previous study and the current study. Besides, different software was used by curcumin diglucoside at an earlier study, which will not allow comparison with the present study to be made. Where else for kirenol, higher binding affinity was observed in the current study, compared to the previous research by the same AutoDock Vina software, as different protein structure was used in both studies. Therefore, the aim to compare binding ability and hence, validation of docking parameters cannot be concluded as different results were obtained as compared to the previous study.

The best-docked configuration with the highest binding affinity was chosen for protein interaction analysis using PyMol. The protein interactions for both previous and current studies were shown in Table 3 below. Lawsone has the lowest binding affinity and the least protein interactions to IL-1α as compared to curcumin diglucoside and kirenol. This might be attributed to the smaller size of lawsone molecular structure compared to curcumin diglucoside and kirenol.

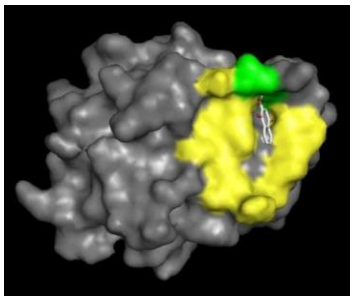
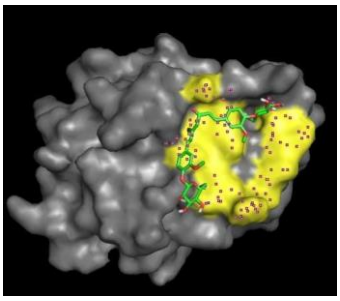
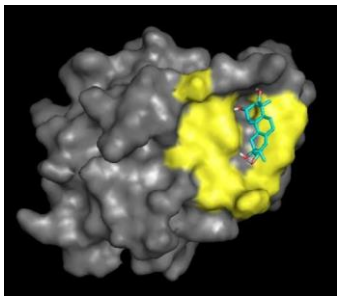
To conclude the findings, results from molecular docking study were compared to previously reported findings on IL-1α binding interactions *in vitro* and *in silico*. Previous studies by (Ozbabacan et al., 2014) which used a protein-protein interaction prediction algorithm named PRISM (Protein Interaction by Structural Matching), was then integrated with the findings that based on analysis of oligonucleotide-directed mutagenesis in *Escherichia coli* expression plasmid for human Il-1α as a way to find common interaction sites (Labriola-Tompkins et al., 1993). From here, a few common interaction sites were determined: Arg16, Ile18, Ile68 and Trp113; and another three binding residues, which were Asp64, Asp65 and Lys100 that were located nearby the binding interface. After comparison, three out of seven binding sites were found to be similar as exhibited by lawsone, kirenol and curcumin diglucoside; which were Asp65, Ile68 and Trp113. Therefore, it can be concluded that Asp65 and Ile68 are among the key residues that commonly involve in the binding interaction to Il-1α. This validated the interaction of lawsone to Il-1α, which is a pro-inflammatory cytokine that predominates this epidermis skin layer and plays a significant role in regulation of skin inflammation.

DISCUSSION

Ethanol exposure is a common and widespread occurrence that people encounter daily. It can be found in many situations such as in hospital settings, household products cosmetics products and manufacturing of medicine (Lachenmeier, 2008; Thind et al., 2015). Ethanol has been associated with various skin inflammatory conditions such as psoriasis, eczema and superficial infection (Lachenmeier, 2008). Neuman et al. (2010) had speculated that even the smallest amount of ethanol could cause damage in A431 human epidermoid skin cancer cell lines.

Table 3

Comparison study of lawsone, curcumin diglucoside and kirenol

Ligand	Lawsone	Curcumin diglucoside	Kirenol
Best binding afinity	Current docking result: -5.2 kcal/mol	(Xu et al., 2018): -138.50 (software: MolDockScore) -115.43(software: RerankScore) Current docking result: -7.0 kcal/mol	(Wu et al., 2017): -4.9 kCal/mol (software: AutoDock 4.2) Current docking result: -6.4 kcal/mol
Top binding afinity	 Binding Affinity: -5.2 kcal/mol	 Binding Affinity: -7.0 kcal/mol	 Binding Affinity: -6.4 kcal/mol
Binding interaction Sites	Ile 68, Asp 65	Ile18, Lys19, Lys63, Asp64, Lys67, Ile68, Thr69, Trp113, Trp115, Glu56	Asp65, Lys67, Ile68
Previously reported interaction sites	None	Interaction sites: Glu21, Arg34, His46, Asn47, Asp49, Glu50, Ser75	Interaction sites: Gln38, Thr82, Ala83, Gln84, Leu91, Leu92, Leu93, Asn108
Similar binding sites (in comparison with (Ozbabacan et al. 2014) and (Labriola-Tompkins et al. 1993)	Ile 68, Asp 65	Ile68, Trp113	Asp65, Lys67, Ile68
Common interaction sites		Ile 68, Asp 65	

*Notes: Comparison were made with: *(Xu et al. 2018), **(Wu et al. 2017), ***(Ozbabacan et al. 2014) and (Labriola-Tompkins et al. 1993)*

A study had reported that ethanol disrupts the integrity of the skin membrane by inducing the formation of pores in the phospholipid bilayer of the skin membrane (Thind et al., 2015). This kind of breach in the skin lipid barrier then allows the penetration of pathogens and infectants. Furthermore, ethanol also alters the metabolic activity of keratinocyte and the signalling of a pro-inflammatory cytokine such as Il-1α, Il-6 and TNF-α (Cartner et al., 2017; Neuman et al., 2002). In this experiment, ethanol was used as an inducer to induce inflammation in epidermoid cells. The concentration used to cause inflammation was 50 mM, which is considered as low dose compared to the previously reported study, which employed 100 mM concentration (Neuman et al., 2010). From the study, it can be concluded that the use of ethanol even at a low dose and in acute condition was able to induce skin inflammation in epidermoid cells. It is speculated that long term exposure to ethanol will subsequently damage the epidermoid layer of the skin and produce more chronic skin inflammatory condition.

Based on Figure 2, it was shown that lawsone has anti-inflammatory effects towards A431 human epidermoid carcinoma skin cell line depicted by the increment in the proliferation of the cell and survival when compared to the negative control group. The result is supported by a few studies (Sattar et al., 2023; Lozza et al., 2019; Ali et al., 1995) which reported that lawsone has excellent anti-inflammatory, analgesic and antipyretic activities in experimental rats. Lawsone (at 500 mg/kg) was found to have similar anti-inflammatory effect in comparison to the reference drug phenylbutazone (100 mg/kg). Moreover, in the work of Biradar and Veeresh (2013) that study on L-arginine induced acute pancreatitis in rats, demonstrated that lawsone has anti-inflammatory activity by decreasing the levels of pro-inflammatory cytokines including TNF-α, and IL-6, as well as the levels of serum amylase and lipase which characterised the inflammation of the pancreas. Furthermore, another study also explained that henna leaves extract was shown to have excellent wound healing properties which majorly involves in the inflammation process, with lawsone was found as the principal active constituents of the *L. inermis* (Yousefi et al., 2017). This speculation is further strengthened by a study that incorporated lawsone to stimulate tissue repair for wound healing and showed significant wound repair results (Adeli-Sardou et al., 2019). Besides, the IC_{50} value of the lawsone indicates that the cytotoxic effect of lawsone occurs only at higher concentration. This shows that lawsone has low cytotoxic property hence, making it a right candidate for the development of skin anti-inflammatory drug. Moreover, based on Figure 2, the effect of anti-inflammatory occurs at 9.375 μM, which is a much lower concentration compared to the values calculated in the cytotoxicity assay. The result means that only a small amount of lawsone is needed to achieve a significant anti-inflammatory effect, which is favoured for this study. Lawsone is a natural dye and has a strong reddish-orange pigment; thus, the use of low concentration of lawsone preparation for topical application on epidermis skin layer can mimic the natural skin colour, which upon topical application can prevent the skin from being stained strongly with the pigment and thus suitable to be developed as skin-based preparation and topical use.

In line with lawsone IC_{50} result which exhibited the detection of IC_{50} value only at higher doses, skin anti-inflammatory activity of lawsone was detected only at low concentration. Lawsone works better in low dose but not effective at higher doses. This can be due to low therapeutic window possess by lawsone, where lawsone did not behave in a dose-dependent manner. This is supported by studies such as Vandenberg et al. (2012) and Moldovan et al. (2018), where the effectiveness of drugs or chemicals they reported occurs only at lower doses. As reviewed by Vandenberg et al. (2012), ample examples and plausible mechanisms to explain the occurrence of low doses effects of disturbing endocrine chemical was published. It is evidenced that the effects of the low doses and the non-linear relationship between dosage of the drug is possible in clinical studies. Meanwhile, Moldovan et al. (2018) proved that patients contracted with essential tremor disorder shows significant positive outcome with lesser side effects when treating with a shorter pulse width of deep brain stimulation compared to the standard pulse width which is higher in terms of treatment dosage. Hence, it can also be predicted that at higher concentration of lawsone, the pharmacokinetic and pharmacodynamics of lawsone are shifted to toxicokinetics and toxicodynamics, thus producing overdose toxicity manifestation in the epidermoid cell line.

One of the inflammation immunological responses is the signalling of a pro-inflammatory cytokine such as TNFα, IL-1, IL-6, IL-17 (Medhitov, 2010). In this study, the focus is on the potential inhibitory effect of lawsone towards IL-1α to control inflammatory skin conditions. Many studies show blocking the signalling of IL-1α can reduce the inflammatory response (Dinarello, 2009; Zheng et al., 2013; Ren et al., 2017). IL-1α was chosen as a target protein for the *in silico* study as IL-1α is a cytokine that predominates in human skin epidermal cells and plays a significant role in skin inflammation (Feldmeyer et al., 2010). As the type of skin cell used in the study was A431, which was an epidermoid carcinoma cell line derived from epidermal layer of skin, the selection of IL-1α as the target protein for the current study was regarded as accurate. The objective of the second part of the experiment was to determine binding interaction and to evaluate the inhibitory activity of lawsone on IL-1α via *in silico* molecular docking technique. This part of the study will support the *in vitro* inhibition study of lawsone and provide a prediction on the mechanistic study of lawsone towards IL-1α as the most abundant interleukin in the epidermis layer of the skin.

There were limited studies have been done to discover the structure of IL-α, and to date, there is only one high-resolution crystal structure of IL-α available in Protein Data Bank (PDB ID:5UC6) (Ren et al., 2017). Consequently, many of the molecular docking studies that had been done on IL-1α did not state the potential or specific inhibitory sites on the IL-1α protein. Most papers explained that the molecular docking was computed based on the software predicted binding sites (Wu et al., 2017) or just by blind docking (Xu et al., 2018).

Our study was the first to dock a ligand, in this case, lawsone to a specific hydrophobic pocket on the surface of IL-1α structure. This is important because IL-1α and IL-1β share homologous structure, and researchers found that they have similar epitopes at the receptor. This specific inhibitory site is found to be specific only for IL- 1α and comprises of ten amino acids residues which are Met15, Arg16, Ile18, Lys60, Ser61, Asp65, Ala66, Lys 67, Ile68 and Trp113. The sites are specific towards only IL- 1α because IL-1β lacks the first β strand that is evidenced in IL-1α, with the presence of protein residues of Met15, Arg16, and Ile18 (Ren et al., 2017).

As depicted in Table 2, the best binding affinity for docking of lawsone is -5.2 kcal/mol, which is considered as moderately strong interaction. The top binding affinity may be explained by two interactions that occur between lawsone and IL-1α (Figure 6a). The first interaction is hydrogen bonding between the hydroxyl group of the lawsone to the carbonyl group of the IL-1α at Asp65 residue. The distance of the hydrogen bonds is 2.9Å which is considered as moderate binding interactions (Jeffrey, 1999). The second interaction is hydrophobic interaction

where the aromatic ring of the lawsone resides at the hydrophobic pocket at Ile68 of IL-1α.

As mentioned above, the intermolecular interactions between lawsone and IL-1α can be considered as moderately strong protein interaction. This might be due to the size of the lawsone molecule itself which is small. Thus, less interaction can occur between the lawsone and IL-1α. However, it has both hydrogen bonding and hydrophobic interactions, and these two interactions have moderate strength and are important for reversible drug-target interactions (Katzung, 1998). These findings give insight into why IL-1α can be considered as a valuable target in the development of potential anti-inflammatory agents for inflammatory skin conditions, where further biological testing is needed to prove the hypothesis.

Validation of molecular docking result via control docking cannot be performed on the crystallographic structure of IL-1α (PDB ID: 5UC6) due to the occurrence of multi-ligand interactions at the binding sites (Ren et al., 2017). Thus, another alternative via comparison has been done as an attempt to support the docking analysis on IL-1α, where detection of similar binding residues between previously studied IL-1α inhibitors and lawsone may act as an indicator for result validation. The two different ligands used for comparison are curcumin diglucoside (Xu et al., 2018) and kirenol (Wu et al., 2017), which had been previously studied for molecular docking towards IL-1α on different IL-1α crystal structures. This alternative is hoped to predict meaningful interaction of the compounds binding to IL-1α.

Based on the result in Table 3, the binding affinity of kirenol is better in comparison with its result in previous studies. In Wu et al. (2017) the best binding affinity for kirenol was -4.9 kcal/mol while in the present study, the best binding affinity was higher at -6.4 kcal/mol. This may be due to the protein crystal structure that is used in the current study, which has a higher resolution compared to the previous study, thus giving better inhibitory results when tested *in silico*. Besides, determination of large binding sites of the aptamer to IL-1a captured in the crystalised structure of 5UC6 allows three-dimensional mapping of a large number of active binding sites of the IL-1α protein, thus giving the higher possibility of ligand binding to the active sites. Nevertheless, the binding comparison of curcumin diglucoside between the previous and the current studies cannot be made, as the previous study used different docking parameters, although the present study showed a high binding affinity of curcumin diglucoside to IL-1α at -7.0 kcal/mol.

On the other hand, further analysis of the docking showed that the binding interactions of the key protein residues are similar for all ligands when tested in the present study. The binding happens on related binding sites on the IL-1α protein for all ligands including lawsone, curcumin diglucoside and kirenol which are Asp 65, Lys 67, and Ile 68 protein residues. This result suggested common binding sites of ligands to IL-1α *in silico*, which may be useful as guidelines for further biological binding interaction analysis.

The result found in the present study is in accordance with a previous study by Ozbabacan et al. (2014). In the study, the interactions between IL-1α and its receptor, IL1R1 was predicted to determine their binding interfaces by using a protein-protein interaction prediction algorithm named PRISM (Protein Interaction by Structural Matching), which integrated related pathways and data from previous biological literature to produce the interaction results. PRISM is a tool that can predict, model and assemble the structure of large-scale protein-protein interaction, that had been successfully applied to several pathways, e.g. apoptosis. IL1R1 is the key receptor which initiates the Il-1α signalling pathway, where only the structures of their extracellular domains have been deposited in the PDB. In the study, previously reported oncogenic mutations and single nucleotide polymorphism (SNP) were mapped onto the binding interfaces residues and their nearby residues of both predicted and experimental interactions in the IL-1α-IL1R1 interaction. The statistical significance of these mappings was calculated. The result revealed that the predicted structure of IL-1α-IL1R1 is essential as it contains eleven SNPs and five mutations of the interface and nearby residues, where the previously reported biological data significantly corresponded to the computational result of the interface, adjoining residues and hot spot residues of the IL-1α- IL-R1 interactions.

The predicted complex structure of Il-1α-IL1R1 was confirmed after the comparison of the predicted binding interface to experimentally reported interaction previously carried out by Labriola-Tompkins et al. (1993). The work was based on oligonucleotide-directed mutagenesis in *Escherichia coli* expression plasmid for human Il-1α, where the binding site of human Il-1α for the human type IL1R1 receptor had been analysed. It was found that a successful binding to the IL-1 receptor requires seven amino residues which are Arg12, Ile14, Asp60, Asp61, Ile64, Lys96 and Trp109. Lacking any one of these seven residues will cause significant reduction of the binding activity to the IL1R, thus highlighted essential residues on the binding site of human Il-1α for IL1R1 binding.

Ozbabacan et al. (2014) then integrated the findings from Labriola-Tompkins et al. (1993) on PDB structures used in the study by mapping those seven residues and compared them to their predicted binding interface of Il-1α-IL1R1. The result from comparison reveals several amino acids that were in common which are Arg16, Ile18, Ile68 and Trp113 and another three protein residues, which were Asp64, Asp65 and Lys100 that were located near the binding interface.

Therefore, in agreement with the discovery from Ozbabacan et al. (2014), three out of seven proteins were found to be similar to the present findings of the protein residues involved in the interaction, which were Asp65, Ile68 and Trp113. Hence, this further supported our inference that Asp65 and Ile68 are among the key residues that involve in the binding interaction to Il-1α. Therefore, it can be concluded that the interaction of lawsone to the key amino acid residues of Il-1α can result in protein-ligand binding, thus inhibition of the Il-1α protein expression.

CONCLUSION

Lawsone possesses anti-inflammatory activity, and this highlights the compound potential to be developed as an anti-inflammatory drug for inflammatory skin conditions. Lawsone can be designed as the alternative treatment for skin inflammatory conditions to replace the current steroid-based topical cream that comes with undesirable side effects. Even though only one concentration of lawsone shows the significant anti-inflammatory effect of lawsone using 50 mM ethanol inducer *in vitro*, many studies have been reported to support that lawsone does have anti-inflammatory properties (Adeli-Sardau et al., 2019; Neuman et al., 2002; Singh & Narke, 2015). In addition, the findings from *in silico* suggest that lawsone can bind and inhibit Il-1α, a pro-inflammatory cytokine, with meaningful interactions that exist in the binding was evidenced. Nevertheless, several chemical modifications and structure-activity relationship studies might need to be performed on the molecular structure of lawsone to produce more effective analogues. All of these discoveries imply the potential of lawsone to be developed as an anti-inflammatory drug by targeting the specific protein, IL-1α. This can be seen on how lawsone can promote the viability and proliferation of the A431 cell-induced with acute ethanol exposure. In conclusion, it is believed that lawsone has promising anti-inflammatory properties against ethanol-induced inflammation on A431 human epidermoid carcinoma cell line and inhibits the signalling of IL-1α. This emphasizes the potential of lawsone to be developed into an anti-inflammatory agent with lesser side effects.

AUTHOR CONTRIBUTIONS

Hani Syahirah Zulkefle was responsible for writing the manuscript, performing the experiments, and conducting data analysis. Shazleen Sofea Abdullah help to carried out the experiments and conducted data analysis. Muhammad Alif Mohammad Latif planned the experiments, conducted *in silico* data analysis, and provided supervision. Siti Farah Md Tohid was in charge of project implementation and planning, drafting the manuscript, conducting data analysis, and providing supervision. All authors discussed the results and contributed to the final manuscript

ETHICS APPROVAL

Not applicable.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest in this work.

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