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Research

In Vitro and *In Silico* Studies of Lawsone on Inflammation-Induced Skin Cells for Development of Skin Anti-Inflammatory Treatment

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ABSTRACT

Lawsone, an active phytoconstituent of *Lawsonia inermis* sp., is proposed as a safer alternative for the current skin anti-inflammatory treatments that are mainly steroidal with unwanted side effects which does not conform to the concept of halalan toyyiban in medicine. This study aims to investigate the inhibitory mechanism of lawsone on pro-inflammatory cytokine, TNF (tumour necrosis factor)-α through *in silico* and *in vitro* methods. The inhibitory mechanism of lawsone were investigated via molecular docking and molecular dynamics which were performed via AutoDock Vina and GROMACS softwares, respectively. The results were then analysed via PyMol, Proteins Plus, RMSD and RMSF graphs. Subsequently, cytotoxicity of lawsone towards A431 skin epidermoid and 3T3 fibroblast cells, was determined via MTT assay. Lawsone was further tested on A431 cells stimulated with ethanol (200 mM) or hydrogen peroxide (250 μM) for 24 hours (acute) and 48 hours (chronic) to induce pro-inflammatory cytokine (TNF-α) release. Cell viabilities were determined via MTT assay and expression of TNF-α were measured via ELISA. *In silico* works predicted lawsone's ability to bind to TNF-α with good binding affinity without disruption to the protein's structure stability.Lawsone is proven to be safe towards non-pathological cells. Lawsone exhibited significant anti-inflammatory effect on inflammation induced A431 epidermoid cells. ELISA results were not as expected as compared to previous *in silico* study, but an anti-inflammatory pattern can be observed in the chronic ethanol-induced treatment groups. In conclusion, lawsone is shown to have potential to be developed as a halalan-toyyiban alternative for skin anti-inflammatory treatment.

Key words: ELISA, lawsone, molecular docking, molecular dynamics, skin inflammation, TNF-α

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INTRODUCTION

Halal pharmaceutical is defined as pharmaceutical products or medicines that comply with the halal requirements contain permissible ingredients and are produced according to Islamic rules and conditions. The halalan-toyyiban concept in Islamic medicine requires that the drugs or medications not only be free of forbidden ingredients but also safe for consumption, non-poisonous, and non-hazardous to the consumer (Saha *et al*. 2019, Malaysian Standards of Halal Pharmaceutical MS2424:2012).

According to the Global Burden of Disease Morbidity and Mortality Report 2013, skin diseases or conditions were the fourth leading cause of non-fatal disease burden with a total contribution of 1.79% to the total global burden of disease and dermatitis carries the greatest burden out of 15 categories of skin conditions assessed (Pulsipher *et al*. 2021). Skin inflammation is defined as a condition where the skin is impaired, damaged, or injured leading to the development of inflammatory conditions that are presented by the known cardinal signs of inflammation namely redness, heat, swelling, pain, and loss of function. The skin inflammation can be further categorized as acute and chronic

skin inflammation. Schwingen *et al*. (2020) reviewed that atopic dermatitis and psoriasis are two major skin inflammatory diseases that affect worldwide. According to the WHO Global Report on Psoriasis (2016), psoriasis is a serious, chronic non-communicable skin disease that affects 2% to 3% of the world population or at least 100 million people worldwide. As for atopic dermatitis, approximately 15% to 20% of children and 1% to 3% of adults from the world population are afflicted with this skin disease (Avena-Woods, 2017).

Steroid-based medications are commonly prescribed medication in treating skin inflammatory conditions as it is most effective and less time-consuming. However, despite the effectiveness, it is also widely known to cause local and systemic adverse effects (Coondoo *et al*. 2014; Ghosh & Coondoo, 2017) which does not compliant with the halalan-toyyiban concept in Islamic medicine. Thus, the journey of finding safer alternative skin anti-inflammatory treatment began, and Lawsone (Figure 1) is the compound deemed to be a potential candidate to be developed as such treatment.

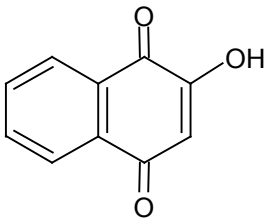

Fig. 1. Structure of lawsone (2-hydroxy-1,4-naphthoquinone)

Cytokines, one of the inflammatory mediators, are soluble factors that play a major role in the communication of cells and tissues and major involvement in immune and inflammatory responses. As keratinocytes act as sensors of invasion of pathogens and initiators of inflammatory response, the cells are predominated by proinflammatory cytokines, majorly TNF-α and IL-1α (Wolff *et al*., 2011; Abdallah, Mijouin & Pichon, 2017). TNF-α is identified as one of the key players in psoriasis, which is a chronic skin inflammatory condition and its inhibition is known to modulate a broad range of genes involved in inflammatory responses, directly or indirectly. Some of the genes that are the direct targets of TNF-α include IL-8, IL-1β, CXCL10, and CXCL11. Anti-TNF-α agents have been proven to be effective as a treatment for several immune diseases, including psoriasis (Langkilde *et al*., 2016; Schwingen, Kaplan & Kurschus, 2020). This study focuses on the inhibition of TNF-α as a mode for the treatment of inflammation due to its evident role in inflammatory responses and the effectiveness of current anti-TNF-α agents for the treatment of inflammatory diseases.

Lawsone (2-hydroxy-1,4-naphthoquinone) is an active phytoconstituent derived from a plant, *Lawsonia inermis* sp. (family Lythraceae). Scientifically, lawsone possesses biological properties such as anti-bacterial, anti-fungal, anti-inflammatory, antioxidant, and more (Badoni Semwal *et al*., 2014). However, little research has been done on the anti-inflammatory effects of Lawsone on skin inflammatory conditions. Therefore, the present study aimed to investigate the ability of lawsone to be developed as a potential, safer, halalan-toyyiban alternative to skin anti-inflammatory medication through *in vitro* and *silico* methods by targeting a pro-inflammatory cytokine, TNF-α.

MATERIALS AND METHODS

Receptor and ligand input preparation

The crystal structure of TNF-α with PDB ID 4G3Y was retrieved from the Protein Data Bank. Water molecules were discarded, polar hydrogen atoms were added while nonpolar were merged and Gasteiger charges were applied. The crystal structure was refined and missing residues were added. The antibody structure that was bound to the protein structure was deleted, leaving only the structure of TNF-α. The original structure of TNF-α was retained as its interaction with Infliximab did not cause any significant structural changes in TNF-α. This was confirmed by comparing the structure to the structure of wild type TNF-α (RMSD 1.4 Å). The ligand structure, lawsone, was obtained from PubChem (compound CID: 6755). As for the ligand input, lawsone, the structure was obtained from PubChem in the form of a '.sdf' file which is then converted to '.pdb' format via software OpenBabel.

Molecular docking

Molecular docking was performed by using MGLTools of AutoDock 2.3 (Trott & Olson, 2010). The crystal structure's coordinates used for docking calculation were saved in pdbqt format. A grid box of centers 40 Å × 32 Å × 20 Å along with dimensions – 14.183 Å × -4.576 Å × -24.377 Å, was centered on

the targeted binding sites of TNF-α. The pose with the highest binding affinity was selected for further analysis. The protein-ligand interaction was analyzed by using PyMol, PoseView, and Protein-Ligand Profiler.

Molecular dynamics

Gromacs 4.5.6 software package was used to perform MD simulation (Bauer *et al*., 2022). Protein-ligand conformation with the best binding pose obtained from the previous docking studies was used for the simulation. Protein topology was generated by 54a7 force-field and ligand topology was generated using PRODRG server. Then, the protein-ligand complex was placed in a triclinic box and solved with simple point charge (SPC) (spc216) water. The system is then neutralized by the addition of two NA+ ions. Subsequently, an energy minimization process was carried out to relax and set the system at an energy minimum. When the system was relaxed, then only simulations could be carried out. Before the production MD run, the system was equilibrated with the desired temperature and pressure. Once equilibrated, a 10-ns production MD simulation was executed. Then, RMSD and RMSF graphs are generated and analyzed. The MD simulation results were examined and graphs were plotted via the XMGrace tool.

Cell culture

Two cell lines used in this study (A431 skin epidermoid & 3T3 fibroblast cell lines) were generous gifts from Prof. Dr. Tan Wen Siang from the Faculty of Biotechnology, Universiti Putra Malaysia and Prof. Madya Dr. Abdah Md Akim from Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Both A431 and 3T3 cell lines were originally purchased from the American Type Culture Collection (ATCC). Both cell lines were maintained in complete growth media encompassing DMEM (high glucose) supplemented with 10% FBS and 1% antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin). The cell lines were grown in a sterile humidified CO_2 incubator with 5% CO2 at 37°C.

Cell viability assay

Cell viability assays were performed on A431 and 3T3 cells by MTT colorimetric assay. Cells were seeded in 96-well plates at 1×10^5 cells/mL in DMEM supplemented with 10% FBS and incubated for 24 hr at 37°C in 5% CO_2. Different concentrations of lawsone (9.77 µM – 5000 µM in media with less than 0.1% of DMSO) were added to the plate in triplicates and incubated for 24 hr. Cells treated with media containing 0.1% DMSO served as a normal control group. Then, the previous media was discarded and replaced with 100 µL of fresh DMEM medium supplemented with 10% FBS. 20 µL of MTT reagent (5 mg/mL) were added to the cells in dark condition and further incubated for 4 hr. MTT was aspirated and 100 µL of DMSO was added into each well to dissolve the formazan precipitate. The absorbance of the formazan product was then measured at 570 nm, with reference wavelength at 630 nm via a microplate reader (Biotek, USA) to determine the relative number of viable cells. Half maximal inhibitory concentration (IC_{50}) was obtained directly from the fit standard curve of cell viability percentage against lawsone concentrations. The IC_{50} obtained was used as a reference dose for the subsequent skin anti-inflammatory assay.

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Skin anti-inflammatory assay

Skin anti-inflammatory assay was performed following the procedure adopted by Neuman *et al*. (2011). A431 epidermoid cells were seeded in a 96-well plate at 1×10^5 cells per well and incubated at 37°C for 24 hr. Difference concentrations of lawsone (1000 µM – 7.81 µM) were added to the plate in triplicates, along with inflammation inducers, ethanol (200 mM), and hydrogen peroxide (250 µM). Cells, with only an inducer, served as a negative control group. As for positive control, cells were treated with 0.5 µg/mL hydrocortisone, a commercial drug. Cells were incubated for 24 hr and 48 hr to mimic the acute and chronic skin inflammatory conditions. Next, old media is replaced with fresh medium DMEM supplemented with 10% FBS, followed by adding 20 µL MTT reagent onto the treated cells and incubating for 4 hr at 37°C. Then, 100 µL DMSO was added into each well to dissolve the formazan product which was then measured at 570 nm with reference wavelength at 630 nm via microplate reader to determine the relative number of viable cells.

TNF-α expression level measurement

A431 epidermoid carcinoma cells were seeded in 24-well plates at 1.5×10^5 cells per well. The cells were treated with the same treatment groups in the previous skin anti-inflammatory assays for 24 (acute) and 48 (chronic) hr. Conditioned media were used instead of a complete medium, where the

serum content was reduced to 5%. The cell culture supernatants were harvested after the incubation periods. Bradford assay was performed on samples to predetermine the protein concentration in each sample. A human TNF-α ELISA kit (Catalogue number: E-EL-H0109, Elabscience, USA) was used to measure the TNF-α level of expressions according to the manufacturer's protocol. Absorbance was recorded at 450 nm using the microplate reader.

RESULTS

Molecular docking

AutoDock Vina had predicted that the ligand, lawsone was best docked to the target protein, TNF-α with a binding affinity of -4.7 kcaL/moL. Further visualization via PyMol revealed that Lawsone was docked on the hydrophobic pocket of TNF-α (Figure 2). Then, a two-dimensional (2D) diagram of the docked pose generated via PoseView revealed that lawsone interacted with leucine 43, a binding residue from the hydrophobic pocket of TNF-α with a distance of 3.7 Å (Figure 3).

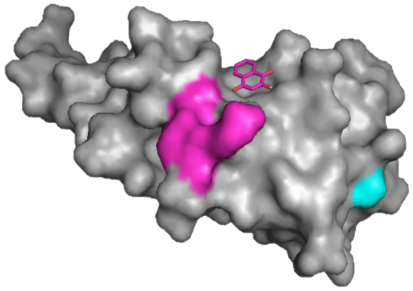

Fig. 2. View of lawsone binding pose on the hydrophobic pocket of TNF-α via PyMol.

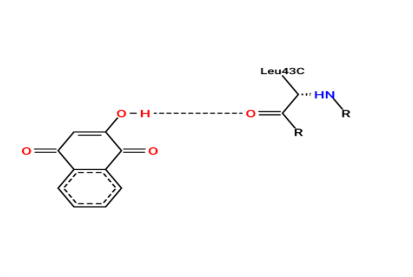

Fig. 3. 2D diagram of lawsone interaction to the binding residue of TNF-α, leucine 43.

Molecular dynamics (MD) simulation

Molecular dynamics was carried out on the best protein-ligand binding mode predicted via molecular docking to simulate the interaction of the complex in vivo condition. The ability to analyze protein-ligand systems in an aqueous solution resembling the real physiological condition is a distinctive feature of the method. Water is known to play a crucial role in protein dynamics. Therefore, the presence of water and ions at room temperature can further correct the previously obtained docking results. Changes in structure or conformation in the active binding site of TNF-α protein against lawsone have been analyzed. A 10 nanoseconds MD simulation was performed on protein only and protein-ligand complex. The stability of the structures was studied by Root-mean-square deviation (RMSD) and Root-mean-square fluctuation (RMSF).

Root-mean-square deviation (RMSD)

Root-mean-square deviation (RMSD) curve of protein backbone atoms was calculated using the g_rms tool of GROMACS software and plotted into a graph (Figure 4). RMSDs were calculated and generated to determine the protein structural stability throughout the 10000 picoseconds simulation. From the graph, it can be observed that the protein-ligand complex follows similarly the overall pattern of the original protein structure simulation, with some fluctuations in some parts of the simulation. Huge fluctuation can be seen during the initial part of the simulation, between 0 to 3500 ns which might be due to the protein adjusting to the presence of the ligand. After 3500 ns, the graph can be seen to decline and more stable, which indicates that the protein has eventually settled down and adjusted to Lawsone.

Towards the end of the simulation, it can be observed that the graph of the protein-ligand complex follows closely the pattern of the graph of the original protein structure. This indicates that this ligand, lawsone, does not introduce any drastic structural instability or changes toward the protein structure. Therefore, the result suggests that Lawsone's inhibitory mechanism is not through destruction or destabilization of the protein structure. It is predicted that lawsone blocks the protein's activity without destroying it.

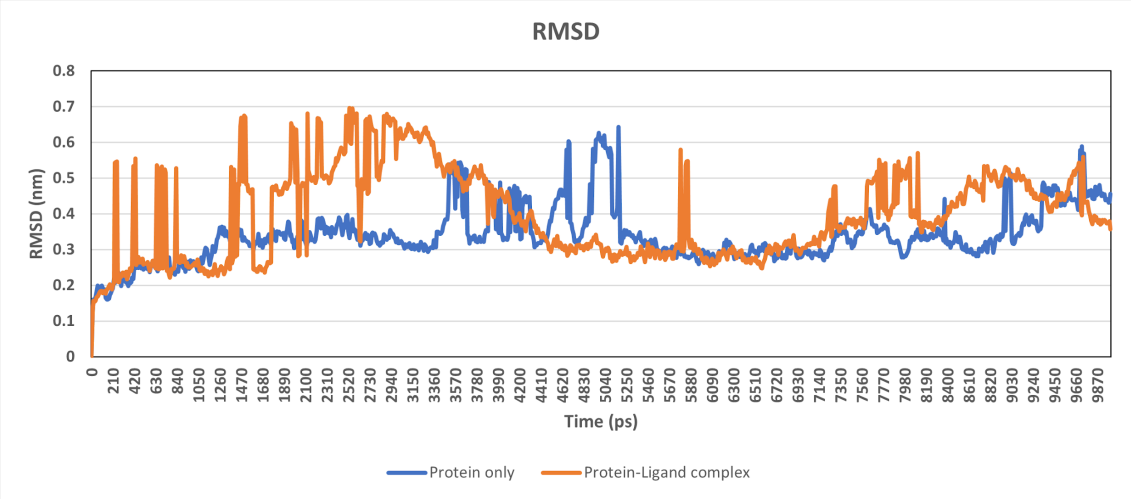

Fig. 4. The Root-Mean-Square Deviation (RMSD) plots of the protein structures throughout 10000 ps simulation.

Root-mean-square fluctuation (RMSF).

Root-mean-square fluctuation (RMSF) of the protein structure was analyzed to dictate the ligand binding effect on the protein structure stability throughout the 10000 picoseconds MD simulation. By utilizing the g_rmsf tool in the GROMACS software, the RMSF of the protein backbone residues were calculated and graphs were generated (Figure 5). Similar to the RMSD graph above, the RMSF graph patterns between the protein-ligand complex and protein only were similar, and only small fluctuations were observed. This supports the conclusion made from the RMSD graph where the ligand did not introduce any drastic changes that disrupt the protein structure stability. The protein-ligand complex showed fluctuations between residue 20 – 25, residue 82, residue 85 – 87, residue 100 – 107, and residue 143 – 149. Using PyMol, each of the fluctuated residues was highlighted and it can be observed that fluctuations mostly occurred in the loops and turns of the proteins, as compared to the helical and sheet structures. Fluctuations in these residues indicate flexibility in the said residues. The binding site residues (Leu29, Arg31, Arg32, Ser52, Tyr56, & Tyr87) in the structure of the protein displayed stability even after the docking of lawsone to the protein. Overall, the RMSF graph indicates that the protein's residues were stable upon the introduction of the ligand. Therefore, it can be similarly concluded as the findings from RMSD graphs where Lawsone does not introduce any drastic changes and works through inhibiting the activity of the protein without destructing the protein.

Cell viability assay

Lawsone was tested upon 3T3 fibroblast and A431 epidermoid cells with different concentrations ranging from 9.77 µM to 5000 µM. At the concentration of 1000 µM, the cell viability was observed to be around 50 to 60%. In this assay, the cytotoxicity of lawsone against A431 epidermoid cells (pathological) and 3T3 fibroblast cells (normal, non-pathological) were compared (Figure 6). The percentage of viable 3T3 cells was constantly above 50% even at high concentrations. However, the same could not be said for A431 cells. the viability percentage of A431 cells remained constant at the range of 70% to 75%, and the trend began decreasing at the concentration of 625 µM, where the viability percentage was around 60% and at 1250 µM, which was a moderately high concentration, the viability percentage of A431 cells were below 50%. Therefore, lawsone was demonstrated to be non-toxic towards normal and non-pathological cells, hence, safe to be used. Subsequently, IC_{50} of lawsone was determined at 1000 ± 6.82 µM (Figure 7). The concentration was used as a reference dose for the subsequent skin anti-inflammatory assay.

Skin anti-inflammatory assay

Anti-inflammatory effect of Lawsone on ethanol-induced inflammation against A431 cells

To observe the anti-inflammatory effects of Lawsone, skin anti-inflammatory assays were adapted on A431 skin cells. A431 epidermoid cells were selected for skin anti-inflammatory study as skin cells were known to produce cytokines. According to Balkrishna *et al*. (2020), their study has proven A431 epidermoid cells' ability to the expression of pro-inflammatory cytokines resembling psoriatic-like inflammation. Besides that, A431 cells were found to be used in other skin inflammatory studies which include studies done by Neuman *et al*. (2011) and Balkrishna *et al*. (2020). Ethanol was used to induce A431 cells into an inflammatory state, as it possesses the ability to induce damage to epidermoidal cell lines and exert cytotoxic effects which include releasing pro-inflammatory cytokines and skin cell apoptosis. Ethanol concentration was priorly determined by MTT assay and 200 mM seems to be the most acceptable concentration as an inducer for its ability in causing 50% of cell viability.

Ethanol exposure to untreated cells acted as a negative control in this assay, which had significantly reduced cell viability after 24-hr and 48-hr exposure, therefore indicating an exhibition of inflammatory activity. Hydrocortisone, a standard topical corticosteroid drug was used as a positive control, which showed marked inhibition of inflammation and a significant increase in cell viability in both, 24-hr and 48-hr treatment (P<0.001).

It was observed that lawsone at the concentration of 15.63 µM showed a significantly higher percentage of cell viability when compared to the negative control, in both 24-hr (Figure 8) and 48- hr (Figure 9) treatments. Lawsone at the concentration of 62.5 µM, too, showed a significantly higher percentage of cell viability in the 24-hr treatment, but not in the 48-hr treatment. There are significant differences between 15.63 µM, and 62.5 µM in comparison to positive control. Meanwhile, other concentrations did not exhibit significant therapeutic effects against ethanol exposure, and the cell viability was significantly reduced.

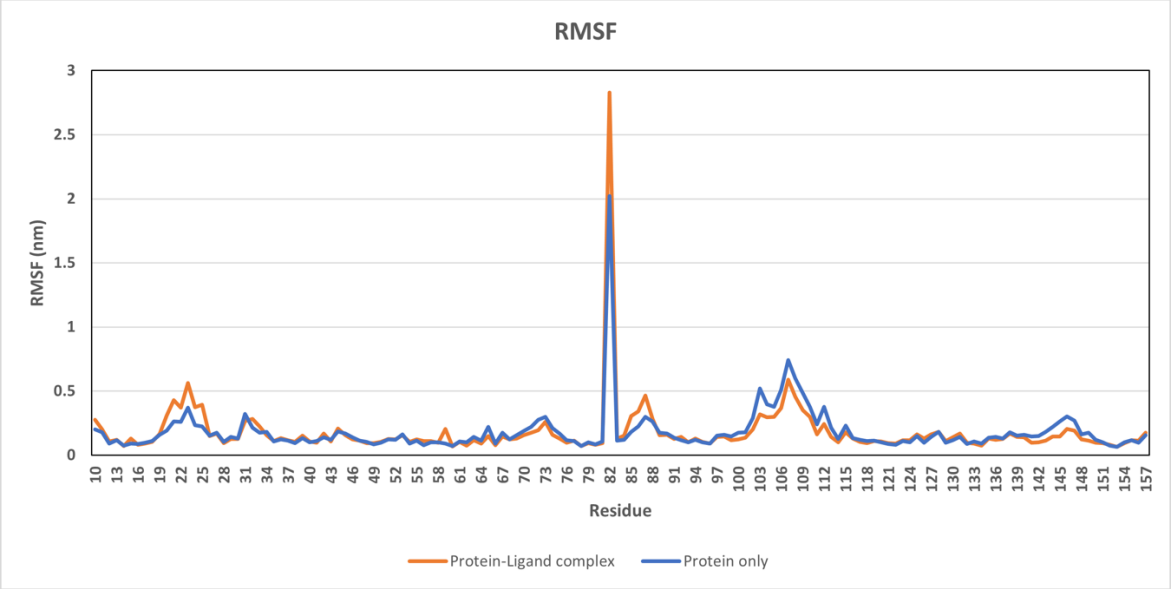

Fig. 5. The Root-Mean-Square Fluctuation (RMSF) plots of the protein structure throughout 10000 ps simulation.

Anti-inflammatory effects of Lawsone on hydrogen peroxide-induced inflammation against A431 cells

Hydrogen peroxide was used to induce A431 cells into an inflammatory state. Hydrogen peroxide concentration was priorly determined by MTT assay and 250 µM seems to be the most acceptable concentration as an inducer for its ability in causing 50% of cell viability. In the negative control treatment group, significant reductions in cell viability after 24-hr and 48-hr exposure were observed, therefore indicating the exhibition of inflammatory activity by the inducer. Marked inhibition and a significant increase in cell viability in both 24 hr and 48 hr of treatment (P>0.001) were observed in the positive control treatment group.

It was observed that lawsone at the concentration of 15.63 µM showed a significantly higher percentage of cell viability when compared to negative control, in both 24-hr (Figure 10) and 48-hr (Figure 11) treatments. Lawsone at the concentration of 125 µM, too, showed a significantly higher percentage of cell viability in 24-hr treatment, but not in 48 hr. There is no significant difference between 15.63 µM, and 125 µM in comparison to positive control. Meanwhile, other concentrations did not show a significant therapeutic effect against hydrogen peroxide exposure and the cell viability was significantly reduced.

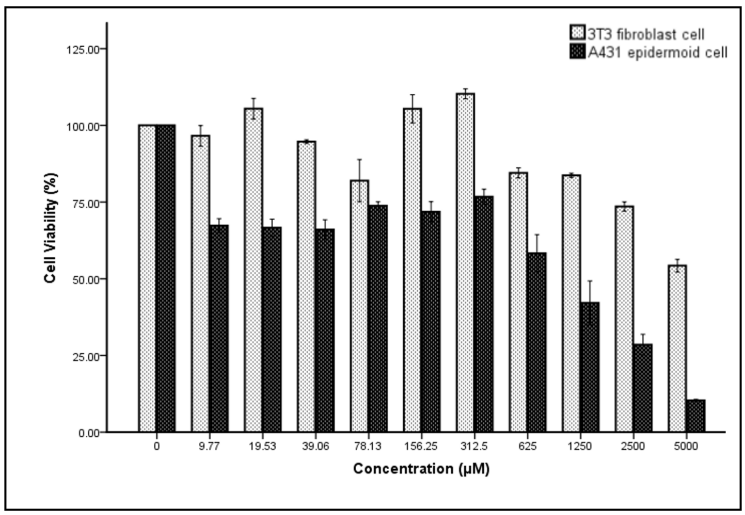

Fig. 6. Cell viability of lawsone-treated A431 epidermoid cells and 3T3 fibroblast cells for 24 hr. Results were expressed as mean ± SEM of at least three independent experiments. The values were expressed as mean ± SEM from three independent experiments.

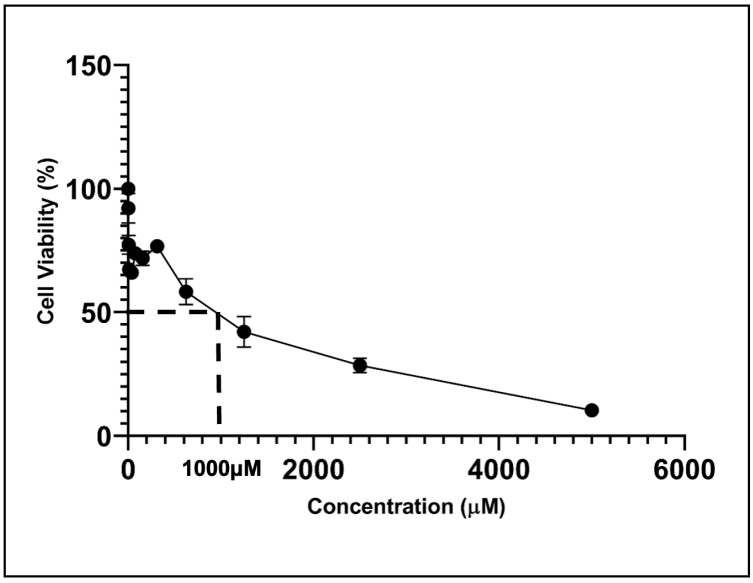

Fig. 7. IC_{50} of lawsone against A431 epidermoid carcinoma cells after 24 hr of exposure was obtained at 1000 ± 6.82 µM. The values were expressed as mean ± SEM from three independent experiments.

TNF-α expression level measurement

In this study, the anti-inflammatory activity of lawsone against the release of pro-inflammatory cytokine, primarily TNF-α, induced by ethanol and hydrogen peroxide in acute and chronic conditions was expected to be observed. According to results from the Bradford assay that was carried out beforehand, each sample contained a high level of protein concentration in the range of 0.5 mg/mL to more than 1.0 mg/mL, however, after running of ELISA, results mainly showed that the level of TNF-α expressions were either technically too low to be detected or not present in the samples, which contradicts to the results obtained from Bradford assay.

According to results from ELISA (Table 1), certain treatment groups did show expressions of TNF-α while some did not, which is utterly unexpected as TNF-α was among the targeted pro-inflammatory cytokines to be released upon inflammation. However, there is a need to emphasize the existence of a pattern that can be observed in the chronic ethanol-induced treatment groups. It can be observed that lawsone (15.63 µM) treatment samples showed the same level of TNF-α expressions as the positive control group, of which both are lower than the level of expressions in the negative control group.

Although the significance was unable to be proven due to the differences in readings between replicates which hindered statistical analyses, it surely displayed the potential of lawsone in exhibiting anti-inflammatory effect through inhibition of TNF-α expressions.

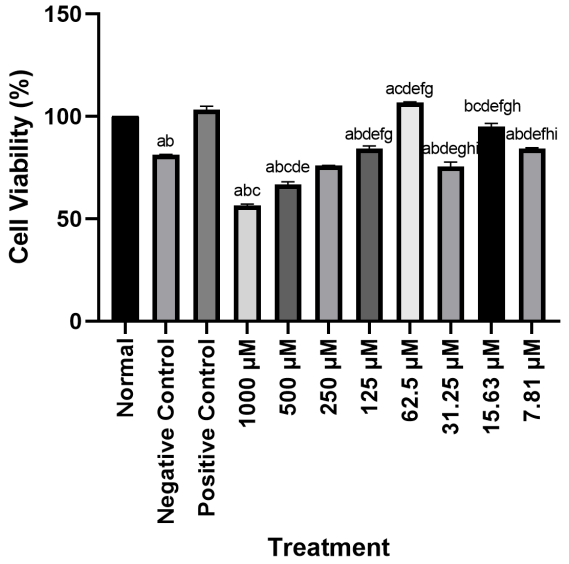

Fig. 8. Cell viability (percentage of control) of lawsone-treated (1000 µM, 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15.63 µM & 7.81 µM) A431 cells with exposure to ethanol for 24 hr. Results were expressed as mean ± SEM from three independent experiments. One-way ANOVA was used to perform the statistical analysis followed by Dunnett's Test as the post-hoc test.

[a]Significantly different (P<0.05) compared to the normal group, [b]Significantly different (P<0.05) compared to the positive control group, [c]Significantly different (P<0.05) compared to the negative control group, [d]Significantly different (P<0.05) compared to lawsone (1000 µM), [e]Significantly different (P<0.05) compared to lawsone (500 µM), [f]Significantly different (P<0.05) compared to lawsone (250 µM), [g]Significantly different (P<0.05) compared to lawsone (125 µM), [h]Significantly different (P<0.05) compared to lawsone (62.5 µM), [i]Significantly different (P<0.05) compared to lawsone (31.25 µM), [j]Significantly different (P<0.05) compared to lawsone (15.63 µM), [k]Significantly different (P<0.05) compared to lawsone (7.81 µM)

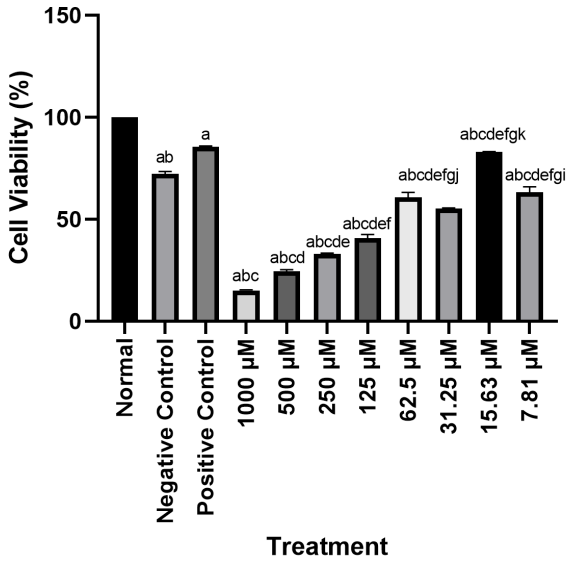

Fig. 9. Cell viability (percentage of control) of lawsone-treated (1000 µM, 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15.63 µM, & 7.81 µM) A431 cells with exposure to ethanol for 48 hr. Results were expressed as mean ± SEM from three independent experiments. One-way ANOVA was used to perform the statistical analysis followed by Dunnett's Test as the post-hoc test.

[a]Significantly different (P<0.05) compared to the normal group, [b]Significantly different (P<0.05) compared to the positive control group, [c]Significantly different (P<0.05) compared to the negative control group, [d]Significantly different (P<0.05) compared to lawsone (1000 µM), [e]Significantly different (P<0.05) compared to lawsone (500 µM), [f]Significantly different (P<0.05) compared to lawsone (250 µM), [g]Significantly different (P<0.05) compared to lawsone (125 µM), [h]Significantly different (P<0.05) compared to lawsone (62.5 µM), [i]Significantly different (P<0.05) compared to lawsone (31.25 µM), [j]Significantly different (P<0.05) compared to lawsone (15.63 µM), [k]Significantly different (P<0.05) compared to lawsone (7.81 µM)

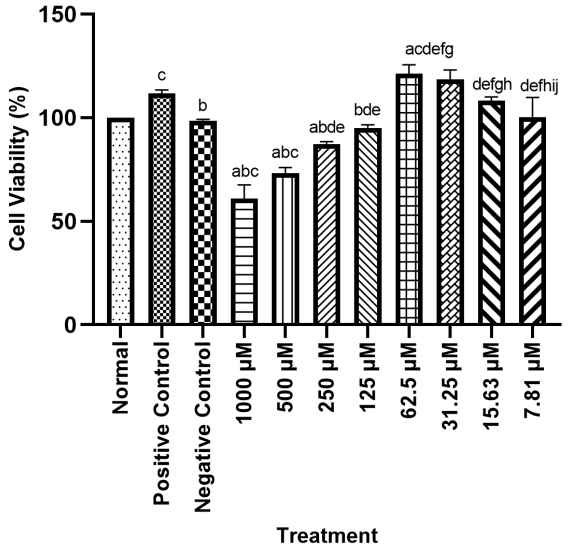

Fig. 10. Cell viability (percentage of control) of lawsone-treated (1000 µM, 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15.63 µM & 7.81 µM) A431 cells with exposure to hydrogen peroxide for 24 hr. Results were expressed as mean ± SEM from three independent experiments. One-way ANOVA was used to perform the statistical analysis followed by Dunnett's Test as the post-hoc test.

[a]Significantly different (P<0.05) compared to the normal group, [b]Significantly different (P<0.05) compared to the positive control group, [c]Significantly different (P<0.05) compared to the negative control group, [d]Significantly different (P<0.05) compared to lawsone (1000 µM), [e]Significantly different (P<0.05) compared to lawsone (500 µM), [f]Significantly different (P<0.05) compared to lawsone (250 µM), [g]Significantly different (P<0.05) compared to lawsone (125 µM), [h]Significantly different (P<0.05) compared to lawsone (62.5 µM), [i]Significantly different (P<0.05) compared to lawsone (31.25 µM), [j]Significantly different (P<0.05) compared to lawsone (15.63 µM), [k]Significantly different (P<0.05) compared to lawsone (7.81 µM)

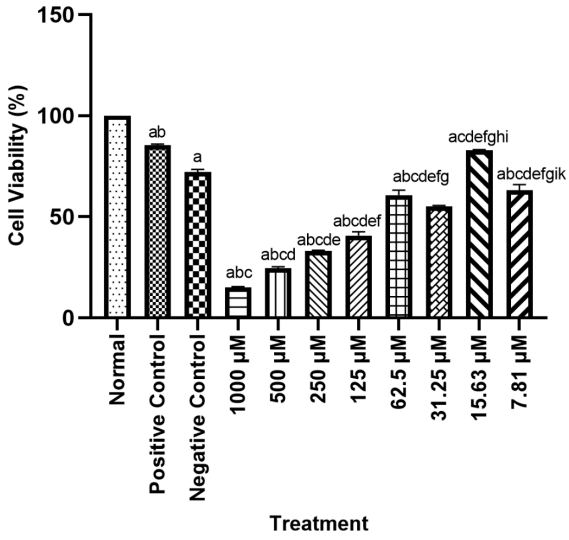

Fig.11. Cell viability (percentage of control) of lawsone-treated (1000 µM, 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15.63 µM & 7.81 µM) A431 cells with exposure to hydrogen peroxide for 48 hr. Results were expressed as mean ± SEM from three independent experiments. One-way ANOVA was used to perform the statistical analysis followed by Dunnett's Test as the post-hoc test.

[a]Significantly different (P<0.05) compared to the normal group, [b]Significantly different (P<0.05) compared to the positive control group, [c]Significantly different (P<0.05) compared to the negative control group, [d]Significantly different (P<0.05) compared to lawsone (1000 µM), [e]Significantly different (P<0.05) compared to lawsone (500 µM), [f]Significantly different (P<0.05) compared to lawsone (250 µM), [g]Significantly different (P<0.05) compared to lawsone (125 µM), [h]Significantly different (P<0.05) compared to lawsone (62.5 µM), [i]Significantly different (P<0.05) compared to lawsone (31.25 µM), [j]Significantly different (P<0.05) compared to lawsone (15.63 µM), [k]Significantly different (P<0.05) compared to lawsone (7.81 µM)

Table 1. Protein expressions quantitated via enzyme-linked immunosorbent assay (ELISA)

Groups		TNF-α (pg/mL)			
		Ethanol-induced inflammation		Hydrogen peroxide-induced inflammation	
		24 hr	48 hr	24 hr	48 hr
I	Vehicle control	2.907 ± 2.907	2.907 ± 2.907	2.907 ± 2.907	2.907 ± 2.907
II	Positive control (Hydrocortisone 0.5 µg/mL)	2.907 ± 2.907	5.407 ± 5.407	0	5.407 ± 5.407
III	Negative control	0 ± 0	10.81 ± 10.81	0	2.483 ± 2.483
IV	Lawsone (15.63 µM)	0 ± 0	5.407 ± 5.407	0	8.476 ± 8.476

DISCUSSIONS

In Silico studies

Based on docking studies, lawsone was predicted to be able to dock in a hydrophobic pocket of TNF-α with a top binding affinity of -4.7 (kcaL/moL) and the distance between the interacting binding residue, leucine 43, measured at 3.7 Å. This was considered as a rather moderate binding affinity between the ligand and protein; however, binding affinity data solely did not indicate the overall effectiveness of the drug or compound, rather it was a combination of the ligand's binding affinity and efficacy. A ligand's efficacy is defined as the ligand's ability to exhibit a biological response upon binding to the target protein. For moderate or low binding affinity ligands, a higher concentration is needed for maximal occupation at the protein's binding site and maximal physiological response of the ligand (Kenakin, 2021).

Then, molecular dynamics studies were performed to analyze the structural stability of protein-ligand interaction in an aqueous system resembling the real physiological conditions. In this simulation, water is added to the system as it plays a crucial role in the structure and dynamics of proteins (Qaiser *et al*., 2020). The overall structural stability of TNF-α upon interaction with lawsone was analyzed through RMSD and RMSF graphs. RMSD analysis measures and determines the difference between the initial conformation of the protein backbone to the final position throughout the time course of 10000 ps, of which more fluctuations indicate more instability (Jade *et al*., 2020). In Figure 4, although there were huge fluctuations in the first 3000 seconds as the protein still adjusted to the presence of lawsone, however, throughout the rest of the simulation, the graph has become more stable indicating the protein's successful adjustment to the ligand. The pattern of the protein-ligand complex also follows closely the curve of protein only. This indicates that the protein maintains structural integrity upon docking of Lawsone throughout the simulation. Similarly can be said on the RMSF graph generated. No drastic changes can be observed between the graph of protein only and the protein-ligand complex. Fluctuations were minor and occurred in areas of loops and turns of the proteins, where the residues might possess more flexibility. Therefore, this supports the findings from RMSD analysis, where the protein structural integrity was not disrupted. It is, therefore, safe to conclude that lawsone can bind and interact with TNF-α and its inhibitory mechanism of action does not involve disruption or destruction of protein structure.

In Vitro studies

Cytotoxicity of lawsone was tested on A431 cells and 3T3 cells, where 3T3 cells represent the normal, healthy cell population and A431 cells represent the pathological population. Our results displayed that the percentage of cell viability of lawsone-treated normal 3T3 fibroblast cells remained above 50% even at higher concentrations. While in lawsone-treated A431 epidermoid carcinoma cells, the percentage of cell viability declined at higher concentrations. Therefore, it was determined that lawsone was demonstrated to be non-toxic towards healthy and non-pathological cells and, hence, safe to use. The IC_{50} value of lawsone was then determined at 1000 ± 6.82 µM. This concentration was used as a reference dose for the subsequent anti-inflammatory assay.

The subsequent skin inflammatory assay proceeded on A431 cells due to the potential of A431 cells to imitate the psoriatic-like inflammatory condition (Balakrishna *et al*., 2020). In the skin inflammatory assay, lawsone at the concentration of 15.63 µM stood out as it showed a significant therapeutic effect in the inhibition of inflammation against both ethanol- and hydrogen peroxide-induced inflammation in A431 epidermoid cells for both 24 and 48 hr. However, it could be observed that the effects were either on par or lesser than the commercial treatment of skin inflammatory diseases, hydrocortisone, as the percentage of cell viability showed no significant difference or was significantly lesser when compared

to the positive control. The effects of Lawsone were shown to be dose-dependent as significant differences in cell viability percentages could be seen from highest to lowest concentration. The cell viability percentages increased as the concentration decreased, with its peak at 15.63 µM, however began to decrease at 7.81µM, which therefore led us to the term 'therapeutic window'. According to the Merriam-Webster medical dictionary, a therapeutic window is defined as the range of concentration or dosage of a drug that exhibits a safe, effective therapeutic effect.

According to the definition of therapeutic window, it is understood that a higher or lower concentration than the therapeutic window will not show effective therapeutic effects. Evident reduction can be observed in cell viabilities of A431 epidermoid carcinoma cells in exposure to inflammation inducer for 48 hr when compared to the ones exposed for 24 hr. Therefore, it is highly suggested that lawsone concentrations outside of the therapeutic window probably work synergistically with ethanol and hydrogen peroxide in exhibiting inflammatory activity.

Following the significant findings obtained from the anti-inflammatory assay, samples were harvested for TNF-α expression level measurement. TNF-α is a pro-inflammatory cytokine targeted to be expressed in this study as it is identified as among the key players in psoriasis and is known to be involved in inflammatory responses (Schwingen *et al*., 2020). Besides that, TNF-α is known to be one of the predominant pro-inflammatory cytokines in keratinocytes (Wolff *et al*., 2011). Before the ELISA run, a preliminary test to determine protein concentration in samples was carried out via Bradford assay have showed a good level of protein concentrations in each sample tested, therefore providing the confidence in proceeding with ELISA.

However, the results obtained from ELISA were unexpected as the level of TNF-α expressions was technically too low to be detected which contradicts the results obtained from the anti-inflammatory assay that clearly showed the significant potential of anti-inflammatory activity, as well as preliminary protein concentrations determined from Bradford assay. This has certainly raised confusion and questions as to the causes that could have led to these occurrences. Although most of the readings obtained from ELISA were too low, readings from samples that showed expressions should not be discarded. As certain samples or replicates did show levels of TNF-α expressions, therefore the chances that TNF-α is being expressed, only that they were expressed at really low levels, should not be disregarded.

Certain treatment groups displayed expressions of TNF-α, particularly chronic ethanol-induced treatment groups that stood out as they displayed a pattern in their level of TNF-α expressions. Based on the previous anti-inflammatory assay, lawsone at the concentration of 15.63 µM was believed to possess anti-inflammatory activity and was within the therapeutic window, therefore this concentration was selected to be studied further in ELISA. In the chronic ethanol-induced treatment group, it was observed that lawsone (15.63 µM) expressed the same level of TNF-α as the positive control group, which was at 5.407 pg/mL, of which both were lower than the negative control group, which was at 10.81 pg/mL. This pattern points out the possibility of the anti-inflammatory activity of lawsone against TNF-α. Besides that, a high level of protein concentration as detected in the Bradford assay might indicate the presence of other inflammatory cytokines, of which the concentration might be more predominant than TNF-α. Examples of other inflammatory cytokines that could be present include interleukin-1α (IL-α), interferon-gamma (IFN-Y), interleukin-17a (IL-17a), and more. IL-1α and TNF-α are among the major pro-inflammatory cytokines that predominate keratinocytes (Wolff *et al*., 2011). Besides that, keratinocytes also express other inflammatory mediators such as interleukin-1 beta (IL-1β) and vascular endothelial growth factor (VEGF), which could also be included as targets in future experiments (Sangiovanni *et al*., 2019).

CONCLUSION

Lawsone had demonstrated anti-inflammatory activity in both, *in vitro* and *silico*. Through *in silico* methods, the mechanism of inhibition of lawsone against TNF-α was studied. According to the results of *in silico* studies, lawsone displayed the ability to interact with TNF-α with moderate binding affinity and did not cause any drastic changes in the protein's structure and conformation. It is predicted that the inhibitory mechanism of lawsone does not involve protein destruction. From *in vitro* studies, lawsone was proven to be safe and non-toxic towards normal, healthy cells and displayed capabilities in inhibiting inflammation at a therapeutic concentration of 15.63 µM, which was suggested to be the therapeutic window for lawsone. Meanwhile, the potential inhibitory mechanism of lawsone on TNF-α can be observed in ELISA, although its significance is unable to be proven it did provide insights on ways to improve results in the next study.

The present study has provided valuable information, especially on the potentiality of anti-inflammatory of lawsone and insights on the possible mechanism of inhibition of lawsone through

interaction with pro-inflammatory cytokine, particularly TNF-α. Considering the limitations of this study, future research which includes a more sensitive ELISA study and determination of lawsone inhibition on other biomarkers are anticipated to enhance the robustness of the present study.

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ETHICAL STATEMENT
Not applicabe.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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