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THE EFFECT OF VITEXICARPIN FROM VITEX ROTUNDIFOLIA ON LIPOPOLYSACCHARIDES AND CARRAGEENAN INDUCED ACUTE INFLAMMATION

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Abstract: *Vitex rotundifolia* or locally known as "Legundi" is a coastal plant that has been traditionally used to treat a variety of ailments. This study investigated the anti-inflammatory properties of vitexicarpin, an isolated flavonoid compound, on macrophage activation *in vitro* and *in vivo*. In addition, the role of peroxisome proliferator activator receptor alpha (PPAR-α) in mediating the anti-inflammatory activity of vitexicarpin was evaluated. Results showed that vitexicarpin prevented lipopolysaccharide-induced RAW264.7 from activating into macrophages in a dose-dependent manner (0.625 – 2.5 µg/mL) which was manifested a reduction in cell size, morphology, and the production of nitric oxide (NO). Meanwhile, vitexicarpin's anti-inflammatory activity was tested *in vivo* on carrageenan-induced paw oedema in Sprague-Dawley rats (n=48) for 24 hours. Results revealed that, pre-injecting vitexicarpin (125µg/ 50µl) 30 min before injecting carrageenan was able to prevent the development of paw oedema at a specific time (2-6 hours post injection). Further investigation using peripheral blood analysis and histopathology showed that vitexicarpin was associated with a reduction of inflammatory cells that infiltrated the paw and directly inhibited the release of NO in the paw tissues. Moreover, the anti-inflammatory properties of vitexicarpin was not blocked by PPAR-α antagonist GW6471. In conclusion, vitexicarpin has the potential to be a cutting-edge analgesic and anti-inflammatory agent in pharmaceutical products. The exact anti-inflammatory mechanism and the vitexcarpin pathway is still to be explored.

Keywords: Vitexicarpin, *Vitex rotundifolia*, macrophages, carrageenan, LPS, inflammatory, pain.

Introduction

Inflammation is a biological immune response that can be triggered by a variety of factors such as injury, infection by pathogens, damaged cells, and toxic compounds, which can lead to acute and/or chronic inflammation (Villanueva *et al.,* 2017 & Huang *et al.,* 2018). Acute inflammation is regarded as a component of innate immunity and acts as the first line of defence to eliminate foreign invaders and it must be resolved for tissue repair to restore homeostasis (Sansbury *et al.*, 2016; Ahmed, 2018). In acute inflammation, the first immune cells to arrive at the injured tissue are circulating immune cells such as neutrophils, which kills pathogens and remove dead tissue. Certain inflammatory conditions extend the life span of these immune cells and the release of pro-inflammatory mediators that can further promote the recruitment of other inflammatory cells such as monocytes/ macrophages, which might favour the chronicity of inflammatory diseases (Caielli, 2012). Activated macrophages in particular release a high number of pro inflammatory mediators such as cytokines (TNF-α, IL-1β & IL-6), chemokines, degradation enzyme (collagenase, elastase, and matrix metalloproteinase) and microbiocidal molecules (nitric oxide) (Gensel & Zhang, 2015). However, uncontrolled inflammation can lead to multiple inflammatory events and chronic

diseases. Non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac, mefenamic acids, and ibuprofen are currently used to treat chronic inflammatory diseases and auto-immune diseases (Klein & Eliakim, 2010 & Orchard, 2012). Unfortunately, the long-term consumption of these medications can cause gastrointestinal bleeding, cardiovascular side effects and nephrotoxicity, in severe cases. There has been an uptick in research to find alternative natural products to treat inflammatory diseases that have fewer side effects than NSAIDs. One natural product of interest is the fruit *Fructus viticis* of the coastal plant, *Vitex rotundifoli*a. Although it has a long history of use in traditional medicine in several Asian countries, it has not been tested clinically and its efficiency has yet to be proven. Linnaeus (1753) was the first to describe the genus Vitex with the discovery of four species which are *V. agnus-castus*, *V. trifolia*, *V. negundo* and *V. pinnata*. Additionally, *V. rotundifolia* is another species that has been discovered and is said to possess largely similar characteristics to *V. trifolia* (Munir, 1987). The phytoconstituents of *V. rotundifolia* are flavones (vitexicarpin, luteolin and artemetin), labdane diterpenes (vitexilactone and previtexilactone), monoterpenes, alkaloids, sesquiterpenoids and glucosides. Vitexicarpin, or also known as casticin, is one of flavonoid derivatives (Azizul e*t al.,* 2021). Therefore, this study investigated the anti-inflammatory effect of isolated vitexicarpin on acute inflammation using known Toll Like Receptor 4 ligands such as lipopolysaccahrides (LPS) and λ-carrageenan *in vitro* and *in vivo* respectively. In addition, the role of peroxisome proliferator activator receptor alpha (PPAR-α) in mediating the anti-inflammatory effect of vitexicapin on immune cell activation and the production of pro-inflammatory mediators such as nitric oxide was also investigated using a potent PPAR-α antagonist

Materials and Methods

Plant materials

V. rotundifolia fruits were collected at Penarik Beach in Setiu, Terengganu. The fruits were taken to the Institute of Marine Biotechnology at Universiti Malaysia Terengganu and dried at 30^{o}C. The dried fruits were fed into a mechanical grinder at the laboratory to obtain 1.5 kg of fine powder. The species of the plant was identified by the Universiti Sains Malaysia Herbarium at Pusat Pengajian Sains KajiHayatin Penang and tagged with the Herbarium Voucher No:11865.

Extraction and fractionation

All 1.5 kg of the powdered *V. rotundifolia* fruits were macerated in acetone and methanol, consecutively. Each of acetone and methanol extract (8L/ extraction, 3x) was filtered using Whatman filter paper. The extract was further concentrated in a vacuum with a rotary evaporator (Büchi, Switzerland) to give 60.2542 g. The crude extracts were kept at 4ºC prior to analysis. 10g of the crude extract of *V. rotundifolia* was fractionated by vacuum liquid chromatography (VLC) containing silica gel 60 GF_{254} Merck (230-450 mesh) (Merck, art. 7747) with gradient elution using hexane-ethyl acetate-methanol that yielded 24 fractions. Each sub-fraction was further concentrated using a rotary evaporator. Meanwhile, a thin layer chromatography (TLC) was performed on all fractions 1-24 to monitor the UV active compounds, while the non-UV active compounds were monitored using *p*-anisaldehyde-sulphuric acid spray reagent (Sigma Aldrich, UK) and heated with a heating gun HL 2010 E (Steinel, Germany) at 170 ºC. The retention factor value, $R_f$was used to quantify the metabolite separation. Active fractions were further isolated using several chromatographic techniques to isolate the target vitexicarpin compound.

Cell Culture

The murine macrophage RAW 264.7 cell line was purchased from American Type Culture Collection (ATCC, Manassa, VA, USA). The cells were cultured in a complete media consisting of DMEM high glucose cell culture medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% (v/v) FBS (Tico Europe, Amstelveen, Netherlands), and 1%

(v/v) penicillin-streptomycin (Sigma-Aldrich, USA). The RAW 264.7 cells were incubated at 37 ºC with 5% CO_2.

In Vitro Anti-inflammatory Activity on LPS-induced Macrophages

The effect of vitexicarpin on the nitric oxide (NO) production from lipopolysaccharides (LPS) induced RAW 264.7 cells was evaluated. Approximately, 2 x 10^5 cells/well of RAW 264.7 cells were placed on a culture plate for 2 hours. After that, the isolated vitexicarpin (0.156 to 50 µg/mL) was added into the cells. To study the effect of PPAR-α in mediating the anti-inflammatory effect of vitexicarpin, a potent PPAR-α antagonist GW6471 was mixed in with the vitexicarpin prior to LPS. Then, 1 g/mL of LPS was added to each well and incubated for half an hour. For positive control, 100 µM of NG-Methyl-L-arginine acetate salt (L-NMMA) (Sigma Aldrich, USA) was used in this study. After 24 hours, the culture media was collected by centrifugation at 1000 rpm for one minute. The media was then transferred into a new 96-well plate and 100 µL of Griess reagent (Sigma Aldrich, USA) and was added into each well. The plate was further incubated in the dark at room temperature for 20 minutes. The absorbance was recorded at 540 nm using Varioskan Flash Spectral Scanning Multimode Reader (Thermo Scientific, USA). The changes on the macrophages size and morphology upon activation and treatments were observed under an inverted microscope (Olympus IX51, Tokyo, Japan) and post analysis was done using ImageJ software.

Effect of Vitexicarpin on Carrageenan-induced Acute Paw Oedema

In the *in vivo* study, 8-week-old male Sprague-Dawley (SD) rats weighing between 220 and 250 g were used. The animals were purchased from the Animal Research and Service Centre (ARASC) at Universiti Sains Malaysia, Health Campus in Kelantan. Animal ethics approval was obtained from Animal Ethics Committee USM (USM/IACUC/2019/(117)(983) prior to experimentation. A total of 48 rats were divided into six different group as showed in Table 1. Prior to inflammation and treatments, normal paw thickness and pain threshold were measured. Approximately, 20 g/kg of anaesthetic agent, sodium pentobarbital was given intraperitoneally. Once, the animal was fully anesthetised, 50 µL of treatment/intervention was administered subcutaneously into the intra plantar of right hind paw (ipsilateral). All treatments were given 30 minutes prior to the induction of the carrageenan injection. Paw oedema (swelling) and pain behaviour was measured using a digital vernier calliper and Randall Selitto machine respectively. All measurements were recorded at 30 minutes, 1, 2, 4, 6, 8, 12, 24 hours post-carrageenan/saline injection. In addition, to study the effects of the treatment on circulating leucocytes, blood sample were taken directly from the heart with 3 mL syringe and 25 gauge; 25 mm needle (Terumo, Belgium). The collected blood samples were sent to the BP Clinical Lab in Kota Baru in Kelantan, Malaysia for full blood count (FBC) analysis.

Table 1: Group of treatments

Group	Treatment	No of animal (n)
A	DMSO (50 µL) + Saline (100 µL)	8
B	DMSO (50 µL) + Carrageenan (100 µL)	8
C	Vitexicarpin (50 µL) + Saline (100 µL)	8
D	Vitexicarpin (50 µL) + Carrageenan (100 µL)	8
E	Vitexicarpin + GW6471 (50 µL) + Carrageenan (100 µL)	8
F	L-NMMA (50 µL) + Carrageenan (100 µL)	8

Paw Skin Tissue Collection

The rat's paw skin tissue was collected 24 hours after the carrageenan injection. Both ventral ipsilateral and contralateral paws were collected for nitric oxide determination and histopathological analysis. For nitric oxide determination, the paw skin tissue was placed in cryovials and stored at -80ºC prior to use. For histopathological analysis, the paw skin tissue was stored in specimen containers with 10% buffered formalin (R&M Chemicals, Semenyih, Selangor).

Statistical Analysis

All data were presented as mean values ± standard error mean (S.E.M) and analysed by using Graph Prism version 8.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). All tests were two-tailed, and the significant value was set at $p < 0.05$.

Results and Discussion

Chemical Profiling

The TLC plate of fractions 6 and 7 showed the UV active compound with the R_f value is the same as that of commercially available vitexicarpin [Figure (1A)]. The fractions 6 and 7 turned yellow after being sprayed with *p*-Anisaldehyde-sulphuric acid reagent and heated, that indicated the presence of flavonoid compounds [Figure (1B)] (Mesaik *et al.,* 2009 & Kim *et al.,* 2012). Meanwhile, the fractions 6 and 7 also showed the non-UV active metabolites. Further isolation work on fractions 6 and 7 was carried out to obtain pure metabolite vitexicarpin.

Anti-inflammatory Activity of Vitexicarpin and GW6471 on LPS-induced Macrophages

The anti-inflammatory effect of the isolated vitexicarpin is shown in Figure 2. Results revealed that vitexicarpin significantly reduced NO production in LPS-stimulated RAW 264.7 cells in a concentration-dependent manner starting at a concentration of 0.625 µg/ml to 2.5 µg/ml [Figure 2 (A)]. As expected, the positive control used, L-NMMA, a potent NO inhibitor significantly reduced the LPS-induced NO production ($p < 0.05$). Moreover, the 0.5% DMSO used as solvent did not trigger any inflammation in the RAW 264.7 cells as the NO concentration was low and was comparable to untreated cells. These findings agree with previous studies, which showed that vitexicarpin isolated from the Fructus viticis also exhibited anti-inflammatory properties reducing NO release in LPS-induced macrophage RAW 264.7 cells with IC_{50} of 4.23 µM (approximately 1.58 µg/mL) (Liou, 2018). The anti-inflammatory effect of vitexicarpin might be related to its chemical structure. According to (Lee *et al.,*

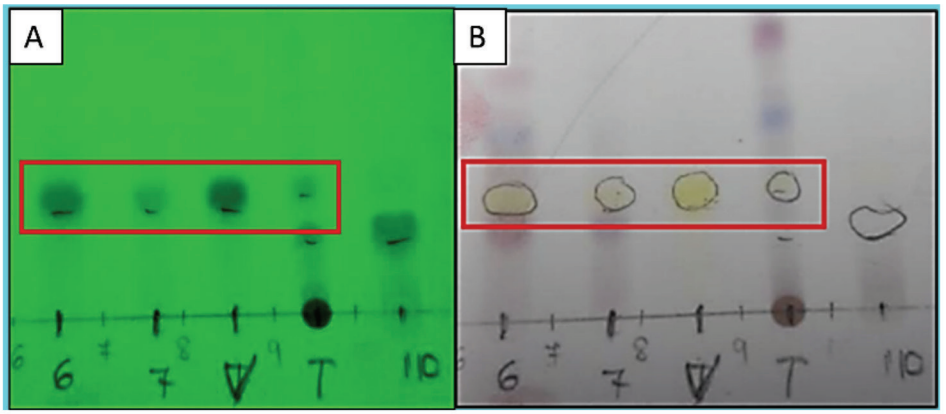

Figure 1: (A) The TLC plate is monitored for UV active compounds; and (B) TLC plate after sprayed with p-Anisaldehyde-sulphuric acid reagent for non-UV active compounds

2013), among the compounds which were isolated from Fructus viticis that played an important role in the inhibiting NO production are flavonoids and labdane-type diterpenoids possessing butenolide ring, neolignane and five- and six-membered cyclic acetal group. Apart from that, in this study, a mix of isolated vitexicarpin and potent PPAR- α antagonist, GW6471 was also tested on LPS-activated RAW 264.7 cells. Results revealed that the GW6471 did not reverse all the anti-inflammatory effects of isolated vitexicarpin [Figure 2 (B)]. These results suggest that PPAR-α activation is not a mechanism by which vitexicarpin exerts its anti-inflammatory effects.

Effect Vitexicarpin on Macrophages Activation

Interestingly, the effect of vitexicarpin on cell activation, under microscopic observation has revealed that there was a significant difference between the cells size and observed morphological changes between LPS-induced macrophages that were treated. The average cell size of untreated RAW 264.7 was between 7 and 8 µm with normal morphological appearance. having a circular shape with little or no extension, of pseudopodia, as shown in Figure 3 (A) & 3 (B). However, the cells became three times bigger when LPS was introduced, induced and observed over 24 hours with average cell sizes of 23.3 ± 0.4 µm that reached a maximum size of 50 µm [Figure 3 (B)] on average. The results revealed that vitexicarpin significantly reduced macrophage activation, which was evidenced by a significant reduction in cell sizes to an almost normal size (LPS: 23.3 ± 0.4 µm vs vitexicarpin: 14.6 ± 0.3 µm) as shown in Figure 3 (B). The results also suggest the ability of vitexicarpin to inhibit NO production is strongly associated with its ability to reduce or delay the activation of monocytes, which turns into inflammatory macrophages. In a different study, *Citrus aurantium* L. (CME) methanolic crude extract suppressed the expression of inducible enzymes and proinflammatory cytokines including iNOS and decreased the level of cell spread and the formation of pseudopodia in macrophages (Kang *et al*., 2011). The results of this study

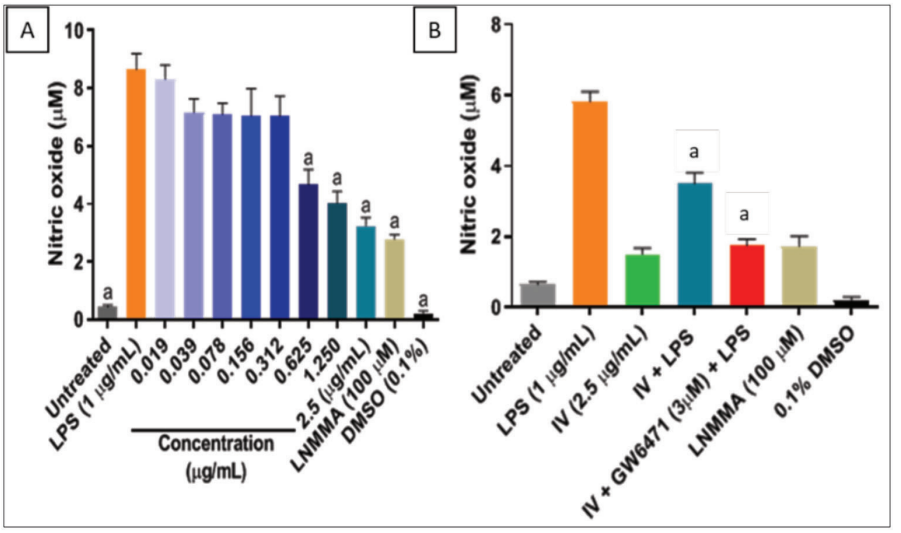

Figure 2: The effect of different concentration of vitexicarpin (A) and the role of PPAR-α antagonist GW6471 in mediating anti-inflammatory effects of vitexcarpin (B) on the NO production in LPS-stimulated RAW 264.7 cells. Griess assay was used to evaluate the anti-inflammatory properties. Data are presented as a mean value of NO concentrations ± SEM and a comparison was done between the treated and untreated groups using a one-way ANOVA test, which was followed by a Tukey multiple comparison test (n=6). $^{a}p <$ 0.05 was considered significantly different between LPS (1 µg/mL) and the treated group that was tested

demonstrated that vitexicarpin is not a natural ligand of PPAR-α as its anti-inflammatory activity on activated RAW 264.7 cells was not blocked by the potent PPAR-α blocker, GW6471. However, this needs to be verified and investigated further using receptor-ligand binding assays or using various concentrations of both substances.

In vivo: Effect of Vitexicarpin on Carrageenan Induced Paw Oedema

The basal measurement of paw thickness in all of the rats pre-injection was between 5.1 and 5.3 ± 0.1 cm (n=8). However, as expected 30 minutes post-injection all of the animals in all of the groups showed increasing paw thickness which was due to the trauma as a result of the introduction of a needle into the paw. Figure 4 (A) shows animal group treated with vehicle + saline did not show any further development of paw oedema starting from 2 hours to 24 hours with a constant paw thickness of 6.0 ± 0.1 mm. AN almost similar pattern was observed in the group treated with isolated vitexicarpin + saline starting from 1 hour (6.1 ± 0.1 mm) up to the next 24 hours (5.7 ± 0.1 mm) indicating isolated vitexicarpin did not cause any inflammation prior to carrageenan injection. When the rats were injected with 2% carrageenan, the mean value of paw thickness increased significantly at the 30-minute mark when compared with vehicle + saline (7.8 ± 0.2 mm; $p < 0.01$) and the oedema remained the same at 8.6 ± 0.1 mm; $p > 0.0001$ for the first 24 hours. Interestingly, animals treated with vitexicarpin + carrageenan showed significant delay in the development of paw oedemas as compared with those in the group with vehicle + carrageenan at 4 hours (8.0 ± 0.1 mm vs 9.6 ± 0.2 mm; $p < 0.0001$), 6 hours (8.1 ± 0.1 mm vs 10.1 ± 0.2 mm; $p < 0.0001$), 8 hours (8.3 ± 0.1 mm vs 10.1 ± 0.2 mm; $p < 0.0001$), 12 hours (8.5 ± 0.3 mm vs 9.7 ± 0.2 mm; $p < 0.0001$), and 24 hours (7.8 ± 0.2 mm vs 8.6 ± 0.1 mm; $p < 0.01$). A co-treatment of isolated vitexicarpin and GW6471 did not reverse the anti-inflammatory effect of isolated vitexicarpin. There was no significant difference in paw thickness at all-time points between treatment with isolated vitexicarpin + GW6471 + carrageenan and treatment isolated vitexicarpin + carrageenan. This result confirmed that the anti-inflammatory effect of casticin is not mediated in the activation of a PPAR-α pathway. Animals treated with potent NO inhibitor; L-NMMA showed significant reduction of paw oedema development starting

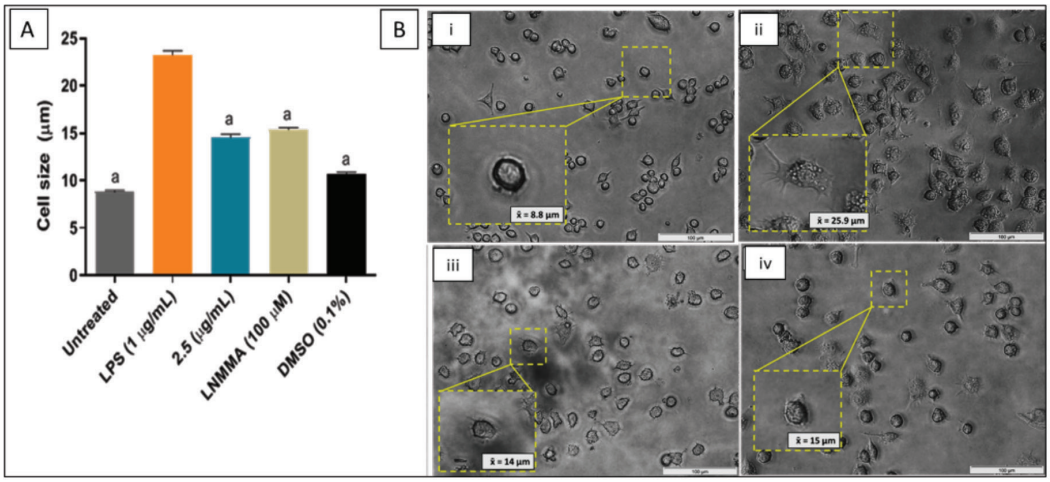

Figure 3: The effect of vitexicarpin at 2.5 µg/ml on cell size of LPS-induced RAW264.7 activation into macrophages (A). Photomicrograph at 20X magnification of the effect of vitexicarpin on the activation of macrophages (B; i) untreated cells, ii) 1 µg/ml of LPS, iii) 2.5 2.5 µg/ml + LPS and iv) L-NMMA + LPS). [a]$p < 0.05$ was considered significantly different between LPS (1 µg/mL) and treatment group tested

from 30 minutes up to 24 hours when compared with those in group vehicle + carrageenan with a final paw thickness of 7.4 ± 0.2 mm as opposed to 8.6 ± 0.1 mm at the 24 hour mark. Figure 4 (B) showed the representative of the gross observation of paw oedema and treatments intervention at 24 hours injection. A few studies reported the effect of vitexicarpin on paw oedema development using a different inflammogen model. Vitexicarpin from Fructus viticis significantly reduced egg-albumin induced paw oedema in rats that were given between 25 and 50 mg/kg of vitexicarpin orally (Lin *et al.,* 2007). 50 mg/kg showed significant inhibition of the paw oedema development at the 3 hour, 4 hour and 6 hour mark, while 25 mg/kg grow showed a reduction in inflammations at the 3 and 4 hour mark post-treatment.

Histology Analysis of the Effect of Isolated Vitexicarpin

Figure 5 (A) shows the histological analysis of all treatment groups on the paw tissue collected 24 hours post-injection. The group that was treated with a vehicle + saline had a normal appearance of paw tissue with no infiltration of inflammatory cells, no redness, and no destruction of paw tissue. The paws of the group treated with isolated vitexicarpin + saline showed an almost similar pattern to those in the vehicle + saline group where there was a low infiltration of inflammatory cells. The group treated with vehicle + carrageenan, that received a 2% carrageenan injection showed massive infiltration of inflammatory cells and were dense at the dermis as shown in Figure 5 (A). However, paw tissues treated with isolated vitexicarpin + carrageenan along with a mix of isolated vitexicarpin + GW6471 showed less infiltration of inflammatory cells into the paw dermis. The inflammatory cells also became more scattered around the tissue and less dense compared with those that were treated with a vehicle + carrageenan. This indicates co-mixture treatment with GW6471 did not block the inflammatory effect of isolated vitexicarpin. Furthermore, tissues treated with L-NMMA + carrageenan showed more scattered inflammatory cell infiltration when compared with tissue treated with isolated vitexicarpin. Quantification of the results further confirmed

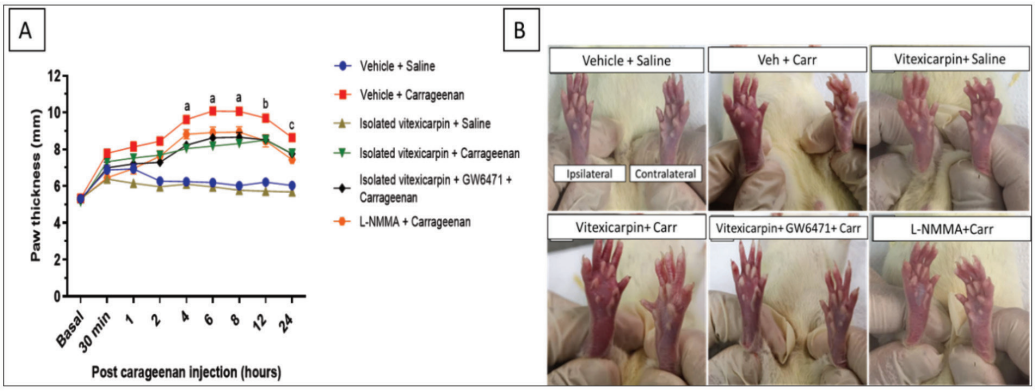

Figure 4: Comparison of paw thickness (mm) measured with a digital vernier calliper at different time intervals between different treatment groups (A). Paws injected with 2% carrageenan caused intense paw swelling development at all-time interval when compared with paws injected with saline only. Isolated vitexicarpin displayed clear anti-inflammatory effects significantly slowing down the development of carrageenan-induced paw oedemas from four to 12 hours. Gross observation of the effect of treatment on oedema development in hind paw (B). Data are expressed as mean values of paw thickness ± SEM and were compared between isolated vitexicarpin + carrageenan and vehicle + carrageenan using a Two-Way ANOVA test that was followed by a Bonferonni's multiple comparison tests with (n=8). $^{a}p < 0.0001$, $^{b}p < 0.001$ and $^{c}p < 0.05$

the anti-inflammatory effects of vitexicarpin that inhibited inflammatory cells in the hind paw as shown in Figure 5 (B). The anti-inflammatory effects of vitexicarpin were also reflected in the reduction of inflammatory mediators such as TNF-α, NO, IL-1β and IL-6 (Lin *et al.,* 2007) and immune cells like neutrophils, macrophages, and lymphocytes (Liou *et al.,* 2014). Based on the histology analysis and quantification of immune cells infiltration in the paw skin tissue in this study, isolated vitexicarpin has significantly reduced the percentage of area covered by inflammatory cells, which supports the findings in other studies where vitexicarpin was seen to reduce inflammation by inhibiting proinflammatory mediators and immune cells (Lee *et al.,* 2012).

Effect of Vitexicarpin on NO & Immune Cells in Hind Paws

In this study as expected, carrageenan injection has significantly ($p < 0.001$) elevated the NO by almost 2.5-fold with average of NO concentrations of 14.8 ± 1.3 µM in comparison with the saline treated group [Figure 6 (A)]. Meanwhile, animals pre-treated with vitexicarpin prior to carrageenan had significantly inhibited NO production levels (9.3 ± 0.4 µM; $p < 0.001$) when compared with vehicle. This study would be the first to report that vitexicarpin also managed to lower NO levels in paw tissue, indicating the very potent anti-inflammatory property of the compound. As demonstrated earlier in the *in vitro* study, GW6471 did not block the anti-inflammatory activity of isolated vitexicarpin as the NO measured in the co-mixture of vitexicarpin and PPAR-α antagonist GW6471 had significantly lower NO concentrations at 6.7 ± 1.8 µM; $p < 0.001$ compared with this treated with a vehicle + carrageenan [Figure 6 (A)], which leads to a solid conclusion that the anti-inflammatory activity of vitexicarpin is not modulated by PPAR-α. Meanwhile the Full Blood Count (FBC) analysis, results demonstrated that generally speaking, there were no significant effects in all treatments on the white blood cell (WBC) count as shown in Figure 6 (B). However, it was observed that the WBC in the vehicle + saline and isolated vitexicarpin + saline groups were slightly lower than that of the vehicle + carrageenan at 5.6 ± 0.6 x 10^9/L, although the drop was not significant. In addition, the rats treated with carrageenan showed an increase in PMNs count at 42.5 ± 12.0% compared with

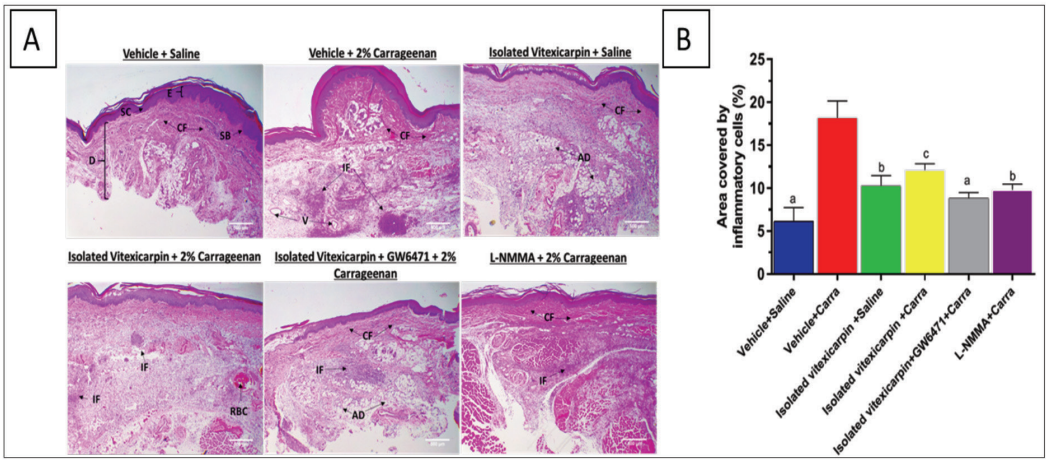

Figure 5: Photomicrograph of rat paw tissue cross section from all treatment group at 24 hours stained with standard haematoxylin and eosin staining (A). Quantification of inflammatory cells in the hind paw (B). Note: D; dermis, E; epidermis, SC; stratum corneum, SB; stratum basale, CF; connective fibres, SG; sweat glands, AD; Adipose, V; venules, IF; inflammatory cells & RBC; red blood cells). Photos taken at 4X magnification

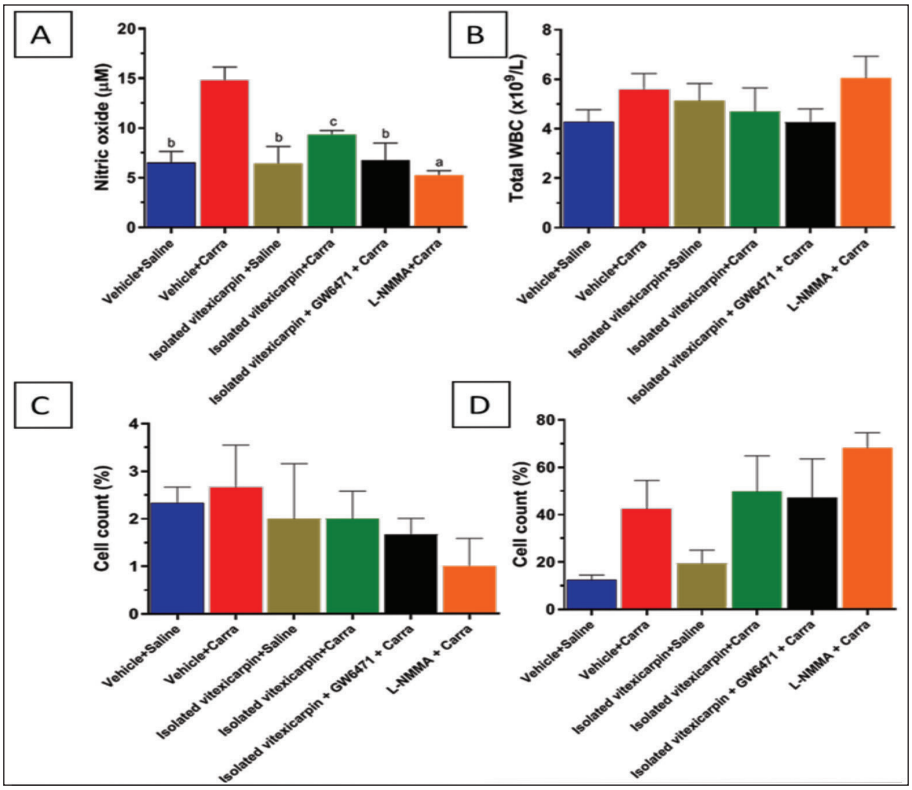

Figure 6: Effect of different treatment groups on NO concentration in paw tissue harvested after 24 hours post-injection (A). The effect of isolated vitexicarpin on circulating immune cells in hind paw: total white blood cells (C), polymorphonuclear neutrophils (PMNs) (C) and monocytes/macrophages (D). Data are expressed as mean ± SEM and were compared between all treatment group with Vehicle + carrageenan using One-Way ANOVA test followed by Dunnett's multiple comparison tests with (n=4). $^{a}p < 0.0001$, $^{b}p < 0.001$ and $^{c}p < 0.01$, were considered significantly different in all treatment groups when compared with the vehicle + carrageenan group

saline group at 12.3 ± 2.2% [Figure 6 (C)]. Like PMNs, the FBC results on the monocyte count revealed that there is insignificant difference between all treatment groups as shown in Figure 6 (D). However, it can be observed that isolated vitexicarpin + carrageenan group had slightly lower monocyte percentages at 2.0 ± 0.6% as compared with the vehicle + carrageenan group at 2.7 ± 0.9% as shown in Figure 6 (D). The ability of vitexicarpin to reduce NO levels might be related to its ability to inhibit the influx of inflammatory cells into the paw such as monocytes or activated macrophages. Macrophages are known to induce an inflammatory response as they produce potent pro inflammatory mediators such as TNF-α, ROS, IL-1β, and IL-6 (Ma *et al.,* 2018).

Conclusion

In conclusion, isolated vitexicarpin from fruits of coastal medicinal plant *Vitex rotundifolia* showed a potent anti-inflammatory effect associated with its ability to prevent the activation of immune cells which subsequently reduced the production of pro inflammatory mediators such as nitric oxide *in vitro* and *in vivo*. However, the anti-inflammatory effect of vitexicarpin is not likely to be mediated by a PPAR-α and further investigation using different

isotypes of PPARs and assays is required to test and ascertain if this is true. Overall, vitexicarpin shows promise as an anti-inflammatory agent to be developed and used in future pharmaceutical products.

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References

Ahmed, A. U. (2018). An overview of inflammation: Mechanism and consequences. *Frontier in Biology*, *6*(4), 274-281. https://doi.org/10.1007/s11515-011-1123-9

Azizul, N. H., Ahmad, W. A. N. W., Rosli, N. L., Muhammad, A. H. M. A., Choo, E. L., Noor, W. M, & Suvik, A. (2021). The coastal medicinal plant *Vitex rotundifolia*: A mini review on its bioactive compounds and pharmacological activity. *Traditional Medicine Research. 6*(2), 1-10. http://dx.doi.org/10.12032/TMR20201128209

Caielli, S., Banchereau, J., & Pascual, V. (2012). Neutrophils come of age in chronic inflammation. *Current Opinion in Immunology*, *24*(6), 671-677.

Gensel, J. C. & Zhang, B. (2015). Macrophage activation and its role in repair and pathology after spinal cord injury. *Brain Research*, *1619*, 1-11.

Huang, C., Li, W., Zhang, Q., Chen, L., Chen, W., Zhang, H., & Ni, Y. (2018). Anti-inflammatory activities of Guang-Pheretima extract in lipopolysaccharide-stimulated RAW 264.7 murine macrophages. *BMC Complementary and Alternative Medicine***,** *18*(1), 1-11.

Kang, S. R., Han, D. Y., Park, K. I., Park, H. S., Cho, Y. B., Lee, H. J., Lee, W. S., Ryu, C. H., Ha, Y. L., Lee, D. H., Kim, J. A., & Kim, G. S. (2011). Suppressive Effect on Lipopolysaccharide-Induced Proinflammatory Mediators by Citrus aurantium L. in Macrophage RAW 264.7 Cells via NF-κB Signal Pathway. *Evidence-based complementary and alternative medicine: eCAM, 2011*, 248592. https://doi.org/10.1155/2011/248592

Kim, H., Yi, J. M., Kim, N. S., Lee, Y. J., Kim, J., Oh, D. S., Oh, S.M., Bang, O.S., & Lee, J. (2012). Cytotoxic compounds from the fruits of *Vitex rotundifolia* against human cancer cell lines. *Journal of the Korean Society for Applied Biological Chemistry*, *55*(3), 433-437.

Klein, A. & Eliakim, R. (2010). Non steroidal anti-inflammatory drugs and inflammatory bowel disease. *Pharmaceuticals (Basel, Switzerland), 3*(4), 1084-1092.

Lee, C., Lee, J. W., Jin, Q., Lee, H. J., Lee, S. J., Lee, D., Lee, M.K., Lee, C.K.L., Hong, J.T., Lee, M.K., & Hwang, B. Y. (2013). Anti-inflammatory constituents from the fruits of *Vitex rotundifolia*. *Bioorganic & Medicinal Chemistry Letters*, *23*(21), 6010-6014.

Lee, S. M., Lee, Y. J., Kim, Y. C., Kim, J. S., Kang, D.G., & Lee, H. S. (2012). Vascular protective role of vitexicarpin isolated from *Vitex rotundifolia* in human umbilical vein endothelial cells. *Inflammation*, *35*(2), 584-593.

Lin, S., Zhang, H., Han, T., Wu, J. Z., Rahman, K., & Qin, L. P. (2007). *In vivo* effect of casticin on acute inflammation. *Zhong xi yi jie he xue bao (Journal of Chinese integrative medicine*), *5*(5), 573-576.

Liou, C. J., Cheng, C. Y., Yeh, K. W., Wu, Y. H., & Huang, W. C. (2018). Protective effects of casticin from Vitex trifolia alleviate eosinophilic airway inflammation and oxidative stress in a murine asthma model. *Frontiers in pharmacology, 9*, 635.

Liou, C., Len, W., Wu, S., Lin, C., Wu, X., & Huang, W. (2014). Casticin inhibits COX-2 and iNOS expression via suppression of NF- κ B and MAPK signalling in lipopolysaccharide stimulated mouse macrophages. *Journal of Ethnopharmacology*, *158*, 310-316.

Ma, J. Yin, G. Lu, Z. Xie, P. Zhou, H. Liu, J., & Yu, L. (2018). Casticin prevents DSS induced ulcerative colitis in mice through inhibitions of NF-κB pathway and ROS signaling. *Phytotherapy Research, 32*(9) 1770-1783.

Mesaik, M. A., Murad, S., Khan, K. M., Tareen, R. B., Ahmed, A., & Choudhary, M. I. (2009). Isolation and immunomodulatory properties of a flavonoid, casticin from *Vitex agnus-castus*. *Phytotherapy Research, 23*(11), 1516-1520.

Munir, A. A. (1987). A taxonomic revision of the genus *Vitex* L. (Verbenaceae) in Australia. *Journal of the Adelaide Botanic Garden*, *10*(1), 31-79. http://www.jstor.org/stable/23890926

Orchard, T. R. (2012). Management of arthritis in patients with inflammatory bowel disease. *Gastroenterology & Hepatology, 8*(5), 327-329.

Sansbury, B. E. & Spite, M. (2016). Resolution of acute inflammation and the role of resolvins in immunity, thrombosis, and vascular biology. *Circulation Research, 119*(1), 113-130.

Villanueva, J. R., Esteban, J. M., & Villanueva, L. R. (2017). Solving the puzzle: What is behind our forefathers' anti-inflammatory remedies? *Journal of Intercultural Ethnopharmacology, 6*(1), 128-143. https://doi.org/10.5455/jice.20161204021732