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Evaluation of *Trametes Lactinea* Extracts on the Inhibition of Hyaluronidase, Lipoxygenase and Xanthine Oxidase Activities *in Vitro*

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Abstract: *Trametes lactinea (T. lactinea), a macrofungus belonging to the family of Polyporaceae was investigated for its ability to inhibit hyaluronidase, lipoxygenase and xanthine oxidase activities in vitro. Results showed that extracts from the supernatant and mycelia of T. lactinea significantly inhibited the activities of hyaluronidase and lipoxygenase with more than 80% inhibition. The presence of total phenolics and flavonoids were also determined in the fungus extracts. The highest total phenolics and flavonoids were found in the supernatant of T. lactinea culture. Linear regression analysis carried out has found no correlation between the bioactive compounds (phenolics and flavonoids) and the inhibition of the enzyme activities, $p > 0.05$. The gas chromatography mass spectrometry (GCMS) analysis carried out has identified the presence of other bioactive compounds in the extracts that may act as the enzyme inhibitors.*

Keywords: submerged fermentation, *Trametes lactinea*, fungus extraction, anti inflammatory, GCMS

1. INTRODUCTION

Lipoxygenases (LOX) enzyme was reported to convert the arachidonic, linoleic and other polyunsaturated fatty acid into biologically active metabolites involved in the inflammatory and immune responses.[1] In their study, Pidgeon et al.[2] noted that 5-LOX and platelet type 12-LOX are generally considered as pro-carcinogenic, 15-LOX-2 suppresses carcinogenesis while 15-LOX-1 remains controversial. According to Pontiki and Hadjipavlou-Litina,[3] the enzymes are correlated with inflammatory and allergic reactions because of the formation of the leukotrienes (LTs). High levels of LTs could be observed in the case of asthma, psoriasis, allergic rhinitis, rheumatoid arthritis and colitis ulcerosa.[4] The production of LTs can be prevented via inhibition of the lipoxygenase pathway.[5] Pidgeon et al.[2] also suggested that lipoxygenase inhibitors may lead to the design of biologically and pharmacologically targeted therapeutic strategies inhibiting

LOX isoforms and/or their biologically active metabolites which may be useful in cancer treatment.[2]

The overall amount of hyaluronic acid in human is approximately 15 g (for a 70 kg individual), with the largest portion (ca. 50%) being found in the dermis and epidermis of the skin.[6,7] It is also the most important component in blood vessel walls. It can be degraded by hyaluronidase enzyme which causes high capillary permeability thus leading to edema.[8] Arct and Pytkowska[8] suggested that inhibition of hyaluronidase activity can decrease capillary permeability in blood vessel. Further, Maeda et al.[9] claimed that inhibition of hyaluronidase activity can also be used to evaluate the anti allergic reaction.

Another common disease is gout, affecting 1–2% of adults and this number keeps increasing over the past two decades.[10] This disease occurs when xanthine oxidase catalyses the metabolism of hypoxanthine and xanthine into uric acid leading to gouty arthritis and uric acid nephrolithiasis.[11] The excess productions of uric acid lead to the deposition of urate crystals especially in the joints between two bones causing swelling, heat and pain.[12] Kong et al.[13] suggested that to block the production of uric acid, the xanthine oxidase inhibitors should be used in the gout treatment. A well-known xanthine oxidase inhibitor is allopurinol. However, its use on patients can cause severe adverse effects.

The fungal kingdom with many species has unique and unusual biochemical pathways for secondary metabolites production.[14] According to Zjawiony,[15] numerous cytotoxic triterpenoids have been isolated from various species of the *Polyporaceae* fungi. Bioactive metabolites including phenolic compounds, flavonoids, polysaccharides, proteoglycan, triterpenoids, terpenes, lectin, β-glucan, schizophyllan, alkaloid, steroids, etc., from fungi can be isolated from fruiting bodies, pure culture mycelia and culture broth.[16,17] According to Dimitrijević et al.,[18] the presence of total phenolics and flavonoids content in these fungi extract may be contributing in antibacterial, antifungal, antiviral, antioxidative, antidiabetic, anticarcinogenic, antiallergic, antimutagenic and anti inflammatory activities.[19,20] However, fruiting bodies cultivation is a long-term process and may take about several months depending on species and substrates used.[21] In contrast, cultivation in submerged (liquid) culture allows rapid growth and attaining maximum yield is possible with constant composition.[21]

Trametes lactinea (*T. lactinea*), a species belonging to the family of *Polyporaceae*, was evaluated to inhibit the activities of hyaluronidase, lipoxygenase and xanthine oxidase *in vitro*. To the authors' knowledge, no work has been reported on its biological activities. The total phenolics and flavonoids content in the extracts were determined. Further, the identification of bioactive

compounds presents in the extracts was analysed using gas chromatography mass spectrometry (GCMS).

2. EXPERIMENTAL

2.1 Chemicals

Soybean lipoxygenase, linoleic acid, hyaluronidase, hyaluronic acid, xanthine oxidase, xanthine, allopurinol, apigenin, potassium phosphate, sodium phosphate, Dimethyl sulfoxide (DMSO), Folin Ciocalteau reagent, aluminum chloride, gallic acid and quercetin were purchased from Sigma-Aldrich and Merck (Malaysia). All other chemicals and reagents used were of the highest commercially available purity.

2.2 Microorganism

The main stock culture of the macrofungus *T. lactinea* was obtained from the Forest Research Institute of Malaysia (FRIM), Kepong, Selangor, Malaysia. Mycelia from stock culture was cultured in malt extract agar (MEA) plate at 30°C for 7 days, and maintained on agar plate before any subsequent studies.

2.3 Preparation of Mycelia Suspension

Mycelia suspension or inoculum for shake flask studies was prepared by inoculating a stock culture of *T. lactinea* onto malt extract agar-plates and incubated at 30°C for 6 days. The mycelial mat formed was scraped off using a sterile blade and mixed with 10 mL of sterile Tween 20 (Sigma) solution prior to putting it into a sterile bottle (100 mL). The sampling bottle was vortexed for 3 min so that the mycelia were evenly distributed in the liquid.

2.4 Growth of Selected Fungi in Shake Flask

Shake flask experiments were carried out in a 250 mL Erlenmeyer flask with a working volume of 100 mL. 5 mL of mycelia suspension was inoculated into the flask containing 95 mL of the production media comprised of (g L^{-1}): glucose 20, yeast extract 10 and malt extract agar 10.[22] Prior to incubation, the production media was sterilised at 121°C (150 kN m^{-2}) for 15 min. Cells were then left in contact with the media in a rotary shaker at 30°C, 150 rpm for 7 days. After 7 days, the culture broth was harvested from the flask and centrifuged at 20°C, 4000 rpm for 5 min. The extract obtained were then prepared for the hyaluronidase, lipoxygenase, and xanthine oxidase activities, total phenolic and total flavonoid content, respectively.

2.5 Batch Extraction Process

Extraction process was carried out using three different types of solvents: acetone, distilled water and ethanol:

- Maceration extraction was carried out by soaking the sample (mycelia and supernatant) in ethanol and acetone in a ratio of 1:10 (w/v) at 27°C for 24 hr.
- For ultrasonic extraction, the sample (mycelia and supernatant) was immersed in distilled water in a ratio of 1:10 (w/v) and extracted at 35°C, 20 kHz for 15 min.

Crude extract obtained from maceration and ultrasonication was dried using rotary evaporator and freeze dryer. The extract was kept at 4°C for further analysis.

2.6 *In-vitro* Enzyme Assays

2.6.1 Hyaluronidase inhibition assay

The assay was performed following the method suggested by Sigma protocol with slight modifications. Stock solutions of the tested samples and apigenin (a control) at concentration of 5 mg mL^{-1} were prepared by dissolving the extracts in DMSO. The assay medium consisted of 100 µL of hyaluronidase (4 U mL^{-1}), 100 µL of sodium phosphate buffer (200 mM, pH 7, 37°C) with 77 mM sodium chloride and 0.01% BSA were mixed with 25 µL of sample solution and incubated at 37°C for 10 min. The reaction was then initiated by the addition of 100 µL of the substrate in the form of hyaluronic acid (0.03% in 300 mM sodium phosphate, pH 5.35) solution and incubated at 37°C for 45 min. The undigested hyaluronic acid was precipitated with 1 mL acid albumin solution made up of 0.1% bovine serum albumin in 24 mM sodium acetate and 79 mM acetic acid, pH 3.75. After leaving the mixture at room temperature for 10 min, the absorbance of the reaction mixture was measured at 600 nm using a spectrophotometer (model XMA 1200V). All solutions were prepared fresh before enzyme assay was performed. The absorbance in the absence of enzyme was used as control value for maximum inhibition. Apigenin (5 mg mL^{-1}) was used as the positive control in this assay. All the reactions were performed in triplicates. The percentage of inhibition was calculated as:

$$\%Inhibition = \frac{Ab_S}{Ab_C} \times 100\% \quad (1)$$

where

Ab_C = absorbance of control
Ab_S = absorbance of the tested sample

2.6.2 Lipoxygenase inhibition assay

Lipoxygenase inhibition activity was determined using a spectophotometric method with slight modifications.[23] Stock solutions of the tested samples and quercetin (positive control) at concentration of 10 mg mL^{-1} and 100 μg mL^{-1} were prepared by dissolving the extracts and quercetin in DMSO. Sodium phosphate buffer 2.46 mL (100 mM, pH 8), 10 μL of test samples and 20 μL of soybean lipoxygenase solution (167 U mL^{-1}) were mixed and incubated at 25°C for 10 min. The reaction was then initiated by the addition of 10 μL of the substrate in the form of sodium linoleic acid solution. The enzymatic conversion of sodium linoleic acid to form (9Z, 11E)-(13S)-13-hydroperoxyoctadeca-9,11-dienoate was measured by monitoring the change of absorbance at 234 nm over a period of six min using UV-vis spectrophotometer (Model Evolution 201). Another reaction mixture (a negative control) was prepared by replacing 10 μL samples with 2.47 mL mixture of sodium phosphate buffer (5 mL) and DMSO (25 μL) into the quartz. All the reactions were performed in triplicates. The percentage of inhibition was calculated as:

$$\%Inhibition = \frac{Ab_C - Ab_S}{Ab_C} \times 100\% \quad (2)$$

where

Ab_C = absorbance of control
Ab_S = absorbance of the tested sample

2.6.3 Xanthine oxidase inhibition assay

Xanthine oxidase inhibition activity was determined according to Sigma protocol with slight modifications. Stock solutions of test samples and allopurinol (as a control) at concentration of 10 mg mL^{-1} were dissolved in DMSO. Potassium phosphate buffer 2.38 mL (0.05 M, pH 7.5), 10 μL of test solution and 10 μL of xanthine oxidase solution were mixed and incubated at 25°C for 10 min. The reaction was then initiated by the addition of 100 μL of the substrate in the form of xanthine solution. The enzymatic conversion of xanthine to form uric acid and hydrogen peroxides was measured at 295 nm using UV-vis

spectrophotometer (model Evolution 201). Another reaction mixture (control) was prepared by replacing 10 µL of the tested solution with 2.39 mL mixture of sodium phosphate buffer (5 mL) and DMSO (25 µL) in order to obtain maximum uric acid formation. The performance of the assay was verified using allopurinol as the positive control. All the reactions were performed in triplicates. The percentage of inhibition was calculated as in Equation 2.

2.7 Determination of Total Phenolics Content

The total phenolics content was determined using Folin Ciocalteau method as described by Andrew[24] with slight modification. An aliquot of the extract (20 µL) was mixed with 1.68 mL of Folin-Ciocalteu reagent (diluted with water in a ratio 1:10) and 300 µL (75 g L^{-1}) of sodium carbonate in test tubes. The tubes were vortexed for 15 s and allowed to stand at 40°C for 30 min for colour development. Absorbance was then measured at 765 nm using the spectrophotometer (model XMA 1200V). All measurements were carried out in triplicates. The total phenolics content was expressed as gallic acid equivalents (mg GAE g^{-1}).

2.8 Determination of Total Flavonoids Content

The total flavonoids content was determined using the method of Ordoñez et al.[25] with slight modification. Standard curve of quercetin was prepared by weighing 10 mg quercetin, dissolved in 85% ethanol to obtain concentration range of 6.25–100 µg mL^{-1}. Sample (100 mg) was diluted in 85% ethanol and 0.5 mL of the diluted sample was pipetted into 0.5 mL of 2% $AlCl_3$ ethanol solution (2 g in 100 mL ethanol). Ethanol was used as blank. After 30 min at room temperature, the absorbance was measured at 420 nm using a spectrophotometer (model XMA 1200V). A yellow color indicated the presence of flavonoids. All measurements were carried out in triplicates. The total flavonoids content was expressed as quercetin equivalents (µg QE g^{-1}).

2.9 GCMS Analysis

In this study, GCMS was used to analyse the sample qualitatively. Gas chromatography analyses were performed using Perkin Elmer Clarus 600 gas chromatograph equipped with an ELITE-5MS (L 30 m ID 0.25 mm DF 0.25) column. The gas chromatography was coupled to the Perkin Elmer Clarus 600 mass spectrometer. The oven temperature was programmed at 65°C for 4 min and then increased to 280°C at a rate 8°C min^{-1}. Samples were prepared and injected in triplicates.

2.10 Statistical Analysis

The experimental results are expressed as the mean ± standard deviation of three parallel measurements. The results were subjected to one way analysis of variance (ANOVA) and the significance of differences between sample means was analysed. $P < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

3.1 Inhibition of Hyaluronidase, Lipoxygenase and Xanthine Oxidase Activities by *T. Lactinea* Extracts

Figure 1 shows the hyaluronidase activity of *T. lactinea* extracts [supernatant (Sn) and mycelia (M)] using three different types of solvent. The concentration of all extract tested was at 100 μg mL^{-1}. The highest hyaluronidase inhibition was recorded in the aqueous extract of supernatant (W.Sn) with 88.6 ± 0.11% followed by acetone extract of mycelia (Ace.M) with 88.3 ± 0.14%. The positive control (apigenin) inhibited the hyaluronidase activity with 87.4 ± 0.03%. For lipoxygenase activity (Figure 2), the acetone extract of mycelia (Ace.M) showed a strong inhibition on lipoxygenase activity with 89.1 ± 0.007% inhibition followed by aqueous extract of supernatant (W.Sn) with 88.2 ± 0.015%. Quercetin (100 μg mL^{-1}) used as a positive control inhibited the activity of lipoxygenase with 89.4 ± 0.01%. According to Fujitani et al.,[26] the anti allergic compound of the microalgae has strong inhibitory effects on the activation of hyaluronidase enzyme. Meng et al.[27] in their studies on the fungus claimed that the fruiting bodies of *P. citrinopileatus* which was tested inhibited the hyaluronidase activity that can potentially be used as an ingredient in skin cosmetics. For the lipoxygenase enzyme, the role of lipoxygenase inhibitors is important in preventing carcinogen-induced DNA damage, further preventing cancer development.[28] However, all extracts tested in the present study were found to be poor inhibitors for xanthine oxidase enzyme. The inhibition for all extracts were recorded less than 10% inhibition (figure not shown).

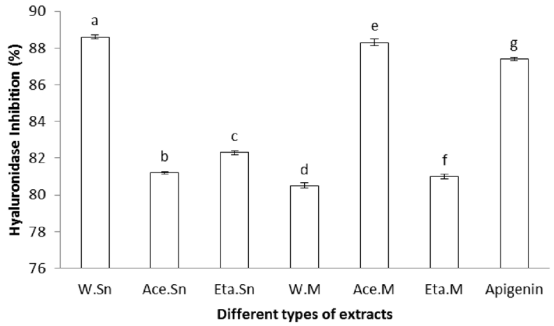

Figure 1: Hyaluronidase inhibition by the supernatant and mycelia extracts of *T. lactinea* at concentration of extract and apigenin 100 µg mL^{-1}. The column containing different letter was significantly different, $p < 0.05$).

Note: Sn = supernatant (without extraction); W.Sn = aqueous extract of supernatant; Ace.Sn = acetone extract of supernatant; Eta.Sn = ethanol extract of supernatant; W.M = aqueous extract of mycelia; Ace.M = acetone extract of mycelia; and Eta.M = ethanol extract of mycelia.

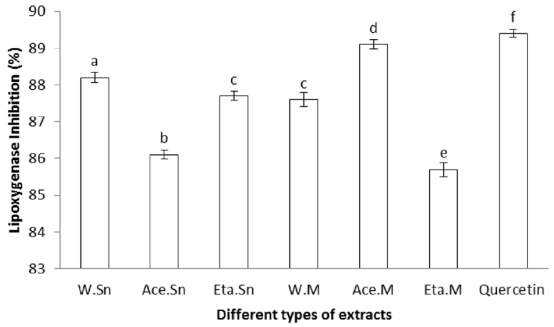

Figure 2: Lipoxygenase inhibition by the supernatant and mycelia extracts of *T. lactinea* at concentration of extract and quercetin 100 µg mL^{-1} (The column containing different letter was significantly different, i.e., $p < 0.05$).

Acetone and distilled water were found to be good solvents to extract hyaluronidase and lipoxygenase inhibitors from the tested macrofungus *T. lactinea* for both the supernatant and mycelia (Figure 1 and 2). As reported in the literature,[29] for bioactive compound extraction, the choice of solvent is very important as they must be able to dissolve the compound in addition to being non toxic, non flammable and easy to remove from the extracted sample. The recovery of anti inflammatory compound from raw materials is also influenced by the solubility of the bioactive compounds in the solvent used for the extraction process. Meng et al.[27] also reported that the solvents used have shown a significant effect on the inhibition of hyaluronidase activity by *P. citrinopileatus*; thus, showing that solvent is an important chemical in the extraction process. Nevertheless, more studies on the extraction using water as a solvent should be

carried out as they are safer for human consumption and handling. In fact, the disposal of toxic wastes into the environment could be reduced accordingly.

3.2 Total Phenolics and Total Flavonoids Content of *T. Lactinea* Extracts

Literature has reported on the ability of phenolics and flavonoids content to inhibit the enzyme activities.[8,30,31] In this work, the total phenolics content of the extracts were determined using Folin Ciocalteau method as described in Section 2.7. Table 1 recorded that the supernatant of *T. lactinea* (without extraction) had the highest phenolics content followed by aqueous extract of supernatant (W.Sn). The phenolics content decreased more than 50% in the acetone and ethanol extracts of the supernatant (Ace.Sn and Eta.Sn). A reverse trend was observed in aqueous extract of mycelia (W.M) as it showed the lowest phenolics content compared to acetone and ethanol extracts of mycelia (Ace.M and Eta.M). This study also revealed that the total phenolics content was the highest in supernatant and can be obtained using a polar solvent, water. Moreover, the total phenolic content was higher in the supernatant of *T. lactinea* than in its mycelia.

Table 1: The total phenolic and total flavonoid content in different treatment methods of *T. lactinea* extracts.

Extracts	Total phenolic content (GAE mg g^{-1})	Total flavonoid content (QE μg mg^{-1})
Sn	36.36 ± 0.04	123.5 ± 0.10
W.Sn	22.14 ± 0.04	95.42 ± 0.05
Ace.Sn	11.94 ± 0.02	91.67 ± 0.07
Eta.Sn	16.68 ± 0.06	67.92 ± 0.21
W.M	3.23 ± 0.03	81.17 ± 0.04
Ace.M	6.08 ± 0.05	91.67 ± 0.02
Eta.M	11.06 ± 0.07	79.58 ± 0.02

Note: Data are mean ± SEM (n = 3). Sn = supernatant (without extraction); W.Sn = aqueous extract of supernatant; Ace.Sn = acetone extract of supernatant; Eta.Sn = ethanol extract of supernatant; W.M = aqueous extract of mycelia; Ace.M = acetone extract of mycelia; and Eta.M = ethanol extract of mycelia.

The total flavonoids content was also determined in all extracts. The results showed that the supernatant of *T. lactinea* (without extraction) had the highest flavonoids content followed by the aqueous supernatant (W.Sn). The total flavonoids content in descending order of: Sn > W.Sn > Ace.Sn > Eta.Sn > Ace.M > W.M > Eta.M. The flavonoids content was the highest in the supernatant extracted using a polar solvent, water. For the less polar solvent, acetone, the highest total flavonoid content was found in the mycelia. Table 1

also showed that the extraction of phenolics and flavonoids content in the supernatant and mycelia of *T. lactinea* is depending on the extracting solvents.

The relationship between these bioactive compounds and the inhibition of enzyme activities can be studied using linear regression analysis. However, the linear regression analysis obtained (figure not shown) found that there were no correlation between the inhibition of enzyme activities and total phenolics/flavonoids content in this study, $p > 0.05$. Yu et al.[32] suggested that it is necessary to run a composition analysis to identify the presence of other compounds that may act as enzyme inhibitors.

3.3 GCMS Analysis

The GCMS analysis was carried out to identify the presence of other bioactive compound in the extracts. The interpretation on mass spectrum GCMS on the extracts with highest anti inflammatory activity (W.Sn and Ace.M) were conducted using the database of National Institute Standard and Technology (NIST) library version 2.0. First, blank was injected and followed by sample injection. Figure 3 and 4 shows the GCMS chromatogram of the aqueous extract of supernatant (W.Sn) and acetone extract of mycelia of *T. lactinea* (Ace.M). The corresponding major components that are present in these extracts based on the GCMS spectrum is listed in Table 2 [(a) and (b)].

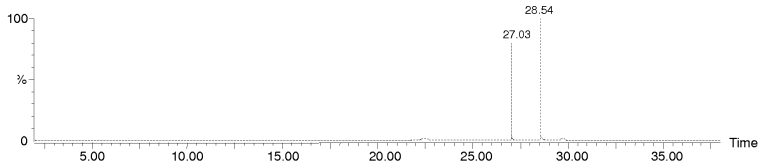

Figure 3: Chromatogram of aqueous extract of supernatant (W.Sn) from *T. lactinea*.

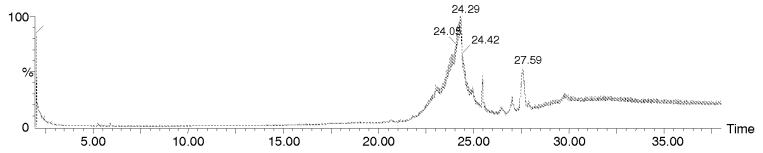

Figure 4: Chromatogram of acetone extract of mycelia (Ace.M) from *T. lactinea*.

The main component in the aqueous extract of supernatant (W.Sn) of *T. lactinea* was linoleic acid chloride (9,12-Octadecadienoyl chloride, (Z,Z)-) with area peak 18.120% [Table 2(a)]. This compound was believed to have influence in the inhibition of hyaluronidase and lipoxygenase activity as it showed more than 80% inhibition. Although there are no reports on its biological activity, this

compound has been reported present in the extract of *Cynodon dactylon* (L.) Pers. (family – Poaceae) which was used as anti inflammatory agent for kidney problems, urinary disease, gastrointestinal disorder constipation, abdominal pain and also as a blood purifying agent.[33,34] The strong inhibition of enzyme activity was also due to the presence of compound 9,12-Octadecadienoic acid (Z,Z)- with area peak 17.014%, n-Hexadecanoic acid and oleic acid. In the acetone extract of mycelia (Ace.M) of *T. lactinea* [Table 2(b)], the presence of oleic acid and 9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester was identified. As reported by Syazana et al.,[35] the presence of oleic acid in the methanolic extract of honey has contributed to the inhibition of proliferative effect in keloid fibroblasts.

Table 2(a): Chemical component identified in the aqueous extract of supernatant (W.Sn) of *T. lactinea* using GCMS.

Aqueous extract of supernatant (W.Sn)				
Chemical compound	Retention time (min)	Area (%)	Activity	References
n-Hexadeca-noic acid	22.036 22.257 22.479	2.445 4.528 13.555	Antioxidant, hypocholesterolemic, nematicide, pesticide, antiandrogenic, flavour Hemolytic, 5-Alpha reductase inhibitor.	33
Oleic acid	22.847 23.013 23.189 29.761	1.880 1.014 0.844 8.380	Antioxidant, cancer preventive, nematicide, lubricant, hypocho-lesterolemic.	34
9,12-Octade-cadienoyl chloride, (Z,Z)-	28.539	18.120	No activity reported.	33

Table 2(b): Chemical component identified in the acetone extract of mycelia (Ace.M) of *T. lactinea* using GCMS.

Aqueous extract of mycelia (Ace.M)				
Chemical compound	Retention time (min)	Area (%)	Activity	References
Oleic acid	23.018	2.160	Antioxidant, cancer preventive, nematicide, lubricant, hypocho-lesterolemic,	34
	23.110	2.338		
	23.405	1.831		
	23.479	1.444		
	23.571	1.009		
	23.663	2.020		
	23.737	2.228		
	23.829	1.206		
	23.885	2.074		
	23.903	2.253		
	24.501	2.969		
	24.272	11.711		
	24.548	1.146		
9,Octadecenoic acid (Z)-,2-hydroxy-1-(hydroxymethyl)ethyl ester	27.591	4.533	Inhibition of proliferative effect in keloid fibroblasts.	35

4. CONCLUSION

Of all the extract tested, the aqueous and acetone extract of supernatant and mycelia of *T. lactinea* at 100 µg mL^{-1} (W.Sn and Ace.M) significantly inhibited the activity of hyaluronidase and lipoxygenase enzyme. However, all extracts were found to be poor inhibitors to xanthine oxidase activity.

- The phenolics and flavonoids contents were determined in all extracts. However, no correlation was found between them and inhibition of hyaluronidase and lipoxygenase activity, $p > 0.05$.
- The CGMMS carried out has identified the presence of other bioactive compounds including linoleic acid chloride, 9,12-Octadecadienoic acid (Z,Z)- with area peak 17.014%, n-Hexadecanoic acid, oleic acid and 9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester that may act as the hyaluronidase and lipoxygenase inhibitors.

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