Trop J Nat Prod Res, May 2021; 5(5):850-856
ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Determination of a Chemical Marker in *Dipterocarpus alatus* Oleoresin Samples and Bioactivity Screening via Antioxidants, Nitric Oxide Inhibition on Murine RAW 264.7 Cells, and Collagen Production on Normal Human Dermal Fibroblasts

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ARTICLE INFO

Article history:
Received 17 February 2021
Revised 24 April 2021
Accepted 25 May 2021
Published online 03 June 2021

ABSTRACT

Oleoresin from the many species of Dipterocarps consist of several group of compounds such as monoterpenes and diterpene resin acids, sesquiterpenes and essential oils which presented various bioactivities. In this research, the 5 samples of *D. alatus* oleoresin from the different sources and preparation methods were evaluated for the chemical contents and biological activities. The oleoresin samples obtained from Roi Et province, Thailand (OD1), heating oleoresin (OD2), supernatant and precipitate after degumming oleoresin (OD3 and OD4) and the oleoresin from Surat Thani Province, Thailand (OD5) were determined for total phenolic compounds (TPC) and (-)-*α*-gurjunene using validated HPLC-UV technique. The biological activities such as, antioxidation, inhibition of nitric oxide (NO) production on murine RAW 264.7 cells, and collagen production activity on normal human dermal fibroblasts (NHDFs) were also studied. The results indicated that the major component of all oleoresin samples was (-)-*α*-gurjunene. OD5 established the highest (-)-*α*-gurjunene content and TPC. All samples demonstrated the potential to inhibit NO production on RAW 264.7 cells comparable to positive control (L-NAME), OD3 showed the highest activity with the NO inhibition as 71.89%. In addition, the OD2 could stimulate higher collagen production on NHDFs than positive, ascorbic acid. Therefore, different oleoresin preparation methods and different sources affected the chemical contents and biological activities which are nitric oxide inhibition and collagen production. Our results suggested that *D. alatus* oleoresin has potential to be developed as an anti-inflammatory or wound healing product(s), however, its efficacy requires further investigation.

Keywords: *Dipterocarpus alatus* oleoresin, (-)-α-gurjunene, Anti-inflammatory, Collagen production activity.

Introduction

Oleoresin is produced in specialized epithelial secretory cells in the bark or wood of tree stem, root, or the needles of plants. It is a potent defence mechanism against insects and fungi. It consists of monoterpenes and diterpene resin acids and smaller amounts of sesquiterpenes which are both volatile and non-volatile components. It may be solid or semi-liquid and is always water insoluble.[1] Oleoresin from the many species of *Dipterocarps* is locally referred to gurjan oil (India), minyak keruing (Western Malesia) and kanyin oil (Burma).[2]

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Citation: Longsri B, Kanpipit N, Puthongking P, Mahakunakorn P, Tiyaworanant S, Sungthong B, Thapphasaraphong S. Determination of a Chemical Marker in *Dipterocarpus alatus* Oleoresin Samples and Bioactivity Screening via Antioxidants, Nitric Oxide Inhibition on Murine RAW 264.7 Cells, and Collagen Production on Normal Human Dermal Fibroblasts. Trop J Nat Prod Res. 2021; 5(5):850-856. doi.org/10.26538/tjnpr/v5i5.10

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Bioactivities of oleoresin such as antibacterial, antifungal, anticancer, anti-inflammatory activity, insecticidal activity, antioxidant activities, antiarthritic agent,[3] including its use for the treatment of skin allergies, diarrhea, dysentery, astringency have been reported.[4,5] The essential oils, components separated from oleoresins were utilized in pharmaceutical industry, perfume industry, food, drink additives and other chemical industries.[6]

The *D. alatus* oleoresin turns brown after standing at room temperature for a few days while *D. alatus* oleoresin after boiling appears as a clear homogeneous yellow liquid and are more stable without a precipitate. The oleoresin could improve the quality by degumming under alkaline condition at temperature of 95 ºC for 30 min according to the announcement of the Department of Energy, Thailand.[7] The degumming process facilitates the removal of impurities or gum from the oil to prevent crude oil settling during storage or transport. However, the alkali degumming products could change the chemical composition subsequently affect biological activities. The properties of *D. alatus* oleoresin were found to have potential as an alternative liquid biofuel and (-)-*α*-gurjunene was found as the major compound in oleoresin by GC-MS analysis.[8] The major compound in *D. alatus*–oleoresin is (-)-α-gurjunene which can be used as a chemical marker. *Copaifera langsdorffii* was used to treat rat skin wounds and also had potential in healing wounds of the skin.[9]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

C. langsdorffii oleoresin has also been investigated and shown to have non-cytotoxic effect on fibroblasts and improved wound healing.[10] A 10%copaiba oleoresin cream was used for treatment of injured rats and found to promote wound healing by regulating MMP-2 and MMP-9 activities to stimulate collagen synthesis and promote tissue remodelling and reepithelialization.[11]

Thus, our study used various samples of *D. alatus* oleoresin from different preparation methods and different sources to determine their (-)-*α*-gurjunene content by a validated HPLC-UV method. The antioxidant activities and the effects on RAW 264.7 cells and normal human dermal fibroblasts (NHDFs) of oleoresin samples of *D. alatus* oleoresin samples were investigated for their potential anti-inflammatory activity as well as wound healing potential which might be useful for pharmaceutical or cosmetic product development in the near future.

Materials and Methods

Plant materials

Oleoresin from *D. alatus* (Yang-Na in Thailand) were collected from Roi Et province and Surat Thani province, Thailand, in June 2017. The trunks of Yang-Na with age more than 3 years were selected for tapping oleoresin.[8] The *D. alatus* was identified by Suppachai Tiyaworanant and a voucher specimen was deposited in the Faculty of Pharmaceutical Sciences, Khon Kaen University, under registration NO. KKPSH010102992 to KKPSH010102996.

Sources of chemicals and cell lines

The standard (-)-α-gurjunene (97.0%) and other reagents and chemicals, such as DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-tripyridyl-S-triazine), ABTS, 2-thiobarbituric acid, ascorbic acid and Folin-Ciocalteu reagent, 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) and standard trolox (6-hydroxy-2,5,7,8-tetramethyl chromane 2-carboxylic acid) were purchased from Sigma Chemical Co., USA. The RAW 264.7 cells, a murine macrophage cell line, were obtained from Dr. Pramote Mahakunakorn. Normal human dermal fibroblast cell lines (NHDF) were purchased from ATCC (PCS-201-012, USA). Roswell Park Memorial Institute medium (RPMI–1640), Fetal Bovine Serum (FBS) and 1%(v/v) penicillin–streptomycin and Dulbecco's Modified Eagle's Media (1:1) containing 10%fetal bovine serum and 1% (v/v) penicillin–streptomycin were purchased from Gibco, USA. TLC Silica gel 60 F254 (20x20 cm) was purchased from Merck Millipore, Germany.

Sample preparation from the D. alatus oleoresin

The five samples of *D. alatus* oleoresin were prepared using the following procedure. The 4 oleoresin samples from Roi Et province, Thailand were crude *D. alatus* oleoresin (OD1), which were heated at 95°C for 30 min (OD2). The supernatant from alkaline degumming sample (OD3) was obtained by heating oleoresin with water at 95°C for 30 min which was then neutralized by addition of 6%NaOH. The gum residue (OD4) was obtained from alkaline degumming sample, and the final sample was crude oleoresin from Surat Thani Province, Thailand (OD5).

Sample characterization

All samples (OD1-OD5) were dissolved in 95%ethanol and the pH was determined in triplicates. The absorbance of all samples was measured by UV-Visible spectrophotometer in the range of 200-800 nm. The samples were further determined for TLC fingerprint by normal phase (silica gel 60 F_{254}, (Merck)). The solvent system was hexane: ethyl acetate (8:2) which was subsequently detected under UV at wavelength 254 nm and sprayed with anisaldehyde sulfuric acid reagent on a heating plate at 105°C for 1-2 min.[12]

Method validation of (-)-α-gurjunene

The validation method of (-)-α-gurjunene was performed using HPLC equipped with UV detector and linearity, accuracy, precision, the limit of detection (LOD). Limits of quantitation (LOQ) were evaluated according to the International Council for Harmonisation (ICH) guidelines.[13] Linearity was evaluated by analyzing different concentrations of the standard solution of the (-)-α-gurjunene in the range from 10-50 µg/mL. Precision was evaluated by measurement of the peak area which was expressed as %RSD. The five different concentrations of standard (-)-*α*-gurjunene were determined intra-day for repeatability and inter-day for reproducibility. Accuracy was evaluated by %recovery of (-)-*α*-gurjunene. The values of %RSD of repeatability, reproducibility and accuracy which must be less than 2. The values of LOD and LOQ were calculated according to the standard deviation of the response (σ) of the curve, and the slope of the calibration curve (S) at levels resembling the LOD according to the formula: LOD = 3.3(σ/S). For the LOQ according to the formula: LOD = 10(σ/S). The standard deviation of the response was determined based on the standard deviation of y-intercepts from the regression lines.

Determination of (-)-α-gurjunene in D. alatus oleoresin samples (OD1-OD5)

All samples (OD1-OD5) were analyzed (-)-α-gurjunene by HPLC-UV using Phenomenex® column RP-18 (250 cm X 4 mm, 5 µm), the mobile phase was 90%acetonitrile, with the flow rate 1.5 mL/min. The injection volume was 20 µL. The column temperature was 25°C with UV detection at 220 nm. The (-)-α–gurjunene in sample was calculated according to the calibration curve of standard (-)-α–gurjunene in the range of 10-50 µg/mL.

Determination of total phenolic compounds (TPC)

An aliquot of 20 µL of 100 µg/mL *D. alatus* oleoresin samples (OD1-OD5) were mixed with 100 µL of Folin-Ciocalteu phenol reagent which was previously diluted 10-fold with distilled water. After 5 min, 80 µL of 7.5%sodium carbonate was added to the mixture and incubated in the dark at room temperature for 90 min. Subsequently, the absorbance of sample mixture was measured at 765 nm using microplate reader. Total phenolic content of samples was calculated by the standard curve of gallic acid (0-50 µg/mL) and stated in terms of mg gallic acid equivalent (GAE) per gram of sample.[14]

Determinations of antioxidant activities

DPPH Radical Scavenging assay

An aliquot of 150 µL of 0.1 mM DPPH methanolic solution was mixed with 50 µL of 2-500 µg/mL *D. alatus* oleoresin samples (OD1-OD5) or (-)-α-gurjunene in methanol. The mixture was left at the room temperature for 30 min in a dark place. The absorbance was measured at 517 nm using the microplate reader.[15] The standard Trolox was used as a positive control. The inhibition percentage of the DPPH radical scavenging activity was calculated as follows with the equation below:

$$\%\text{Inhibition} = (1 - \frac{A_{sample}}{A_{control}}) \text{x } 100$$

Where $A_{control}$ is the absorbance of control sample and A_{sample} is the absorbance of sample.

ABTS radical scavenging assay

A 7 mM ABTS solution was mixed with 2.45 mM potassium persulfate solution in the dark at room temperature for 12-16 h. The $ABTS^+$ solution was then diluted by 50%methanol to obtain the absorbance of 0.70±0.02 at 734 nm using the spectrophotometer. ABTS radical scavenging activity was measured by 150 µL of the $ABTS^+$ working solution was reacted with 50 µL of 2-500 µg/mL *D. alatus* oleoresin samples (OD1-OD5) or (-)-α-gurjunene in methanol and kept in the dark for 30 min. After that, their absorbance was carried at 734 nm.[16] The standard Trolox was used as a positive control. The inhibition percentage of the ABTS radical scavenging activity was calculated based on the following equation below:

$$\%\text{Inhibition} = (1 - \frac{A_{sample}}{A_{control}}) \text{x } 100$$

Where $A_{control}$ is the absorbance of control sample and A_{sample} is the absorbance of sample.

Ferric reducing antioxidant power (FRAP) assay

The FRAP reagent was prepared fresh daily by mixing of 25 mL acetate buffer, 2.5 mL of 10 mM TPTZ solution, and 2.5 mL of 20 mM $FeCl_3$ $6H_2O$ solution and warmed to 37°C in a water bath until used for the experiments.[17] The 50 µL of 250 µg/mL *D. alatus* oleoresin samples (OD1-OD5), Trolox or 100 µg/mL of standard (-)-

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

α-gurjunene in methanol were mixed with 150 µL of the FRAP reagent for 30 min in the dark condition. The absorbance variance was read at 594 nm and the antioxidant activity was calculated with the standard curve of $FeSO_4$ in the range of 0.02-0.10 mM. The results were stated as mmol Fe (II) per gram of sample.

Cell culture assay

Inhibition of nitric oxide production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells

RAW 264.7 cells were cultured in RPMI 1640 media, Fetal bovine serum (FBS), and 10% FBS and 1% (v/v) penicillin–streptomycin and incubated at 37°C, 5%CO_2 for 24 h. For all the experiments, cells were grown at a density of 1×10^4 cells/well before the experiments. The culture medium was removed and then 100 µL of 0.625, 1.25 and 2.5 µg/mL of samples, (-)-α-gurjunene or 1 mM L-NAME (positive control) in culture media without or with 1 µg/mL LPS were added into cells. The cells were incubated for another 24 h. Cell viability was assessed using an MTT assay. The culture medium was removed and then 100 µL of 5 mg/mL MTT was added. After incubation for 2.5 h, the culture medium was removed and 100 µL DMSO was added.[18] The absorbance at 550 nm was measured with microplate readers (Bio-Rad Model 680, Tokyo, Japan). The percentage cell viability was calculated by the following equation:

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

Where $A_{control}$ is the absorbance of non-treated cell and A_{sample} is the absorbance.

The cultured media from the cells which were treated by the samples and control and stimulated with LPS (1 µg/mL) were further determined for inhibition of NO production.[19] The quantity of nitrite accumulated in the culture medium was measured to indicate NO production. Briefly, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent (1%sulphanilamide and 0.1%naphthylethylenediamine dihydrochloride in 2.5%phosphoric acid) (Sigma-Aldrich, Germany). The mixture was incubated at room temperature for 30 min and the absorbance was measured at 520 nm using a microplate reader. Fresh culture medium was used as a blank. The percentage of NO inhibition was calculated by the following equation.

$$\%\text{NO inhibition} = (1 - \frac{A_{sample}}{A_{control}}) \times 100$$

Where $A_{control}$ is the absorbance of non-treated cell and A_{sample} is the absorbance of sample.

Determination of collagen stimulating activity in normal human dermal fibroblasts (NHDFs)

Cell proliferation assay of NHDFs was performed by the MTT assay method.[20] NHDFs were cultured in Dulbecco's Modified Eagle's Media containing 10%fetal bovine serum and 1%(v/v) penicillin–streptomycin and incubated at 37°C, 5% CO_2 for 24 h. For all experiments, cells were grown at a density of 1×10^4 cells/well before the experiments. The medium was removed and 100 µL of 0.625, 1.25 and 2.5 µg/mL of samples, (-)-α-gurjunene or positive control (50 µg/mL ascorbic acid) were added and incubated at 37°C, 5% CO_2 for 24 h. Subsequently, medium was removed for collagen content determination by Sirius red assay. Then 100 µL of 5 mg/mL MTT in PBS pH 7.4 was added to cells and incubated at 37°C, 5% CO_2 for 2.5 h. A 100 µL DMSO was added and the absorbance at 550 nm was then measured. The non-treated medium was the negative control. Percent Cell viability was calculated with the following equation:

$$\%\text{Cell viability} = A_{sample}/A_{control} \times 100$$

Where $A_{control}$ is the absorbance of non-treated cell and A_{sample} is the absorbance of sample.

Collagen content determination was performed by Sirius red assay.[21] The 100 µL of treated medium from NHDFs after incubation at 37 °C, 5%CO_2 for 24 h was transferred into 1.5 mL microcentrifuge tube to which 100 µL of 0.5 M acetic acid with 1 mL of dye solution (0.02 g Direct red 80 in picric acid saturated 30 mL) was added. The mixture was sonicated for 30 min and then centrifuged at 10,000 rpm for 5 min. The supernatant was removed and 1 mL of 0.1 M HCl was added. The mixture was further mixed and centrifuged at 10,000 rpm for 15 min. Finally, the supernatant was removed and 1 mL of 0.5 N NaOH was added. The mixture was mixed and measured the absorbance at 540 nm. The standard collagen type I was used to perform the calibration curve.

Statistical analysis

Data were expressed as Mean ± Standard Deviations (SD). Results of the activity were evaluated by analysis of variance (ANOVA) using SPSS software version 26.0 (KKU software support). P-values of less than 0.05 and 0.001 were considered to indicate a significant difference. The multiple comparison was performed by Duncan or Dunnett's test ($p < 0.05$).

Results and Discussion

Sample characterization

The characteristics of all samples (OD1-OD) are shown in Figure 1. All samples showed the same maximum UV absorption at 217 nm (Figure 2). The pH of all samples ranged from 5.75 to 9.82. The gum residue (OD4) showed the highest pH at 9.82. The normal phase TLC fingerprint of the sample *D. alatus* oleoresin (OD1-OD5) also presented the major compound as (-)-α-gurjunene with Rf-value of 0.83 (Figure 3).

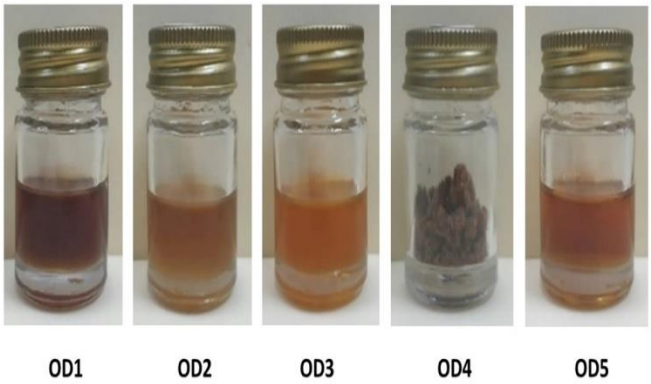

Figure 1: Characteristics of *D. alatus* oleoresins (OD1-OD5)

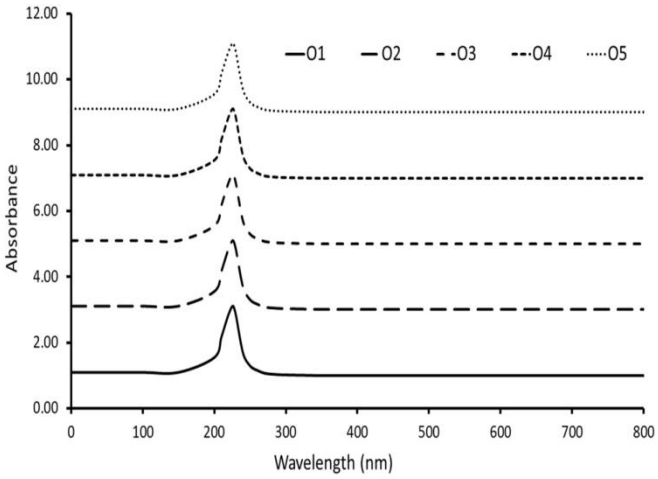

Figure 2: The UV-Vis spectra of the samples from *D. alatus* oleoresins (OD1-OD5) 100 µg/mL (wavelength range 0-800 nm)

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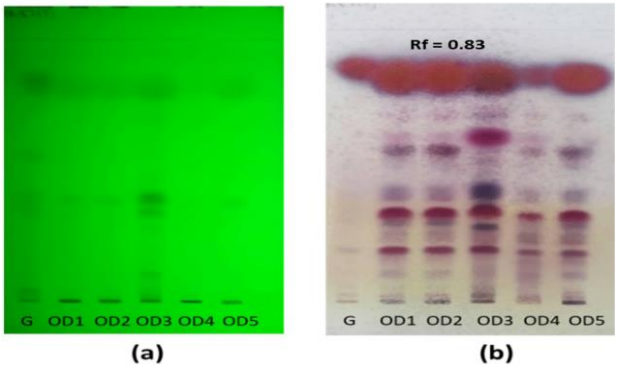

Figure 3: The normal phase TLC fingerprint of the *D. alatus* oleoresins detected under wavelength 254 nm (a) and spraying with anisaldehyde sulphuric acid reagent (b) using TLC normal phase TLC and mobile phase was hexane: ethyl acetate (8:2); Lane 1 is the standard (-)-α–gurjunene (G) and lane 2-5 were *D. alatus* oleoresins (OD1-OD5), respectively. The major compound in all sample is (-)-α–gurjunene with the R_f value at 0.83.

Table 1: Validation methods of (-)-α-gurjunene by HPLC-UV

Parameters	(-)-α-gurjunene
Linearity (n = 5)	
-linear equation	Y = 23419x – 60176
Coefficient of determination(R^2)	0.998
Range(µg/mL)	10-50
LOD (µg/mL) (n = 9)	1.15 ± 0.21
LOQ (µg/mL) (n = 9)	3.49 ± 0.63
Precision(%RSD)	
Intra-day (n = 3)	0.17-1.91
Inter-day (n = 5)	0.11-1.82
Accuracy (%Recovery) (n = 7)	95.49-101.71

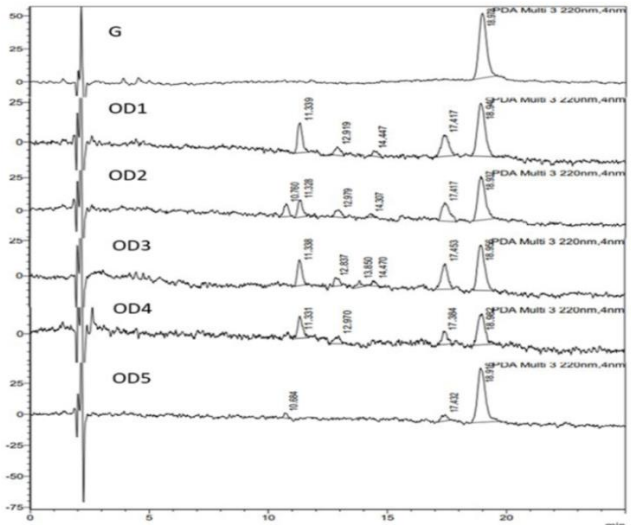

Figure 4: The HPLC chromatogram profile of the standard (-)-α-gurjunene(G) and test samples oleoresin (OD1-OD5) using an isocratic elution of 90%acetonitrile with a flow rate of 1.5 mL/min, injection volume 20 µL, column temperature 25°C and UV detections at 220 nm. The retention time of (-)-α–gurjunene in all oleoresin samples were in the range of 18.916-18.982 min.

Determination of (-)-α-gurjunene in D. alatus oleoresin and total phenolic compounds

The validated HPLC method was utilized for (-)-α-gurjunene determination. The HPLC chromatogram of standards (-)-α-gurjunene showed the retention time at 18.978 min. The results of validation are shown in Table 1. At the concentration range of 10-50 µg/mL provided a good linear relationship and the regression equation was y = 23419x – 60176 (R^2 = 0.998). The percentage recovery of (-)-α-gurjunene ranged from 98.95% to 101.71% (%RSD < 2). The precision was presented as the values of %RSD in the range of 0.17 to 1.91 under repeatability of the standard solution of the (-)-α-gurjunene at five different concentration (10-50 µg/mL). The reproducibility was shown as %RSD in the range of 0.11 to 1.82 (%RSD < 2).

The HPLC chromatogram profile of all oleoresin samples (OD1-OD5) showed that (-)-α-gurjunene was found as the major compound in OD1-OD5 (Figure 4) with the retention time in the range of 18.916-18.982 min. The results among five samples (OD1-OD5) are shown in Table 2, the crude oleoresin from Surat Thani Province (OD5) showed the highest TPC and (-)-α-gurjunene level at 95.69±0.29 mg GAE/g sample and 17.35±0.02 mg/g sample, respectively whereas the crude oleoresin from Roi Et province (OD1) was 50.12±2.94 mgGAE/g sample and 13.78±0.05 mg/g sample, respectively. The various preparations and different sources of oleoresin provided the different level of TPC content and (-)-α-gurjunene.

From comparison of the different preparation methods of oleoresin from Roi Et province (Table 2), the highest TPC was from the crude oleoresin (OD1, 50.12±2.94 mg GAE/g sample) and the lowest TPC was from its supernatant after the degumming process (OD3, 34.34±3.09 mg GAE/g sample), whereas heating oleoresin (OD2, 42.50±2.49 mg GAE/g sample) provided no significant difference of TPC from gum residue after degumming (OD4, 41.96±2.94 mg GAE/g sample). For (-)-α-gurjunene content, OD4 presents the lowest (-)-α-gurjunene level (7.32±0.01 mg/g sample) whereas OD3 shows the highest (-)-α-gurjunene level (13.97±0.04 mg/g sample) which are slightly higher than OD1 (13.78±0.05 mg/g sample) and OD2(13.60±0.05 mg/g sample). Therefore, the various preparation method for oleoresin could provide different TPC and (-)-α-gurjunene level.

However from antioxidant capacity study by DPPH, ABTS and FRAP assay, OD1-OD5 showed the low capacity on these 3 models, only FRAP assay could be reported and OD5 was found to have the highest FRAP value (0.40±0.00 mmole Fe(II) /g sample) which may relate to its highest TPC and (-)-α-gurjunene content.

For heating oleoresin (OD2) and degumming with high temperature might cause degradation or evaporation of some volatile phenolic compounds so the TPC became reducing. However, the crude oleoresin causes the unstable performance and properties of oleoresin such as color change and precipitate formation in storage duration. Therefore, heating of oleoresin could help to prevent precipitation incidence. Furthermore, the degumming process of oleoresin was also performed for the good quality, stability of oleoresin. The degumming process helps to separate phospholipids from the oil in oleoresin.[22] Phospholipid molecules are known to improve the oxidative stability of the vegetable oils, and synergists of phenolic compounds and possibly other constituents with antioxidant activity were partly destroyed during degumming process.[23] Based on the results, the degumming method significantly caused reduction of TPC, and some active compounds which also affect biological activity. However, the gum residue (OD4) provided high TPC whereas supernatant (OD3) after degumming provided higher (-)-α-gurjunene level (Table 2).

For cell assays, the samples (OD1-OD5) at 0.0625, 1.25 and 2.5 µg/mL were determined for inhibition of nitric oxide production on LPS treated murine RAW 264.7 cells compared with positive control (L-NAME) (Figure 5). Most of the samples at 0.0625, 1.25 and 2.5 µg/mL caused non-cytotoxicity on RAW 264.7 cells except 2.5 µg/mL of OD1 and OD2. However, all samples showed %NO inhibition less than 80 (Figure 6). The highest %NO inhibition with non-cytotoxicity on RAW 264.7 cells was from 2.5 µg/mL of OD3 (71.89%), whereas

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

%NO inhibition of L-NAME was 77.23. The OD3 might contain most of the essential oils or terpenes and (-)-α-gurjunene as the major compound. Therefore, the supernatant after degumming (OD3) was important for anti-inflammatory activity. Like the former researches, anti-inflammatory activities were obtained from the essential oils and sesquiterpenes from various plants.[24–27] However, NO scavenging activity does not only depend on (-)-α-gurjunene or phenolic compounds level but the other compounds in oleoresin could be involved in this activity. Chemical composition analysis of *D. alatus* oleoresin was investigated by GC-MS and showed various sesquiterpenes that α-gurjunene (30.31%) was the major compounds.[5] The various groups of compounds in crude *D. alatus* oleoresin might cause antagonistic effects. Therefore, the OD3 which was the separated gum residue could show better nitric oxide inhibition on RAW 264.7 cells. To understand the important components necessary for activity, further studies should be conducted.

All oleoresin samples (OD1-OD5) at 0.625, 1.25 and 2.5 µg/mL showed non-cytotoxicity on NHDFs as well as (-)-α-gurjunene and ascorbic acid (Figure 7). The OD2 at 0.625, 1.25 and 2.5 µg/mL compared with negative control showed higher collagen content ratio than 3.00 (3.18,3.21, 3.08, respectively). Whereas the collagen content ratio of 50 µg /mL of ascorbic acid was 2.82 (Figure 8). Heating of oleoresin (OD2) which increased the solubility or provide the change of chemical composition in oleoresin could enhanced collagen production on NHDFs. The positive effects on skin regeneration and wound healing in human skin were also obtained from of essential oil of *Artemisia montana*.[28] Moreover, the sesquiterpenes in the essential oil extracted from *Eugenia dysenterica* leaves indicated as a potent natural wound healing compound.[29] The collagen stimulating property of oleoresin would be beneficial to wound healing capacity which should be examined in more detail.

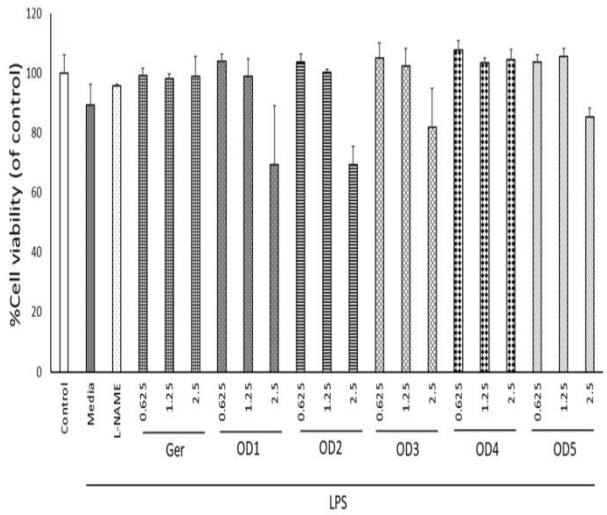

Figure 5: Effects of 0.625, 1.25, 2.5 µg/mL oleoresins (OD1-OD5) of *D. alatus*, standard (-)-α-gurjunene (Ger) and L-NAME (positive control) on LPS treated RAW 246.7 cells. Data are representatives of three replicates and shown as mean ± SD (control is the media from untreated group).

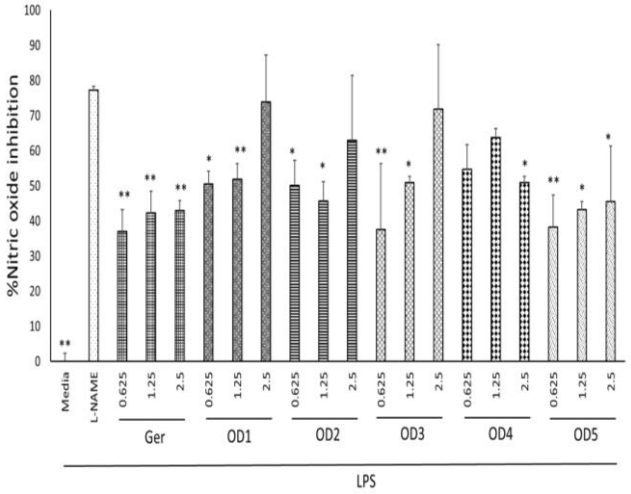

Figure 6: %NO inhibition of 0.625, 1.25, 1.25 µg/mL oleoresins (OD1-OD5) of *D. alatus*, standard (-)-α-gurjunene (Ger) and 1 mM L-NAME (positive control) on LPS treated RAW 246.7 cells. Data are representatives of three replicates and shown as mean ± SD; * $p < 0.05$, ** $p < 0.001$ (compared to L-NAME).

Table 2: Total phenolic content and (-)-α-gurjunene content, % ABTS, DPPH scavenging assay, FRAP assay of oleoresin samples (OD1-OD5) compared with (-)-α-gurjunene and Trolox

Sample	TPC (mg GAE/g sample	(-)-α-gurjunene (mg/g sample)	DPPH method IC_{50} (µg/mL)	ABTS method IC_{50} (µg/mL)	FRAP Value (mmole Fe(II) /g sample)
OD1	50.12 ± 2.94^{b}	13.78 ± 0.05^{c}	$>1000^{d}$	$>500^{d}$	0.38 ± 0.01^{c}
OD2	42.50 ± 2.49^{c}	13.60 ± 0.05^{d}	$>1000^{d}$	$>500^{d}$	0.31 ± 0.00^{e}
OD3	34.34 ± 3.09^{d}	13.97 ± 0.04^{b}	$>1000^{d}$	$>500^{d}$	0.35 ± 0.01^{d}
OD4	41.96 ± 2.94^{c}	7.32 ± 0.0^{e}	$>1000^{d}$	$>500^{d}$	0.22 ± 0.00^{f}
OD5	95.29 ± 4.11^{a}	17.35 ± 0.02^{a}	$>1000^{d}$	400.67^{c}	0.40 ± 0.00^{c}
(-)-α-gurjunene	-	-	$>50^{c}$	$>50^{b}$	0.78 ± 0.01^{b}
Trolox	-	-	2.82^{a}	11.82^{a}	2.36 ± 0.04^{a}

Results are expressed as mean ± SD (n = 3). [a, b, c, d, e, f] letters indicate significant differences in the same column at p-value < 0.05 (Post hoc test by Duncan). For FRAP assay, 250 µg/mL of OD1-OD5, Trolox and 100 µg/mL of (-)-α-gurjunene were used.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

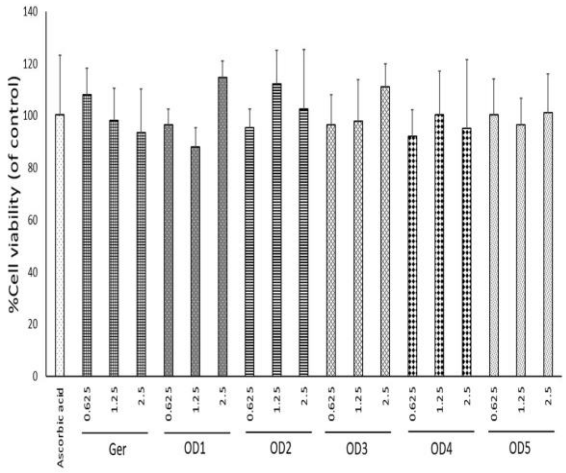

Figure 7: Effect of 0.625, 1.25, 2.5 µg/mL oleoresins (OD1-OD5) of *D. alatus*, standard (-)-α-gurjunene (Ger) and ascorbic acid 50 µg/mL (positive control) on NHDF cells. Data are representatives of three replicates and shown as mean ± SD.

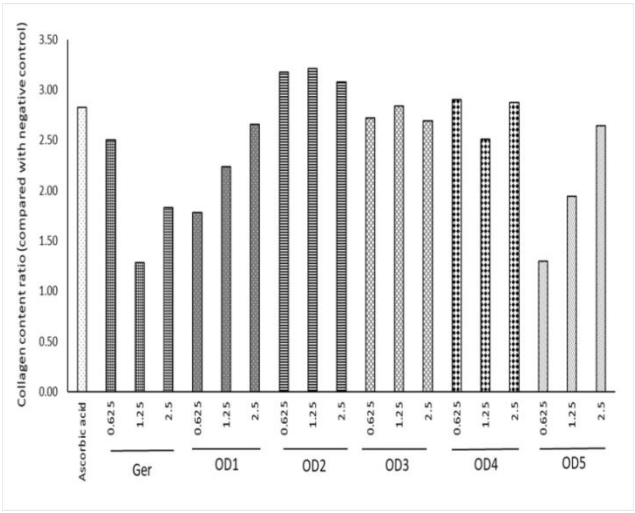

Figure 8: Collagen content ratio from standard (-)-α-gurjunene 0.625, 1.25 and 2.5 µg/mL of oleoresins (OD1-OD5) of *D. alatus* compared with negative control treated on NHDF cells.

Conclusion

The different sources and different sample preparations affected to the chemical constituents in *D. alatus* oleoresin and the biological activities. The oleoresin samples showed high potential of nitric oxide inhibition on RAW 264.7 cells, especially OD3 at 2.5 µg/mL. Whereas OD2 at 0.625-2.5 µg/mL caused increasing collagen production of NHDFs greater than ascorbic acid. In addition, the HPLC method could be successfully utilized for determination of (-)-α-gurjunene which is a chemical marker in *D. alatus* oleoresin. From these results indicated that *D. alatus* oleoresin could provide a potential on anti-inflammatory and stimulation of collagen production involved in wound healing. Furthermore, the preparation process of oleoresin also improved the biological activities of the samples. Therefore, comprehensive investigations of *D. alatus* oleoresin should be conducted to determine its potential usefulness in health products in the future.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

This work was supported by the Plant Genetic Conservation Project under the royal initiative of Her Royal Highness Princess Maha Chakri Sirindhorn, Khon Kaen University Research Fund targeted on Yang-Na, 2016 and 2017 under Grant number KKU-YN59-08 and KKU-YN-60-013; the Program Management Unit for Human Resources & Institutional Development, Research and Innovation targeted on Yang-Na, KKU [Fiscal years 2020, grant number B05F630053/630000050103]; the National Research Council of Thailand (NRCT) for graduate student Year 2018 for the master student (Miss Bantharawan Longsri) under Grant number KKU 9/2561; and the Center for Research and Development of Herbal Health Products (CRD-HHP), Faculty of Pharmaceutical Sciences, KKU, Thailand. We would like to acknowledge Prof. Dr. Ross Hector Andrews from Publication Clinic KKU, Thailand for editing this manuscript.

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