Dayak Onions (*Eleutherine Americana* L Merr) reduced mesothelial detachment after laparoscopic

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ABSTRACT

Background: Laparoscopy induces mesothelial structure changes and detachment. The studies about preventing the mesothelial cell detachment were rarely found. Dayak tribe uses the Dayak onion (*Eleutherine americana* L. Merr) as a wound-healing agent due to its anti-inflammatory and antioxidant activity. This study aimed to prove the anti-inflammatory and antioxidant activity of Dayak onion to prevent mesothelial cells damage after laparoscopy.
Methods: Thirty males Sprague-Dawley *Rattus norvegicus* were divided into five groups (n = 6 per group): (a) control, (b) mediclor, (c) Dayak onion 30 mg/kg, (d) Dayak onion 60 mg/kg, and (e) Dayak onion 90 mg/kg body weight. Transforming growth factor-beta (TGF-β) and total oxidant status (TOS/H_2O_2) from peritoneal fluid was determined 24 h after laparoscopy. Histopathological analysis of mesothelial cell numbers in the peritoneum, small intestine, greater omentum, and liver was performed 7 days after the procedure.
Results: In-silico analysis result suggested one of the Dayak onions' active compounds, eleutherine, has a potential activity as an anti-inflammatory and modulator of TGF-β. Also, the TGF-β level, the number of mesothelial cells detachment, and ZO-1 expression were significantly decreased following Dayak onion administration ($p<0.05$).
Conclusions: Our study showed that Dayak onion administration in rats reduced TGF-β level, number of mesothelial cells detachment, and ZO-1 expression following laparoscopy.

Keywords: Laparoscopy, mesothelial cell, inflammatory response, Dayak onion

BACKGROUND

The usage of laparoscopy tends to increase, especially since the discovery of robotic surgery (Koh *et al*., 2018; Peters *et al*., 2018). The previous study showed that CO_2 insufflation resulted in changes in mesothelial structure (Sampurno *et al*., 2019). Laparoscopy causes mesothelial cell detachment and extracellular matrix (ECM) exposure (Davey *et al*., 2013).

The mesothelium is a dominant structure, equals to the body surface, and has functions in protection, transport, and immunology of the abdominal cavity (Mutsaers *et al*., 2016). Insufflation causes mesothelial cell damage (Peters *et al*., 2018). Zone Occludin 1 (ZO-1) is a protein that constructs the mesothelial cell junction (Kim *et al*., 2019). Reactive oxygen species (ROS) and inflammatory response trigger ZO-1 structural changes and cellular disruption. Adverse effects occur when mesothelial cell

detachment does not recover properly (Sampurno *et al*., 2019). Studies on the prevention of mesothelial cell detachment are rarely found.

Dayak onions (*Eleutherine americana* L. Merr) or Bawang Dayak/BD are inedible wild plants, and the Dayak tribe has been using it as a wound-healing agent (*bahimang*) (Poerwosusanta *et al*., 2019). This study aimed to prove the anti-inflammatory and anti-oxidant effects of Dayak onions in preventing mesothelial cell damage after laparoscopy.

METHODS

Dayak onions extract preparation

Dayak onions bulb were harvested at 12 weeks from the Pulang Pisau area, Central Kalimantan. The species of *Eleutherine americana* L. Merr bulb was determined at the UPT Materia-Medica Batu, Malang, East Java, Indonesia. Ethanol extract of the Dayak onion bulbs was made from the simplicial. The simplicial was macerated and analyzed at the Laboratory of Polytechnic Chemical Engineering Malang using Liquid Chromatography-Mass Spectrometry (LCMS) techniques. The LCMS was used to analyze the active compounds of *Eleutherine americana* L. Merr. The basic principle is to separate ionized extracts between the static and the mobile phase. Active compounds were recorded according to the mass-to-charge ratio (m/z) and retention time (rt) and compared with references (Ardrey, 2003).

Animal model experiments

Our study used Sprague-Dawley rats (with bodyweight between 200-250 g and aged between 20–25 weeks) from the Abadi Jaya farm in Yogyakarta, Indonesia. Thirty male rats were used according to the Federer formula (Federer, 1966). Rat males are stronger and have a higher blood volume, and the estrus phase can be avoided. Experimental animals were treated according to animal research standards (3R5F) (van den Bos *et al*., 2012). The rats were provided with 7-days acclimation, health evaluation, and then randomly classified into 5 groups: (a) control, (b) mediclor, (c) Dayak onion 30 mg/kg, (d) Dayak onion 60 mg/kg, and (e) Dayak onion 90 mg/kg body weight. The Dayak onion extract was given orally at an appropriate dose for 54 days before surgery.

With CO_2 insufflation, laparoscopy was performed for one hour after Dayak onion extract administration. Anesthesia was provided using intra-muscular ketamine 50 mg/kg body weight. The control group was insufflated at 10 mmHg because it is adequately high for rats and is comparable to 15 mmHg pressure in humans (Avital *et al*., 2009). The Mediclor group was insufflated at 10 mmHg and was given intraperitoneal mediclor 5 mg/kg body weight immediately after surgery. The Dayak onion 30 mg/kg group was insufflated at 10 mmHg and given Dayak onion extract with the dose of 30 mg/kg body weight; the Dayak onion 60 mg/kg group was insufflated at 10 mmHg and given Dayak onion extract with the dose of 60 mg/kg body weight and the Dayak onion 90 mg/kg group was insufflated at 10 mmHg and given Dayak onion extract with the dose of 90 mg /kg body weight.

After 24 hours, the TGF-β level and total oxidant status (TOS) in the peritoneal fluid were measured. TGF-β level was measured with the Cloud Clone ELISA (Enzyme-linked immunosorbent assay) kit for Transforming Growth Factor (TGF-β1) species of *Rattus norvegicus* (rat) (SEA124Ra), and TOS was assessed using the colorimetric method with Biovision Hydrogen Peroxide Colorimetric/Fluorometric Assay Kit (K265-200). ZO-1 expression in the parietal peritoneum tissues was evaluated using immunohistochemically anti-ZO-1 tight junction antibodies (Santa Cruz ZO-1 antibody Sc-33725) and quantified with ImageJ software. After 7 days, Hematoxylin Eosin specimens of the liver, parietal peritoneum, small intestine, and omentum were evaluated independently by three pathologists who were blinded to the group allocation, in 1 μM^2 (100×magnification). Mesothelial cells were counted, and the average count was calculated. TGF-β was stated in ng/ml, TOS in nmol/µl, mesothelial cell count in cells/µM2, and ZO-1 expression in % at $1\mu M^2$.

Statistical analysis

Data were presented in mean ± standard deviation, and values between groups were compared. The distribution normality and homogeneity were tested with the Kolmogorov-Smirnov or Shapiro-Wilk test and Levene's test of variance, respectively. In the normally distributed and homogeneous, the analysis was performed with one-way ANOVA and post-hoc LSD tests. If the data were normally distributed but non-homogeneous, the analysis was performed with the Welch Robust Test of Equality of Means and the post-hoc Games-Howell test. If the data were non-normally distributed, the analysis was performed with Kruskal-Wallis and post-hoc Mann-Whitney test. The confidence level was 95%.

In silico analysis

The study was started by searching for amino acid sequences and the structure of active components of Dayak onions (*Eleutherine americana* L. Merr). The amino acid sequences of Nrf2 (GI: 693842), Keap1 (GI: 22027642), ubiquitin (GI: 340068), NF-kB (GI: 1018443262), IkB kinase-β (IKK-β) (GI: 4185275), I-kB kinase-α (IKK-α) (GI: 4185273), TGF-β (GI: 339564) and TGF-βR (GI: 270048022) were obtained from the National Center for Biotechnology Information (NCBI) database, United States National Library of Medicine (NLM), National Institute of Health (NIH) (https: //www.ncbi.nlm.nih.gov). A search in the PubChem Open Chemistry Database resulted in 14 3D structured active compounds of *Eleutherine americana* L Merr, namely: eleutherinone/eleutherinol (CID 15559106), dihydroeleutherinol (CID 102473740), eleutherol (CID 120697), eleutherine (CID 10166), elecanacin (CID: 102091822), isoeleutherol (CID 10800314), isoeleutherine (CID 10445924) eleutheroside A (CID 101855622), eleutheroside B (CID 95224384), anthraquinone (CID 6780), naphthol (CID 8663), naphthoquinone (CID 8530), hongconin (CID 110108147) and triterpenoid (CID 451674). The 3D structures of active components in *Eleutherine americana* L Merr were obtained in the form of *.sdf file format and then converted to *.pdb file using OpenBabel software (O'Boyle *et al*., 2011). The target protein 3D Structure Modeling was predicted using the SWISS-MODEL web server (Arnold *et al*., 2006; Kiefer *et al*., 2009: Setiawan *et al*., 2019) with the homology modeling method, and validated using Ramachandran plot analysis. Docking simulations between the active components of *Eleutherine americana* L Merr and target proteins used HEX 8.0 software (Macindoe *et al*., 2010). The three stages docking protocol of visualization was the minimization of rigid-body energy, semi-flexible repairs, and finishing refinement in explicit solvents. The docking results were visualized with Chimera 1.6.2 software and Discovery Studio 4.1 (Setiawan *et al*., 2019).

The docking analysis was visualized using Discovery Studio 4.1, LigPlot + software (Laskowski *and* Swindells, 2011), and LigandScout 3.1 (Wolber *and* Langer, 2005). Interactions between proteins and ligands were analyzed to assess the number and type of bonds, namely hydrogen bonds, hydrophobic bonds, and van der Waals bonds. Pharmacophore analyzed and saw the residues that directly involved in the interaction, energy minimization analysis. Pharmacophore also improves the structure and shape of molecules at the time of interaction.

RESULTS

The LCMS Dayak onion crude extract study found 3 dominant compounds, namely, isoeleutherol (m/z 244.50; rt 4.00), eleutherol (m/z 245.00; rt 4.53) and eleutherine (m/z 272.00; rt 4.53) (Figure 1).

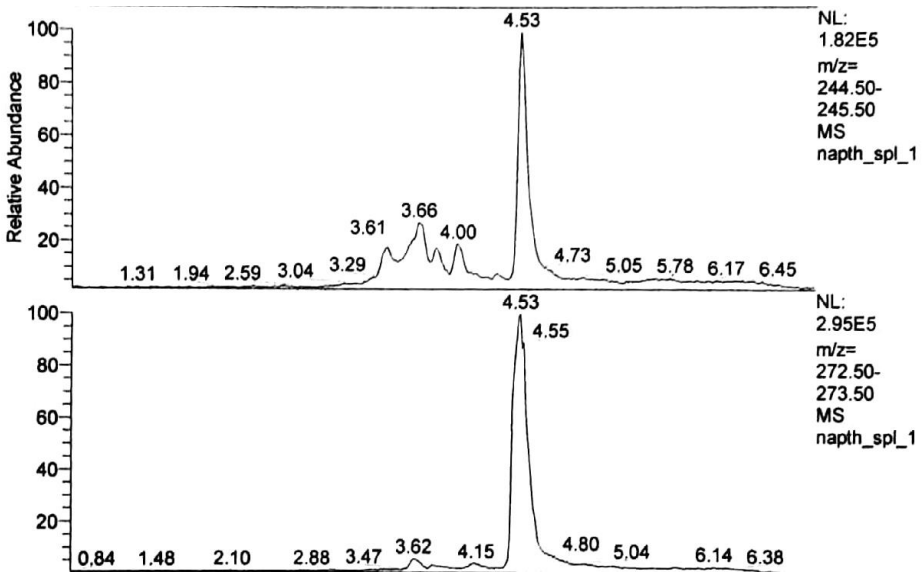
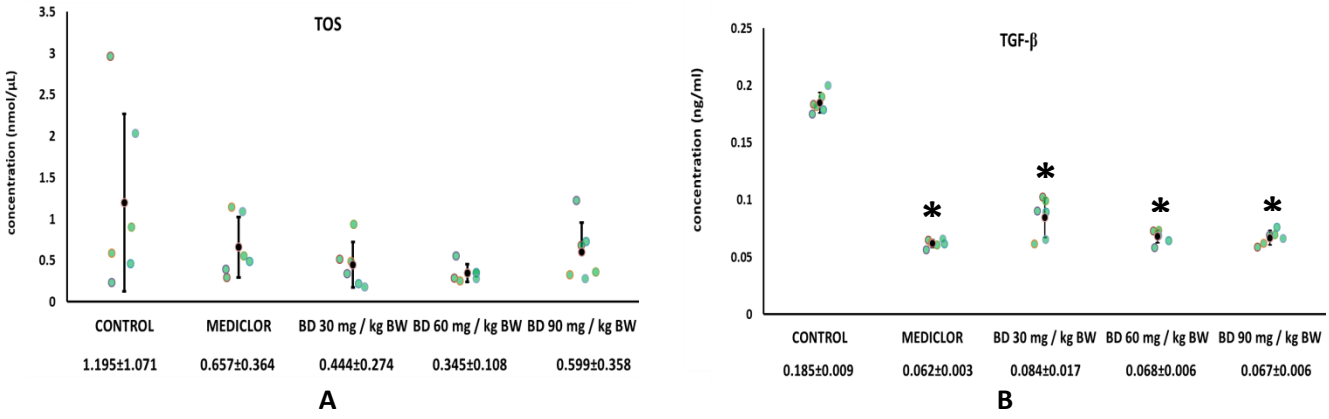


Figure. 1 **The LCMS analysis of 14 active compounds in Dayak onions:** isoeleutherol at rt 4.53 and eleutherol at rt 4.55 (bottom panel); eleutherine at rt 4.53 (top panel).

Total Oxidant Status (TOS)

There was a reduction in TOS levels in the treatment groups, but the difference was not statistically significant. Sequentially the TOS levels in the control, mediclor, Dayak onion 30 mg/kg, Dayak onion 60 mg/kg and Dayak onion 90 mg/kg were 1.195 ± 1.07, 0.657 ± 0.364, 0.444 ± 0.274, 0.345 ± 0.108, 0.599 ± 0.358 nmol / µL ($p > 0.05$), respectively (Figure 2A).



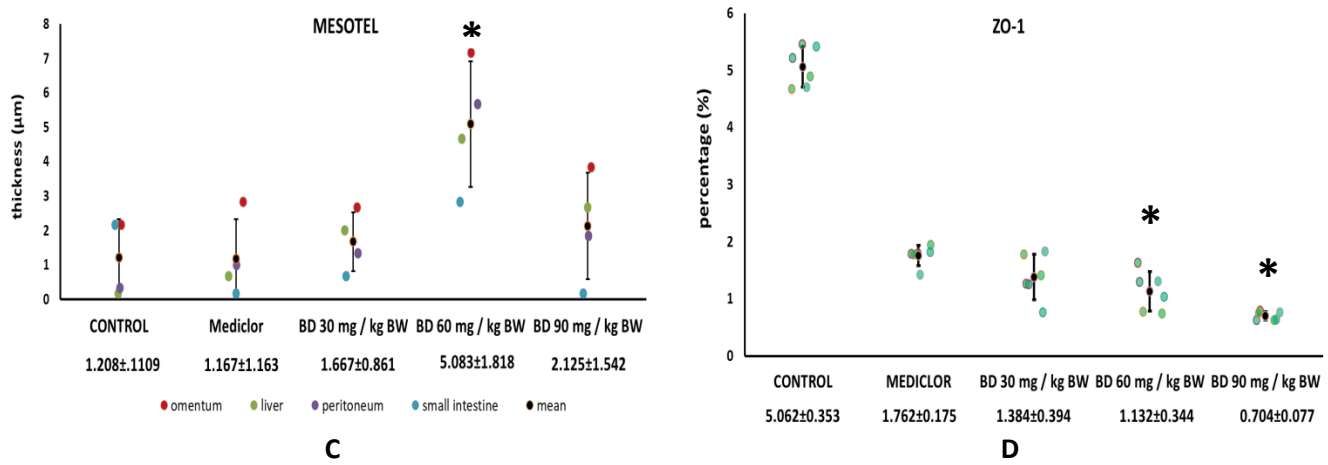


Figure 2 **A) TGF-β profile, B) TOS profile, C) Mesothelial cell profile,** N = 6, with the Welch Robust Test of Equality of Means and post-hoc Games-Howell test, *p < 0.05 vs. control. **D) ZO-1 expression profile,** N = 6, with ANOVA and post-hoc LSD test, *p < 0.05 vs control.

Transforming Growth Factor-β (TGF-β) expression

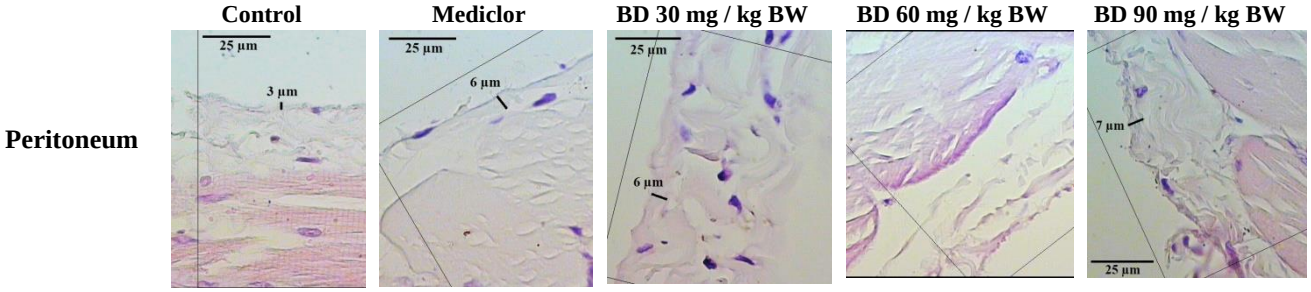
There was a significant reduction of TGF-β levels in four other groups compared to the control. The levels of TGF-β in control, mediclor, Dayak onion 30 mg/kg, Dayak onion 60 mg/kg, Dayak onion 90 mg/kg were 0.185 ± 0.009, 0.062 ± 0.003, 0.084 ± 0.017, 0.068 ± 0.006, 0.067 ± 0.006 (ng/ml), respectively ($p < 0.05$) (Figure 2B).

Mesothelial Cell Detachment

There was an increase in intact mesothelial cells number after administration of 60 mg/kg Dayak onions. The number of intact mesothelium were 1.208 ± 1.109, 1.167 ± 1.163, 1.167 ± 0.861, 5.083 ± 1.818, 2.125 ± 1.542 (µm) in the control group, mediclor, Dayak onion 30 mg/kg, Dayak onion 60 mg/kg, Dayak onion 90 mg/kg, respectively ($p < 0.05$) (Figure 2C; Figure 3).

Zone Occludin-1 (ZO-1) expression

There was a significant reduction of ZO-1 expression after the administration of Dayak onions 60 mg/kg and 90 mg/kg body weight. The ZO-1 expression were 5.062 ± 0.353, 1.762 ± 0.175, 1.384 ± 0.394, 1.132 ± 0.344, 0.704 ± 0.077 (%) in the control, mediclor, Dayak onion 30 mg/kg, Dayak onion 60 mg/kg, Dayak onion 90 mg/kg, respectively ($p < 0.05$) (Figure 2D; Figure 4).



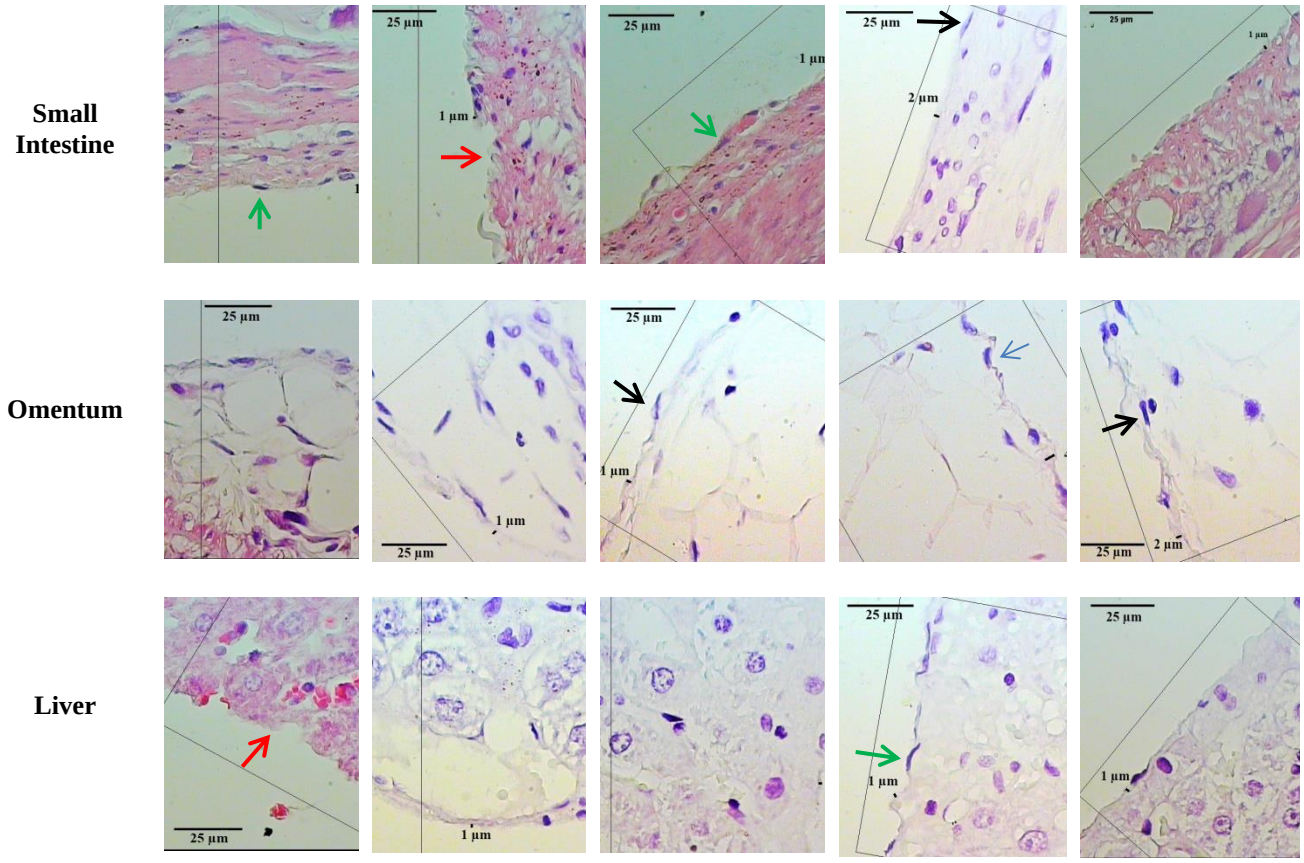
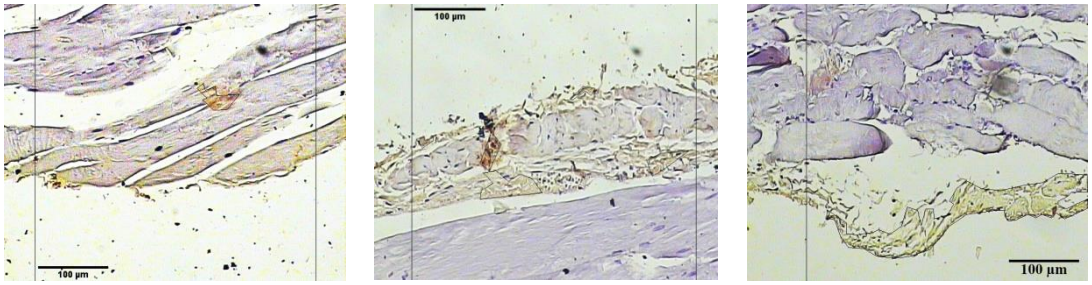
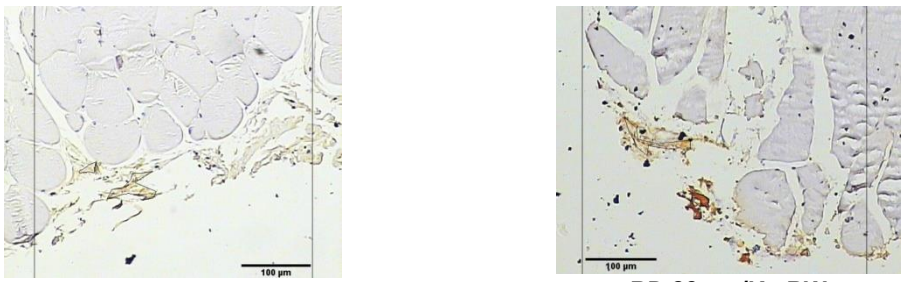


Figure 3 **The mesothelial cell numbers**. There was an increase in intact mesothelial cells after Dayak onions extract administration. Black arrows for intact, red arrows for the damage and green arrows indicate the normal mesothelial cells.



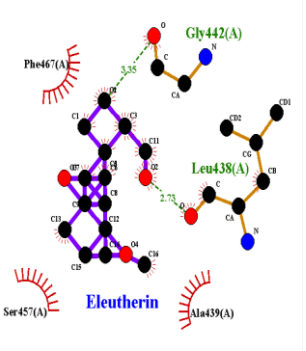
Control Mediclor BD 30 mg/Kg BW



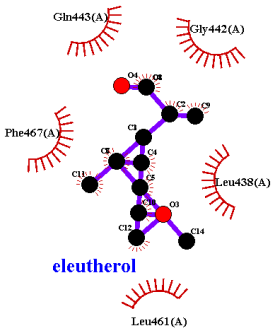
BD 60 mg/Kg BW BD 90 mg/Kg BW

Figure 4 **The ZO-1 expression**. Dayak onions extract 60 mg/Kg BW administration reduced ZO-1 expression and strengthened mesothelial cells bonds.

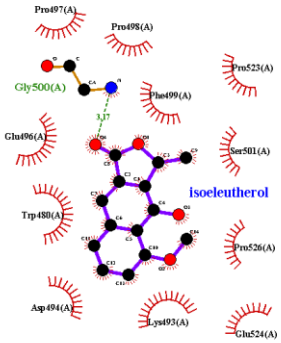
IN-SILICO ANALYSIS



TGF-β – TGF-βR
E total: -1124.66 kJ/mol

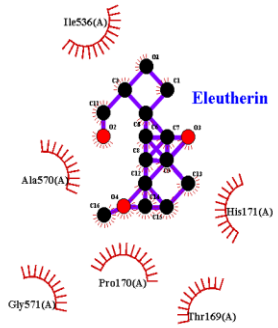


TGFβ-TGFβR, eleutherin
E total: -1030.10 kJ/mol
(Gly442, Leu438, Ala439. Ser457, Phe467)

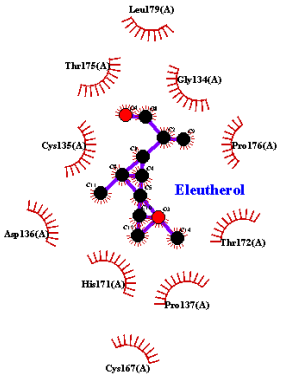


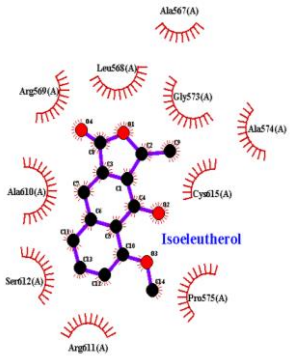
TGFβ-TGFβR, eleutherol
E total: -949.17 kJ/mol
(Gly442, Leu438, Leu461, Phe467, Gln443)

TGFβ-TGFβR, isoeleutherol
E total: -1034.32 kJ/mol
(Gly500, Pro497, Pro498, Phe499, Pro523, Ser501, Pro526, Glu524, Lys493, Asp494, Trp480, Glu496)



NFkB/IkB-IKK
E total: -363.5 kJ/mol

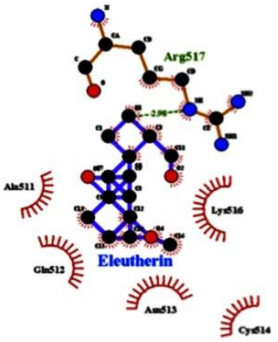




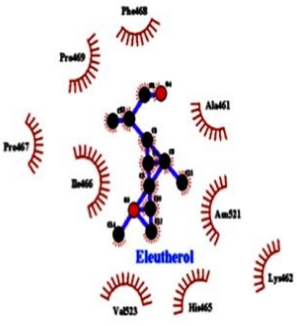
NFκB/IkB-IKK-eleutherine
E total: -366.51 kJ/mol
(Ile536, Ala570, Gly571, Pro170, Thr169, His171)

NFκB/IkB-IKK-eleutherol
E total: -362.48 kJ/mol
(Leu179, Thr175, Cys135, Asp136, His171, Cys167, Pro176, Gly134)

NFκB/IkB-IKK-isoeleutherol
E total: -362.55 kJ/mol
(Leu568, Ala567, Gly573, Cys615, Pro575, Arg611, Ser612, Ala610, Arg569)



NRF2/Keap1-Ub
E total: -658 kJ/mol



NRF2/Keap1-Ub-eleutherine
E total: -624.53 kJ/mol
(Arg517, Ala511, Gln512, Asn513, Cys514, Lys516)

NRF2/Keap1-Ub-eleutherol
E total: -629.31 kJ/mol
(Ile466, Pro469, Phe468, Pro467, Val523, His465,

NRF2/Keap1-Ub-isoeleutherol
E total: -647.90 kJ/mol
(Lys516, Cys514, Arg515, Asn513, Ala511, Gln512,

Figure 5 **The in silico Analysis of Dayak onions.** The Dayak onion's anti-inflammatory inhibited the binding of TGF-β with the receptor, which is characterized by an increase in binding energy. The anti-oxidant capacity through inhibition of NFR2 proteasome degradation and characterized by a decrease in the NRF2-Keap1complex binding energy.

Inflammation is a response to tissue damage marked by vasodilation and the recruitment of immune cells and plasma proteins to the site of infection (Chen *et al*., 2018). TGF-β increases in inflammation. The transforming growth factor-beta (TGF-β) pathway induces the epithelial transition to mesenchyme and plays an essential role during inflammation (Frangogiannis, 2017) Our study found that binding of the active compound *Eleutherine americana* L Merr to TGF-βR can inhibit the binding of TGF-β by increasing the binding energy of interactions.

In addition to the TGF-β pathway, our study examined the potential activity of *Eleutherine americana* L Merr on the activity of nuclear factor-kB (NF-kB), a transcription factor involved in different processes of the immune and inflammatory responses. Upon activation, IKK phosphorylates IkB and thereby triggers ubiquitin-dependent degradation, resulting in transient nuclear translocation of NF-kB (Liu *et al*., 2017). Our in-silico study showed that the binding of IKK to NF-kB/ IkB complex required binding energy about -363.5 kJ/mol, meanwhile in the presence of eleutherine, the binding energy reduced to -366.51 kJ/mol and increased in the presence of eleutherol (-362.48 kJ/mol) and isoeleutherol (-362.55 kJ/mol).

The reactive oxygen species (ROS) overproduction generates oxidative stress in cells and resulting in numerous pathophysiological conditions. The Keap1-Nrf2 is a signaling pathway that plays an essential role in protecting cells against oxidative stress. Nrf2 is negatively regulated by Keap1, which targets Nrf2 for ubiquitination and degradation (Villeneuve *et al*., 2010). Our study found that the binding of eleutherine, eleutherol, and isoeleutherol to Nrf2 increases the binding energy for ubiquitin (Ub) to binds to Nrf2/Keap1 complex. In the absence of the active compounds of *Eleutherine americana* L Merr, binding of Ub to Nrf2/Keap1 complex require -658 kJ/mol, meanwhile, in the presence of eleutherine, eleutherol, and isoeleutherol, the binding energy increased to -624.53 kJ/mol, -629.31 kJ/mol, and -647.90 kJ/mol, respectively. This result suggested that the active compounds of *Eleutherine americana* L Merr could support the Nrf2 activity by inhibiting the degradation of Nrf2 at the proteasome level pathway.

DISCUSSION

Based on the previous studies, laparoscopy causes oxidative stress (Cevrioglu *et al*., 2004; Baysal *et al*., 2009; Veekash *et al*., 2010). By Hagen-Poiseuille's law, CO_2 insufflation will reduce the diameter of blood vessels, then oxygen flow in desufflation causes ROS formation (reperfusion injury). Oxidative stress may lead to mesothelium damage. Laparoscopy at 10 mmHg blocks redox signaling and causes tissue damage (Raffaeli *et al*., 2018). Antioxidant mechanism of action of natural ingredients is generally through the activation of the Keap1/Nrf2 pathway (Babu *et al*., 2017, Sznarkowska *et al*., 2017), hydrogen donors (scavenging ROS) by hydroxyl chains and electron acceptors by oxygen chains. Our in-silico study proved that the Dayak onions have the antioxidant capacity in degrading the Keap1-Nrf2 complex. The migration of Nrf2 to the nucleus increases the formation of antioxidant genes.

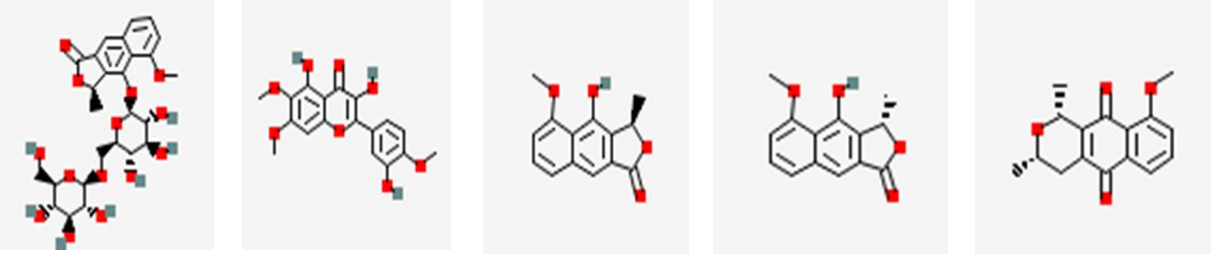


Figure 6 **Electron acceptor and ROS scavenging mechanism.** The antioxidant ability of Dayak onions through hydrogen donors (scavenging ROS) by hydroxyl chains ( ) and electron acceptors through oxygen chains (and). Eleuthoside B and flavonoids have more hydroxyl and oxygen chains. They have a higher antioxidant ability. Contrast to eleutherol, iso-eleutherol and eleutherine contain fewer of these chains (The PubChem Open Chemistry Database)

Pneumo-peritoneum, which is higher than portal venous pressure, results in hypoxia, reperfusion injury, oxidative stress (Sammour *et al*., 2009, Patel *and* Yadav, 2016), and abdominal cavity cell injury (Sammour *et al*., 2009). As a response to cell injury, our body activates the inflammatory response to eliminate damaged cells (Arung *et al*., 2011, Sammour, 2011). Inflammatory resonance triggers TGF-β production (Maciver *et al*., 2011; Sammour, 2011). ROS activates and induces TGF-β gene expression. Instead, TGF-β triggers ROS (Liu *and* Desai, 2015). Mesothelial system damage secretes Damage Associated Molecular Patterns (DAMPs) and triggers an inflammatory response for homeostasis (Mueller *et al*., 2012, Baldwin *et al*., 2016). Our study proved that the administration of Mediclor and Dayak onions could reduce TGF-β levels. Dayak onions showed as a potent anti-inflammatory agent. Anti-inflammatory effects through eleutherine, eleutherol, and isoeleutherol may interact with each other in the NFkB-IkB-IKk and TGF-β pathways.

The mesothelial cells attach to the basement membrane. They are supported by MES, connective tissue, fibroblasts, blood vessels, and lymphatics (Mutsear *et al*., 2016). Hypoxia triggers anaerobic respiration resulting in ATP deficiency and disrupts the dependent ATPase cell membrane canal. This condition disrupts water, ion, and cell homeostasis (Krystel-Whittemore *et al*., 2016, Spoerl *et al*., 2017). Laparoscopy causes reperfusion injury and oxidative stress. ROS induces mesothelial cell damage through lipid peroxidation, protein peroxidation, and DNA peroxidation (Sammour, 2011). Similar to previous research, Similar to previous research, laparoscopy triggers detachment (Peng *et al*., 2009) and damage of the mesothelial cells (Neuhaus *et al*., 2001, Davey *et al*., 2013). Our study proved there was a significant increase in intact mesothelial cells. Dayak onions administration may reduce TGF-β levels and decrease ROS levels. Attach mesothelial cell is characterized by decreased ZO-1 expression.

CONCLUSION

Our study proved that there was a significant decrease in the TGF-β level and an increase in intact mesothelial cell count after Dayak onion administration. Dayak onions gave orally take a longer time to achieve the anti-inflammatory and antioxidant effects. For future research, assessment of the same effects of Dayak onion extract given intra-peritoneal needs to be studied, since the surgery conducted in humans is unpredictable. Studies about the mechanism of Dayak onions on inhibition of Nrf2 degradation in proteasome levels needs to be done.

Declaration

Ethics approval and consent to participate

This research has been approved by the Animal Experimentation Ethical Committee, Research Center, Faculty of Medicine, Universitas Lambung Mangkurat, Banjarmasin, Indonesia (No.282/KEPK-

FK.UNLAM/EC/VII/2019). The experiments were conducted in the Chemical/Biochemical Laboratory, Faculty of Medicine, Universitas Lambung Mangkurat, Banjarmasin, Indonesia.

Consent for publication
Not applicable.

Availability of data and material
The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare no conflicts of interest.

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Authors' contributions
HP, AY, FRPD, KNB, KM, ASB, NSP, NA, GF, AF, AR, EE, DA, and ZN conceived the study. HP, AY, and FRPD drafted the manuscript, KNB, KM, ASB, NSP, NA, GF, AF, AR, EE, DA, and ZN critically revised the manuscript for valuable intellectual content. All authors read and approved the script. They agreed to be accountable, ensuring that questions related to the accuracy or integrity of any part of the work appropriately investigated and resolved.

Acknowledgements

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