



# Dayak Onions (*Eleutherine americana* L Merr) Reduced Mesothelial Cell Detachment After Laparoscopy in Rats

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## Abstract

**BACKGROUND:** Laparoscopy induces changes and detachment of mesothelial structure. Studies on the prevention of mesothelial cell detachment are rarely found. The Dayak tribe uses the Dayak onion (*Eleutherine americana* L. Merr) as a wound-healing agent due to its anti-inflammatory and antioxidant activities.

**AIM:** This study aimed to prove the anti-inflammatory and antioxidant activities of Dayak onions in preventing mesothelial cell damage after laparoscopy.

**MATERIALS AND METHODS:** Thirty male Sprague-Dawley rats were classified into five groups (n = 6 per group), namely: (a) Control, (b) Mediolore, (c) Dayak onion, 30-, (d) 60-, and (e) 90 mg/kg body weight, respectively. The transforming growth factor-beta (TGF- $\beta$ ) and total oxidant status (TOS) in the peritoneal fluid were determined 24 h after laparoscopy. Histopathological analysis of mesothelial cell numbers and the protein Zone Occludin-1 (ZO-1) expression in the peritoneum, small intestines, greater omentum, and liver was performed 7 days after the procedure. An *in silico* study was conducted to analyze the anti-inflammatory effects of the components of Dayak onions.

**RESULTS:** The *in silico* study showed that one of the Dayak onion active compounds, eleutherine, had a potential anti-inflammatory effect and acted as a modulator of TGF- $\beta$ . Following Dayak onion administration, the TGF- $\beta$  level, the number of mesothelial cell detachments, and ZO-1 expression were all significantly reduced (p < 0.05), whereas the TOS level was not (p > 0.05).

**CONCLUSIONS:** Our study showed that Dayak onion administration reduced TGF- $\beta$  level, number of mesothelial cell detachment, and ZO-1 expression following laparoscopy.

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## Introduction

Laparoscopy is becoming more popular, especially with the invention of robotic surgery [1], [2]. CO<sub>2</sub> insufflation during laparoscopy caused alterations in mesothelial structure, according to a recent study [3]. Extracellular matrix (ECM) exposure and mesothelial cell detachment are both caused by laparoscopy [4].

The mesothelium is a major structure that covers the whole-body surface and is responsible for the protection, transport, and immunology of the abdominal cavity [5]. Mesothelial cells are damaged by insufflation [2]. The protein Zone Occludin-1 (ZO-1) is responsible for the formation of the mesothelial cell junction [6]. ZO-1 structural alterations and cellular disruption are triggered by reactive oxygen species (ROS) and inflammatory reactions. When mesothelial cell detachment does not heal correctly, adverse effects

result [3]. There are few studies concerning how to avoid mesothelial cell detachment.

The Dayak tribe has been utilizing Dayak onions (*Eleutherine americana* L. Merr) as a wound-healing remedy (*bahimang*) for centuries [7]. The major goal of the research was to show that Dayak onions can prevent mesothelial cell damage, and the secondary goal was to show that they had anti-inflammatory and antioxidant properties following laparoscopy.

## Materials and Methods

The experimental and *in silico* phases of this investigation were conducted separately. The experimental study followed the ethical guidelines for animal research, and the Animal Experimentation Ethical

Committee, Research Center, Faculty of Medicine, Universitas Lambung Mangkurat, Banjarmasin, Indonesia (No.282/KEPK-FK.UNLAM/EC/VII/2019) granted ethical approval to both experiments. The tests were done in the Faculty of Medicine, Universitas Lambung Mangkurat, Banjarmasin, Indonesia, in the Chemical/Biochemical Laboratory. The *in silico* research was conducted at Universitas Airlangga's Department of Biology, Faculty of Science and Technology, Surabaya, Indonesia.

### **Dayak onions extract preparation**

In the Pulang Pisau district of Central Kalimantan, Dayak onion bulbs were collected at 12 weeks. The UPT Materia-Medica Batu in Malang, East Java, Indonesia, determined the species of the *E. americana* L. Merr bulbs. The simplicia was used to produce an ethanol extract of Dayak onion bulbs. Liquid chromatography-mass spectrometry (LCMS) methods were used to macerate and analyze the simplicia at the Laboratory of Polytechnic Chemical Engineering Malang. The active compounds of *E. americana* L. Merr were determined using the LCMS. Separating ionized extracts into static and mobile phases is the fundamental idea. The mass-to-charge ratio (*m/z*) and retention time (*rt*) of active compounds were measured and compared to those of known compounds [8].

### **Animal model experiments**

Sprague-Dawley rats (male, 200–250 g body weight, 20–25-weeks-old) from the Abadi Jaya farm in Yogyakarta, Indonesia, were utilized in this investigation. According to the Federer formula, thirty male rats were used [9]. Rats were chosen because their physiological characteristics are comparable to those of humans. Because male rats have a larger blood capacity, they can escape the estrus phase. The experimental animals were housed and bred in accordance with animal research guidelines (3R5F) [10]. The rats were given a 7-day acclimation period before being randomly assigned to one of five treatment groups (all *n* = 6): (a) Control, (b) Mediclore, (c) Dayak onion 30 mg/kg, (d) Dayak onion 60 mg/kg, and (e) Dayak onion 90 mg/kg body weight. The study's ill and dead rats were replaced with healthy rats. Mediclore (CGBO-C1025061523-Korea) is a commercial intra-abdominal adhesion, sol-gel transfer formula including poloxamer-chitosan, a water-soluble polymer, a highly biocompatible solution, and it was administered intraperitoneally soon after laparoscopy.

Laparoscopy was conducted 1 h later using CO<sub>2</sub> and automated insufflators (Gimmi, Gimmi® GmbH, Germany, 2000) [11]. Povidone-iodine is used in aseptic operations. Intramuscular ketamine hydrochloride (KTM-10; PT Guardian Pharmatama, No. Reg. DKL0408013443B1) was used in anesthesia at a dose of 10 mg/kg body weight. The control group was insufflated

at a pressure of 10 mmHg, which is appropriate for rats and similar to 15 mmHg pressure in people [12]. The intervention group was also given a 10 mmHg inhalation. Mediclore was administered intraperitoneally at a dose of 5 mg/kg body weight just after surgery. Dayak onion extract was administered orally to the Dayak onion 30, 60, and 90 groups at doses of 30, 60, and 90 mg/kg body weight, respectively. Before surgery, the Dayak onion extract was taken daily for 54 days [13].

The total oxidant status (TOS) and transforming growth factor-beta (TGF- $\beta$ ) levels in the peritoneal fluid were determined after 24 h. The Cloud Clone ELISA (Enzyme-linked immunosorbent assay) kit for TGF- $\beta$ 1 species of *Rattus norvegicus* was used to determine TGF- $\beta$  levels (SEA124Ra). The Biovision Hydrogen Peroxide Colorimetric/Fluorometric Assay Kit was used to measure TOS using the colorimetric technique (K265-200). On the 7<sup>th</sup> day, the rats were anesthetized and euthanized through neck dislocation. (<http://risetcenterfk.ulm.ac.id/euthanasia/>). Hematoxylin-Eosin specimens from the liver, parietal peritoneum, small intestine, and omentum were assessed in 1 M2 (100 magnification) by three blinded pathologists who were unaware of the group allocation. The average was derived after counting the mesothelial cells. Anti-ZO-1 tight junction antibodies (Santa Cruz ZO-1 antibody Sc-33725) were used to assess ZO-1 expression in parietal peritoneum tissues, and ImageJ software was used to quantify it. TGF- $\beta$  was stated in ng/ml, TOS in nmol/ $\mu$ l, mesothelial cell count in cells/ $\mu$ m<sup>2</sup>, and ZO-1 expression in % at 1  $\mu$ m<sup>2</sup>.

### **Statistical analysis**

The data were displayed as mean  $\pm$  standard deviation, and the values of the groups were compared. The Kolmogorov–Smirnov or Shapiro–Wilk test, as well as Levene's test of variance, was used to determine the normality and homogeneity of the distribution. One-way ANOVA and *post hoc* LSD tests were used to analyze normally distributed and homogenous data. The Welch Robust Test of Equality of Means and the *post-hoc* Games–Howell test were utilized if the data were normally distributed but not homogeneous. Kruskal–Wallis and a *post hoc* Mann–Whitney test were performed if the data were not normally distributed. The study was conducted using IBM SPSS version 23.0 and Microsoft Excel 2010, with a 95% confidence level (CI) and significance set as *p* < 0.05.

### **In silico study**

The study began by searching for amino acid sequences and the structures of active components of Dayak onions (*E. americana* L. Merr). The amino acid sequences of nuclear factor erythroid 2/Nrf2 (GI: 693842), Kelch-like erythroid cell-derived protein-1/Keap1 (GI: 22027642), ubiquitin (Ub)

(GI: 340068), NF- $\kappa$ B (GI: 1018443262), inhibitor of nuclear factor kappa B ( $\text{I}\kappa\text{B}$ ) kinase-b(IKK-b) (GI: 4185275), I- $\kappa$ B kinase-a (IKK-a) (GI: 4185273), TGF- $\beta$  (GI: 339564), and TGF- $\beta$  receptor (TGF- $\beta$ r) (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 *E. 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) eletheroside A (CID 101855622), eletheroside 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 *E. americana* L Merr were obtained in the form of \*.sdf file format and converted to \*.pdb file using OpenBabel software [14]. The target protein 3D Structure Modeling was predicted using the SWISS-MODEL web server [15], [16], [17] with the homology modeling method and validated using Ramachandran plot analysis. Docking simulations between the active components of *E. americana* L Merr and target proteins used HEX 8.0 software [18]. The three stages of docking visualization protocol were 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 [17].

The docking analysis was visualized using Discovery Studio 4.1, LigPlot + software, [19] and LigandScout 3.1 [20]. Interactions between proteins and ligands were analyzed to assess the number and type of bonds, namely, hydrogen, hydrophobic, and van der Waals bonds. The pharmacophore was analyzed and observed the residues directly involved in the interaction and energy minimization analysis. Pharmacophores also improve the structure and shape of molecules at the time of interaction.

## Results

The LCMS Dayak onion crude extract study found three 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).

### TOS

There was a reduction in TOS levels in the treatment groups, but the difference was not

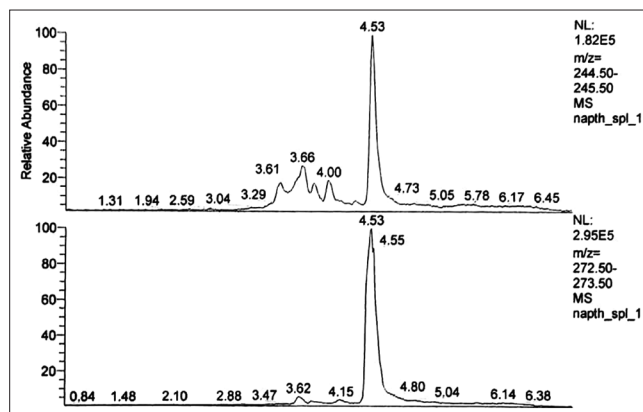


Figure 1: The LCMS analysis of 14 active compounds in Dayak onions: isoeleutherol at rt 4.53 and eleutherol at rt 4.55 (bottom panel); eleutherine at rt 4.53 (top panel)

statistically significant. TOS levels were 1.195 1.071, 0.657 0.364, 0.444 0.274, 0.345 0.108, and 0.599 0.358 nmol/L ( $p > 0.05$ ) in the control, Mediclore, Dayak onion 30 mg/kg, Dayak onion 60 mg/kg, and Dayak onion 90 mg/kg, respectively (Figure 2a).

### TGF- $\beta$ expression

TGF- $\beta$  levels were significantly lower in four treatment groups compared to controls. The TGF- $\beta$  levels were 0.185 0.009, 0.062 0.003, 0.084 0.017, 0.068 0.006, and 0.067 0.006 (ng/ml) in the control, Mediclore, Dayak onion 30 mg/kg, Dayak onion 60 mg/kg, and Dayak onion 90 mg/kg, respectively ( $p < 0.05$ ) (Figure 2b).

### Mesothelial cell detachment

There was a significantly higher number of undetected mesothelial cells after the administration of 60 mg/kg Dayak onion. The number of undetached mesothelial cells was  $1.208 \pm 1.109$ ,  $1.167 \pm 1.163$ ,  $1.167 \pm 0.861$ ,  $5.083 \pm 1.818$ , and  $2.125 \pm 1.542$  ( $\mu\text{m}$ ) in the control group, Mediclore, and Dayak onion 30 mg/kg, Dayak onion 60 mg/kg, and Dayak onion 90 mg/kg, respectively ( $p < 0.05$ ) (Figure 2c and 3).

### ZO-1 expression

There was a significantly lower ZO-1 expression after Dayak's onion administration, 60 mg/kg, and 90 mg/kg body weight. The ZO-1 expression was  $5.062 \pm 0.353$ ,  $1.762 \pm 0.175$ ,  $1.384 \pm 0.394$ ,  $1.132 \pm 0.344$ , and  $0.704 \pm 0.077$  (%) in the control, Mediclore, and Dayak onion 30 mg/kg, Dayak onion 60 mg/kg, and Dayak onion 90 mg/kg, respectively ( $p < 0.05$ ) (Figure 2d and 4).

### In silico study

Vasodilation, immune cells, and plasma protein recruitment are all signs of inflammation in response to



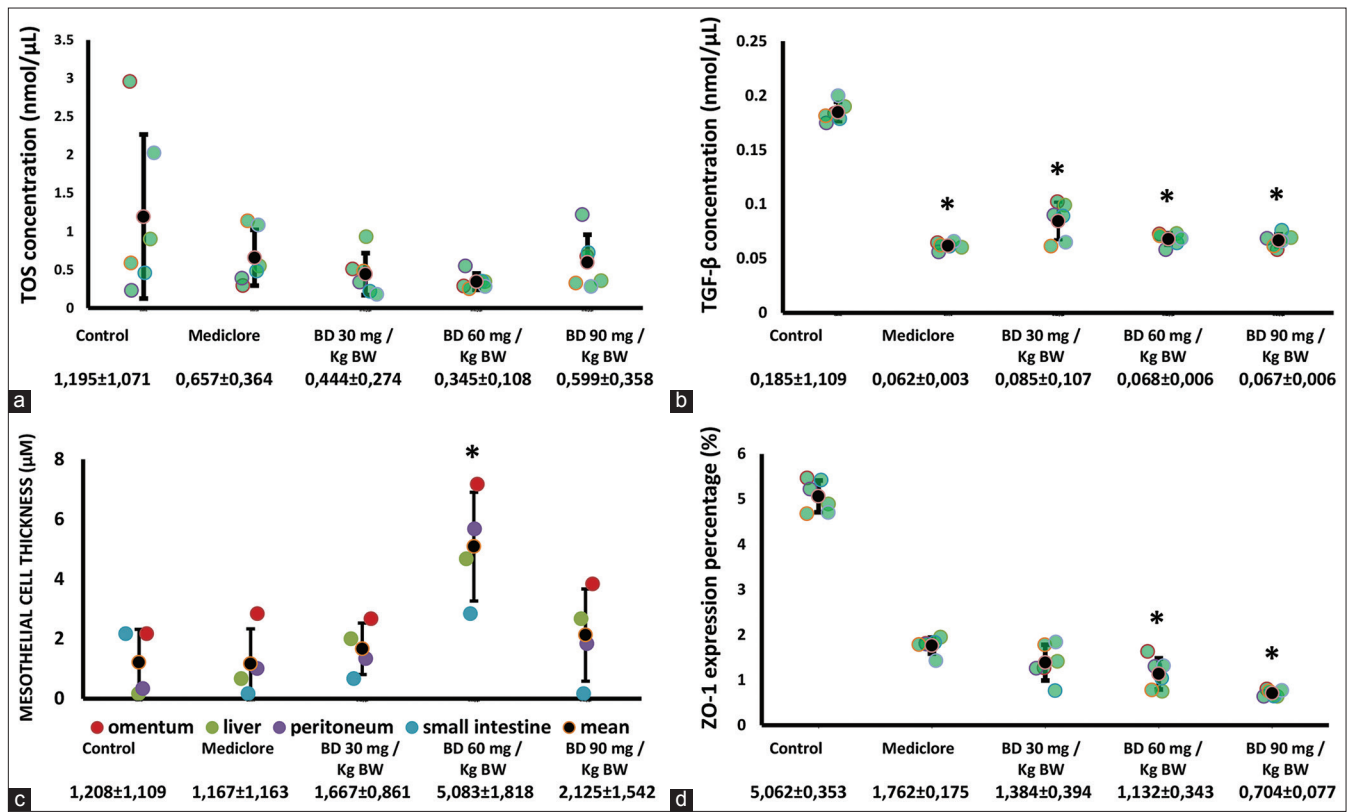


Figure 2: (a) TOS profile, (b) TGF-β profile, (c) Mesothelial cell profile, n = 6, with the Welch Robust Test of Equality of Means and post hoc Games-Howell test, \*p < 0.05 versus control. (d) ZO-1 expression profile, n = 6, with ANOVA and post hoc LSD tests, \*p < 0.05 versus control

tissue injury [21]. The epithelial to mesenchymal transition is induced by inflammation and the TGF-β pathway,

which plays a crucial role during inflammation [22]. According to our findings, the active components of *E.*

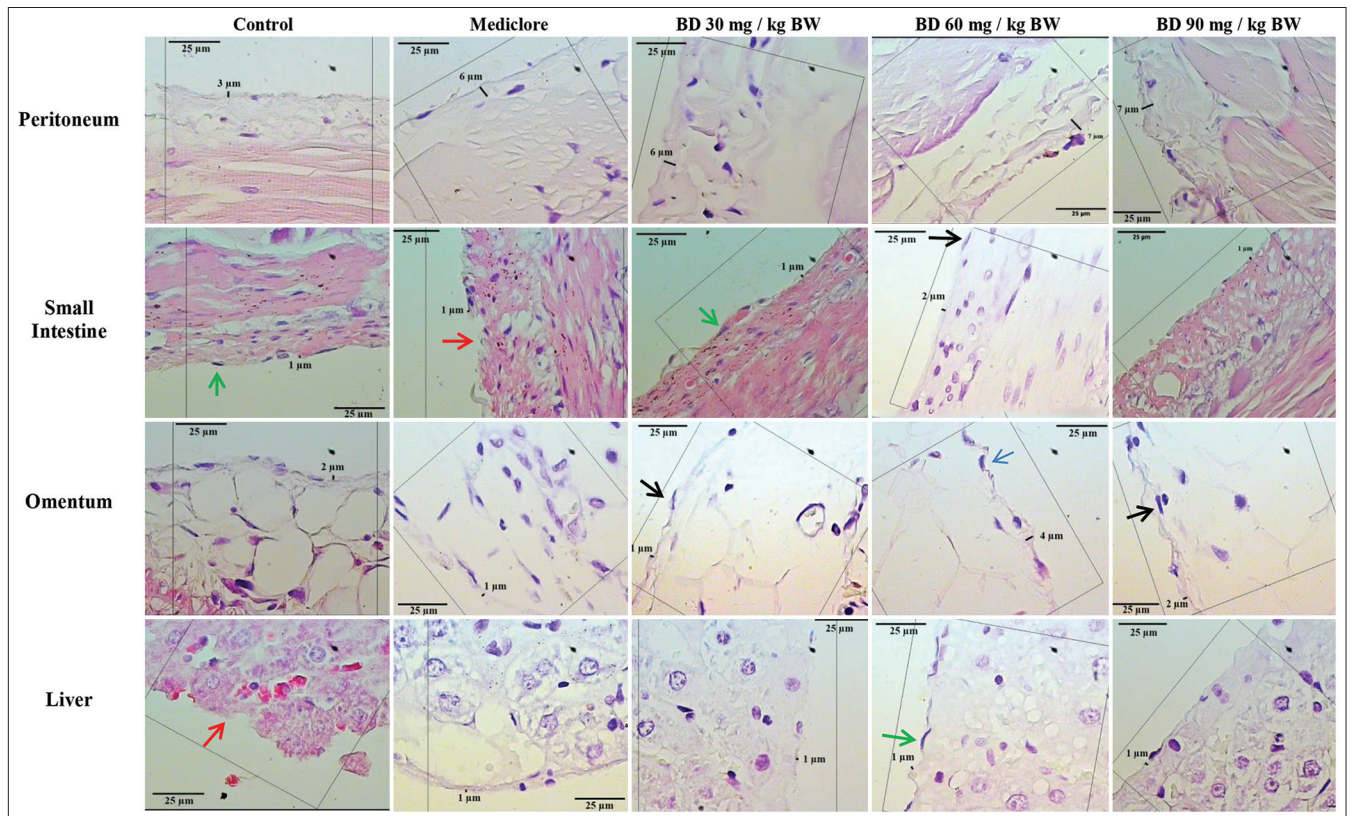


Figure 3: The mesothelial cell counts. There was an increase in intact mesothelial cell counts after the administration of Dayak onion extract. Black arrows indicate the intact cells, red arrows indicate the damaged cells, and green arrows indicate the healthy mesothelial cells

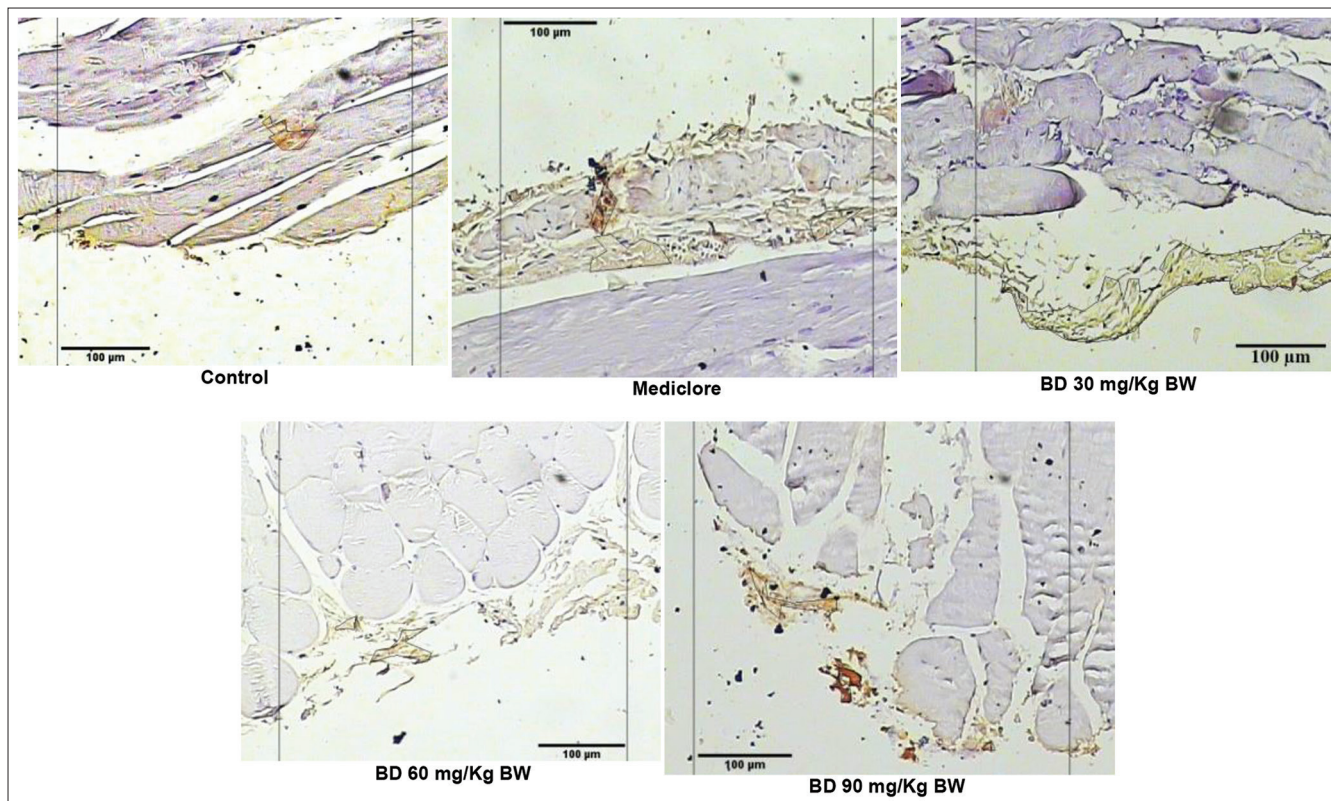


Figure 4: The ZO-1 expression. The administration of Dayak onion extract 60 mg/kg reduced ZO-1 expression (in brown) and strengthened mesothelial cell bonds

*americana* L. Merr inhibit TGF-R binding by raising the binding energy of contacts (Figure 5).

We studied the influence of *E. americana* L. Merr on the activity of nuclear factor-kappa beta (NF- $\kappa$ B), a transcription factor implicated in many immunological and inflammatory responses, in addition to the TGF- $\beta$  pathway.

I kappa B kinase (IKK) phosphorylates I $\kappa$ B on activation, resulting in Ub-dependent degradation and temporary nuclear translocation of NF- $\kappa$ B [23]. The binding energy of IKK to the NF- $\kappa$ B/I $\kappa$ B complex was around -363.51 kJ/mol in the presence of eleutherine, and it increased in the presence of eleutherol (362.48 kJ/mol) and isoeleutherol (362.55 kJ/mol).

Overproduction of ROS causes oxidative stress in cells, resulting in a variety of pathophysiological diseases. The Keap1-Nrf2 signaling pathway protects cells from oxidative stress and is crucial for cell survival. Keap1, which targets Nrf2 for ubiquitination and degradation, has a detrimental effect on Nrf2 [24]. The binding of eleutherine, eleutherol, and isoeleutherol to Nrf2 enhances the binding energy of Ub to the Nrf2/Keap1 complex, according to our findings. The binding of Ub to the Nrf2/Keap1 complex requires a binding energy of -658 kJ/mol in the absence of the active components of *E. americana* L. Merr. Meanwhile, the binding energy of eleutherine, eleutherol, and isoeleutherol rose to -624.53 kJ/mol, -629.31 kJ/mol, and -647.90 kJ/mol, respectively, in the presence of eleutherine, eleutherol, and isoeleutherol. These

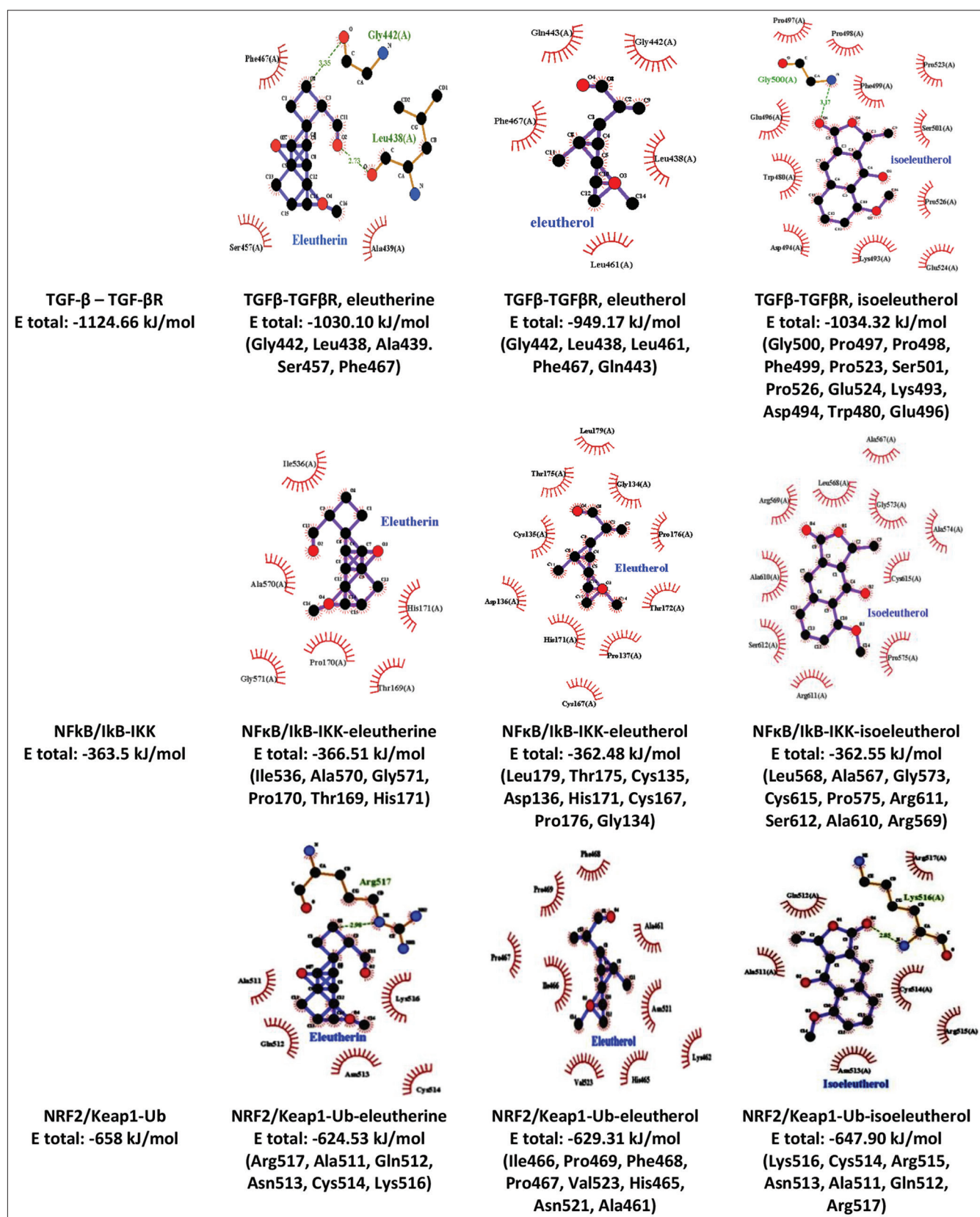
findings show that the active chemicals in *E. americana* L. Merr might help Nrf2 function by preventing it from being degraded at the proteasome level.

## Discussion

Laparoscopy promotes oxidative stress, according to prior research [25], [26], [27], [28]. CO<sub>2</sub> insufflation reduces blood vessel width, while oxygen flow in desufflation induces ROS production, according to Hagen's law. Poiseuille's (reperfusion injury) damage to the mesothelium may result from oxidative stress. At 10 mmHg, laparoscopy inhibits redox signaling and damages tissues [27]. The activation of the Keap1/Nrf2 pathway [29], [30] by hydroxyl chains (scavenging ROS) and electron acceptors by oxygen chains is how the Dayak Onion antioxidant mechanism of natural components works (Figure 6). Dayak onions have the antioxidant potential to destroy the Keap1-Nrf2 complex, according to our *in silico* investigation. The production of antioxidant genes is increased when Nrf2 migrates to the nucleus.

Hypoxia, reperfusion damage, oxidative stress [31], [32], and abdominal cavity cell injury are all caused by pneumoperitoneum, which is greater than portal venous pressure [32]. Our bodies trigger the inflammatory response in response to





**Figure 5:** The *in silico* analysis of Dayak onions. The Dayak onion's anti-inflammatory effect inhibited the binding of TGF- $\beta$  with the receptor, which is characterized by an increase in the binding energy. The antioxidant capacity occurred through inhibition of NRF2 proteasome degradation and was characterized by a decrease in the NRF2-Keap1 complex binding energy

cell injury to destroy the damaged cells [33], [34]. The TGF-production is triggered by inflammatory resonance [34], [35]. TGF-gene expression is activated

and induced by ROS. Meanwhile, TGF- $\beta$  causes the production of ROS [36]. The production of damage associated molecular patterns and the activation of an

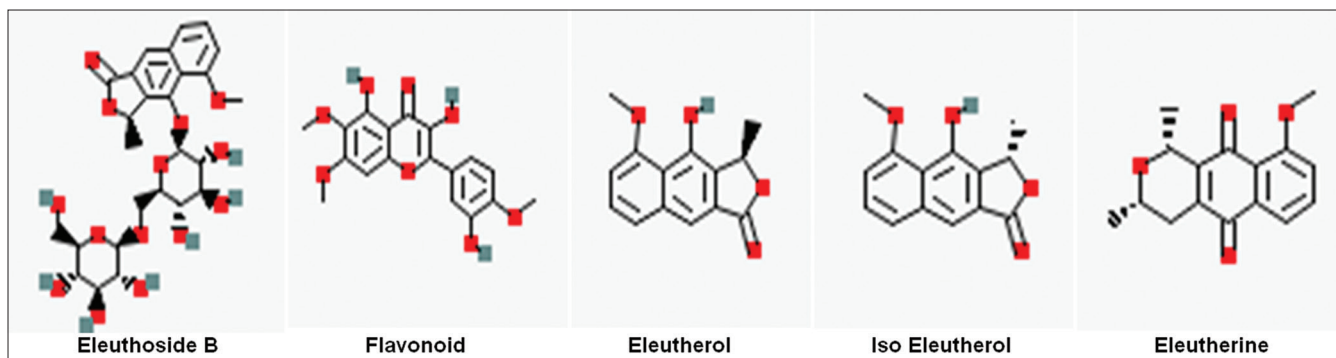


Figure 6: Electron acceptor and ROS scavenging mechanism. The antioxidant ability of Dayak onions works through hydrogen donors (scavenging ROS) by hydroxyl chains (O) and electron acceptors through oxygen chains (O). Eleuthoside B and flavonoids have more hydroxyl and oxygen chains. They have a higher antioxidant ability. Contrasting to eleutherol, iso-eleutherol and eleutherine contain fewer of these chains (The PubChem Open Chemistry Database). Different from previous studies, Eleuthoside B and flavonoids levels in the specimen Dayak onions were low

inflammatory response for homeostasis occur when the mesothelial system is damaged [37], [38]. The administration of Mediclore and Dayak onions reduced TGF- $\beta$  levels, according to our findings. The Dayak onion was demonstrated to have anti-inflammatory properties. Its anti-inflammatory actions through eleutherine, eleutherol, and isoeleutherol might interact in the NF $\kappa$ B-I $\kappa$ B and TGF- $\beta$  pathways.

ECM, connective tissue, fibroblasts, blood vessels, and lymphatics assist mesothelial cells as they adhere to the basement membrane [5]. Hypoxia causes anaerobic respiration, resulting in adenosine triphosphate (ATP) deficit and disruption of the cell membrane canal of the dependent ATPase. Water, ion, and cell homeostasis are all disrupted by this disease [39], [40]. Reperfusion damage and oxidative stress are both caused by laparoscopy. Through lipid peroxidation, protein peroxidation, and DNA peroxidation, ROS damages mesothelial cells [34]. Laparoscopy causes separation [41], as well as the killing of mesothelial cells, according to prior study [42], [43], [44]. There was a considerable increase in intact mesothelial cells, according to our findings. TGF- $\beta$  and ROS levels may be reduced when Dayak onions are consumed. Less mesothelial cell injury is associated with a reduction in ZO-1 expression.

## Conclusions

After Dayak onion administration, we discovered a substantial reduction in TGF- $\beta$  and ZO-1 expression, as well as an increase in the number of intact mesothelial cells. The anti-inflammatory and antioxidant benefits of Dayak onions were delayed when taken orally. Because human surgery can be unpredictable, the future study should focus on determining the precise effects of Dayak onion extract administered intraperitoneally. Furthermore, research

into the mechanism by which Dayak onion antioxidants prevent Nrf2 degradation at the proteasome level is required.

## Author Contributions

HP, AY, FRPD, KNB, ASB, NSP, NA, GF, AF, AR, EE, DA, and ZN compiled the research plan. HP, KNB, ASB, NSP, NA, GF, AF, AR, EE, and DA conducted the study and supervised at the laboratory. HP, AY, and FRPD compiled the manuscript. ZN and AY critically revised the manuscripts for valuable intellectual content. All writers read and agreed on the manuscript.

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