

# INCISION-WOUND HEALING ACTIVITY OF SUNFLOWER SEED OIL (*HELIANTHUS ANNUUS* L.: *IN VIVO* AND *IN SILICO* STUDY

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## Abstract

Sunflower seed oil (SFO) (*Helianthus annuus* L.) is rich in oleic acid and unsaturated fatty acids and can potentially treat various ailments. To validate the ethno-therapeutic claims of the SFO in skin diseases, the incision wound healing activity was studied. The objectives of this study were to evaluate the chemical content and incision wound-healing activities of SFO. Twenty-seven adult male Wistar rats were used, and a 2 cm length and 2 mm depth incision was made on the back of the rat. The rats were administered SFO, povidone iodine (PI), or were left untreated as a control group. Histological evaluations were conducted on postoperative wound tissue biopsies on days 0, 5 and 10. The SFO compounds were assessed using GC/MS analysis, and a subsequent *in silico* investigation was conducted based on the GC/MS findings. The binding affinity of these compounds to TNF- $\alpha$ , VEGFR1, IGFR1 and TGF- $\beta$ 1 was evaluated. On day 10, sunflower seed oil topical application accelerated wound healing by decreasing wound area and increasing wound contraction compared to untreated and PI-treated wounds. Moreover, granulation tissue expanded more, and the epidermis fully recovered. Twenty-four active compounds were detected using GC/MS, and six are responsible for lipid metabolism regulation and anti-inflammatory activity. The *in silico* study showed that 9,10-Secocholesta-5,7,10(19)-trien-3,24,25-triol (3 $\beta$ ,5Z,7E) had a high affinity of -8.5 kcal/mol for TNF- $\alpha$ . The conclusion of this study shows that SFO has incision wound healing activity *in vivo* and *in silico* by controlling the inflammatory phase and proangiogenic factors with a high affinity to bind to TNF- $\alpha$  in the skin tissue.

## Rezumat

Uleiul din semințe de floarea-soarelui (*Helianthus annuus* L.) (SFO) este bogat în acid oleic și în acizi grași nesaturați și prezintă potențialul terapeutic. Obiectivele acestui studiu au fost de a evalua conținutul chimic și efectul cicatrizant al SFO asupra rănilor provocate prin incizie. Au fost utilizați 27 de șobolani Wistar masculi adulți, iar pe spatetele fiecărui animal s-a realizat o incizie de 2 cm lungime și 2 mm adâncime. Șobolanilor li s-a administrat SFO, iod-povidonă (PI) sau au fost lăsați netratați. S-au efectuat evaluări histologice pe biopsiile postoperatorii ale țesutului plăgii în zilele 0, 5 și 10. Compușii prezenți în SFO au fost evaluați cu ajutorul analizei GC/MS, iar pe baza rezultatelor s-a efectuat o investigație *in silico*. Astfel, a fost evaluată afinitatea de legare a acestor compuși pentru TNF- $\alpha$ , VEGFR1, IGFR1 și TGF- $\beta$ 1. În ziua 10, aplicarea topică a SFO a accelerat vindecarea prin scăderea diametrului rănilor și creșterea contracției în comparație cu rănilor netratate sau tratate cu PI. Mai mult, țesutul de granulație s-a extins mai mult, iar epiderma s-a recuperat complet. Un număr de 24 de compuși activi au fost detectați cu ajutorul GC/MS, iar 6 dintre ei sunt responsabili de modularea metabolismului lipidic și de activitatea antiinflamatoare. Studiul *in silico* a arătat că 9,10-secocholesta-5,7,10(19)-trien-3,24,25-triol, (3 $\beta$ ,5Z,7E) a avut o afinitate ridicată pentru TNF- $\alpha$  (-8,5 kcal/mol).

**Keywords:** SFO, incision, wound healing, TNF $\alpha$

## Introduction

Wounds can arise from various sources, including chemical, physical, thermal, microbiological, or immunological factors. These wounds are distinguished by the disruption of cellular and anatomical integrity, as well as the impairment of protective or physiological functions within the affected tissue [1-3]. Wound healing is a normal biological response that restores injured tissues' structural and functional integrity. The process is multifaceted and dynamic, encompassing the replacement of devitalized and missing cellular structures and tissue layers [4, 5]. About 1 - 2% of

the population suffers from a chronic wound at some point in their lives, and this is especially prevalent in developing countries like Sub-Saharan Africa and Southeast Asia, where wound infections are common due to poor hygienic conditions [6]. Over 80% of the world's 14 million annual burn and wound patients reside in low- and middle-income countries. According to the World Health Organisation, chronic wounds cause over 300,000 deaths yearly [7-9]. Wound healing involves repairing damaged epidermis and other soft tissues following an injury. The cells beneath the dermis (the deepest layer of skin) increase collagen (connective tissue) production

in response to a skin injury-induced inflammatory response, and the epithelial tissue (outer epidermis) regenerates over time. This process consists of three stages: inflammation, proliferation and remodelling [10, 11].

Essential oils, extracted from various plant components, are often used as first-aid treatments for wounds due to their pharmacologically active compounds [12]. Sunflower seeds are rich in oleic acid and unsaturated fatty acids, particularly linoleic acid, a precursor to arachidonic acid. The skin contains a significant amount of arachidonic acid, a polyunsaturated fatty acid. Its primary function is to produce biologically active mediators such as prostaglandins, thromboxane and leukotrienes. These compounds function as agents that induce inflammation, thereby promoting the growth of new blood vessels in the affected area, facilitating the movement of cells, encouraging the proliferation and differentiation of fibroblasts, and promoting the synthesis of extracellular matrix. Research has demonstrated the antibacterial properties of sunflower oil [13, 14]. Several wound healing studies involving sunflower seed oil (SFO) have been reported. Topical application of SFO was beneficial in the healing process of experimentally induced skin wounds in horses [15, 16], goats [17] and in human organotypic skin explant culture (hOSEC) [18].

There have not been many reports on the involvement of biochemical components in the wound healing activity of SFO. This study aimed to assess the chemical content and wound-healing activities of SFO to determine the potential application of the sunflower receptacle in medicinal domains. The wound healing ability of SFO was evaluated and validated by *in silico* and *in vivo* experiments. The histological repercussions of the SFO application were performed, and metabolic profiling was carried out to highlight the bioactive chemicals involved in the activity. All the discovered components were subjected to *in silico* molecular docking research and evaluated against four different protein targets implicated in wound healing, namely tumour necrosis factor (TNF), vascular endothelial growth factor-1 (VEGF1), insulin-like growth factor-1 receptor (IGF1R) and transforming growth factor-1 (TGF1).

## Materials and Methods

### *Ethical considerations*

Ethical Clearance (EC) approval using animal subjects with No. 012111084 has been approved by the research ethics committee (KEP UAD) of Ahmad Dahlan University Yogyakarta, Indonesia.

### *Plants material and sunflower seed oil extraction*

The seeds of sunflowers were collected in September 2021 from Greenara, North Jakarta, in Indonesia. Thirty grammes of dried sunflower seed flour were

extracted by the Soxhlet method in hexane at 70 - 75°C. The hexane extract was concentrated under a vacuum to yield a viscous syrupy residue.

### *GC/MS analysis of SFO*

In this research, the Thermo Scientific™ TRACE 1310 Gas Chromatography and Thermo Scientific™ ISQ LT Single Quadrupole Mass Spectrometer were performed. The components of SFO were separated using a fused silica capillary column TR-5, which had dimensions of 7 m in length, 0.32 mm in internal diameter and a film thickness of 0.25 mm. The gas chromatograph was operated using the following conditions: the injector temperature was set to 250°C, while the oven temperature was initially set to 50°C for a duration of 1 minute. Subsequently, the oven temperature was programmed to increase at a rate of 5°C *per* minute until it reached 260°C. Finally, the oven temperature was maintained at a constant 260°C for a period of 5 minutes. The carrier gas used was helium UHP (99.99%) with a constant flow rate-total flow of 50 mL/min and column flow of 3 mL/min. The total running time of the GC-MS programme was 60 minutes. SFO components were identified by comparison of their retention indices (RI) relative to mass spectral data (MS) from the National Institute of Standards and Technology (NIST) 14 library (National Institute of Standard and Technology, US) and WILEY mass spectral library database (similarity index > 90%).

### *Prediction of the biological activity*

SFO's biological activity was digitally tested using the PASS platform. The produced activity predictions were then tested for biological activity. This neural networking-based screening technology does large-scale pharmacophore-based virtual screening with over 3000 biological activities. The generated data were presented as Pa scores (probably active scores), with compounds with Pa values greater than 0.7 having a high likelihood of exhibiting biological activity *in vitro*. The opposite is true for compounds with Pa values of 0.7 [29].

### *Molecular docking study*

*In silico* molecular docking of the identified compounds of SFO against four different wound-healing related proteins. The protein crystal structure involved in the wound healing process was obtained from the Protein Data Bank with PDB ID 2AZ5 for TNF- $\alpha$ , 3HNG for VEGFR1, 5HZN for IGF1R and 1VJY for TGF- $\beta$ 1. The target protein was prepared by removing water, adding polar hydrogen, energy minimisation and Gasteiger charges using Autodock Tools. A selection of six molecules exhibiting potential biological activity in the wound-healing process in SFO were identified as ligands. Preparing ligands for docking involves downloading ligand structures from the PubChem database and executing structural and file-format docking preparations. The compounds and protein structure were previously saved in PDBQT

file format. Native ligand compounds from each protein structure were used as a positive control and validation of the molecular docking method by redocking the protein's crystal structure and native ligand. Molecular docking of the SFO compounds to the target proteins was performed using Autodock Vina. Then in the settings of the grid box, the results of the validation method were used, where the size of the grid box was 20 x 20 x 20 with coordinates X = -19.515, Y = 74.84, Z = 33.894 for TNF- $\alpha$  protein, X = 4.7, Y = 17.8, Z = 33.4 for VEGFR1 protein, X = 23.394, Y = 2.607, Z = 11.568 for IGF1R protein and X = 15.299, Y = 66.923, Z = 4.522 for TGF- $\beta$ 1 protein. The default distance used was 0.375 Å. The assessment of the docking outcomes revealed that the conformation with the greatest significance was determined based on the binding energy score ( $\Delta G$ ), which exhibited the lowest inhibition constant (Ki). Additionally, the Discovery Studio Visualiser identified functionally important amino acid connections that were found to be influential in the docking interactions.

#### *Wound healing animal model*

In this study, ten-week-old male Wistar rats weighing 250 - 270 g were purchased from Pondok Tikus (Rat House), Muaro Palam, West Sumatra, Indonesia. The rats were kept at a constant room temperature (20 - 25°C) throughout the experiment, with free access to water and a regular meal. The rats were anaesthetized with 1.5% halothane in an induction chamber, and pentobarbital (0.5 mL/kg) was administered intraperitoneally. Following hair removal and cleansing with 70% ethanol, a 2 cm length and 2 mm depth incision was made on the back of a rat using a sterile scalpel (no. 11) [19] to perform the sling procedure. Thirty-six (n = 36) rats were subjected to random allocation into three distinct groups consisting of twelve rats (n = 12), namely: (1) the untreated group, which underwent wound surgery exclusively, treated only with 0.9% sterile sodium chloride; (2) the control group, which had their wounds topically treated with povidone iodine (PI) and (3) the SFO group, which had their wounds topically treated with SFO. Subsequently, a volume of 50  $\mu$ L of every solution was administered onto the wound site immediately following the surgical procedure, and each intervention was sustained on alternate days. Four rats were sacrificed on days 1, 5 and 10 post-surgery from each group for histopathological, haematological and IL-10 measurements.

#### *Histopathological examination*

The wound area was digitally imaged using a digital camera at 1, 5 and 10 days following wound surgery, and the area was measured using Image J (NIH). The measuring findings are presented as a wound area in cm<sup>2</sup>. The wound area is subsequently converted to a wound healing percentage based on the previous experiment [20]. To collect tissue samples from the skin, rats were sacrificed via intraperitoneal administration of an overdose of pentobarbital.

After removal, the rat skin was cleaned in ice-cold phosphate-buffered saline (PBS) and fixed in a 10% buffered formalin solution. Following 48 hours, the organs underwent trimming and preparation utilising a tissue processor manufactured by Thermo Scientific®. The tissues that underwent processing were subsequently immersed in paraffin wax and sliced into 4 - 5  $\mu$ m-thick sections using a microtome. These sections were then processed by haematoxylin and eosin (H&E) staining. The tissue sections were examined using a light microscope (Olympus BX-50, Olympus Corporation, Japan) linked to a computer monitor.

#### *Haematological Measurements*

The blood profile was analysed using an automated haemology analyser against several parameters, such as total white blood cells (WBC), red blood cells (RBC), haemoglobin (HGB) and haematocrit (HCT). The rats were subjected to anaesthesia before collecting blood samples via heart puncture. These samples were then placed into EDTA bottles and used for haematological studies and IL-10 analysis.

#### *IL-10 Determination*

The *in vitro* quantification of IL-10 was conducted using ELISA kits (BMS629 Rat IL-10 Elisa Kit, Thermo Fisher, USA) according to the instructions provided by the manufacturer. The data was provided as pg/mL  $\pm$  SD for triplicate measurements.

#### *Statistical analysis*

SPSS 22 was used to analyse all the data. If the data were normally distributed and homogeneous, they were analysed with one-way ANOVA ( $p < 0.05$ ) and Duncan's post-hoc test to determine whether there were significant differences [21]. The standard deviation (SD) provides quantitative information.

## **Results and Discussion**

#### *Determination of phytochemical compounds in SFO using GC-MS*

Different components with different retention durations were identified in the GC-MS spectra. Compounds were recognised by comparing the mass spectral data bank with NIST11 libraries, checking for registered ranges with a similarity index (SI) of 80% or higher, and then comparing those values to those found in the scientific literature. The active compounds of SFO extract had many components, as evidenced by GC-MS results. Table I presents the formula, molecular weight (g/mol), retention time (RT), similarity index (SI) and percentage peak area (%) for the active constituents of SFO.

The extract contained six major compounds, accounting for over 4% of the total composition. These compounds were identified as 10-octadecenoic acid, methyl ester (4,9%) (Peak No 7), 9,12-octadecadienoic acid (Z, Z)-2,3-dihydroxypropyl ester (7,78%) (Peak No 12), 9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester (Z, Z, Z)- (10,82%) (Peak No 14), Glycidyl

oleate (12,86%) (Peak No 15), Oleic acid, 3-(octadecyloxy)propyl ester (5,51%, 6,93%, 7,01%, 9,57% and 8,48%) (Peak No 16, 18, 20, 22 and 24) and Ethyl iso-allocholate (5,86%) (Peak No 5,96%). The main chemical components were oleic acid and octadecatrienoic acid. Fatty acids (FAs) make up around 90% of SFO, and most of them are unsaturated fatty acids such as oleic and linoleic acid [22, 23].

The previous study showed that SFO contains a high concentration of oleic acid [24, 25]. Sunflower oil provides nutraceutical and therapeutic potential due to its composition, enhanced bioactive components and antioxidant activity [26, 27]. Then, active plant components can be employed as natural antioxidants in vegetable oils [28, 29].

**Table I.**  
Identification of phytochemical compounds in SFO extract by using GC-MS

No. crt.	Name of compounds	Formula	Mol. Weight	RT	SI	Rel. Area (%)
1.	Acetylhydrazide, 2--tert-butoxycarbonylamino-N2-benzyloxycarbonyl-	C <sub>15</sub> H <sub>21</sub> N <sub>3</sub> O <sub>5</sub>	323	5,08	685	1,33
2.	Bicyclo[2.1.1]hexan-2-ol, 2-ethenyl-	C <sub>8</sub> H <sub>12</sub> O	124	5,27	672	1,17
3.	Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate	C <sub>16</sub> H <sub>28</sub> O <sub>3</sub>	268	27,10	673	0,53
4.	Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl)methyl]-cyclopropyl]methyl]cyclopropyl]methyl]-, methyl ester	C <sub>25</sub> H <sub>42</sub> O <sub>2</sub>	374	31,77	677	1,94
5.	Atis-16-ene, (5 $\beta$ ,8a,9 $\beta$ ,10a,12a)-	C <sub>20</sub> H <sub>32</sub>	272	34,02	717	1,63
6.	Linoleic acid ethyl ester	C <sub>20</sub> H <sub>36</sub> O <sub>2</sub>	308	34,94	696	1,31
7.	10-Octadecenoic acid, methyl ester	C <sub>19</sub> H <sub>36</sub> O <sub>2</sub>	296	35,05	759	4,90
8.	Ethyl iso-allocholate	C <sub>26</sub> H <sub>44</sub> O <sub>5</sub>	436	35,53	675	1,25
9.	Ethyl iso-allocholate	C <sub>26</sub> H <sub>44</sub> O <sub>5</sub>	436	36,79	670	0,83
10.	Ethyl iso-allocholate	C <sub>26</sub> H <sub>44</sub> O <sub>5</sub>	436	38,48	691	2,34
11.	Oleic acid, 3-(octadecyloxy)propyl ester	C <sub>39</sub> H <sub>76</sub> O <sub>3</sub>	592	40,14	668	2,46
12.	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester	C <sub>21</sub> H <sub>38</sub> O <sub>4</sub>	354	40,59	674	7,78
13.	Ethyl iso-allocholate	C <sub>26</sub> H <sub>44</sub> O <sub>5</sub>	436	41,13	672	0,88
14.	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	C <sub>21</sub> H <sub>36</sub> O <sub>4</sub>	352	41,41	741	10,82
15.	Glycidyl oleate	C <sub>21</sub> H <sub>38</sub> O <sub>3</sub>	338	41,49	721	12,86
16.	Oleic acid, 3-(octadecyloxy)propyl ester	C <sub>39</sub> H <sub>76</sub> O <sub>3</sub>	592	41,92	681	5,51
17.	Spirost-8-en-11-one, 3-hydroxy-, (3 $\beta$ ,5a,14 $\beta$ ,20 $\beta$ ,22 $\beta$ ,25R)-	C <sub>27</sub> H <sub>40</sub> O <sub>4</sub>	428	42,99	651	0,79
18.	Oleic acid, 3-(octadecyloxy)propyl ester	C <sub>39</sub> H <sub>76</sub> O <sub>3</sub>	592	44,14	690	6,93
19.	Ethyl iso-allocholate	C <sub>26</sub> H <sub>44</sub> O <sub>5</sub>	436	45,58	687	1,56
20.	Oleic acid, 3-(octadecyloxy)propyl ester	C <sub>39</sub> H <sub>76</sub> O <sub>3</sub>	592	46,96	669	7,01
21.	9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3 $\beta$ ,5Z,7E)-	C <sub>27</sub> H <sub>44</sub> O <sub>3</sub>	416	47,23	608	2,15
22.	Oleic acid, 3-(octadecyloxy)propyl ester	C <sub>39</sub> H <sub>76</sub> O <sub>3</sub>	592	50,58	684	9,57
23.	Ethyl iso-allocholate	C <sub>26</sub> H <sub>44</sub> O <sub>5</sub>	436	51,72	710	5,96
24.	Oleic acid, 3-(octadecyloxy)propyl ester	C <sub>39</sub> H <sub>76</sub> O <sub>3</sub>	592	55,31	671	8,48

#### Biological activity prediction

In order to forecast the compounds with a high likelihood of exhibiting biological activity on wound healing processes at SFO, we utilised the PASS (prediction of activity spectra of substances) platform, which employs neural networking-based algorithms for biological activity prediction [30]. The structures of all dereplicated compounds were included in the analysis. With an average accuracy of above 95%, PASS Online is made to predict the biological activity spectra of organic compounds based on their structural formulas for more than 4000 different categories of biological activity. A study of the structure-activity relationships in the training set, which contains data on the structure and biological activity of more than 300,000 organic compounds, served as the foundation for the prediction. The

retrieved result for each structure was provided as a Pa score. Structures with a Pa score > 0.7 have a high probability of showing biological activity. Six compounds are responsible for the observed biological activities, such as lipid metabolism regulators and anti-inflammatory wound healing (Pa score > 0.7) (Table II). Lipid metabolism plays an important role in the wound-healing process. Several lipid regulators are implicated in several stages of wound healing, including inflammation, proliferation and tissue remodelling [31]. Lipid-based signalling controls various cellular functions during wound healing, including intercellular communication and tissue regeneration [32]. Changes in cell membrane fatty acid content affect inflammation in different ways. These compositions affect membrane fluidity, cell signalling, gene expression and lipid mediator synthesis [33, 34].

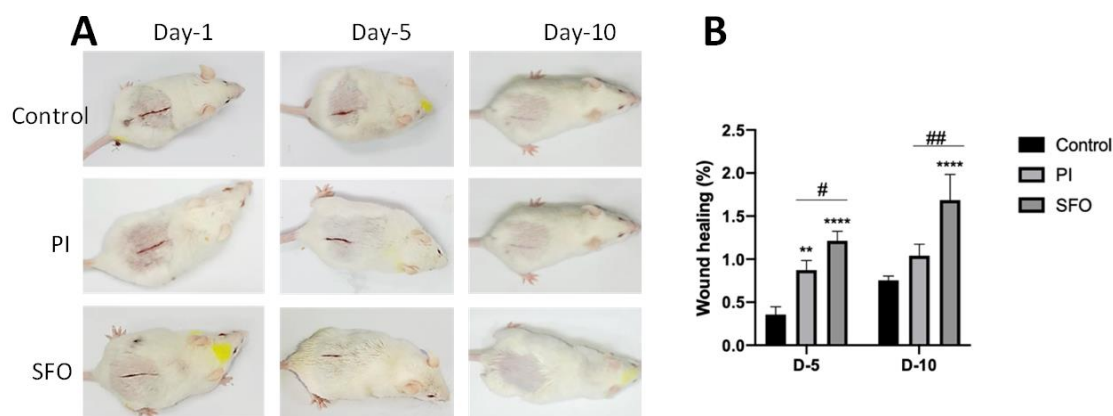
**Table II.**

Predicted potential biological activity of SFO				
Number of compounds	Name of compounds	Pa	Pi	Biological activity
14	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	0.977	0.002	Lipid metabolism regulator
		0.796	0.007	Anti-inflammatory
11	Oleic acid, 3-(octadecyloxy)propyl ester	0.836	0.005	Lipid metabolism regulator
21	9,10-Sesta-5,7,10(19)-triene-3,24,25-triol, (3R,5Z,7E)- ecochol	0.793	0.007	Anti-inflammatory
12	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester	0.975	0.002	Lipid metabolism regulator
		0.746	0.011	Anti-inflammatory
7	10-Octadecenoic acid, methyl ester	0.895	0.004	Lipid metabolism regulator
		0.718	0.014	Anti-inflammatory
6	Linoleic acid ethyl ester	0.943	0.003	Lipid metabolism regulator
		0.764	0.009	Anti-inflammatory

### Histopathological Study

The macroscopic images of the experiment are shown in Figure 1. There was no clinical abnormality or gross sign of infection found during the experiment, suggesting the absence of pathophysiological deformities that could affect the common process of wound healing. To assess the effects of SFO extracts on the laceration wounds, we measured the wound closure rate at different time intervals. Our analysis showed SFO could significantly decrease the wound size as compared to the negative control group. Moreover, this ability

of SFO was significantly higher than that of PI (positive control) at all the time intervals evaluated. It is noteworthy that the wound healing efficacy of SFO remains effective even after a period of 10 days following surgery, but the wound healing activity of PI has exhibited a deceleration by day 10. The closure of the wound was observed to be complete in the group of rats treated with SFO; however, in the group of rats treated with PI, the wound did not exhibit complete closure.

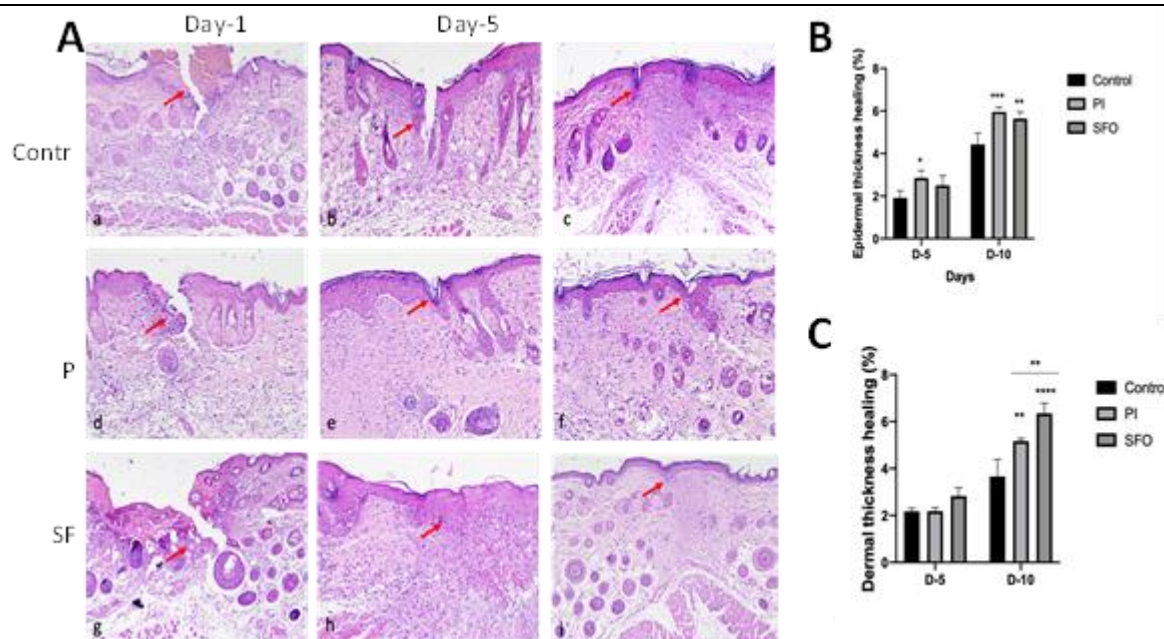
**Figure 1.**

Photographical trends represent the contraction wound healing rate. (A). The macroscopic image of wound healing rate on untreated (control), PI-treated (PI) and SFO-treated (SFO) rat skin on days 1, 5 and 10. (B). The percentage of wound closure rate on days 5 and 10 after injury were calculated

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$  versus control; #  $p < 0.05$ , ##  $p < 0.01$  versus positive control (povidone iodine). Data is presented as the mean  $\pm$  SD,  $n = 4$

The rats were then sacrificed to observe the histological differences at different time intervals using standard haematoxylin and eosin (HE) staining of wound area tissue samples. Figure 2A depicts different healing processes observed microscopically in the treatment and control groups. In the negative control group, both the epidermis and dermis were not regenerated thoroughly. Even after 10 days since the incision, the wound has not completely closed. High infiltration of inflammatory cells was clearly seen in the dermis surrounding the detached skin appendages, and

scattered collagen bundles could be observed. However, in PI-treated (positive control) and SFO-treated groups, skin tissue damages were decreased to some extent in day 5 and almost abolished completely at day 10, especially in the SFO group. We also calculated the percentage of epidermal and dermal thickness from those three groups, and the result showed that SFO indeed accelerates healing. This microscopic result is relevant to what appears macroscopically and suggests that SFO works better than PI in curing the wound.



**Figure 2.**

Microscopic staining of skin wound healing. (A) Haematoxylin and eosin stains of rat skin wound healing in untreated (a, b, v), PI-treated (d,e,f) and SFO-treated (g,h,i) on day 1 (a,d,g), day 5 (b,e,h) and day 10 (c,f,i).

(A) HE staining. (B) The percentage of epidermal thickness from HE staining, calculated on day 5 and 10. (C) Dermal thickness from HE staining calculated on day 5 and 10. Nine animals were used for each group and the significance differences were evaluated

The data is presented as the mean  $\pm$  SD. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ ,  $n = 4$

Sunflower oil is an example of other vegetable oils that encompass essential fatty acid constituents with significant implications for the process of wound healing. SFO has been reported to be beneficial for several skin diseases, such as atopic dermatitis. Prior research has investigated the wound healing capabilities of SFO and demonstrated its potential impact on chronic, extensive wounds [35]. Our findings represent a pioneering demonstration of the therapeutic efficacy of SFO during the initial stages, surpassing that of povidine iodine [13, 36].

Among the several antimicrobial agents that are readily available, povidone iodine has consistently been preferred due to its notable attributes that make it highly effective for therapeutic purposes. These attributes include a wide-ranging antibacterial spectrum, absence of resistance development, strong efficacy, acceptable tolerability and its ability to reduce inflammation. Previous experiments showed that PI enhanced wound healing by stimulating alpha smooth muscle actin ( $\alpha$  SMA), TGF- $\beta$ , new blood vessel formation and re-epithelialization [37]. Microscopic images from our experiment also show that on day 5, high inflammatory cells, new blood angiogenesis and granulation tissue started to show in the SFO and PI groups [38]. These images are in accordance with important histological features of the proliferative phase. The wound-healing process consists of different highly integrated and overlapping phases, namely haemostasis, inflammation, proliferation and tissue

remodelling phases. In the proliferative phase, growth factors will stimulate the proliferation of two cells: fibroblasts and endothelial cells [39, 40]. The fibroblast is involved in the arrangement of the extracellular matrix that guides the healing process, while the latter will initiate neovascularization, both of which are critical for a successful healing process. Proving that SFO has better potential to heal laceration wounds [41, 42].

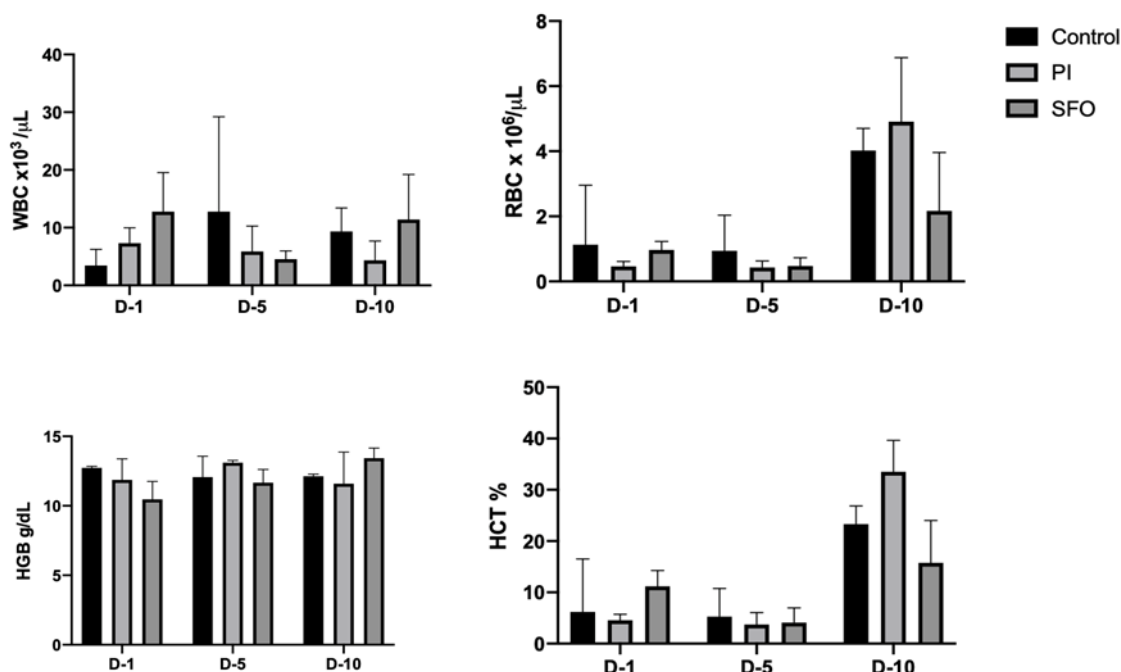
Interestingly, there is an eventful trend between SFO and the positive control group in the healing process of the scar between the epidermis and dermis. The epidermis started to thicken in the rats treated with povidone iodine on day 5 and intensified on day 10. While the SFO-treated group did not show any epidermal thickening on day 5, it started to extend on day 10. But the dermis layer did not thicken in both groups on day 5 until it accelerated on day 10, especially in the SFO group. Previous reports about the ability of the PI to repair the wound undeniably showed the potential in the epithelial layer. This means that while SFO has better potential for curing the laceration, it works on a different pathway than PI. Further studies are still needed to explore this mechanism [38].

#### *Haematological and IL-10 measurements*

Because of the involvement of many cells during normal wound repair, we investigated the systemic effect of SFO using haematological parameters. All components of blood have important roles in the

healing cascade; platelets play the most crucial role in the first and shortest phase of homeostasis to stop bleeding [43]. Platelets will trigger the next, acute inflammation phase by recruiting the WBC to disinfect and remove the dead tissues from the wound [43, 44]. While other blood cells' roles are not clearly explained, it is believed that enhancing the oxygen-

carrying capacity of blood could improve the healing process [44]. All of these processes are essential for a normal, healthy state of healing [45]. We examined the hematologic parameters in the serum of rats at different intervals to see the systematic effect of SFO. We found no significant differences in blood components between the groups.

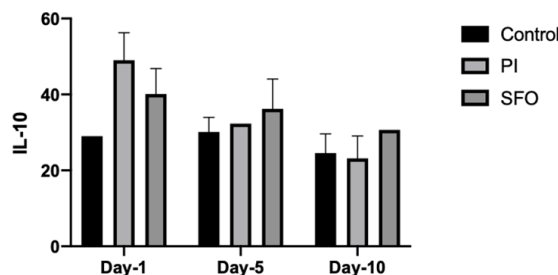


**Figure 3.**

Haematological parameters: (A) white blood cells (WBC); (B) red blood cells (RBC); (C) haemoglobin (HGB) and (D) haematocrit (HCT) on serum rat treated with PI and SFO extract compared with the control. No significance difference in WBC, RBC, HGB and HCT count between groups. Data presented as mean ± SD, n = 4

The experimental results indicated a slight increase in systemic WBC levels in the group treated with SFO starting from day 1. This suggests that SFO may induce an early phase of inflammation. Although the result is not significant, this might be due to the fact that the low dose of topical SFO did not exert enough forces to influence the systemic effect. The result is also in line with the microscopic image, where it showed an early increase of high inflammatory cells compared to another group. The leukocyte count reduced on day 5, probably because of the high content of flavonoids in SFO that reduced the pro-inflammatory mediator, then rose again because of the potential residual WBC after the peak of the inflammatory phase. This is different from the PI group, which did not influence the WBC [46]. A previous study by Wang *et al.* stated that PI promotes healing by alleviating acute inflammation [38], proving another pathway of healing between SFO and PI. Notably, the newest evidence found the great flexibility of WBC in driving signals in wound microenvironments, which means that it does not always give a robust inflammatory response. Moreover, although platelets are important in the early phase of healing, we did not

investigate the platelet count as the short window period of the homeostasis phase made it difficult to acquire the sample [47]. Further investigation to clearly explain the mechanism is needed.



**Figure 4.**

The effect of SFO on anti-inflammatory IL-10. Expression of IL-10 in the serum of a rat wound healing model treated with SFO extract was compared with untreated and PI data from days 1, 5 and 10. There is no significant difference in IL-10 expression between groups. Data presented as mean ± SD, n = 3

The enzyme-linked immunosorbent assay (ELISA) was employed to investigate whether SFO affects the

expression of interleukin 10 (IL-10) in the serum of rats during the wound healing process. In this present study, we found that SFO promotes higher IL10 expression in wound healing rat models at early stages, although not significantly. Higher IL-10 expressions were observed in the SFO-treated group compared to the control; it also increased IL-10 expressions compared to PI standard wound treatment, except on day 1, where PI treatment produced the highest IL-10 protein expression among the 3 groups. As a key anti-inflammatory cytokine, IL-10 can suppress the inflammatory response of both the innate and adaptive immune systems. It is produced mainly by macrophages and regulatory T cells but also by non-leukocyte cell populations, such as epithelial and keratinocytes, as a response to the inflammation process. Several studies have proven the role of IL-10 in wound healing processes [48]. Interleukin-10-overexpressed mice indicated that lower wound pro-inflammatory mediator expression and inflammatory cell recruitment resulted in reduced scar formation after 3 weeks [49, 50].

Although PI demonstrated greater potency than SFO 24 hours after treatment, SFO exhibited higher efficacy in stimulating IL-10 production in the subsequent days. However, IL-10 expression in both PI and SFO groups showed a decreasing trend from day 1 to day 10. PI is a standard for wound treatment used commonly. The differences in carrier molecules and physicochemical properties between these substances might cause a different phenomenon, as PI is a drug that contains a pure and concentrated active compound.

While inflammation is required for the normal wound healing process, prolonged and excessive inflammation can lead to re-epithelialization failure, which is responsible for chronic wound formation [50].

#### Molecular docking studies

Molecular docking analysis was performed for the selected compounds to determine probable binding interactions and affinities. Six compounds were evaluated using the computer programme Autodock Vina against four different protein targets that were significantly included in the wound healing process. The free energy values of the binding interactions between the molecules have been determined. The native ligand and the six compounds were evaluated for validation. Docking into the receptor's active site predicts the compounds' actions. Four different protein receptors have been found to play a crucial role in wound healing. The SFO active compounds analysed in the present study were docked to each of the four proteins for wound healing, such as TNF $\alpha$  (PDB ID: 2AZ5), VEGFR1 (PDB ID: 3HNG), IGF1R (PDB ID: 5HZN) and TGF- $\beta$ 1 (PDB ID: 1VJY). Moreover, the inhibition constant (Ki) was calculated according to each ligand-receptor binding energy ( $\Delta G$ ) in the following equation:

$$\Delta G = RT \ln K_i,$$

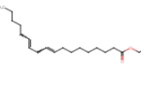
where, R is the gas constant (1.987 cal/K.mol) and T is the temperature (298.15 K) [51]. The ligand molecule was re-docked into the protein active site, and the observed binding interactions were compared to six SFO compounds (Table III and Table IV).

**Table III.**

Data molecular docking for the six molecules of SFO based on ligand-receptor interaction with target protein in the wound healing process

Compound	Information	2D Chemical Structure	Binding Energy ( $\Delta G$ ) (Kcal/mol)				Constant Inhibition (Ki) (uM)				
			TNF- $\alpha$	VEGF1	IGF1R	TGF- $\beta$ 1	TNF- $\alpha$	VEGF1	IGF1R	TGF- $\beta$ 1	
14	9,12,15-octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)	MW = 352.51 g/mol MF = C21H36O4 H-bond Donor = 2 H-bond Acceptor = 4 Pubchem ID = 5367328		-6.2	-7.6	-6.7	-7.0	28.5	2.68	12.25	7.38
11	oleic acid, 3-(octadecyloxy)propyl ester	MW = 593.02 g/mol MF = C39H76O3 H-bond Donor = 0 H-bond Acceptor = 3 Pubchem ID = 21159937		-5.5	-6.8	-5.5	-5.6	92.90	10.35	92.90	78.48
21	9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol, (3 $\beta$ ,5Z,7E)	MW = 416.64 g/mol MF = C27H44O3 H-bond Donor = 3 H-bond Acceptor = 3 Pubchem ID = 5283748		-8.5	-8.6	-7	-8.3	0.58	0.50	7.39	0.82
12	9,12-octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester	MW = 354.52 g/mol MF = C21H38O4 H-bond Donor = 2 H-bond Acceptor = 4 Pubchem ID = 5283469		-5.6	-7.2	-6	-6.4	78.48	5.27	39.95	20.34
7	10-octadecenoic acid, methyl ester	MW = 312.49 g/mol MF = C19H36O3 H-bond Donor = 1 H-bond Acceptor = 3 Pubchem ID = 12559718		-5.8	-6.9	-5.9	-6.9	55.99	8.74	47.30	8.74



Compound	Information	2D Chemical Structure	Binding Energy ( $\Delta G$ ) (Kcal/mol)				Constant Inhibition (Ki) (uM)			
			TNF- $\alpha$	VEGF1	IGF1R	TGF- $\beta$ 1	TNF- $\alpha$	VEGF1	IGF1R	TGF- $\beta$ 1
6	linoleic acid ethyl ester MW = 308.50 g/mol MF = C20H36O2 H-bond Donor = 0 H-bond Acceptor = 2 Pubchem ID = 5282184		-5.6	-6.8	-6.2	-6.7	78.48	10.35	28.50	12.25

According to the docking data, compounds no. 14, 11, 12, 7 and 6 exhibit low activity as TNF, VEGFR1, IGF1R and TGF-1 inhibitors. Compound no. 21 (9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol, (3 $\beta$ ,5Z,7E)) has the highest binding affinity score when compared to the other compounds against the four receptor target proteins. The results show that compound no. 21 has the highest binding affinity value compared to the native ligand of the TNF $\alpha$  receptor, 6,7-dimethyl-3-[(methyl{2-[methyl({1-[3-(trifluoro-

methyl)phenyl]-1H-indol-3-yl)methylamino]ethyl]-amino)methyl]-4H-chromen-4-ones (Table IV) which is -8.5 (kcal/mol). The binding involved six hydrogen bond interactions, of which three form H-donors and H-acceptors. The root-mean square deviation (RMSD) of the docked ligand from the original crystal structure was used to measure docking accuracy, and it is generally accepted that an RMSD value is  $\leq 2$  Å [52] (Table IV).

Table IV

Re-docking native ligand-receptor interaction with target protein in the wound healing process

Protein Target	Native Ligand	$\Delta G$ (kcal/mol)	Grid box coordinate	Grid box size	RMSD (Å)
TNF- $\alpha$ (2AZ5)	6,7-dimethyl-3-[(methyl{2-[methyl({1-[3-(trifluoromethyl)-phenyl]-1H-indol-3-yl)methylamino]ethyl}amino)methyl]-4H-chromen-4-one	-8.4	x: -19.515 y: 74.84 z: 33.894	20 x 20 x 20	1.07
VEGFR1 (3HNG)	N-(4-chlorophenyl)-2-[(pyridin-4-ylmethyl)amino]benzamide	-10.6	x: 4.7 y: 17.8 z: 33.4	30 x 30 x 30	0.61
IGFR1 (5HZN)	7-[cis-3-(azetidin-1-ylmethyl)cyclobutyl]-5-[3-(benzyloxy)-phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine	-11.2	x: 23.394 y: 2.607 z: 11.568	30 x 30 x 30	0.93
TGF- $\beta$ 1 (1VJY)	2-[5-(6-methylpyridin-2-yl)-2,3-dihydro-1H-pyrazol-4-yl]-1,5-naphthyridine	-10.3	x: 15.299 y: 66.923 z: 4.522	30 x 30 x 30	0.30

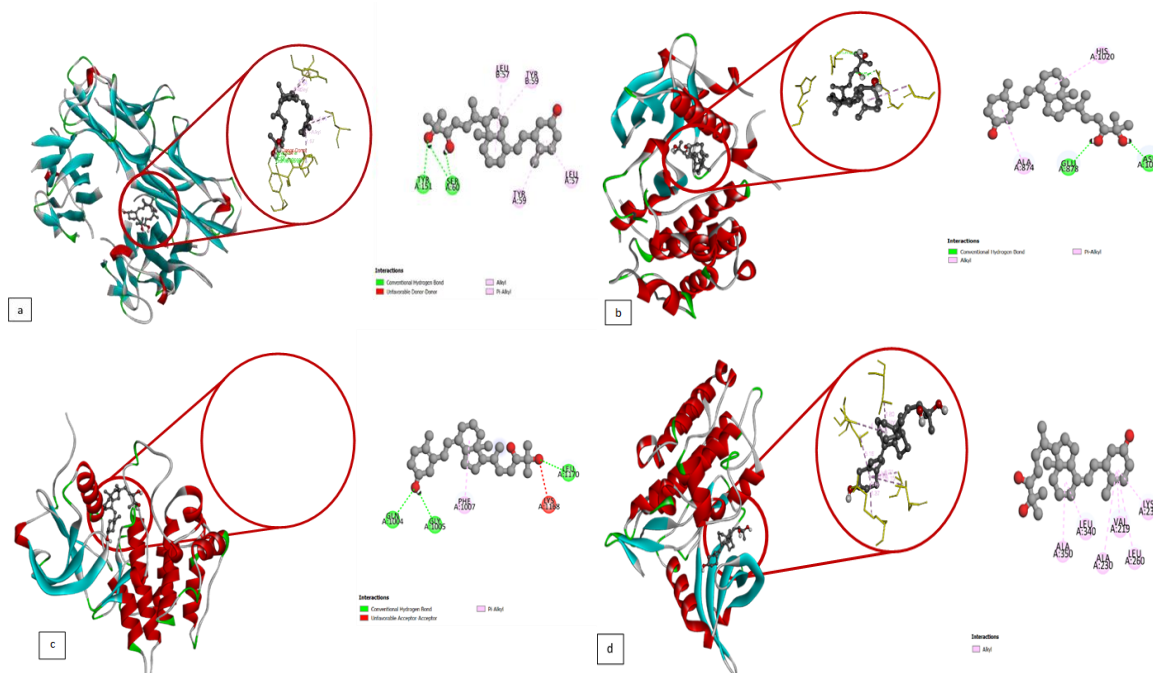


Figure 5.

2D interaction between 9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol, (3 $\beta$ ,5Z,7E) compound (21) with TNF- $\alpha$  (a), VEGFR1 (b), IGF1R (c) and TGF- $\beta$ 1 (d)

Figure 5 shows the interactions between the amino acid residues of the active site of TNF- $\alpha$ , VEGFR1, IGFR1, TGF- $\beta$ 1 and compound no, 21. Moreover, TNF $\alpha$  active sites were marked as residue numbers of LEU57, TYR59, SER60, TYR151; VEGFR1 active sites were marked as residue numbers of HIS1020, ALA 874, GLU 878, ASP 1040; IGFR1 active sites were marked as residue numbers of GLN1004, GLY1005, PHE1007, LYS1168, LEU1170 and TGF- $\beta$ 1 active sites were marked as residue numbers of ALA350, LEU340, VAL219, ALA230, LEU260 and LYS232.

Wounds exhibit significant inflammation due to elevated levels of TNF- $\alpha$ , both locally and systemically. Evidence suggests that the suppression of TNF- $\alpha$  is essential for wound treatment. It promotes wound healing by re-epithelializing the wound, causing inflammation, activating angiogenesis and generating new skin tissue. SFO, a bioactive compound, binds strongly to TNF- $\alpha$ . Several growth factors, including VEGFR1, IGFR1 and TGF-1, play important roles in wound healing [53]. Proangiogenic factors, such as VEGF, increase endothelial cells' survival, migration, self-assembly, differentiation and self-repair [54]. According to the current findings, SFO may have wound healing benefits primarily by controlling the inflammatory phase and proangiogenic factors.

### Conclusions

In this study, the main chemical components of SFO were oleic and octadecatrienoic acids, which have potential biological activities in wound healing by regulating lipid metabolism and being anti-inflammatory. *In vivo* studies proved their ability to heal wounds macroscopically and microscopically, which works better than the standard PI. Subsequent investigation through docking analysis revealed a robust binding affinity between the bioactive compound and TNF- $\alpha$ , a protein target involved in wound healing. Thus, the healing ability of SFO probably works by controlling inflammatory and proangiogenic factors. The limitation of this study is that there is less variation in some data that may affect the end result. Further studies using standardised SFO extract with larger samples are needed to see the true potential of SFO in the wound healing process.

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### Conflict of interest

The authors declare no conflict of interest.

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