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In Vitro and *In Vivo* Evaluation of Photoprotective Effect of *Elephantopus Mollis* Extracts

Verawati Verawati[1,4]*, Almahdy Almahdy[2], Febriyenti Febriyenti[3], Deddi P. Putra[1]

[1]*Department of Biology Pharmacy, Faculty of Pharmacy, Andalas University, West Sumatera-25175, Indonesia*
[2]*Department of Pharmacology, Faculty of Pharmacy,Andalas University, West Sumatera-25175, Indonesia*
[3]*Department of Pharmaceutics, Faculty of Pharmacy,Andalas University, West Sumatera-25175, Indonesia*
[4]*Faculty of Pharmacy, UniversitasPerintis Indonesia, West Sumatera-25586, Indonesia*

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ABSTRACT

Exposure to ultraviolet radiation from the sun can cause various harmful effects such as erythema, wrinkles, dark spots and sunburn that trigger premature aging and skin cancer. This research aimed to explore the photoprotective potential of the nonpolar, semipolar, and polar extracts of *Elephantopus mollis* herbs through *in vitro* and *in vivo* evaluation. *In vitro* evaluation was done by examining antioxidant activity, determination of sun protection factor (SPF) value, total phenolic content, and identification of the chemical components of the extract. The most active extract on *in vitro* evaluation was used for *in vivo* testing with observation parameters of erythema and wrinkle formation and dermal histopathology of mice exposed to radiation from ultraviolet lamps. The *in vitro* examination showed that the polar extract had the best photoprotective potential compared to other extracts, with high antioxidant activity (IC_{50} 46.55 µg/mL), total phenolic content of 109.2 mg/g extract GAE (gallic acid equivalent), and SPF values of 13.5. Topical administration of polar extract with increased doses showed improved skin performance and dermis of mice exposed to ultraviolet radiation. The polar extract can significantly reduce erythema, wrinkles, and dermis histological performance similar to the normal skin structure of mice. LC-HRMS analysis showed that the polar extract contains bioactive components of polyphenols and flavonoids such as caffeic acid, chlorogenic acid, rutin, trifolin, and 3,5-di-O-caffeoylquinic acid. Polar extract from *Elephantopus mollis* exhibited a high phenolic content and good antioxidant activity and was able to protect the skin from the negative effects of repeated UV exposure.

Keywords: *Elephantopus mollis*, Ultraviolet radiation, Antioxidant, Phenolic, Photoprotective

Introduction

Exposure to solar ultraviolet radiation can promote the formation of radical species in the skin and evoke an oxidative stress state responsible for various skin damages.[1,2] Skin exposed to repeated sunlight may suffer from erythema, wrinkles, inflammation, and sunburn resulting in premature aging and skin cancer.[3,4] The adverse effects of sunlight can be overcome through prevention and treatment using products that work with a combination of sunscreen, antioxidant, and agent to repair skin damage.[5,6] Skin protection agents against the adverse effects of sunlight can be sourced from synthetic and natural compounds consisting of organic or inorganic substances.[7] Various commercial sunscreen and antiaging products contain many synthetic ingredients and have several harmful effects.[8] For example, synthetic substances such as benzophenone, octocrylene, and their derivatives are potentially carcinogenic.[8,9] The use of synthetic materials over a long period can cause allergies, pain in the skin, acne, and even tumors or skin cancer.[10] Health-harmful threats from synthetic products provide opportunities for the use of natural ingredients such as plant extracts that contain bioactive substances.[5,11,12]

*Corresponding author. E mail: verawati@upertis.ac.id
Tel: +6281374094606

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Plants from the family Asteraceae are known to be rich in phenolic components that are efficacious as organic sunscreens and active antioxidants.[13,14] One of the Asteraceae plants commonly found in the province of West Sumatra, Indonesia, is *Elephantopus mollis*.
E. mollis has been reported to have anti-inflammatory, anti-tyrosinase, antimelanogenesis, and anticancer properties.[15,16] Previous research, especially antimelanogenesis and antityrosinase activity, has shown the *in vitro* activity of *E. mollis* against certain enzymes found in the skin.[17] There have been no reports of the ability of *E. mollis* extract to protect the skin against UV rays. This study aimed to evaluate the potential bioactivity of non-polar, semi-polar and polar extract of *E. mollis* as photoprotective. The bioactivity evaluation was comprehensively conducted using *in vitro* and *in vivo* examination.

Materials and Methods

Chemicals and reagents

Gallic acid, DPPH, sodium carbonate, reagent Folin Ciocalteu were purchased from Merck, n-hexane, ethyl acetate, methanol, and aquadest are obtained from suppliers of chemicals in Indonesia such as Mutiara Lab Sains, Nitra Kimia, and Bratachem.

Plant material

Elephantopus mollis plant was collected in August 2020 in Pariaman City of West Sumatra Province, Indonesia. This plant was identified in the Herbarium Biology University of Andalas (ANDA)with voucher specimen number ANDA 00038471. The aerial part of the plant was used in this study.

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Procedure extraction of Elephantopus mollis
The aerial parts of plant (5 kg) were air-dried for five days and powdered using a mechanical grinder. The dried powder of the aerial part of *E. mollis* (980 g) was extracted by maceration method using solvents in a stepwise gradient of increasing polarity starting with n-hexane (18 L), followed by ethyl acetate (17 L) and then methanol (17 L). Each maceration step was done for 48 hours with triplicate maceration for each solvent. The filtrate from each extracting solvent was concentrated using a rotary evaporator to obtain the viscous extract, a nonpolar extract from hexane solvent, semipolar extract from ethyl acetate solvent, and polar extract from methanol solvent.

Characterization of extracts
The three types of *E. mollis* extract were characterized by observing organoleptic, phytochemical screening, percentage yield, loss on drying, and total ash content using the methods listed in the Indonesian Herbal Pharmacopoeia.[18]

In vitro evaluation
Determination of phenolic content
Total phenolic content was determined by the FolinCiocalteu method and used gallic acid as a standard compound.[19] Each extract solution (1000 µg/mL) was pippeted (0.5 mL) and mixed with a 5 mL Folin-Ciocalteu (10%) reagent and 4 mL of sodium carbonate solution (7.5%). The mixtures were incubated at room temperature for 15 minutes. The absorbance of test solutions was measured using a spectrophotometer at a wavelength of 762 nm. Gallic acid as a standard solution was prepared in 20, 40, 60, 80, and 100 µg/mL concentration series.

Examination of antioxidant activity
The antioxidant activity of *Elephantopus mollis* extracts was measured by the radical DPPH (1,1-diphenyl-2-picrilhydrazine) scavenging method.[20] The test solutions of extracts were made in various series of concentrations, then pipetted 2 mL and mixed with 4 mL of DPPH solution 50 µg/mL. The control solution using 4 mL of DPPH 50 µg/mLwas mixed with 2 mL of methanol. Gallic acid was used as an antioxidant standard compound and methanol as a blank solvent. All test solutions (extracts, standard, and control) were incubated for 30 minutes in the dark. Then, the absorbance of solutions was measured at a wavelength of 516 nm. Antioxidant activity was expressed as % inhibition and IC_{50} (concentration of compounds that inhibit 50% of DPPH radicals).

Determination of sun protection factor (SPF)
Sun Protection Factor (SPF) values were determined using a spectrophotometer by measuring the absorbance of extract solutions in a cuvette with the thickness of 1 cm.[21] Nonpolar, semipolar and polar extract of *Elephantopus mollis* were prepared in concentrations of 100 µg/mL and 250 µg/mL.The absorbance of each extract solution was examined at a wavelength range of 290 nm – 320 nm at intervals of 5 nm. The value of absorbance was incorporated into Sayre's mathematical equation.

In vivo Evaluation
Experimental animals
Thirty albino male mice (*Mus musculus*) weighing 25 - 35 g were obtained from the Department of Pharmacology of Andalas University. The animals were fed with a standard pellet Bravo 512® (produced by Charoen Pokphand Indonesia Inc.) and water. Animals were acclimated for a week, with a room temperature of 25 ± 2°C.The experimental procedures were approved by The Research Ethics Committee of Medical Faculty Andalas University, Padang, Indonesia (number 104/UN.16.2/KEP-FK/2020).

Treatment of test animals
In vivo testing using animals was continued on *E. mollis* extract, which has the best activity based on the results of the *in vitro* evaluation. Male mice acclimatized for seven days were divided into five groups consisting of 6 mice each; the normal control group, the UV control group, and extract groups.The normal control group consisted of normal or non-radiated mice, while the UV control was the group of mice that were given UV exposure. The extract groups consisted of a 2.5% of extract group (irradiated with UV and treated with an extract concentration of 2.5%), a 5% of extract group (irradiated with UV and treated with an extract concentration of 5%) and a 7.5% of extract group (irradiated with UV and treated with an extract concentration of 7.5%). Each concentration of extract was administered topically on the shaved dorsal part of the mice as much as 200 mg per application, daily for 14 days. Sources of Ultraviolet radiation using 13 Watt Reptile UV lamps (Exo Terra®). The distance of the lamp to the mice was 20 cm to obtain 300 $\mu W/cm^2$ of UV radiation intensity.[22] UV control and extract groups were irradiated for 2 hours,three times a week for two weeks. The mice received energy at2.16 $J/cm^2$for each radiation exposure.[22]Everyday observations were made for erythema and wrinkles on the dorsal area. On the 15th day, the mice were euthanized, the dorsal region was removed, and the skin tissue was prepared for a histopathological examination with Hematoxylin-Eosin staining.

Examination of chemical components with Liquid Chromatography – High Resolution Mass Spectrometry (LC – HRMS)
The chemical components of the active extract *E. mollis* were examined with non-targeted screening by using LC – HRMS instrument. The instrument used for separation was HPLC Thermo Scientific Dionex Ultimate 3000 RSLC nano with a microflow meter. Instrument used for Mass Spectroscopy was High Resolution Mass Spectrometer: Thermo Scientific Q Exactive by using Processing data software: Compound Discoverer with mzCloud MS/MS Library.

Results and Discussion

Elephantopus mollis herb was extracted using a step gradient polarity of extracting solvent that aimed to group compounds in plant based on their polarity. The extraction procedure chosen was maceration because it is simpler and can protect the thermostable compounds. The results of the characterization of three types of *E. mollis* extract can be seen in Table 1. The appearance of organoleptic of all three kinds of extracts looks different and so its physical characters. Polar extract provides a higher percentage yield, loss on drying, and total ash content compared to semipolar and nonpolar extracts. In the observation of phytochemical screening, the polar extract is also rich in phytochemical groups compared to 2 other types of extracts. Research by Kuete *et al*. reports the phytochemical content of the crude extract of *E mollis* showed the presence of flavonoids, steroids, triterpenes, and polyphenols.[23] In this research, the phytochemical components have been distributed based on the polarity of the extract so that the phytochemical profile of each extract becomes different.
Antioxidant activity and total phenolic content of *E. mollis* extracts are shown in Table 2. The result of each extract is remarkably different, and the polar extract indicates the most potential value. Antioxidant activity was tested using the radical method DPPH with the principle of the reaction mechanism is the electron donor of antioxidant substances to DPPH radicals to complete and stabilize DPPH electrons. Visually, antioxidant activity can be observed by discoloring DPPH solutions that are initially dark purple to yellowish after reacting with antioxidant substances. Antioxidant activity is indicated by the value of IC_{50}, which is the concentration of antioxidant substances that can scavenge DPPH radicals by 50%. The lower the IC_{50} value indicates more potent antioxidant activity. The antioxidant activity of each extract was different, and the polar extracts showed excellent antioxidant activity with IC_{50} lower than other extracts. The antioxidant activity of polar extract can be categorized very strongly (< 50 ppm) while semipolar extracts are moderate activity (101 - 250 ppm) and nonpolar extracts are weak (250 - 500 ppm).[24] The highest total phenolic levels obtained in polar extracts were 109.2 mg/g GAE, higher than semipolar and nonpolar extracts. The antioxidant activity and total phenolic content of *E. mollis* extract are higher than the results of a study reported by Ooi*et al*.[25] This may be due to differences in the extraction method used. Ooi *et al* used a Soxhletation method that involves heating while this study used maceration which is a cold extraction method. Heating can decrease total phenolic content and antioxidant activity.[26]

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Table 1: Characterization results of *Elephantopus mollis*extracts

Extract	Characteristics				
	Organoleptic	Yield	Loss on drying	Ash content	Phytochemical compound
Nonpolar extract	Thick, dark green, distinctive odour, slightly bitter taste	2.95 % (28.92 g)	6.73 %	2.31%	Steroids
Semipolar extract	Thick, blackish green, distinctive odourless, slightly bitter taste	3.83 % (37.54 g)	5.68%	4.09%	Terpenoids, phenolics
Polar extract	Thick, yellowish-brown, odourless, slightly bitter taste	4.33 % (42.47 g)	9.40%	6.20%	Alkaloids, phenolic, flavonoids, saponin

Table 2: Test results of antioxidant activity and total phenolic content of *E. mollis* extract

No.	Extracts	IC_{50} value	Total Phenolic Compounds	Pearson's Correlation
1	Nonpolar	363.858 ± 3.046 µg/mL[a]	24.182 ± 0.721 mg/g GAE[a]	-0.864**
2	Semipolar	177.320 ± 1.405 µg/mL[b]	33.187 ± 0.937 mg/g GAE[b]	
3	Polar	46.553 ± 0.149 µg/mL[c]	106.936 ± 0.891 mg/g GAE[c]	

** : significant at $p < 0.01$

Table 3: SPF value of *E. mollis* extract

Wavelength	EE x I	Absorption of extract at a concentration of 100 µg/mL			Absorption of extract at a concentration of 250 µg/mL		
		Nonpolar	Semipolar	Polar	Nonpolar	Semipolar	Polar
290	0.0150	0.196	0.351	0.511	0.471	0.872	1.288
295	0.0817	0.185	0.341	0.534	0.446	0.851	1.352
300	0.2874	0.161	0.341	0.544	0.434	0.844	1.376
305	0.3278	0.173	0.339	0.545	0.424	0.847	1.377
310	0.1864	0.172	0.343	0.548	0.425	0.863	1.390
315	0.0839	0.179	0.348	0.569	0.436	0.868	1.448
320	0.0180	0.184	0.357	0.601	0.451	0.886	1.543
SPF Value		1.72 ± 0.01	3.4 ± 0.01	5.47 ± 0.01	4.31 ± 0.16	8.53 ± 0.03	13.85 ± 0.01

Examination of total phenolic content using the Folin Ciocalteu (FC) method based on a redox reaction mechanism between phenolic substances that reduce the acidic components in the reagent (FC) in an alkaline medium. Phenolic substances turn into phenolic ions, while the acidic components of reduced FC reagent will result in discoloration from yellow to deep blue. This discoloration is stoichiometrically proportional to the number of phenolic substances that react.[27] Pearson's correlation analysis showed a correlation between antioxidant activity and total phenolic levels with a value of -0.864. This negative value indicates that a high phenolic content provides a low inhibition value (IC_{50}). Higher antioxidant activity is indicated by a low IC_{50} value.[13]The group of phenolic compounds is a natural antioxidant whose potency is due to its ability to donate hydrogen atoms to radical compounds. In contrast, the phenolic radicals formed will stabilize through the delocalization of electrons in aromatic ring resonance and provide the formation of a stable diquinone structure.[28] The antioxidant activity of the phenolic will be more potent with the presence of two hydroxyl groups close together at the ortho position.[29] The presence of other substituents in the structure of phenolic compounds and the bond strength of the O-H group also affect its antioxidant activity.[30] SPF values were measured at UVB wavelengths, in the range of 290 nm – 320 nm.Polar extract at a concentration of 250 ppm gives an SPF value of 13.85, the highest compared to other extracts.

SPF values of each extract and absorbant measurements at intervals of 5 nm in the UVB region are shown in Table 3. The SPF ability of polar extract belongs to the category of maximum protection.[31,32] UV radiation can cause acute effects such as erythema, pigmentation, and sunburn. The chronic impact of this radiation is susceptible to individuals who work daily in open areas and are exposed to direct sunlight to suffer the effects of immune system suppression, inflammation, and photoaging.[33] Polar extract *E. mollis* has a high phenolic content and vigorous antioxidant activity and can also act as an organic sunscreen that will reduce the penetration of UV rays into the skin. Organic substances such as phenolic components in plants can serve as sunscreens because they have chromophore groups that absorb sunlight energy.[34] The energy of sunlight will change the molecules to the excited state, and then when returned to the original energy state their release energy in the form of fluorescence or low heat.[35] This process continues to repeat and is influenced by the number of chromophore groups and electron resonance in these organic compounds. Polar extracts that are active antioxidants, sunscreens, and contain the highest levels of phenolics were tested *in vivo* on male albino mice using a topical application at the dorsal area. The test results can be seen in Table 4 and Figure 1. On visual observations, increased doses of polar extract further reduced erythema and wrinkles on the skin of test animals. The highest concentration of extract (7.5 %) showed dermis performance similar to the normal control group. Based on histopathological observations, exposure to UV light leads to increased collagen deposition, collagen compaction, and thickening of the epidermis (acanthosis). Thickening of the epidermis can be caused due to hyperkeratosis (thickening of the stratum corneum), spongiosis (edema containing intercellular fluid). Thickening of the epidermis due to exposure to UV light can reach 12 times compared to normal skin epidermis.[36]In this study, the thickening of the epidermis was three times higher than normal skin.

In general, UV light can result in the degradation of collagen fibers. But at the same time, there is also an increase in collagen synthesis by fibroblasts as feedback to physiological disorders.

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Table 4: Results of examination of skin performance and dermis of test animals

Groups	Collagen score	Fibroblast score	Epitel score	Epidermal thickness	Wrinkle score	Erythema score
Normal control	1.3 ± 0.09^{a}	1.33 ± 0.47^{a}	3 ± 0.00^{a}	23.3 ± 0.65^{a}	0 ± 0.00^{a}	0 ± 0.00^{a}
UV control	2.8 ± 0.16^{d}	3.00 ± 0.00^{c}	2.3 ± 0.47^{b}	69.3 ± 1.82^{d}	2.28 ± 0.71^{d}	3.08 ± 1.09^{d}
2.5 % of extract	2.3 ± 0.25^{c}	$2.67 \pm 0.47^{b,c}$	3 ± 0.00^{a}	35.4 ± 1.86^{c}	1.13 ± 0.48^{c}	1.44 ± 0.77^{c}
5 % of extract	$1.9 \pm 0.25^{b,c}$	$2.00 \pm 0.47^{a,b}$	3 ± 0.00^{a}	29.2 ± 2.13^{b}	0.71 ± 0.39^{b}	0.81 ± 0.49^{b}
7.5 % of extract	$1.5 \pm 0.19^{a,b}$	1.67 ± 0.47^{a}	3 ± 0.00^{a}	25.1 ± 1.80^{a}	0.41 ± 0.23^{b}	$0.43 \pm 0.25^{a,b}$

Values are represented as Mean (±) standard deviation. Values with different alphabets as superscripts for a parameter in a column differ significantly ($p \leq 0.05$)

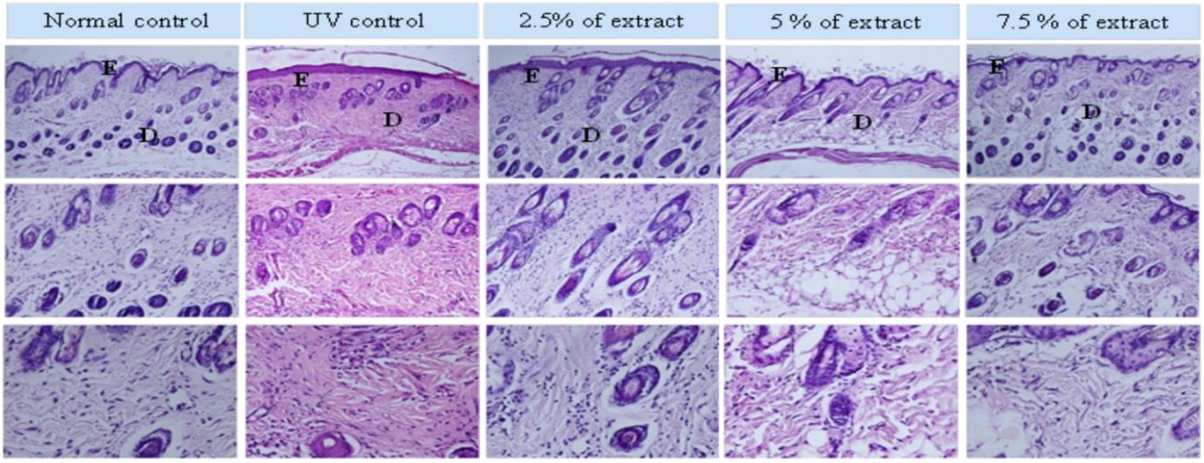

Figure 1: Histology of animal skin tissue shows the epithelium of the epidermis (E) and dermis (D), staining with Hematoxylin-eosin. Top panel objective 10x, middle 20x under objective 40x.

Table 5: Chemical component profile of polar extract *E. mollis* with LC-HRMS

No	Name	Formula	Molecular Weight	RT [min]
1	Chlorogenic acid	$C_{16}H_{18}O_9$	376.07748	0.865
2	Adenine	$C_5H_5N_5$	135.0547	1.165
3	Isoleucine	$C_6H_{13}NO_2$	131.09473	1.51
4	Kynurenic acid	$C_{10}H_7NO_3$	189.04282	4.148
5	(1r,3R,4s,5S)-4-{[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-1,3,5-trihydroxycyclohexane-1-carboxylic acid	$C_{16}H_{18}O_9$	354.09539	4.878
6	2,3,4,9-Tetrahydro-1H-β-carboline-3-carboxylic acid	$C_{12}H_{12}N_2O_2$	216.09001	5.274
7	(1S,3R,4R,5R)-1,3,4-trihydroxy-5-{[(2E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoyl]oxy}cyclohexane-1-carboxylic acid	$C_{17}H_{20}O_9$	368.11098	6.287
8	2-[(4-hydroxy-3,5-dimethoxyphenyl)methoxy]-6-(hydroxymethyl)oxane-3,4,5-triol	$C_{15}H_{22}O_9$	368.11098	6.695
9	Rutin	$C_{27}H_{30}O_{16}$	610.15416	7.007
10	Quercetin	$C_{15}H_{10}O_7$	302.04265	7.309
11	Quercetin-3β-D-glucoside	$C_{21}H_{20}O_{12}$	464.09596	7.312
12	3-{[(2S,3R,4S,5R,6R)-3,5-dihydroxy-6-(hydroxymethyl)-4-{[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy}oxan-2-yl]oxy}-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one	$C_{27}H_{30}O_{15}$	594.15911	7.596
13	Kaempferol	$C_{15}H_{10}O_6$	286.04775	7.606
14	3,5-di-O-caffeoyl quinic acid	$C_{25}H_{24}O_{12}$	516.12699	7.852
15	Trifolin	$C_{21}H_{20}O_{11}$	448.10094	7.894
16	Apigenin 7-O-glucuronide	$C_{21}H_{18}O_{11}$	446.08553	8.151
17	Apigenin	$C_{15}H_{10}O_5$	270.05217	8.153
18	Caffeic acid	$C_9H_8O_4$	180.04235	8.199

Conclusion

This study showed that polar extract *E. mollis* has a high phenolic content and is active as an antioxidant, and organic sunscreen can protect the skin of mice exposed to UV rays. Administration of polar extract *E. mollis* can reduce hyperplasia in the epidermis and decrease disorganized or pathological collagen.Polar extract *E. mollis* rich in polyphenol components can be a natural photoprotector that provides skin protection against UV rays through its activity as an antioxidant and organic sunscreen.

Conflict of Interest

The authors declare no conflict of interest.

Author's 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.

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