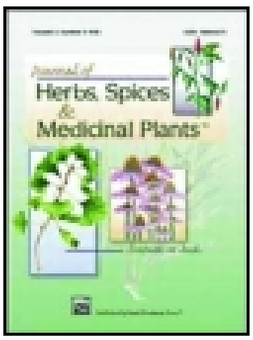

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Phytochemical Screening and the Antimicrobial and Antioxidant Activities of Medicinal Plants of Meru Betiri National Park – Indonesia

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ABSTRACT

The Meru Betiri National Park in Indonesia is host to more than 266 medicinal plants species, of which 10 were investigated for their phytochemicals as well as antioxidant and antimicrobial activities. A majority of the tested plant species contained polyphenols. The crude leaf extracts of *Dioscorea esculenta* possessed the highest antioxidant activity with IC_{50} of 26.8 µg mL^{-1}. *Escherichia coli* was sensitive to *Bryophyllum pinnatum* and *Hibiscus tilliaceus* leaf extracts with similar minimum inhibition concentration (MIC) of 250 µg mL^{-1}. *Klebsiella pneumoniae* was sensitive to *Moringa oleifera* leaf extract with MIC of 125 µg mL^{-1}. *Staphylococcus aureus* was the most sensitive to leaf extracts of *Hibiscus tilliaceus* with MIC of 62.5 µg mL^{-1}, *Pseudomonas aeruginosa* was sensitive to *Lunasia amara* leaf extract with MIC of 125 µg mL^{-1}. Autobiographic TLC confirmed the presence of anti-microbial constituents in *L. amara* leaf extract.

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KEYWORDS
Indonesian medicinal plants; minimum inhibition concentration

Introduction

Indonesia is rich in tropical biodiversity with more than 40,000 flora, and 6,000 medicinal plant species have been recorded.[1,2] Indonesia has 300 diversely ethnic people living in 17,000 different Islands. There are many tribal communities living in remote areas that depend on their indigenous knowledge of herbal medicines, transmitted orally from one generation to another.[2] The first written inscription of the ancient medicament preparation technique was depicted on a relic at the biggest Borobudur Temple dating back to 8th century.[3] "Serat Centhini" is a comprehensive record of Javanese culture written on a request from King of Mataram Kingdom (1600–1800 AD).[4]

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Lifestyle changes resulting in unhealthy diet and increasing life stress[5] and increase of degenerative diseases prevalence including neurodegenerative diseases, cancer, diabetes and heart diseases.[6] Metabolic diseases are correlated to cell metabolism malfunction due to increase of reactive oxygen species in the body.[7] When natural antioxidant in the body is outnumbered, exogenic antioxidant intake from supplement and food diet is necessary.[7] Therefore, the need for exploration for antioxidant is demanded from traditional medicinal plant species which have been passed through many generations.

World Health Organization (WHO) reported 5.7 million mortality caused by diarrhea, mycobacterium tuberculosis and respiratory tract infection.[8] A major problem is due to the pathogenic bacteria adaptation to the current antibiotics.[9] Recently, industries dealing with herbal medicines and conventional pharmaceuticals have shown interest in the development of indigenous medicines[10] and the products manufactured are therefore regulated based on the basis of those forms medicinal dosage such as a simplisia, extract or active fractions in which the latter is categorized as "fitofarmaka." The fitofarmaka form requires the highest quality standard, which is comparable to conventional drug requirements.[10] The only non-regulated traditional medicament is a traditionally prepared medicament, "*Jamu*," which is prepared by water-based extraction from fresh ingredient.[3,11,12]

To date, around 200 species of Indonesian medicinal plants have been studied, and are reflected in about 500 publications.[2] The Indonesian Government has regulated 2,967 million hectares of land in the archipelago as protected pristine forests and parks.[13] The Meru Betiri National Park in East Java are protected since 1931 occupies 540 km^2 of protected land that has coastal, mangrove, swamp, rheofit and lowland tropical forest vegetation [14] (Fig. 1). The forest is managed by nine resort areas and surrounded by 10 remote villages. Previous ethnobotanical survey in this area recorded 266 medicinal plant species belonging to 77 families, of which 10 plant species were selected for this study and collected through a field expedition at Bandealit resort-Meru Betiri National Park (Fig. 1).

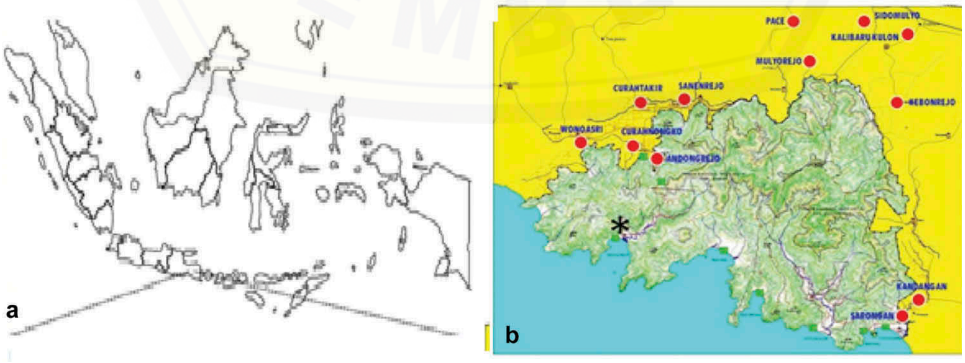

Figure 1. (a) Map of Indonesia; (b) Meru Betiri National Park map[8] and * indicate plants collection site.

Material and Methods

Legal Administration

Research proposal was approved and access permit (SIMAKSI No. SI2409/BTNMB/TU/PPI/11/2016) to collect plant materials were granted by the head office of Meru Betiri National Park – Jember on April 2016. The research permit (No. 1026/STe/UN25.3.1/LP2 M/2016) was granted by the Research Center of University of Jember.

Medicinal Plants

The expedition to Meru Betiri National Park was conducted with assistance from the park forest ranger on May 25, 2016. The plant species *Senna siamea, Bryophyllum pinnatum Dioscorea esculenta, Flagellaria indica, Persea odoratissima, Hibiscus tilliaceus, Moringa oleifera, Lunasia amara, Murraya paniculate*, and *Tetrastigma lanceolarium* were collected (Table 1) and assigned codes as SS, BP, DE, FI, PO, HT, MO, LA, MP, and TL, respectively. Samples were reported to the authority both in the collection site office and the head office of Meru Betiri National Park and transported into the Faculty of Pharmacy University of Jember – Indonesia for further studies. Specimen samples were kept in the Faculty of Pharmacy University of Jember and their copies were sent to Purwodadi Botanical Garden-Indonesian Institute of Science (Pasuruan District-East Java Province, Indonesia) for taxonomical confirmation.

Table 1. Selected medicinal plants of Meru Betiri National Park studied.

Botanical name	Family	Parts used (VSN)*	Local name	Traditional indication	Yields of extracts (%)
Bryophyllum pinnatum	Crassulaceae	Leaves (BP)	Cocor bebek	Boils, sores, and swellings	6.17
Dioscorea esculenta	Dioscoreaceae	Leaves (DE)	Gembili	Dysentery and diarrhea	6.77
Senna siamea	Fabaceae	Leaves (SS)	Johar	Swelling and rheumatism	8.95
Flagellaria indica	Flagellariaceae	Leaves (FI)	Wowo	Gonorrhea and rheumatism	10.72
Persea odoratissima	Lauraceae	Leaves (PO)	Talesan	Headache and fever	18.29
Hibiscus tilliaceus	Malvaceae	Leaves (HT)	Waru	Dysentery and malaria	12.04
Moringa oleifera	Moringaceae	Leaves (MO)	Kelor	Boosting immune system and malaria	12.38
Lunasia amara	Rutaceae	Leaves (LA)	Kemaitan	Malaria	4.96
Murraya paniculata	Rutaceae	Leaves (MP)	Kemuning	Diarrhea and malaria	9.98
Tetrastigma lanceolarium.	Vitaceae	Leaves (TL)	Paleran	Boils	8.12

*VSN: voucher specimen number.

Extraction Method and Preparation of Leaf Extracts

Leaf samples of the selected medicinal plant species were shade-dried and stored in the freezer (−20°C) until further use. A portion of each dried sample (100 g) was ground to make coarse powder, soaked with methanol (500 mL), for 5 days (solvent was renewed three times), filtered and the supernatant obtained was vacuum-dried using rotary evaporator (Steroglass strike 300, Italy) to produce crude methanol extracts.

Phytochemical Screening for Major Classes of Secondary Metabolites

Test Tube Reaction Method

A series of phytochemical screening for alkaloid, flavonoid, polyphenol, tannin, saponin, terpenoid and steroid were carried out. Qualitative screening for phytochemicals was performed based on reaction against specific reagents.[15] Briefly, a portion of crude methanol solution (1 mg in 5 mL ethanol) in a tube was reacted with screening reagents. Alkaloid was detected by reacting the crude extract solution with Dragendorff and Mayer reagents. The presence of alkaloids was indicated by changes in the endpoint color which is reddish and white or turbid colors, respectively. Flavonoid was indicated by reddish, yellowish or orange color after reacting with a mixture of magnesium (0.1 mg), 1-pentanol (4 mL), and HCl:ethanol (1:1, 4 mL). Saponnin was detected by adding one drop of H_2SO_4 into the crude extract solution followed by thorough mixing. Greenish blue color indicated the presence of steroid saponin, reddish color indicated the presence of steroid triterpene and yellowish color suggested the saturated saponin presence. Polyphenol and tannins were evaluated by dissolving crude extract (1 mg) into hot water (5 mL) and cooling to room temperature. NaCl solution (1% in water) was added, mixed, and filtered. $FeCl_3$ solution was added (two drops) to generate color changes. Dark green color suggested the presence of tannins. Polyphenol presence was detected by NaCl and $FeCl_3$ addition to the solution. No precipitation with NaCl and forming greenish blue color upon addition of $FeCl_3$ confirmed the presence of polyphenolic compounds in the solution.

Thin-Layer Chromatography (TLC) Method

The sample was loaded into thin-layer chromatography plate (5×10 cm) followed by development with dichloromethane:methanol (9.5:0.5). Visualization was performed under UV light (254 and 365 nm) and reagent test. Vanillin reagent test was used to see terpenoids components with purple color indication. Dragendorff reagent was able to confirm alkaloids present by orange color.[16]

Antioxidant Activity Test

The antioxidant activity was determined as described.[17] Briefly, methanol extracts of the plants were tested against 2,2-diphenyl-1-picrylhydrazyl (DPPH). A series of concentration of crude extract (0.25 mL) was mixed with DPPH (0.1 mM, 1.0 mL), incubated at room temperature in a dark room for 30 min. Absorbance was measured using a UV spectrometer at λ 513.5 nm. Quercetin was used as a positive control whereas DPPH solution (0.1 mM, 1.25 mL) was used as blanks. Percentage inhibition was calculated as:

$$Percentage\ inhibition = \frac{Abs\ DPPH - Abs\ sample}{Abs\ DPPH} x100\%$$

Linear regression was generated based on percentage inhibition *vs* concentration, and the IC_{50} (the concentration of a substance at which 50% of the target radical is inhibited) was calculated from the concentration curve used to produce 50% inhibition.

Antibacterial Activity

Bacterial Culture Preparation

A colony of each bacterium (Gram-positive bacterium, *Staphylococcus aureus* (SA) and Gram-negative bacteria, *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), and *Klebsiella pneumonia* (KP)) were aseptically transported into Nutrient Agar Slant (NAS, Merck) media followed by incubation at 37°C for 24 h. Each colony was loaded into Nutrient Broth Media (Merck) (4 mL) and homogenized with its turbidity and adjusted to 0.5 Mc Farland standard. Microdilution assay was performed.[18] Briefly, series of concentration (prepared by twofold dilution) of crude leaf extracts were made in DMSO/media solution (1000 ppm, 500 ppm, 250 ppm, 125 ppm, and 31.25 ppm). Antibacterial activity was tested by mixing the leaf extracts bacterial solution and Tryptic soy broth (TSB, Himedia), followed by incubation at 37°C for 24 h. Turbidity on each crude extract concentration were visually evaluated to indicate a minimum inhibition concentration (antibacterial activity).

Bioautographic Thin-Layer Chromatography

Crude methanol extract (20 µL) was loaded into TLC plate and developed with dichloromethane:methanol (9.5:0.5) at 8 cm distance. The TLC was then checked under UV light (254 and 365 nm) prior sterilization under UV light for 30 min in an aseptic chamber laminar air flow. Bacterial suspension was loaded into Miller Hinton Agar plate and the TLC was attached into the agar plate for 60 min prior incubation for 18 h at 37°C. Antibacterial activity of the chromatographic spots was indicated by clean zone on the agar plate.

Results

Phytochemical Screening for Secondary Metabolites

All the plant species tested positive for polyphenols (Table 2). Thin-Layer Chromatographic method revealed all the selected plant species contained terpenoids (Fig. 2).

Antioxidant Activity

D. esculenta demonstrated the strongest antioxidant activity with IC_{50} of 26.8 μg mL^{-1}, followed by *M. paniculate* (IC_{50} of 50.2 μg mL^{-1}) and *H. tilliaceus* (IC_{50} of 56.3 μg mL^{-1}). *T. lanceolarium* had the lowest antioxidant potency (Table 3).

Antibacterial Activity

H. tilliaceus extract exhibited the highest antibacterial activity against *S. aureus* with MIC of 62.5 μg mL^{-1} (Table 4). The plant extracts had moderate to strong antimicrobial effects against gram-positive (*S. aureus*) and gram-negative

Table 2. Phytochemical screening of selected medicinal plants of Meru Betiri National Park, Indonesia.

Botanical name	Alkaloid	Flavonoid	Polyphenol	Tannin	Saponin	Steroid
1. Bryophyllum pinnatum	+	-	+	-	-	-
2. Dioscorea esculenta	-	-	+	-	+	-
3. Senna siamea	+	-	+	-	+	-
4. Flagellaria indica	+	+	+	-	+	-
5. Persea odoratissima	-	-	+	-	+	-
6. Hibiscus tilliaceus.	-	+	+	+	+	-
7. Moringa oleifera	-	-	+	-	+	-
8. Lunasia amara	+	-	+	+	+	-
9. Murraya paniculata	+	-	+	-	+	+
10. Tetrastigma lanceolarium	+	+	+	-	+	-

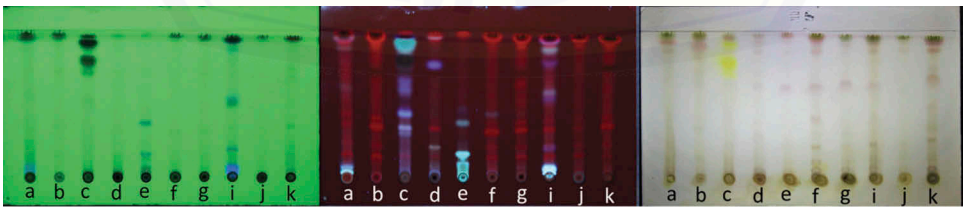

Figure 2. Thin-layer chromatogram of solvent leaf extracts from selected plants developed with dichloromethane:m ethanol (9.5:0.5) and visualized under 254 nm UV light (left), 365 nm UV light (middle) and vanillin reagent (right). a: *Tetrastigma lanceolarium* b: *Persea odoratissima*; c: *Murraya paniculata*; d: *Hibiscus tilliaceus*; e: *Senna siamea*; f: *Moringa oleifera*; g: *Dioscorea esculenta*; h: *Lunasia amara*; i: *Flagellaria indica*; j: *Bryophyllum pinnatum.*

Table 3. Radical scavenging activity of selected medicinal plants of Meru Betiri National Park.

Botanical name	IC_{50} (μg mL^{-1})
Bryophyllum pinnatum	125.1 ± 1.9
Dioscorea esculenta	26.8 ± 0.7
Senna siamea	64.6 ± 3.1
Flagellaria indica	144.1 ± 6.0
Persea odoratissima	79.6 ± 1.9
Hibiscus tilliaceus	56.3 ± 2.2
Moringa oleifera	119.8 ± 4.3
Lunasia amara	474.7 ± 4.5
Murraya paniculata	50.2 ± 0.4
Tetrastigma lanceolarium	551.5 ± 6.1
Quercetin (reference/standard)*	2.2 ± 0.0

Quercetin was used as a standard antioxidant which commonly presents in plants.

Table 4. Antibacterial activity of selected medicinal plants of Meru Betiri National Park.

	MIC (μg mL^{-1})			
Botanical name	EC	KP	SA	PA
Bryophyllum pinnatum	>1,000	>1,000	250	500
Dioscorea esculenta	>1,000	500	250	>1,000
Senna siamea	250	250	125	>1,000
Flagellaria indica	>1,000	>1,000	500	250
Persea odoratissima	>1,000	>1,000	250	250
Hibiscus tilliaceus	250	>1,000	62.5	250
Moringa oleifera	500	125	500	500
Lunasia amara	>1,000	>1,000	1,000	125
Murraya paniculata	1,000	>1,000	>1,000	>1,000
Tetrastigma lanceolarium	1,000	125	>1,000	>1,000
Gentamycin*	na	0.25	na	na

*positive control; na: not available; EC: *Escherichia coli*; SA: *Staphylococcus aureus*; PA: *Pseudomonas aeruginosa*: KP: *Klebsiella pneumonia.*

(*E. coli, P. aeruginosa, K. pneumoniae*) bacteria. The gram-positive bacteria, *S. aureus* was sensitive against the crude extracts of *D. esculenta, H. tilliaceus, B. pinnatum*, and *S. siamea* with MIC value of 250, 62.5, 250, and 125 μg mL^{-1}, respectively. While the gram-negative bacteria, *K. pneumoniae* was sensitive to the crude extract of *M. oleifera* with MIC values of 125 μg mL^{-1}, other gram-negative bacteria, *P. aeruginosa* was sensitive to the crude extracts of *L. amara* and *F. indica* with MIC values of 125 and 250 μg mL^{-1}, respectively. *S. aureus* was moderately sensitive to *S. siamea* extract. While KP was moderately sensitive to *T. lanceolarium* extract with IC_{50} of 125 μg mL^{-1}, PA was moderately sensitive to *L. amara* at similar concentration. Bioautographic TLC confirmed the antibacterial activity of the *L. amara* against *P. aeruginosa.*

Discussion

In this study, 10 medicinal plants used by the communities living around the park for treating various disorders including boils, wounds/sores, gonorrhea, diarrhea, fever, malaria, and rheumatism, which all bore relevance to modern microbial and malarial infections were collected from nearby Bandealit coast. The plants studied showed the presences of varied phytochemicals (Table 2). Plants produce these various chemotyes as secondary metabolites (SM) to heal their injuries, defend themselves against herbivores and safeguard their territory. For example, the alkaloids serve the plants as a feeding deterrent to protect from predators and fungal infections, and consequently they are observed in areas where herbivores usually attack (inflorescence, young plants, and peripheral cell layers of stems and roots).[19]

It is estimated that more than 73% of new chemical entities or pharmaceutical drug entities that were discovered between 1981 and 2014 were isolated from natural sources and that 11% of the 252 essential drugs currently listed by the WHO are exclusively of plant origin.[19] The main categories of plant-derived drugs that are available today are: terpenes (34%), glycosides (32%), polyketides and others (18%), and alkaloids (16%).[20] Most of the Indonesian tropical flora, parasitic and medicinal plants are poorly studied for medicinal applications. This study tested the methanol extracts of selected medicinal plants for their antioxidant and antimicrobial activities. Since all leaf extracts of the selected species tested positive for phenolic compounds, it was suggestive of their potential antioxidant properties.

Studies have shown that intake of natural antioxidants lower the risks of developing several diseases due to free radicals in the human body, especially neurodegenerative disorders, cardiovascular diseases, and cancers.[21] *M. oleifera* which is traditionally used by local community of Indonesia as immune boosting agent tested positive for phenolic compounds, which contribute to radical scavenging properties. necessary in cell adaptation mechanism toward biotic and abiotic stress. The crude methanol extract of *D. esculenta* (IC_{50} value of 26.8 μg mL^{-1}), was the most antioxidant, which is traditionally used for treating dysentery and diarrhea.

Simple phenols are known for their antibacterial activity. For example, divaricatic acid, a depside derivative was indicated as a potential antimicrobial agent for the treatment of methicillin resistant-*S. aureus* (MRSA) infection[22], and glycosylated quercetin to inhibit HIV integrase enzyme.[23] The bioautograph confirmed the crude extract of leaf of *L. amara* to posses antibacterial activity in both polar and less polar constituents indicated by clean zone at the both high and low retention time (Fig. 3). However, anti-*Staphylococcus* of the leaf crude extract of

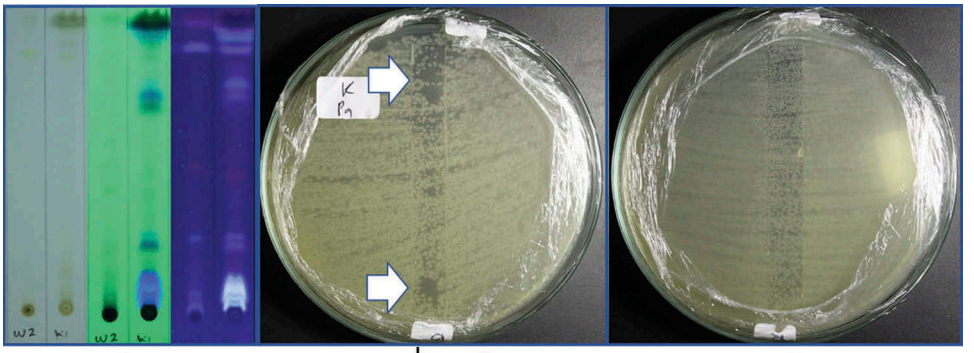

Figure 3. (a) Thin-layer chromatogram (TLC) of crude leaf extract of *Hibiscus tilliaceus* (W2) and *Lunasia amara* (K1) developed with dichloromethane:Methanol (9.5:0.5). (b) Bioautographic TLC of *Lunasia amara* (K1) against *Pseudomonas aeruginosa*. (c). Bioautographic TLC of *Hibiscus tilliaceus L. (W2)* against *Staphylococcus aureus*. Arrow indicated a clean zone representing antimicrobial activity.

H. tilliaceus was absent after chromatographic separation, which may be due the synergism mechanism of the mixed component in the crude extract.

The results of this study are consistent with other reports. For example, *B. pinnatum* crude extract collected from Cameroon was reported to possess good antioxidant activity (IC_{50} 25.31 μg mL^{-1}), and inhibit *Helicobacter pylori* growth with MIC value of 32 μg mL^{-1}.[24] Similarly, the crude extract of *S. siamea* from Thailand exhibited antioxidant activity (in DPPH assay) with IC_{50} value of 39 μg mL^{-1} but not antibacterial against *S. aureus*.[25,26] The essential oil of *P. odoratissima*, from the Himalayas, showed very mild antioxidant activity with IC_{50} value of 675 μg mL^{-1}, and strong antibacterial activity against *E. coli* and *S. aureus* with MIC value of 3.90 and 7.81 μg mL^{-1}, respectively.[27] Acetone extract of *M. oleifera* from China was antioxidant and antibacterial with IC_{50} value of 5.18 μg mL^{-1} and 62.50 μg mL^{-1} (*against S. aureus*), respectively.[28] However, *M. paniculata* extract, from Kalimantan Island-Indonesia, did not show antioxidant activity.[29] The differences in the results may be due to environmental, ecological and geographical conditions that contribute to the diversity of the species and chemotypes.

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Author Contributions

Conceptualization, A.S.N.; methodology, A.S.N., D.K.P., B.T., D.D., V.A.R., I.N., I.P.D., I.P.S.; investigation, A.S.N., A.E.N.P., C.P.K., D.K.P., B.T., D.D., V.A.R., I.N., I.P.D., I.P.S.; data curation, A.E.N.P., C.P.K.; resources, A.S.N., D.K.P., B.T., D.D., V.A.R., I.N., I.P.D., I.P.S.; formal analysis and data interpretation, A.S.N., D.K.P., P.W; original draft preparation, A.S.N., D.K.P., B.T., P.W.; review and editing, A.S.N., P.W.

Declarations of Interest

The authors declare no conflict of interest.

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