Screening of various Ayurvedic medicines for their inhibitory activities on reverse transcriptase and identification of arecatannins and embelin as major inhibitory substances from *Areca catechu* and *Embelia ribes*

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Abstract

Forty-two different kinds of Ayurvedic medicines were tested for *in vitro* effects on reverse transcriptase (RT) from avian myeloblastosis virus, using $(rA)_n \cdot (dT)_{12-18}$ as a template-primer. Of these, methanol and water extracts of *Areca catechu* (seed), *Cinnamomum verum* (bark) and *Terminalia arjuna* (stem bark) and methanol extracts of *Eugenia jambolana* (bark), *Ficus religiosa* (bark), *Punica granatum* (pericarp), *Terminalia chebula* (fruit), and water extracts of *Coleus amboinicus* (leaf), *Rhus acuminata* (gall) and *Saraca indica* (bark) were found to be the most potent RT-inhibitors with IC_{50} of 4-66 μg/ml. By bio-assay directed fractionation, arecatannins B1 and A2 from the *Areca catechu* extract and embelin from the *Embelia ribes* extract were identified as potent RT-inhibitory principles.

Key words Ayurvedic medicines, arecatannin, reverse transcriptase inhibitor.

Abbreviations AMV, avian myeloblastosis virus; DEAE, diethylaminoethyl; DMSO, dimethyl sulfoxide; dTMP, deoxythymidine 5'-monophosphate; DTT, dithiothreitol; dTTP, deoxythymidine 5'-triphosphate; HIV, human immunodeficiency virus; HTLV, human T-cell leukemia virus; MuLV, Moloney murine leukemia virus; RT, reverse transcriptase.

Introduction

Reverse transcriptase (RT) is a retrovirus-specific enzyme which catalyzes the synthesis of DNA, complementary to the viral RNA genome (reverse transcription), to give a DNA - RNA hetero-duplex and the subsequent formation of a DNA duplex through digestion of the RNA strand, followed by complementary DNA synthesis.[1] Since reverse transcription is an essential step in the life - cycle of retroviruses, such as human immunodeficiency virus (HIV) and human T-cell leukemia virus (HTLV), inhibition of RT may be one of the most promising targets of the prevention of retrovirus infection and the subsequent virus production.

As regards naturally - occurring RT inhibitors, quinones,[2-4] alkaloids[5-8] and flavonoids[8-11] have hitherto been reported. In the previous papers,[12-14] we have reported that some dimeric ellagitannins and benzo [c] phenanthridine alkaloids strongly inhibit RT activity possibly through their interactions with nucleic acids used as a template-primer. Furthermore, we have investigated inhibitory effects of 190 flavonoids and 75 alkaloids on avian myeloblastosis virus RT (AMV-RT) and found that highly hydroxylated flavonoids and quaternary alkaloids were potent inhibitors against this enzyme.[8] HIV - RT was also inhibited by these compounds with similar inhibitory potencies but Moloney murine leukemia virus (MuLV) RT was more strongly inhibited compared to HIV-and AMV-RTs.[15]

In the present paper, we report on the inhibitory effects of the extracts of Ayurvedic medi-

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cines on AMV-RT, which is commonly used for general screening tests of RT-inhibitory agents.

Materials and Methods

Plant Materials : All crude drugs used in this experiment were purchased from W. Wilbert and Co. (Colombo, Sri Lanka). The botanical sources of the drugs were identified by Drs. Upali Pilapitiya (Bandaranayake Memorial Ayurvedic Research Institute Nawinna, Sri Lanka) and D. M. R. Dissanayake (Institute of Indigenous Medicine, University of Colombo, Sri Lanka). The voucher specimens were deposited in the Herbarium of Materia Medica of Toyama Medical and Pharmaceutical University.

Preparation of extracts : A pulverized crude drug (5 g) was extracted with water or methanol (100 ml) under reflux for 3 hr. After filtration, the solution was evaporated *in vacuo* to give a water or methanol extract.

Enzyme and chemicals : AMV-RT was purchased from Wako Pure Chemical Industries Co. (Osaka, Japan). RT was diluted with a solution containing 0.2M phosphate buffer (pH7.2), 50 % glycerol, 2mM dithiothreitol (DTT), and 0.2 % Triton X-100 to adjust a concentration of 1 unit/ml. One enzyme unit was defined as the amount of enzyme which catalized the incorporation of 1 nmol of deoxythymidine 5'-monophosphate (dTMP) into a polymer fraction in 10 min at 35°C. Poly riboadenylic acid and oligo deoxythymidylic acid duplex, $(rA)_n \cdot (dT)_{12-18}$ and deoxythymidine 5'-triphosphate (dTTP) was purchased from Pharmacia Co. (Uppsala, Sweden). [methyl-^{3}H]-dTTP was obtained from Amersham-Japan Co. (Tokyo, Japan) and its specific activity was 1.55 TBq/mmol. DEAE-cellulose filter paper discs were obtained from Whatman Ltd. (Maidstone, England). Adriamycin (doxorubicin hydrochloride) was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Embelin was isolated from the fruit of *Embelia ribes* as reported in a previous paper.[16] All other reagents and solvents were of analytical grade.

Inhibitory activity on RT : A reaction mixture (20 μl) contained the following : 50 mM Tris-HCl (pH 8.3), 40 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 5 μg/ml $(rA)_n \cdot (dT)_{12-18}$, 0.1 mM [methyl-^{3}H]-dTTP, an extract dissolved in DMSO and 1 unit of RT. The final DMSO concentration was 10 %. The reaction mixture was incubated for 60 min at 37°C. A 10 μl portion of the mixture was applied to a DEAE-cellulose paper disc (2,3 cm diameter ; Whatman DE 81), and the paper disc was washed in a batch in 3 ml of a 5 % Na_2HPO_4 solution five times, followed by two times each with water and ethanol, and dried. The disc was then transferred into a vial containing ACS-II scintillation fluid (Amersham) and the radioactivity was measured in a liquid scintillation counter. The inhibitory activity was calculated as follows :

$$\left(1-\frac{\text{dpm of an assay with a sample}}{\text{dpm of control}}\right)\times 100$$

The control experiment was carried out without adding a test sample, and *ca.* 15000 dpm of [^{3}H]-dTMP was incorporated into a polymer fraction. Adriamycin (1.0 mM) was used as a positive control for the complete inhibition of the enzyme activity.

Fractionation of a 50 % acetone extract of the seeds of Areca catechu : As reported previously,[17] a 50 % acetone extract (132 g) was suspended in water and extracted with EtOAc. The EtOAc extract (61 g) was divided into hexane-soluble and -insoluble fractions. The hexane-insoluble fraction was partitioned with $CHCl_3$-MeOH-H_2O (3 : 3 : 2). The upper aqueous layer (47 g dry weight) was applied to a Diaion HP-20 column (100 cm×5 cm i.d.) and it was washed with H_2O and eluted with MeOH. A portion of the MeOH eluate was chromatographed on a Sephadex LH-20 column with H_2O and increasing amounts of MeOH, followed by high performance liquid chromatography (HPLC), using a preparative reverse phase column, Develosil ODS-10, Nomura Chemical (Seto, Japan). This gave (+)-catechin (**1**), (−)-epicatechin (**2**), procyanidin B1 [epicatechin (4$\beta \rightarrow$ 8) catechin] (**3**), arecatannin A1 [epicatechin (4$\beta \rightarrow$8) epicatechin (4$\beta \rightarrow$8) catechin] (**4**), arecatannin A2 [epicatechin (4$\beta \rightarrow$8) epicatechin (4$\beta \rightarrow$8) epicatechin (4$\beta \rightarrow$8) catechin] (**5**) and arecatannin B1 [epicatechin (4$\beta \rightarrow$ 8)

epicatechin (4β→6) catechin] (**6**). These compounds were identified by comparing the spectroscopic data with those reported by Nonaka *et al.*[18]

Table I Inhibitory effects of the extracts of Ayurvedic medicines on AMV-RT.

Botanical name	Sinhalese name	Part used	IC_{50} (μg/ml) MeOH extract	Water extract
Aconitum ferox WALL. (Ranunculaceae)	Wachchanavi	tuber	n.d.	>1000
Aegle marmelos CORR. (Rutaceae)	Beli	root bark	>1000	1000
Andropogon muricatus RETZ. (Gramineae)	Sewendara	root	>1000	n.d.
Apium graveolens L. (Umbelliferae)	Asamodagan	fruit	100	200
Areca catechu L. (Palmae)	Punvak	seed	30	42
Azadirachta indica A. JUSS. (Meliaceae)	Kohomba	seed	>1000	>1000
Boerhaavia diffusa L. (Nyctaginaceae)	Sarana	root	>1000	>1000
Caesalpinia bonducella FLEMING (Leguminosae)	Kumburuwel	seed	n.d.	>1000
Cassia fistula L. (Leguminosae)	Ehela	bark	200	130
Cinnamomum verum J. S. PRESL. (Lauraceae)	Kurundu-kola	leaf	200	200
C. verum J. S. PRESL. (Lauraceae)	Kurundu-potu	bark	32	32
Coleus amboinicus LOUR. FL. (Labiatae)	Kapparawalliya	leaf	1000	50
Coscinium fenestratum COLEBR. (Menispermaceae)	Weniwelgeta	gall	200	600
Crataeva religiosa FORST. F. (Capparidaceae)	Lunuwarana	bark	n.d.	140
Elettaria cardamomum MATON (Zingiberaceae)	Ensal	seed	1000	n.d.
Embelia ribes BURM. (Myrsinaceae)	Walagasal	fruit	400	200
Eugenia jambolana LAM. (Myrtaceae)	Madan	bark	18	n.d.
Ficus religiosa L. (Moraceae)	Bo	bark	36	n.d.
Mimusops elengi L. (Sapotaceae)	Muna-kola	leaf	200	200
M. elengi L. (Sapotaceae)	Muna-mal	bark	200	400
Myristica fragrans VAN HOUTT. (Myrticaceae)	Wasawashi	aril	>1000	n.d.
Nigela sativa L. (Ranunculaceae)	Kaluduru	seed	>1000	600
Oldenlandia herbacea ROXB. (Rubiaceae)	Pepiliya	root	n.d.	>1000
Peucedanum graveolens BENTH. (Umbelliferae)	Satakuppa	seed	1000	300
Phyllanthus emblica L. (Euphorbiaceae)	Nelli	fruit	200	500
Piper longum L. (Piperaceae)	Tippili	fruit	>1000	>1000
Pogostemon heynaenus BENTH. (Labiatae)	Kollan-kola	leaf	1000	1000
Pongamia glabra VENT. (Leguminosae)	Karanda	root	>1000	>1000

Table I continued

P. glabra VENT. (Leguminosae)	Karanda-potu	bark	>1000	>1000
Punica granatum L. (Punicaceae)	Delum	pericarp	2	200
Rhus acuminata L.f. (Anacardiaceae)	Kiribaduala	gall	>1000	66
Saraca indica L. (Leguminosae)	Asoka	bark	n.d.	4
Sida cordifolia L. (Malvaceae)	Bebila	root	>1000	>1000
Strychnos potatorum L.f. (Loganiaceae)	Ingini	seed	n.d.	>1000
Tephrosia purpurea PERS. (Leguminosae)	Pila	root	>1000	>1000
Terminalia arjuna WIGHT et ARN. (Combretaceae)	Kumbuk	stem bark	34	34
T. bellerica ROXB. (Combretaceae)	Bulu	fruit	200	1000
T. chebula RETZ. (Combretaceae)	Aralu	fruit	10	400
Trianthema decandra L. (Euphorbiaceae)	Sarana	root	>1000	>1000
Uncaria gambir ROXB. (Rubiaceae)	Kahdir	stem+branch	n.d.	1000
Vitex negundo L. (Verbenaceae)	Nika	root	>1000	>1000
Zingiber officinale ROSCOE. (Zingiberaceae)	Inguru	rhizome	>1000	>1000

The assay was carried out in the presence of $(rA)_n$-$(dT)_{12-18}$ as a template-primer, n.d., not determined.

Results

Screening of various extracts for inhibitory activity on RT

Water and methanol extracts of Ayurvedic medicines obtained in Sri Lanka were screened for their inhibitory activity on AMV RT (Table I). The assay was carried out by measuring incorporation of [methyl-^{3}H]-dTMP residue into a polymer fraction when poly (rA) · oligo (dT) were used as a template-primer. Of the extracts examined, the methanol and water extracts of the seed of *Areca catechu,* the bark of *Cinnamomum verum* and the stem bark of *Terminalia arjuna* ; the methanol extracts of the bark of *Eugenia jambolana,* the bark of *Ficus religiosa,* the pericarp of *Punica granatum* and the fruit of *Terminalia chebula* ; the water extracts of the leaf of *Coleus amboinicus,* the gall of *Rhus acuminata* and the bark of *Saraca indica,* had potent inhibitory activity against RT with 50 % inhibitory concentration (IC_{50}) values of 4-66 μg/ml. On the other hand, methanol and/or water extracts of the fruit of *Apium graveolens,* the bark of *Cassia fistula,* the leaf of *C. verum,* the fruit of *Embelia ribes,* the leaf and bark of *Mimusops elengi* and the fruit of *Phyllanthus emblica* showed moderate inhibition with IC_{50} of 100-200 μg/ml.

Inhibitory principles from the A. catechu extract against RT

Since the extract of the seed of *A. catechu* contains significant amounts of condensed tannins[18] which induce a variety of enzyme inhibitions,[17, 19, 20] we assumed that potent inhibitory action of both water and methanol extracts (more than *ca.* 90 % inhibition at 0.2-1.0 mg/ml) were primarily due to procyanidins. For the purpose of confirming this, bio-assay directed fractionation of a 50 % acetone extract, which had inhibitory activity similar to the methanol extract and is more suitable for the isolation of procyanidins, was carried out. The methanol-eluate from a Diaion HP-20 column (see Materials and Methods) induced potent inhibition at 1.0 mg/ml but stimulated the enzyme activity at a low concentration, similar to the

Table II Percentages of inhibition of the *A. catechu* extracts and the fractions against RT.

Extract and fraction	% Inhibition (mean ±S.E.)		
	Concentration (mg/ml)		
	0.01	0.1	1.0
MeOH extract	-27.3±7.5*	80.8±0.5	99.4±0.1
50% Acetone extract	-36.4±4.8*	76.2±1.3	98.7±0.2
Hexane-soluble	4.9±1.1	1.0±2.4	15.4±1.3
$CHCl_3$-MeOH-soluble	-3.3±1.0	-2.6±0.9	30.4±3.5
MeOH-eluate	-37.0±5.3*	24.6±3.2	98.5±0.3

The values were expressed as averages of 4 or 8 (*) experiments. For fractionation of the 50% acetone extract, see "Materials and Methods."

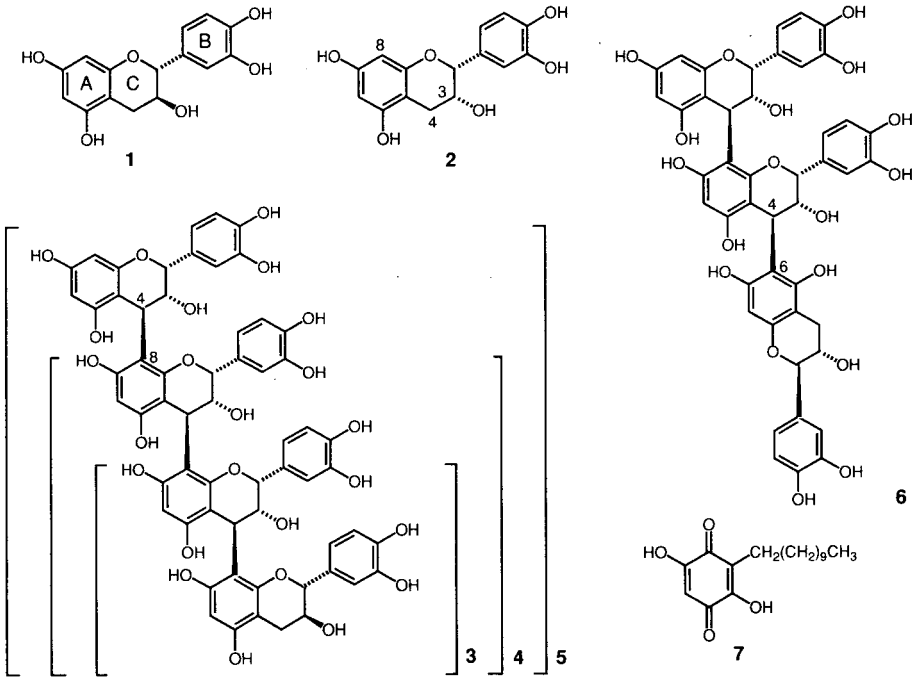

Fig. 1 Structures of procyanidins from *A. catechu* and embelin from *E. ribes*.

1, (+)-catechin ; **2**, (-)-epicatechin ; **3**, procyanidin B1 ; **4** arecatannin A1 ; **5**, arecatannin A2 : **6**, arecatannin B1 ; **7**, embelin

original 50 % acetone extract (Table II). Other fractions showed no significant inhibition at concentrations of 0.1 - 1.0 mg / ml. The methanol - eluate was then subjected to repeated column chromatography on Sephadex LH-20, followed by HPLC to give (+)-catechin (**1**), (−)-epicatechin (**2**), procyanidin B1 (**3**) and arecatannins A1 (**4**), A2 (**5**) and B1 (**6**) (Fig. 1). (+)- Catechin (**1**), (−)-epicatechin (**2**) and procyanidin B1 (**3**) showed no inhibition against RT activity at concentrations of 0-5.0 mM. However, arecatannins A1 (**4**), A2 (**5**) and B1 (**6**) induced appreciable inhibition (Fig. 2).

Of these condensed tannins, arecatannin B1 (**6**) induced the highest inhibition with an IC_{50} of approx. 0.5 mM (Table III).

Table III IC_{50} of components from *A. catechu* and *E. ribes* against RT.

Compound	IC_{50} (mM)
1 (+)-Catechin	>10.0
2 (-)-Epicatechin	>10.0
3 Procyanidin B1	>10.0
4 Arecatannin A1	3.3
5 Arecatannin A2	1.0
6 Arecatannin B1	0.5
7 Embelin	1.0

$(rA)_n \cdot (dT)_{12-18}$ was used as a template-primer and the IC_{50} of adriamycin was 66 μM under the same condition.

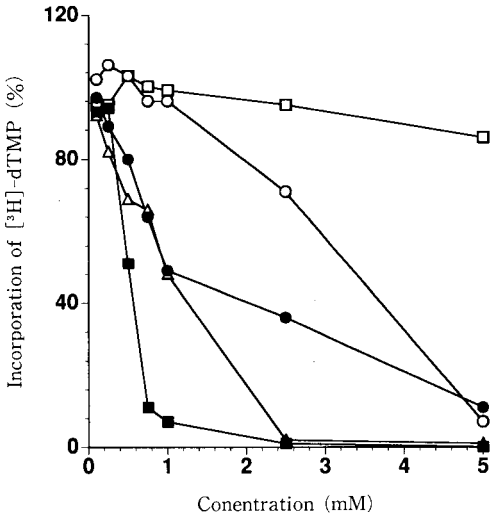

Fig. 2 Effects of increasing concentrations of procyanidins and embelin on RT-catalyzed incorporation of [³H]-dTMP residue into a polymer fraction.

The reaction mixtures containing 50 mM Tris-HCl (pH 8.3), 40 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 5 μg/ml $(rA)_n$-$(dT)_{12-18}$, 0.1 mM [³H]-dTTP and various concentrations of test sample, were incubated for 60 min at 37°C. The RT activities were measured by the incorporation of [³H]-TMP residue into an acid insoluble fraction.

(□), procyanidin B1; (○), arecatannin A1; (△), arecatannin A2; (■), arecatannin B1; (●), embelin

An Inhibitory principle from the Embelia ribes extract against RT

Similarly, bio-assay directed fractionation of the *Embelia ribes* extract (79 and 98 % inhibition at 1.0 mg/ml of the water and methanol extracts, respectively) led to the isolation of embelin (**7**) from the active phenolic-fraction. The incorporation of [³H]-dTMP into a polymer fraction dropped abruptly by increasing the concentrations of embelin up to 1.0 mM, while the incorporation declined gradually at higher concentrations (1.0-5.0 mM; Fig. 2). The IC_{50} value of embelin (**7**) was estimated to be approx. 1.0 mM (Table III).

Discussion

By screening various extracts of the crude drugs available in Sri Lanka, some extracts were shown to inhibit RNA-dependent DNA polymerization in a concentration dependent manner. Most of the extracts which had potent inhibitory action against RT were obtained from the plants rich in tannins.

Bio-assay directed fractionation of the *A. catechu* extract, which showed potent RT-inhibition at 0.2-1.0 mg/ml, led to the isolation of (+)-catechin (**1**), (−)-epicatechin (**2**) and their oligomers [procyanidin B1 (**3**) and arecatannins A1 (**4**), A2 (**5**) and B1 (**6**)]. The inhibitory activities of the oligomers were higher than those of the monomeric units at concentrations of 0-5.0 mM. The higher enzyme-inhibitory potency in the oligomers relative to that of the monomers seems to be a characteristic property of condensed tannins as observed in the inhibition of angiotensin-converting enzyme[19] and of glucosyltransferase.[17] The inhibitory potency increased in the order of dimer (procyanidin B1, **6**), trimer (arecatannin A1, **4**) and tetramer (arecatannin A2, **5**) in a (4β, 8)-linked procyanidin oligomers. Their IC_{50} values were 5.0, 3.3 and 1.0 mM, respectively. On the other hand, arecatannin B1 (**6**), a trimer which possesses both (4β, 8) and (4β, 6)-linkages in the molecule, had the highest inhibition with an IC_{50}= 0.5 mM. Thus, enzyme-inhibitory potency of procyanidins was proportional to their degree of polymerization and was also related to their lin-

kages. These phenomena were also observed for bursting action of *Areca* tannins on dog roundworm larvae (*Toxocara canis*) in the presence of fatty acids.[20]

Both water and methanol extracts of the pericarp of *Punica granatum* or of the fruit of *Terminalia chebula* showed potent inhibitory activities against RT. These drugs contained characteristic ellagitannins; granatins A and B, punicalin and punicalagin from the former[22] and chebulagic acid and chebulinic acid from the latter.[23] Our previous investigation demonstrated that these tannins strongly inhibited RT activity in a non-competitive manner as regards to a template-primer.[12]

Embelin is the major benzoquinone-type constituents present in the fruit of *E. ribes* and has been reported to be an inhibitor of glucosyltransferase from the primarily cariogenic bacterium, *Streptococcus mutans*.[17] However, embelin showed a different RT-inhibitory curve *versus* its concentration, compared to those of arecatannins (Fig. 2). Though the IC_{50} of embelin was approx. 1.0 mM, the complete inhibition was not obtained even at a higher concentration of 5 mM. This may be due to poor solubility of embelin in the medium.

The screening of traditional medicines for RT-inhibitory activities has been a promising trial to find substances that interfere in retroviral replication, consequently controlling the spread of retroviral diseases in humans. At present, there are no available antiviral agents that satisfactorily eradicate, control, or prevent HIV infection. Only a few drugs such as 3'-azidothymidine (AZT), 2',3'-dideoxyinosine (DDI) and 2',3'-dideoxycytidine (DDC) are clinically used in AIDS patients and these are actually RT-inhibitors. Searching for RT-inhibitory substances in traditional medicines is one of the methods worthy to be considered under the circumstances where more effective anti-retroviral drugs are really demanded.

和文抄録

AMV 由来の逆転写酵素 (RT) に対する阻害作用を調べるため，42種のアーユルヴェーダ薬物のスクリーニングを行なった。$(rA)_n \cdot (dT)_{12-18}$ をテンプレート・プライマーに用いた実験の結果，*Areca catechu* の種子，*Cinnamomum verum* の樹皮，*Eugenia jambolana* の樹皮，*Ficus religiosa* の樹皮，*Punica granatum* の果皮，*Rhus acuminata* の虫えい，*Saraca indica* の樹皮および *Terminalia chebula* の果実のエキスによる阻害作用が最も強く，4～66 μg/ml の IC_{50} を示した。さらに，RT 阻害作用を指標にエキスを分画し，*Areca catechu* からは arecatannin B1 および arecatannin A2. *Embelia ribes* からは embelin を阻害活性成分として単離した。

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