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Comparison of cycloartenone from four insecticidal *Kotschya* species (Fabaceae) harvested during dry and wet seasons

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Abstract
Kotschya plant species have been shown to be potential larvicides against *Culex quinquefasciatus* and *Anopheles gambiae* species. Isolation of compounds from larvicidal crude extracts of selected four *Kotschya* plant species namely *K. uguenensis*, *K. strigosa*, *K. speciosa* and *K thymodora* yielded cycloartenone (1) as the major compound. The presence of this compound in the four species was confirmed by detailed spectroscopic methods and the already published data. Quantitative analysis of cycloartenone (1) in the four species collected during wet and dry seasons was compared. Findings indicated higher yields during wet seasons ranging from 68 x 10^{-2} to 120 x 10^{-2} mg/ml than dry seasons which revealed amounts ranging from 0 to 150 x 10^{-2} mg/ml. However, the highest amount of cycloartenone (1) was present in the stem barks of *K. uguenensis* for the dry season whereas the 120 x 10^{-2} mg/ml was observed in the aerial parts of *K. strigosa* collected during wet season. Cycloartenone (1) is being isolated for the first time in the genus *Kotschya* while also revealing mild larvicidal activity against *Anopheles gambiae* with mortality up to 40% below the concentration of 50 ppm.

Keywords: *Cycloartenone*, Mosquito larvicides, *K. uguenensis*, *K. strigosa*, *K. speciosa*, *K thymodora*, wet season, dry season.

Abbreviations: Comparison of cycloartenone from the four insecticidal *Kotschya* species

1. Introduction
The easy availability of pesticidal plants from nature can be a good alternative to synthetic pesticides such as organochlorine, organophosphorous and carbamates which have adverse environmental effects and high level of multi-resistances. In recent years uses of eco-friendly chemicals have been advocated in larviciding mosquito breeding sites in efforts to eradicate malaria and mosquito related diseases [1]. Previous studies reported the potential of *Kotschya uguenensis* plant species as source of growth inhibitory of immature *Anopheles gambiae* mosquitoes upon prolonged exposure time [2, 3]. Further studies of *K. uguenensis* in the emulsion and powder forms at semi-field, were effective in reducing *Anopheles gambiae* population [3]. Another larvicidal investigation of crude ethanol extracts from the roots and stem of *K. thymodora, K. speciosa* and *K. strigosa* were effective against *Culex quinquefasciatus* in the laboratory [4]. These prompted carrying out the isolation of compounds from the four Kotschya species and compare the abundance of the isolated compounds when plant materials were collected during dry or wet season. Thus, chromatography of the ethanol extracts from the root or stem and aerial parts of the four plant species lead to isolation of compound (1) which was used for quantitative comparison as well as tested against *Anopheles gambiae* species. Compound 1 was identified to be cycloartenone upon analysis of its spectroscopic data and comparison with literature [5, 6].

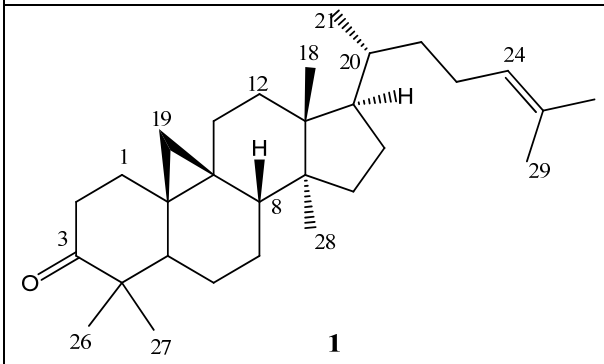
2. Materials and Methods
2.1. Plant Material collection
Collection of plant materials of the aerial parts, stem and root parts for these investigations was done in Iringa and Mbeya Regions, Tanzania in April, 2012 (Wet season) and October, 2013 (Dry season) (Table 1). The plants were authenticated by the Botanist from the Department of Botany, University of Dar es Salaam and specimens deposited at the Herbarium of the Institute of Traditional Medicine.

Table 1: Information of Plant materials collected during dry season

S/No	Botanical name	Voucher specimen No:	Altitude: (GPS)	Place of collection & Habitant
1. -	*Kotschya thymodora (Bak.f) wild:*	FMM3628	1444 m. ($9^0$56' 11.2 E $34^0$34 '52.6)	Mvira village in Njombe district 20 km to Rudewa town in Marshland surrounded by miombo woodland
2. -	*Kotschya speciosa* (Hutch.) Hepper	FMM 3626	1704 m ($8^0$34' 56. 4 E $34^0$58' 56.3)	Iringa along the road of Makambako to Mafinga In *Brachystegia -parinari- protea* woodland associated with a shrub of *Aeschynomene schliebenii* var *mossambicensis*
3. -	*Kotschya strigosa* (Benth). Dewit and Duvign	FMM 3629	1846 m. ($8^0$30' 39.9 E $35^0$10' 10.8)	Mufind District near the Ngwazi lake In Stunted *Braschystegia* woodland on reddish loam soil
4. -	*Kotschya uguenensis* (Taub) F.Whote	FMM 3624	1846 m. ($8^0$30' 39.9 E $35^0$10' 10.8)	Mufindi District Lake Ngwazi in Swampy forest with *Syzygium cordatum and Prunus Africana*

Chromatography of crude extracts and elucidation of isolated compounds. The air-dried plant materials were pulverized and soaked in ethanol (96%) for 72 h and then filtered. Soaking was done twice for every plant part. The crude filtrate was concentrated in *vacuo* using a rotary evaporator while maintaining the bath temperature at 40 ºC or freeze dried in order to remove water. All samples were kept in the refrigerator until time for analyses. The crude ethanol extracts were separately subjected to different chromatographic techniques as required such as Column Chromatography (CC), Vacuum Liquid Chromatography (VLC), Thin Layer Chromatography (TLC) using silica gel Mesh (230-400) nm. A white amorphous which seemed to be a major compound was isolated from several *Kotschya* extracts (Table 2). Different spectroscopic methods such as UV, HRMS, 1D and 2D NMR, and comparison with published data in the Chemfinder database were used to elucidate and confirm the structure of isolated pure compound to be cycloartenone (**1**).

Table 2: Isolation of compounds from ethanol extracts of different *Kotschya* species harvested during wet season

	Extract	Nature of compound	Weight (mg)[#]
 **1**	*Kotschya uguenesis (Kusw)*	White amorphous	54
	Kotschya strigosa (Kstrrw)	White amorphous	200
	Kotschya speciosa (Ksprw)	Cream amorphous	136
	Kotschya speciosa (Kspsw)	White crystals	83
	Kotschya thymodora (KTRW)	Cream amorphous	235

[#]Amount isolated does not necessarily represent abundance of cycloartenone (1) in the species

2.3 Chemical profile of isolated cycloartenone (1) at different collection seasons.

The retention time (R_t) and the amount (% age) corresponding to cycloartenone (1) was compared among different extracts of *Kotschya* species. Equal weight of extracts from different *Kotschya* species was dissolved in methanol to make a concentration of 2 mg/ml. Normal phase Aluminum TLC plates coated with silica gel were used for qualitative analysis of the crude extracts eluting with petroleum ether-dichloromethane (70:30 v/v) solvent system. Sixteen spots were applied on a plate (20×10 cm) at a distance of 1.1cm between spots, each loaded fifteen times with a drop of extract. Also, one spot of the pure cycloartenone (1) and of the dissolving solvent were applied for comparison (Figure 1 A). After elution, the TLC plates were left in open air for drying for about 20 minutes before spraying with Vanillin. The quantitative analysis of cycloartenone (1) was determined after running a calibration curve from the serial dilutions of pure cycloartenone (1) of 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml on the HPTLC. The curve was then used to determine the amount of the compound in the extracts. The retention times and the relevant peak areas corresponding to the pure compound, cycloartenone (1) were calculated and fitted into the regression equation to obtain the concentration of cycloartenone (1) in different individual plant extracts using the regression:

$$y = -834.66x^2 + 5394.3x; R^2 = 0.9967$$

Where x and y represent concentration and peak areas of a sample respectively.

2.4 Mosquito larvicidal studies of isolated cycloartenone (1).

The isolated pure compound cycloartenone (1) was tested for mosquito larvicidal activity against *Anopheles gambiae* species. The aim of the assay was to monitor whether the

larvicidal activity of *Kotschya* species was due to compound 1 or rather was just a major compound in *Kotschya* species. The WHO protocol of 1996, with some modification as described by Innocent *et al*. 2012 was adopted [4]. The Mean mortality of *Anopheles gambiae* after 72 hrs of exposure to cycloartenone at concentration ranged between 3.125 and 50 µg/ml were computed.

3. Results

3.1. Qualitative analysis of chemical constituents of *Kotschya* plant extracts

The TLC plates upon spraying with vanillin/sulfuric acid and then heated at ~120 °C for 3-5 min revealed deep blue and purple colored spots indicating the presence of terpenoids or steroids constituent compounds in the extracts. However, few spots were either yellow or brown (Figure 1 A).

3.2. Quantitative analysis of cycloartenone (1) in the extracts

Generally, quantitative analysis of cycloartenone (1) indicated higher yields, ranging from 68 x 10^{-2} to 120 x 10^{-2} mg/ml during wet seasons than dry seasons which revealed amounts ranging from 0 to 150 x 10^{-2} mg/ml (Figure 1 B). The quantity of cycloartenone (1) in the stem barks of *K. uguenensis* harvested during dry season was determined to be 150 x 10^{-2} mg/ml. This was observed to be twice the amount contained in the roots of *K. uguenensis* and *K. strigosa* for the same season. No cycloartenone (1) was detected in the aerial parts of *K. speciosa* and stem bark of *K thymodora* harvested during dry season (Figure 1 B and Figure 2). On the other hand, analysis of cycloartenone (1) for species collected during wet season revealed the highest quantity of the compound in the aerial parts of *K. strigosa* (120 x 10^{-2} mg/ml) followed by the root barks (111 x 10^{-2} mg/ml). Both the roots and stem barks of *K. thymodora* harvested during wet season contained approximately equal amounts (~100 x 10^{-2} mg/ml) of cycloartenone (1). For the case of *K. uguenensis*, the stem barks contained slightly higher amount (96 x 10^{-2} mg/ml) than the root barks (86 x 10^{-2} mg/ml) whereas the roots of *K. speciosa* had the least amount of compound (1) (Figure 1 B). In general, the quantity of cycloartenone (1) during dry season followed the trend; *K. uguenensis stem barks* > *K. strigosa* root bark > *K. uguenensis* root barks > *K. speciosa* root barks> *K. speciosa* aerial parts/*K. thymodora* root barks. During wet season, the trend was; *K. strigosa* aerial parts >*K. strigosa root barks* > *K. thymodora* root barks > *K. thymodora* stem barks > *K. uguenensis* stem barks > *K. speciosa* root barks > *K. uguenensis* root barks > *K. speciosa* stem barks.

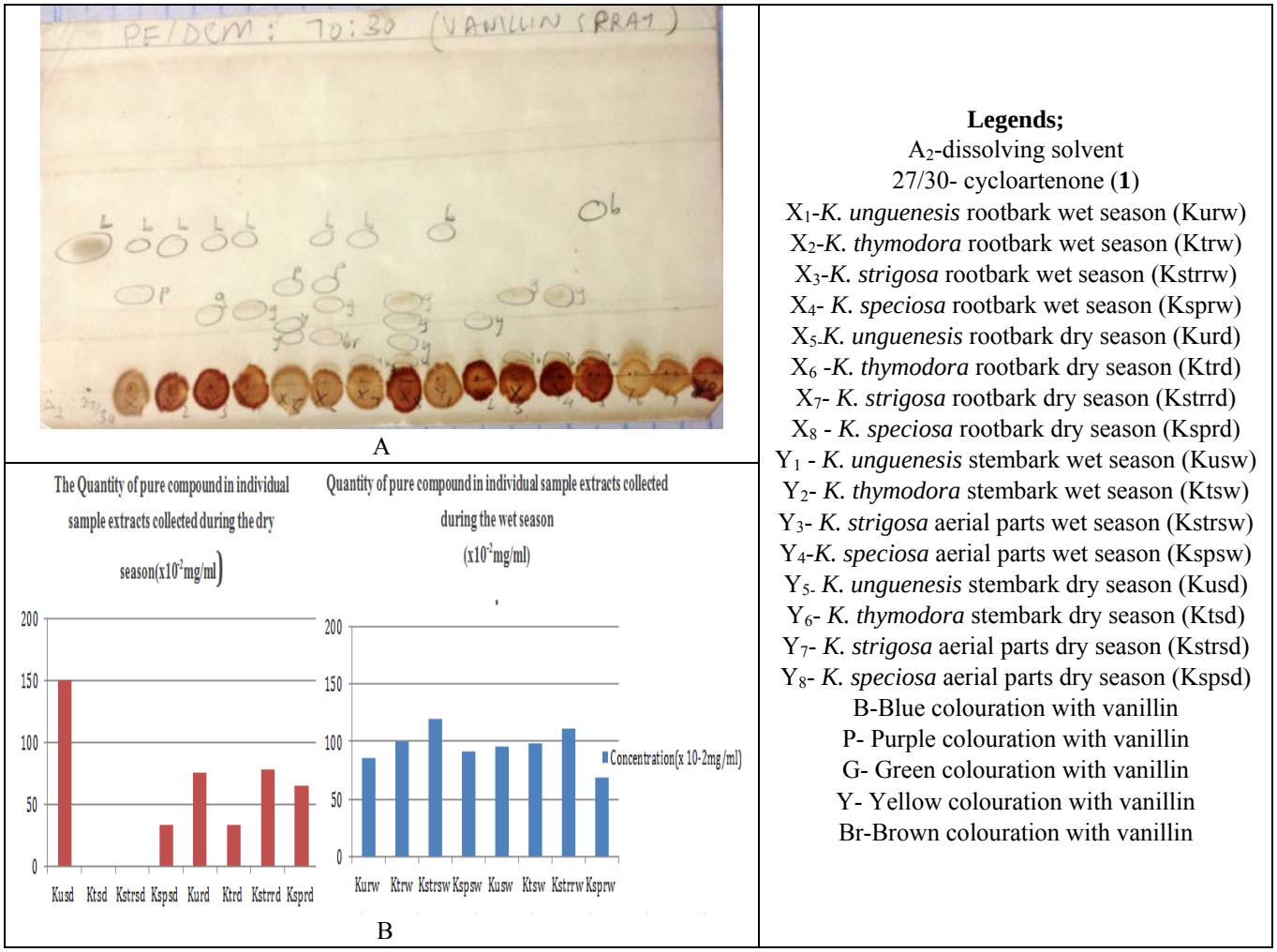


Fig 1: Chemical profiles of *Kotschya* extracts collected during dry and wet seasons showing Qualitative (**A**) and quantitative (**B**) of cycloartenone (1) from sixteen ethanol extracts of four Kotchya species

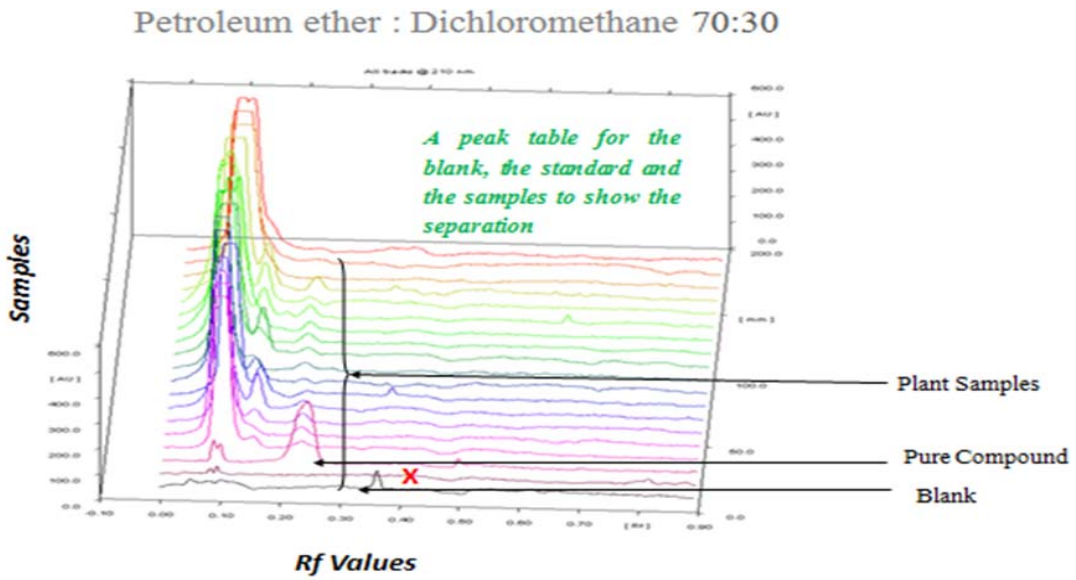


Fig 2: The 3D superimposed densitograms of the pure cycloartenone (1) compound in sixteen *Kotschya* extracts run in petroleum ether: dichloromethane solvent (70:30 v/v).

3.3. Spectroscopic elucidation of isolated cycloartenone (1)

Column chromatograph on silica gel (230-400 Mesh) eluting with 3:7 v/v dichloromethane methanol yield cycloartenone (**1**), as white armophous. The compound absorbed UV radiation both at 254 nm and 366 nm. Table 3 shows different yields spectroscopic data that were extracted from the ^{1}H and ^{13}C NMR and other 2D NMR as well as established literature data [5-6]. MS for cycloartenone *m/z* 426[M+H] $^{+}$; *m/z* 425[M] $^{+}$ observed 425.3757; calc 425.3739, 424[M-H] $^{+}$ (See supplement files).

Table 3: NMR data for cycloartenone (**1**) at AV 600 MHz in CD_2Cl_2

carbon #	Observed			Reported [5,6]
	δ_H (*multiplet*)		δ_c	δ_c
1	1.76 (*m*) 1.47(*m*)	CH_2	33.6	33.4
2	2.63 (*dd*, 13.9,6.4) 2.15(*ddd*, 14.0, 4.4, 2.6)	CH_2	37.7	37.5
3	-	C	216.2	216.6
4	-	C	50.3	50.2
5	1.62 (*dd*, 12.3, 4.4)	CH	48.7	48.4
6	1.47 (*ddd*,13.3, 6.2, 2.7) 0.87 (*dd*, 12.6, 2.5)	CH_2	21.8	21.5
7	1.84 (*m*) 1.23(m)	CH_2	28.3	28.1
8	1.50 (*m*)	CH	48.2	47.9
9	-	C	21.3	21.1
10	-	C	26.2	25.9
11	1.3(m) 1.04 (*dd*, 12.5, 2.7)	CH_2	26.1	25.8
12	1.58 (*m*)	CH_2	33	32.8
13	-	C	45.5	45.3
14	-	C	49	48.7
15	1.24(*m*)	CH_2	35.8	35.5
16	1.98(*m*) 1.1(*m*)	CH_2	26.9	26.7
17	1.54 (*m*)	CH	52.5	52.3
18	1.00 (s)	CH_3	19.3	19.3
19	0.71 (*d*, 4.1) 0.51 (*d*, 4.3)	CH_2	29.7	29.5
20	1.32 (*m*)	CH	36.1	35.8
21	0.93 (s)	CH_3	18.1	18.0
22	1.37 (*m*) 0.97 (*m*)	CH_2	36.5	36.3
23	1.97(*m*) 1.79 (*m*)	CH_2	25.1	24.9
24	5.03 (*dd*, 7.1, 1.4)	CH	125.4	125.2
25	-	C	131	130.9
26	1.53 (s)	CH_3	17.6	17.6
27	1.6 (*d*, 1.0)	CH_3	25.7	25.7
28	0.82(*d*, 6.5)	CH_3	18.3	18.2
29	1.00 (s)	CH_3	20.8	20.7
30	0.93(s)	CH_3	22.2	22.1

3.4 Mosquito larvicidal activity of cycloartenone (1).

The isolated pure compound cycloartenone (1) was tested for mosquito larvicidal activity against *Anopheles gambiae* species for three days. Results indicated that cycloartenone (1) was mild active with mortality up to 40% below the concentration of 50 ppm. The mean mortality was not significantly different to the control up to 25 ppm, but significantly different to 50 ppm (Figure 3).

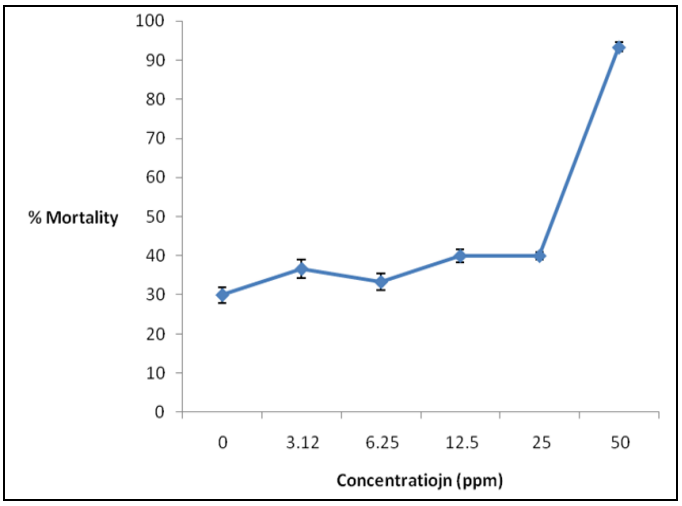


Fig 3: Mean percentage Mortality effect of *A. gambiae* 72 hrs post exposure to cycloartenone (1)

4. Discussion

Previous investigation of the stem and root bark extracts of *Kotschya speciosa, K. thymodora, K. uguenensis* and *Kotschya strigosa* by HPLC indicated similar pattern of profiles [5]. The same study demonstrated that extracts from *K. speciosa, K. thymodora* and *K. strigosa* showed a good mortality with little variations when tested against *Culex quinqefasciatus* larvae. The current study was conducted to isolate some compounds and subject them for bioassays to see whether they contribute to the bioactivity of the extracts. One major compound was isolated while others were not in substantive amounts to enable bioassay investigation. Quantitative analyses showed the amount of cycloartenone (1) comparatively higher in *K. uguenensis* and *K. strigosa* than in *K. thymodora* and *K. speciosa*. The general qualitative and quantitative analysis of cycloartenone (1) indicated more accumulation of the compound during wet seasons than dry seasons. The fact that cycloartenone (1) was only mild active against *A. gambiae* may suggest another compound or combination of several compounds being responsible for bioactivities exhibited by *Kotschya* species.

The cycloartenone (1) has also been isolated from many plant species including *Wrightia tinctoria* [7], *Dillenia indica Linn* [8], *Artocarpus ovatus* [9], *A. integra* [10], *A. heterophyllus* [11] and *Zanthoxylum bungeanum* [6]. The compound has been reported to exhibit antimicrobial activity [11] high estrogenic activity [12], Inhibition of TPA-induced Epstein-Barr virus, activate ERα and ERβ [13] and moderate cytotoxic activity against the human tumor cell lines [14, 15]. The present study has significantly contributed to an understanding that cycloartenone (1) which is biosynthesized in *Kotschya* species as the major compound does not exhibited the IGR property reported for this plant genus when used alone. Thus, further exploration for other chemical constituents to be tested singly or in combination will help to plan further development of larvicidal products from *Kotschya* species.

5. Conclusions and Recommendations

The contribution of other compounds in *Kotschya* species is yet to be determined hence calls another effort for further investigation. The genus *Kotschya* may play a vital role in covering the basic health needs in preventing mosquito transmitted diseases in rural areas where plant resources can be used for intervention.

6. Supplementary Data

EI-MS and NMR spectra of cycloartenone **(1)**

7. Acknowladgements

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8. Conflict Of Interest

Authors declare no conflict of interest

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