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ANTIFUNGAL ACTIVITY OF SOME MEDICINAL PLANTS OF SRI LANKA

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Abstract: Thirty six medicinal plants (63 extracts) have been screened for antifungal activity against *Cladosporium cladosporioides*. Extracts of the following plants have displayed significant antifungal activity: *Butea monosperma, Costus speciosus, Curcuma zedoaria, Eupatorium riparium, Pleiospermium alatum, Theobroma cacao* and *Zingiber zerumbet*. A preformed antifungal constituent of *C. speciosus* has been identified as methyl 3–(4–hydroxyphenyl)–2 (E)–propenoate (1). The antifungal activity of this compound has been evaluated against *Aspergillus niger, C. cladosporioides, Colletotrichum gloeosporioides, Curvularia* sp. and *Penicillium* sp.

1. Introduction

Development of specific fungicides for use in the treatment of plant diseases is important. [1,2] Plants are known to contain antifungal compounds[9], and are a potentially useful source of these compounds. The present study describes the investigation of 63 plant extracts derived from 36 medicinal plants of Sri Lanka. Isolation of an active constituent from the rhizome of *Costus speciosus*[6], a potential source for the diosgenin–derived steroidal drugs[7,11] is also described.

2. Experimental

2.1 Plant Material

Plant materials used in this study, were mature (reproductive maturity) specimens collected (1–5 kg) from different localities of Sri Lanka specially from Central Province (Table 1). All plant varieties collected were true species. Plant specimens were identified by comparison at the National Herbarium, Royal Botanic Gardens, Peradeniya. The specimens were immediately washed in running water to remove contaminated soil etc. and cut into small pieces about 3–6 cm in length. The specimens were immediately air–dried and powdered in a laboratory mill.

2.2 Preparation of Plant Extracts

The air—dried plant materials (100 g) were extracted successively with (500 ml) hot hexane/light petroleum (40—60^{0}C) dichloromethane, ethyl acetate and methanol in a Soxhlet apparatus, or extracted directly with cold ethyl acetate and cold methanol in a bottle shaker, for a period of 48 hrs. The solubles were concentrated to dryness separately using a rotavapor (below 45^{0}C). The extracts were subjected to *Cladosporium* TLC—bioassay for antifungal screening as described below.

2.3 *Cladosporium* TLC—Bioassay

Extracts (2 mg) were spotted on tlc plates (silica gel 60 $PF_{254-366}$, 0.50 mm x 20 cm x 20 cm) and the plates were developed in dichloromethane. After air—drying overnight the plates were sprayed with a suspension of conidia of *Cladosporium cladosporioides* in Czapek—Dox nutrient solution. Plates were then incubated in a moist chamber at 25 ± 2^{0}C for 48 h.[3] Inhibition areas appeared white against a background of green mycelia. The diameter of zones in which the growth was inhibited, which were approximately circular, was measured (in mm). The extracts which showed inhibition are given in the Table 1, with the R_f value and the diameter of the zone of inhibition. Benlate (0.2 mg, in methanol, 50% active ingredient, methyl—1—(butylcarbonyl)—2—benzimidazolecarbamate. Du Pont, USA) was spotted on each tlc plate as the standard fungicide.

Table 1

Plant (Family)	Collection	Part[a]	Solvent[b]	$R_f(CH_2Cl_2)$ diameter (mm)	
Actinodaphne speciosa Nees. (Lauraceae)	Horton Plains	Lf	MeOH[h]	–	
Allaeophania decipiens Thw. (Rubiaceae)	,,	Ap	MeOH[h]	–	
Alpinia abundiflora Burtt & Smith (Zingiberaceae)	,,	Rh	*Pet[h]	–	
			*CH_2Cl_2[h]	–	
			*MeOH[h]	–	
Alpinia fax Burtt & Smith (Zingiberaceae)	Rakwana	Tb	MeOH[h]	–	
Alpinia nigra (Gaertn.) Burtt (Zingiberaceae)	Hakgala	Ft	MeOH[h]	–	
		Tb	MeOH[h]	–	
Anotis richardiana (Arn.) Hook.f. (Rubiaceae)	Pattipola	Wp	MeOH[h]	–	
Artemisia dubia var. *grata* Wall (Compositae)	Ambewela	Lf	MeOH[c]	–	
		Lf	MeOH[h]	–	
		St	MeOH[c]	–	
		St	MeOH[h]	–	
		Rt	MeOH[c]	–	
		Rt	MeOH[h]	0.35 (06)	
Butea monosperma (Lam.) Taub. (Leguminosae)	Mahiyanganaya	St Bk	EtOH[c]	0.15 (20)	0.10 (30)

Cestrum aurantiacum Lindl. (Solanaceae)	Hakgala	Lf	$MeOH^h$	–	
		St	$MeOH^h$	–	
Costus speciosus (Koen.) Sm. (Zingiberaceae)	Kalutara	Rh	$MeOH^h$	0.30 (08)	
Curcuma zedoaria (Berg.) Roscoe. (Zingiberaceae)	Kandy	Tb	$MeOH^h$	1.00 (15)	
Eupatorium riparium Regel. (Compositae)	Hakgala	Wp–Rt	$MeOH^h$	0.72 (26)	
		Rt	Hex^h	0.80 (26)	0.70 (22)
			$CH_2Cl_2{}^h$	0.80 (26)	0.70 (22)
Gaultheria rudis Satpf. (Ericaceae)	Horton Plains	Wp–Lf	$MeOH^h$	–	
		Lf	$MeOH^h$	–	
Gynostemma laxum (Wall.) Cogn. (Cucurbitaceae)	Hakgala	Wp–Rt	$MeOH^h$	–	
Hortonia floribunda Wight ex Arn. (Monimiaceae)	Hakgala	Lf	$MeOH^h$	–	
		St	$MeOH^h$	–	
Hypericum mysurense Wight & Arn. (Guttiferae)	Pattipola	Lf	$MeOH^h$	–	
Leucas biflora (Vahl.) Benth. (Labiatae)	Horton Plains	Wp	$MeOH^h$	–	
Mastixia tetrandra (Wight ex Thwaites) C.B. Clarke (Cornaceae)	Hakgala	Lf	$MeOH^h$	–	
Osbeckia cupularis D.Don ex Wight & Arn. (Melastomaceae)	Horton Plains	Wp	$MeOH^h$	0.10 (06)	0.80 (08)

Pedalium murex L. (Pedaliaceae)	Puttalum	Lf	MeOH[c]	–
Phyllanthus reticulatus Poir. (Euphorbiaceae)	Sigiriya	Lf	MeOH[h]	–
Pleiospermium alatum (Wight & Arn.) Swingle (Rutaceae)	Sigiriya	St Bk	*Hex[h]	0.60 (20)
			*EtOAc[h]	–
		Rt Bk	*EtOAc[h]	0.60 (10)
			*MeOH[h]	0.60 (18)
Pimenta officinalis Lindl. (Myrtaceae)	Kandy	Lf	MeOH[h]	–
		St Bk	MeOH[h]	–
Psychotria bisulcata Wight & Arn. (Rubiaceae)	Horton Plains	Lf	MeOH[h]	–
		St	MeOH[h]	–
Psychotria nigra var. *Coronata* Hk. f. (Rubiaceae)	Horton Plains	Lf	MeOH[h]	–
		St	MeOH[h]	–
Ricinus communis L. (Euphorbiaceae)	Sigiriya	St Bk	MeOH[h]	–
Saprosma foetens (Wight) K.Schum. (Rubiaceae)	Kandy	Lf	MeOH[h]	–
		St Bk	MeOH[h]	–
Sesamum indicum L. (Pedaliaceae)	Rambukkana	Lf	EtOH[c]	–
Solanum giganteum Jacq. (Solanaceae)	Hakgala	Ft	MeOH[c]	–
		Ft	MeOH[h]	–
Strobilanthes auriculatus Nees (Acanthaceae)	Horton Plains	Lf	MeOH[h]	–
		St	MeOH[h]	–
		Rt	MeOH[h]	–

Strobilanthes hookeri Nees (Acanthaceae)	,,	Lf	$MeOH^h$	–	
		St	$MeOH^h$	–	
Strobilanthes viscosa (Nees) T. Anders. (Acanthaceae)	,,	Lf	$MeOH^h$	–	
		St	$MeOH^h$	–	
Theobroma cacao L. (Sterculiaceae)	Kalutara	Ft	$EtOAc^c$	0.71 (21)	
Thespesia populnea (L.) Soland ex Corr. (Malvaceae)	Kandy	Lf	$MeOH^h$	–	
		St	$MeOH^h$	–	
Ulex europaeus L. (Leguminosae)	Horton Plains	Wp	$MeOH^h$	–	
Zingiber zerumbet (L.) Sm. (Zingiberaceae)	Hakgala	Tb	$MeOH^h$	1.00 (30)	0.80 (10)
Standard fungicide				–	
Benlate				(38)	

a Ap = areal part, Bk = bark, Ft = fruit, Lf = leaf, Rh = Rhizome, Rt = root, RtBk = root bark, St = stem, StBk = stem bark, Tb = tuber, Wp = whole plant

b Hex = normal hexane, Pet = light petroleum

* successively extraction
c – cold extraction; h – hot extraction

2.4 Isolation of Methyl 3—(4—hydroxyphenyl)—2—(E)—propenoate (1) from *Costus speciosus*

Dried and powdered rhizome of *C. speciosus* (84 g) was extracted with hot methanol. Removal of solvent *in vacuo* gave a brownish solid (12 g). The extract showed considerable antifungal activity when bio—assayed against *Cladosporium cladosporioides*.

A portion of this solid (8 g) was chromatographed on a silica gel (250 g) column using solvent combinations with increasing polarity of light petroleum, chloroform and methanol. The fractions having similar TLC patterns were combined and new fractions were tested for antifungal activity against *C. cladosporioides* using TLC—bioassay technique. One of the fractions (200 ml, 60% $CHCl_3$—petroleum) produced a distinct inhibition area at R_f 0.30 (CH_2Cl_2) in the *Cladosporium* TLC—bioassay. Repeated purification of this fraction using silica gel (50 g) column chromatography (25% chloroform—n— hexane) yielded a white crystalline solid (21 mg) which was identified as methyl 3—(4—hydroxyphenyl)—2—(E)—propenoate (1) m.p. 133—134^{0}C (acetone—petroleum) $[\alpha]_D$ 0^0 (*c*, 1.0, $CHCl_3$); UV λ_{max} (ethyl acetate) 305 nm;IR ν_{max} (KBr) 3425, 1685, 1620, 1445 and 1210 cm^{-1} ^{1}H NMR (D_6 acetone—$CDCl_3$) δ 8.33(1H,s, OH),7.63(1H,d,*J*=16 Hz, =/), 7.43 (2H,d,*J*=8 Hz, orthocoupled), 6.87(2H,d,*J*=8 Hz, orthocoupled), 6.27(1H,d, *J* = 16 Hz, =/) and 3.77(3H,s, OMe); MS *m/z* 178(M^+ 72.34), 147(100), 119(42.55), 91(42.55), 65(34.04), found M^+ 178($C_{10}H_{10}O_3$ requires 178.0775).

2.5 Chemical Fractionation and Bioassay of Ethanol, Methanol and Dichloromethane Extracts of *C. speciosus*

Dried and powdered rhizome (30 g) of *C. speciosus* was divided into 3 equal portions and were extracted under reflux conditions separately with ethenol, methanol and dichloromethane (300 ml). The weights of the extracts after removing solvents under reduced pressure were 617 mg, 817 mg and 150 mg, respectively. Each extract (2 mg) was subjected to *Cladosporium* TLC—bioassay and the extracts produced prominent inhibition areas at R_f 0.30 ± 0.02 (dichloromethane). Each crude extract, after purification by preparative TLC, (80% chloroform—petroleum) was bioassayed and the active component in each was identified as the methyl 3—(4—hydroxyphenyl)—(E)—2—propenoate (1).

2.6 Bioassay of Extracts from Rhizome of *C. speciosus*

Discs of tissue (10 mm diameter and 2—3 mm thick) were cut from the fresh rhizome of *C. speciosus* using a cork borer. A 10 g portion was dipped in a suspension of conidia (50 x 10^5 conidia per ml) of *Gloeosporium mangiferae*, a non—pathogenic fungus on *C. speciosus*, and another 10 g were placed in sterile distilled water. Two days after incubation at 26^{0}C the

tissue discs were taken out and extracted with hot methanol under the same conditions. Each extract (4 mg) was tested for antifungal activity using the TLC—bioassay technique. Both the extracts produced inhibition areas of similar diameter at R_f 0.28 — 0.29.

2.7 Bioassay of Compound (1) Against *Aspergillus niger*, *Cladosporium cladosporioides*, *Colletotrichum gloeosporioides*, *Curvularia* sp. and *Penicillium* sp.

Test solutions were made by dissolving 4 mg each of methyl 3—(4—hydroxyphenyl)—2—(E)—propenoate and a standard fungicide Benlate, separately in 4 ml of chloroform and water, respectively. Five TLC plates were spotted with 150, 100, 50, 25, and 10 μl aliquots of the above solution and Benlate 100, 50, 25 and 10 μl in each plate using micropipettes. The undeveloped plates were air—dried overnight. Each plate was sprayed with one of the above fungi in Czapek—Dox nutrient solution and kept in a moist chamber at 28 ± 2^0C for 48 hrs. The diameters of the inhibition zones were measured (Figure 1).

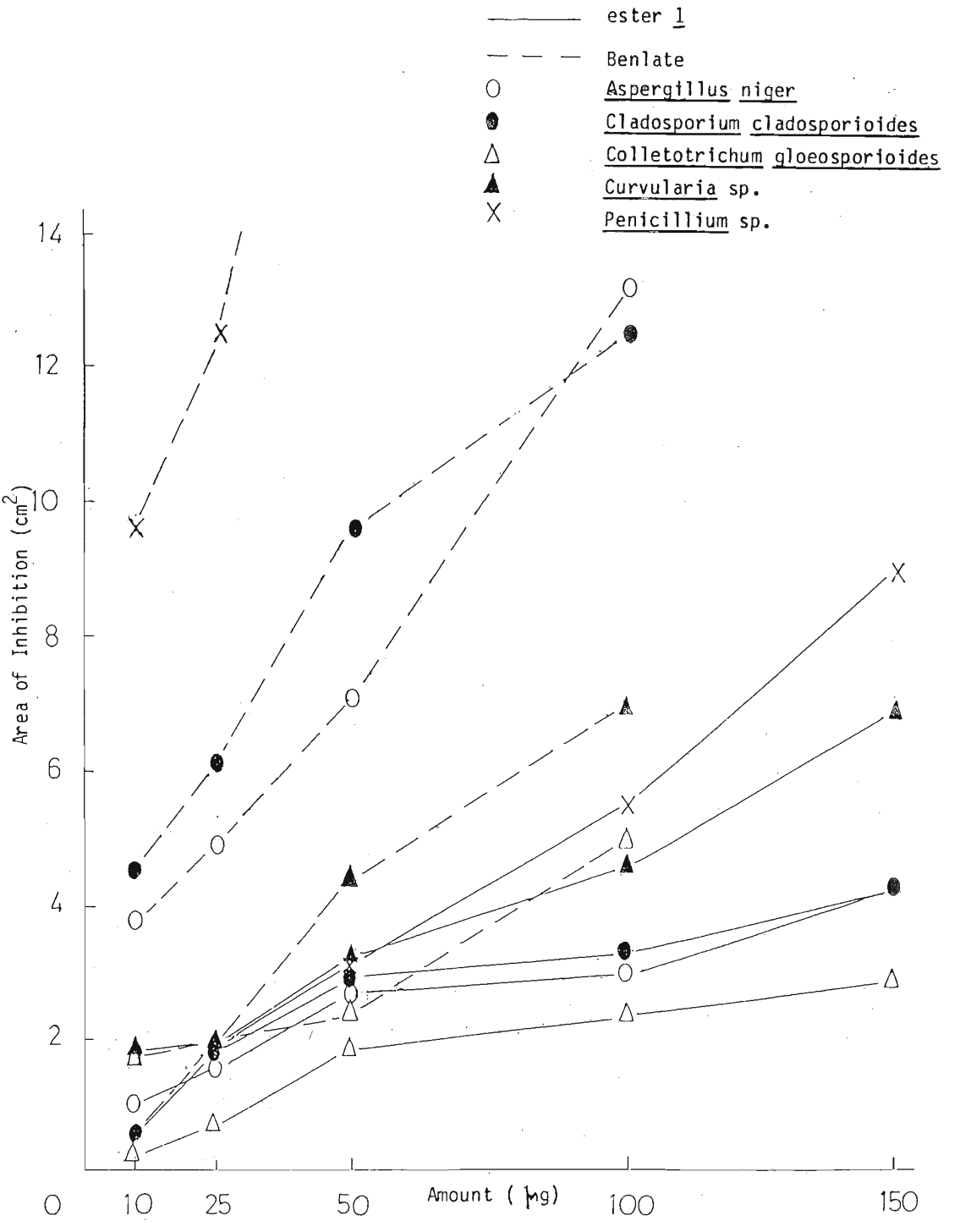
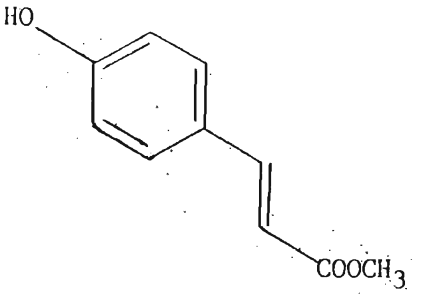
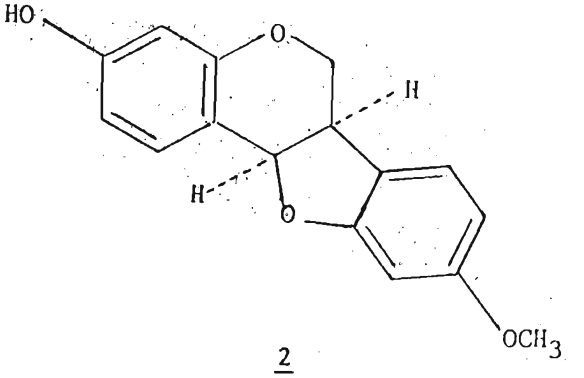


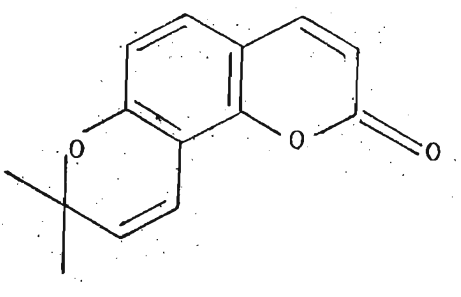
Figure 1. Inhibition of fungal growth by the ester 1 and Benlate



1



2



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3. Results and Discussion

3.1 Biological Screening of the Extracts

Most of the plants listed in Table 1 are used in ethnomedical preparations. Some plants have been selected for the present study on the basis that they are widely distributed and apparently free of pest attack. *Eupatorium* and *Strobilanthes* species, for example, are found abundantly in the hill country. Sometimes selection has been made on the basis that useful compounds and/or biological properties have been reported from the plant previously and information on the antifungal properties would enhance the industrial or medicinal utility of the species. *Costus speciosus* has been, for example, reported to possess antifertility[13], estrogenic[10,14] and anti-inflammatory[5] properties and has been identified as a source of diosgenin.[4,7,8,11]

Of the 36 plants (Table 1) tested for antifungal activity against *Cladosporium cladosporioides* using the TLC—bioassay technique[3], extracts from 10 plants inhibited the growth of the fungus. The extracts of *B. monosperma*, *C. speciosus*, *C. zedoaria*, *E. riparium*, *P. alatum*, *T. cacao* and *Z. zerumbet* displayed significant antifungal activity while those of *A. dubia var. grata*, *L. biflora* and *O. cupularis* only moderate activity.

The low polar antifungal compound in the extracts of *B. monosperma* has been identified as (—)—medicarpin (2).[2] The active constituent in the hexane extract of *P. alatum* root bark has been obtained by chromatographic fractionation and characterized as seselin (3).[1]

3.2 Active Constituent of *Costus speciosus*

Considering the economic potential of *C. speciosus* as a source of diosgenin and its medicinal value, bioassay—directed fractionation of the rhizome extract was carried out in order to isolate the antifungal constituent.

A part of the methanol extract was chromatographed over silica gel. The column fraction eluted with 60% chloroform in petroleum inhibited the growth of *C. cladosporioides*. Further purification of this fraction by column chromatography on silica gel yielded a white crystalline solid, m.p. 133—134°C which was found to be responsible for the antifungal activity. The IR absorption bands of this compound at 3425, 1685 and 1610 cm^{-1} corresponded to -OH, C=O and C=C groups, respectively. The UV λ_{max} at 305 nm suggested the presence of an α,β —unsaturated carboxyl system with extended conjugation. The D_2O exchangeable proton at δ 8.33 in the ^1H NMR spectrum confirmed the presence of a hydroxy group in the molecule. The doublets at δ 7.43 and 6.87 (J=8 Hz) corresponded to two *ortho* coupled protons. Two protons of a *trans* olefinic double bond was indicated by the doublets at δ 7.63 and 6.27 (J=16 Hz). The singlet at δ 3.77 was assignable to

a methoxy group. The molecular ion at m/z 178 in the mass spectrum was consistent with a formula of $C_{10}H_{10}O_3$. The mass peaks at m/z 147 and 119 corresponded M—OCH_3 and M—CO_2CH_3 fragments. The compound was thus identified as methyl 3—(4—hydroxyphenyl)—2—(E)—propenoate (1).

The possibility that this compound (1) was an artifact formed by transesterification of a labile natural product during the methanol extraction was eliminated on the following grounds. The rhizome of *C. speciosus* was separately extracted with methanol, ethanol and dichloromethane under reflux conditions. All three extracts produced an inhibition zone at the same R_f value (0.30 eluant:dichloromethane) in the *Cladosporium* TLC—plate. The three extracts were subjected to repeated TLC separation, and the bioactive fraction compared with an authentic sample of compound (1). The results showed that all three extracts contained the active compound(1).

The fresh rhizome was inoculated with a fungus *Gloeosporium mangiferae* and incubated at 28°C over a period of 2 days. Equal amounts of the inoculated rhizome and the untreated rhizome were separately extracted with hot methanol under identical conditions. Equal amounts of each extract produced inhibition zones of similar area in the *Cladosporium* TLC—plate suggesting that the inoculation had not increased the amount of the active compound. This indicated that the ester (1) was a preformed antifungal compound and not a phytoalexin.

The antifungal activity of the ester 1 against the following five fungi was compared with Benlate a commercial fungicide by using the TLC—bioassay technique (*Aspergillus niger*, *Cladosporium cladosporioides*, *Colletotrichum gloeosporioides*, *Curvularia* sp. and *Penicillium* sp.) Results are given in Figure 1. The ester 1 inhibited the growth of all the fungi tested. Both the natural compound 1 and Benlate displayed comparable activities against the saprophytic soil fungus *Curvularia* sp. and the plant pathogenic fungus *C. gloeosporioides*.

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