Research Article

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Isolation and Purification of Cleistanthin A and B from the leaves of *Cleistanthus collinus* Rob. (Euphorbiaceae)

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Cleistanthus collinus Roxb. (Euphorbiaceae) is a toxic plant which is widely distributed in the southeast part of Asia. This plant is widely used as suicidal and homicidal agent. Toxicity of the plant is mainly because of aryl-napthalide lignan lactones type of glycosides like cleistanthin A and B. An attempt was made to isolate the cleistanthin A and B from the leaves of *Cleistanthus collinus* leaves using chromatographic methods. The structures of the isolated constituents were confirmed by various spectroscopic methods.

Keywords: *Cleistanthus collinus*, cleistanthin A, cleistanthin B.

INTRODUCTION

Cleistanthus collinus Roxb. (Euphorbiaceae) is known as a toxic plant and found in Africa, India, Sri Lanka and Malaysia (1). In India this plant is widely distributed in rural and suburban areas in the states of Pondicherry, Tamil Nadu, Andhra Pradesh and Orissa. This plant is commonly used as a suicidal poison in India. All parts of the plant are reported to be toxic and used as suicidal, homicidal, cattle and fish poison and for inducing criminal abortion (2). Toxicity is mainly due to its diphyllin glycosides like cleistanthin A and B (3). In 1969, a series of constituents were isolated from the hot benzene extract of *Cleistanthus collinus* leaves *viz*., ellagic acid, fatty alcohol, collinusin, cleistanthin, diphyllin (4). The cleistanthin A and B were isolated from chloroform extract of *Cleistanthus collinus* barks and the structures were proposed by Lakshmi *et al.,* in 1970 (5). An attempt was made to isolate cleistanthin A and B from the leaves of *Cleistanthus collinus* using chromatographic techniques and identify the compounds using various spectroscopic methods. Cleistanthin A was isolated from benzene: ethyl acetate (1:1) fraction by modifying the existing method and cleistanthin B was isolated from methanolic fraction by newly developed method.

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MATERIALS AND METHOD

Plant material: The taxonomically identified plant parts were collected in the regions of Puducherry, rural parts of Villupuram, Cuddalore districts of Tamil Nadu and certified by the Botanical Survey of India (BSI), Coimbatore (BSI/SC/5/23/08-09/Tech.241). Leaves of *Cleistanthus collinus* were collected in the month of February 2008. Voucher specimen of the plant is kept in the Department of Pharmacology, JIPMER for further reference.

Determination of extractive value: The shadow, air dried *Cleistanthus collinus* leaf powder was used for extraction. The extractive value of *Cleistanthus collinus* leaf powder was determined with methanol, water, acetone, benzene, ethyl acetate, petroleum ether (40-60ºC), chloroform and n-Hexane.

***Cleistanthus collinus* leaves extraction for isolation of cleistanthin A and B:** The shadow, air dried *Cleistanthus collinus* leaf powder was used for extraction. The powdered leaves were defatted with n-hexane by cold maceration process for 24 h. The marc of the n-hexane was extracted with acetone by cold maceration process for the duration of 36-48 h. The acetone extract was then concentrated. The constituents of the plant extract were identified with primary qualitative analysis and thin layer chromatography (TLC) method for the presence of glycosides (4, 7).

GC-MS analysis of the plant extract: The presence of constituents was analysed with GC-MS spectroscopy (GC Clarus 500 Perkin Elmer

with Mass detector- Turbo mass gold-Perkin Elmer, USA). 2µl of acetone extract of *Cleistanthus collinus* leaves was injected with helium (1ml/min) as carrier gas. The Elite-5 (5% diphenyl 95% dimethyl poly siloxane) (30m × 0.25mm ID × 0.25µmdf) column was used for the analysis. Initially oven temperature was maintained at 110°C for 2 min, and temperature was gradually increased up to 280°C. The sample injector temperature was maintained at 250°C throughout the experiment period. The mass spectroscopic analysis was done with 70eV electron energy level, between 45 m/z to 450 m/z in the duration of 45 min. The separated compounds pack was compared with NIST 2.0 (2005) database.

Column chromatography for acetone fraction: The column was made with neutral alumina and was stabilized with benzene for 3-4 h. The column was prepared by wet packing method and slurry of neutral alumina was made with benzene. The acetone extract was dissolved in benzene and passed through column. The column was eluted with benzene, benzene: ethyl acetate (1: 1) to remove free fatty acids and fatty materials and the same column was eluted with benzene: ethyl acetate (1: 1) and methanol: chloroform (9.5 : 0.5) for isolating fractions of cleistanthin A, cleistanthin B. The fractions were collected (10 ml) and checked for purity by TLC method (7, 8).

Purification of the constituents: The benzene: ethyl acetate (4:1) fraction was eluted with silica gel column using benzene: ethyl acetate (1:1) solvent system and the elute was washed with absolute alcohol to purify cleistanthin A. The methanol: chloroform (9.5 : 0.5) fraction was allowed to natural drying and the dried substance was washed with minimum volume of methanol or chloroform to purify cleistanthin B which was crystallized with methanol.

Spectroscopic analysis of cleistanthin A and B: Infrared analysis of cleistanthin A and B were carried out with Avatar FT-IR 330 by potassium bromide pellet method. NMR spectroscopic analysis of cleistanthin A and B were carried out with Bruker 300 MHz with deuterated acetone ($(CD_3)_2CO$) and deuterated methanol (CD_3OD) as solvent.

RESULT

The methanol (25.06 % w/v), water (14.13 % w/v) and acetone (9.36 % w/v) had given high percentage yield of extractive value. The acetone extract showed a clear compound separation in TLC and hence the acetone extract was used for column chromatography. The qualitative GC-MS analysis of the acetone extract of *Cleistanthus collinus* leaves showed 19 compound separations in spectrum (Table 1). The percentage yield of isolated cleistanthin A and B were 0.9-1.0 % w/w and 0.6-0.8 % w/w receptively.

Purified cleistanthin A was isolated as a white to yellowish white colour substance eluting column with benzene: ethyl acetate (1:1). TLC showed R_f value of 0.37 (chloroform: n-heptane: ethanol 50:50:5). IR (KBr) spectrum of cleistanthin A showed the presence of dimeric OH stretch [3435.60 cm^{-1}], saturated methylene [2956.60 cm^{-1}, 2922.35 cm^{-1}, 2852.80 cm^{-1}], aldehyde carbonyl compound [2956.60 cm^{-1}], conjugated ketone [1645.52 cm^{-1}], carboxylic acid [1757.04], trimethyl group[1377.56] and skeletal C-C vibration between 1241.30 $cm^{-1}$to 738.70 cm^{-1} were observed. ^1H NMR (300 MHz) spectrum of cleistanthin A showed presence of aromatic protons constituted by two singlets [$^1H\delta$=7.239 ppm, $^1H\delta$=7.225 ppm], non chelated hydroxyl group [$^1H\delta$=5.849 ppm] and methylenedioxy groups [$^1H\delta$= 4.549 ppm, 4.565 ppm].

Purified cleistanthin B was isolated as colourless transparent crystals after eluting column with methanol: chloroform (9.5 : 0.5). TLC showed R_f value of 0.63 (chloroform: n-heptane: ethanol 50:50:5). IR spectroscopy of cleistanthin B showed the presence of dimeric OH [3432.98cm^{-1}], saturated methylene [2922.54 cm^{-1}, 2851.60 cm^{-1}], aldehyde [2762.18 cm^{-1}], carboxylate anion [2433.78 cm^{-1}], transition metal carbonyl group [2098.94 cm^{-1}], aromatic carbonate [1790.51 cm^{-1}, 1569.04 cm^{-1}], trimethyl/ tert butyl multiplet C-C skeletal vibrations [1353.13 cm^{-1}], skeletal C-C vibration/ C-O stretch [1039.15 cm^{-1}], epoxy and oxirane ring [836.20 cm^{-1}] were observed. ^1H NMR (300 MHz) spectrum of cleistanthin B showed presence of aromatic protons [$^1H\delta$= δ=8.553ppm], non chelated

hydroxyl group [^{1}Hδ=5.885 ppm] and methylenedioxy group [^{1}Hδ= 4.652].

Table 1: GC GC-MS analysis of acetone extract of leaves of *Cleistanthus collinus*

S.No	RT	Name of the compound	Molecular formula	Molecular weight	Peak Area %
1	15.83	6-Nonen-1-ol, (E)-	$C_9H_{18}O$	142	0.81
2	17.72	4-Octanol	C_8H_8O	130	0.27
3	19.00	2,3-Epoxyhexanol	$C_6H_{12}O_2$	116	2.30
4	20.88	1-Octanol,2-nitro-	$C_8H_{17}NO_3$	175	1.49
5	21.08	Cyclohexanol,5-methyl-2-(1-methylethyl)-,[1R-1α,2β,5α]-	$C_{10}H_{20}O$	156	4.86
6	21.97	Pentanoic acid, 10-undecenyl ester	$C_{16}H_{30}O_2$	254	10.41
7	25.73	Acetic acid, trifuoro-2,2-dimethylpropopyl ester	$C_7H_{11}F_3O_2$	184	0.41
8	27.28	Oxalic acid, allyl pentadecyl ester	$C_{20}H_{36}O_4$	340	1.89
9	28.07	Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390	1.49
10	31.59	Sulfurous acid, dodecyl 2-propyl ester	$C_{15}H_{32}O_3S$	292	7.84
11	31.90	Squalene	$C_{30}H_{50}$	410	3.65
12	33.23	1-cyclohexxylnonene	$C_{15}H_{28}$	208	1.62
13	35.51	Sulfurous acid, hexyl pentadecyl ester	$C_{21}H_{44}O_3S$	376	13.65
14	36.08	Vitamin E	$C_{29}H_{50}O_2$	430	7.97
15	36.77	Sulfurous acid, 2-propyl tridecyl ester	$C_{16}H_{34}O_3S$	306	9.32
116	37.53	Pentadecanal-	$C_{15}H_{30}O$	226	10.68
17	39.97	Oxalic acid, allyl pentadecyl ester	$C_{20}H_{36}O_4$	340	4.86
18	40.23	Cedran-diol,8S, 14-	$C_{15}H_{26}O_2$	238	9.19
19	41.22	9-Octadecenoic acid (Z)-phenylmethyl ester	$C_{25}H_{40}O_2$	372	7.30

DISCUSSION

In 1969, cleistanthin was isolated from hot benzene fraction of cold acetone extract of *Cleistanthus collinus* leaves and purified by TLC method (4). In 1970, cleistanthin A and B were isolated from *Cleistanthus collinus* bark and structures of the compounds were proposed. In the present study, the method was modified and optimized to isolate cleistanthin A from benzene: ethyl acetate (1; 1) fraction of acetone extract and methanolic fraction of acetone extract which was not used before was tried for isolating cleistanthin B. The column was eluted with benzene and benzene: ethyl acetate (4: 1) to remove fatty alcohols and plant secondary metabolites. Qualitative analysis of acetone extract was studied by GC-MS analysis. A trial and error method was used to purify cleistanthin A and B.

The IR spectroscopy of cleistanthin A and B showed the presence of hydroxyl group and aromatic residue as well as lactone carbonyl group. The ^{1}H NMR spectroscopy of cleistanthin A and B showed the presence of aromatic proton, methylenedioxy group, free hydroxyl group and free skeletal carbonyl protons and these observations were supported by ^{13}C NMR. The glycone part of the cleistanthin A and B differs in structure and remaining aglycone part same for both cleistanthin A and B (3,6). Apart from cleistanthin A and B, the plant also contains diphyllin and collinusin (7). Cleistanthin A, cleistanthin B, diphyllin and collinusin are major phytoconstituents present in the plant. Cleistanthin A and B are arylnaphthalide lignan glycosides compounds and it has been

reported that these compounds are responsible for the toxicity. But no scientific data available for toxicity profiles of cleistanthin A and B. Cleistanthin A is one of the major constituents present in *Cleistanthus collinus*, but it is also present in *Phyllanthus toxodiifolius* Beille (Euphorbiaceae) which is used as a traditional diuretic agent in Thailand (9). The pharmacological and toxicological investigations are required to elucidate the biological actions of cleistanthin A and B.

CONCLUSION

This study suggests that apart from cleistanthin A and B, the *Cleistanthus collinus* leaves have many constituents. As there are no scientific data available for toxicity of cleistanthin A and B, the isolation of cleistanthin A and B can lead researchers to explore the preclinical profile of this compound.

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