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Research Article

Isolation and identification of a new molecule from *Curculigo Orchioides* (hypoxidaceae)

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ABSTRACT
The novel compound 2-β-D-glucopyranosyloxy-5-hydroxybenzyl-2', 6'-dimethoxy-3'-hydroxybenzoate, a glucoside of substituted benzyl benzoate isolated from *C. orchioides*. The molecular formula $C_{22}H_{26}O_{12}$ was deduced from HRFAB-MS m/z 505.1323 $[M+Na]^+$ (required 505.1322 for $C_{22}H_{26}O_{12}Na$). Assignments of proton and carbon resonances were deduced from analysis of ^1H-^1H COSY, heteronuclear HMQC, and HMBC 2D chemical shift correlations. The data are supported by 1D and 2D NMR spectra show consistent with a benzyl benzoate structure. The examination of the data suggested the presence of two aromatic rings, of which one ring A, is trisubtituted with three aromatic protons at δ 6.70 (1H, dd, J=8.8Hz and 3.0Hz, H-4), δ 7.08 (1H, d, J=8.8Hz, H-3) and δ 6.92 (1H, d, J=3.0 Hz, H-6). The other aromatic system, ring B, is tetrasubstituted.

KEY WORDS:*Curculigo orchioides*,Curculigoside, Curculigoside-2, New molecule

INTRODUCTION

Tuberous roots of *C. orchioides* are widely used as tonic for health, vigour and vitality. The plant material has been used along with other plants, viz., *Asparagus ascendense, A. racemosus, Chlorophytum borivilianum* and *Withania somnifera* in several pharmaceutical formulations which are used as metabolic enhancer and aphrodisiac[1]. Plant extract of *C. orchioides* showed hypoglycemic, spasmolytic and anticancer properties[2]. Pharmacological studies in China showed several active effects of alcoholic extract of roots such as adaptogenic, anti-inflammatory, anticonvulant, sedative, androgenic and immunopromotion activities[3]. The rhizome is prescribed for asthma, piles, jaundice, diarrhoea, colic and gonorrhoea. It is considered to be demulcent, diuretic, tonic and aphrodisiac and is often combined with aromatics and bitters[4,5]. In Chinese traditional medicine it is used as tonic for the treatment of decline in physical strength[6]. Presence of flavanone glycoside, steroids, terpenoids, phenolic glycosides and other compounds have been reported in the species[3,7-12].Steroids and triterpenoids: Three steroids, sitosterol, stigmasterol[8] and yuccagenin[7] have been isolated from *C. orchioides*. Out of six triterpenes isolated, one triterpene is of ursane series 31-methyl-3-oxo-20-ursen-28-oic acid [13] and rest of them are cycloartene series namely cycloartenol[8] , curculigol [14], curculigenin A[9,11], Curculigenin B and curculigenin C[10] .Glycosides and saponins: Xu and co-workers[3,10-11] characterized thirteen saponins from *C. orchioides* rhizomes and named them curculigosaponin A-M.phenolic compounds. Five phenolic compounds have been isolated and characterized from *C. orchioides*. These are curculigoside (5-hydroxy-2-O-□-D-glucopyranosyl benzyl-2,6-dimethoxy benzoate) [15], curculigine A and orcinol glucoside[16-17] , corchioside A[8] and flavanone glycoside–I (glycoside–5,7-dimethoxy-dihydromyricetin-3-O-α-L-xylopyranosyl (4-1)-β -D-glycopyranoside) [18].

Nitrogenous constituents: N-acetyl-N-hydroxy-2-carbamic acid methyl ester, 3-acetyl-5-carbomethoxy-2H-3,4,5,6,-tetrahydro-1,2,3,5,6-oxotetrazine, N,N,N',N'-tetramethyl succimmide have been isolated from the rhizome of *C. orchioides*[19]. Lycorine is the only alkaloid isolated and known so far in *C. orchioides*[7].

Aliphatic hydroxy ketones: A number of fatty acids have been isolated from root oil of *C. orchioides* by gas liquid chromatography[20]. They are palmitic, oleic, linoleic, arachidic and behenic acid. Later on, Hentriacontanol[8], 3-methoxy-5-acetyl-31-tritriacontane, 4-acetyl-2-methoxy-5-methyltriacontane and 25-hydroxy-33-methylpentatriacontan-6-one[21-22] were identified from the rhizome. Misra *et al*. [23-24] have reported 27-hydroxy triacontan-6-one, 23-hydroxytriacontan-2-one, 21-hydroxytetracontan-20-one, and 4-methyl heptadecanoic acid from the rhizome of *C. orchioides.*

In addition to these, the plant also contains glucose, mannose, xylose, glucoronic acid, resin, tannin, fat, starch and good deal of mucilage[3,7,8,9,10,11,12,15]. In related species, benzylbenzoate and norlignan glucosides have

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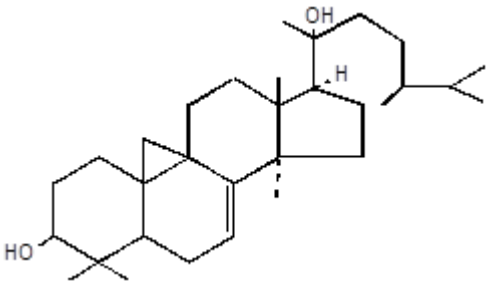
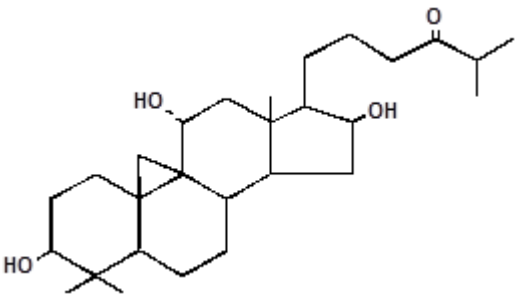
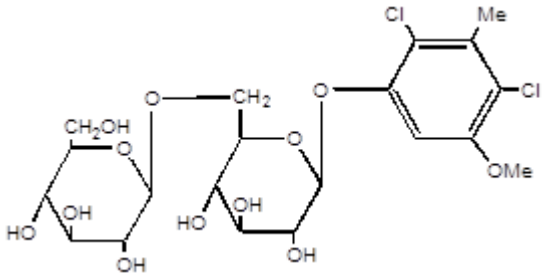
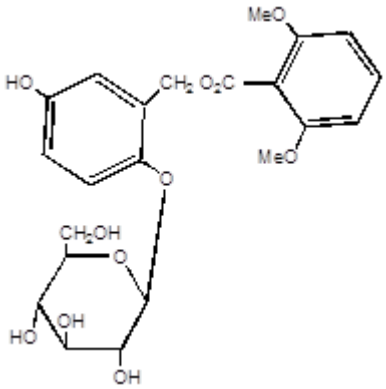


Curculigol

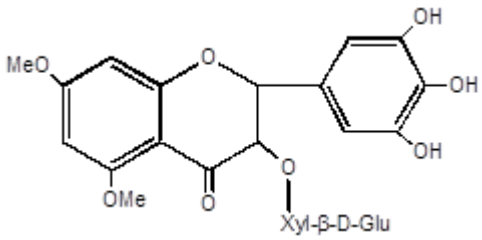


Curculigenin A

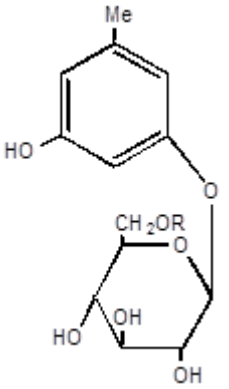




Curculigine A



Curculigoside



5,7-dimethoxydihydromyricetin-3-O-α-L-xylopyranosyl-4-O–β-D-glucopyranoside

R=H=Orchinol glucoside
R=β-D-Xylose=Corchioside A

Figure : 1 Structures of some of the metabolites isolated from *C. Orchioides*

been identified in *C. pilosa*[25] and curculin, a sweet-tasting and taste-modifying protein (a non-functional mannose binding lectin) in *C. latifolia*[26].

Overexploitation of plant associated with poor seed set and germination made it an endangered species[27]. Micropropagation methods have been developed in the laboratory using leaf explants grown on static[28-29] and in liquid shake flask cultures[30]. It is of interest to study the phytochemical properties of the cultures and evaluate the isolated molecules for their pharmacological properties. In the present communication we report the isolation and identification of a new compounds from *in vitro* cultures grown as bulbils in shake flasks. Chemical investigations carried out by Indian and Chinese workers demonstrated presence of various compounds in *C. orchioides* (Figure-1).

Group	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Fraction No.	10-11	12-17	18-19	20-25	26-45 **(72 mg)**	46-77	78-99	99-end

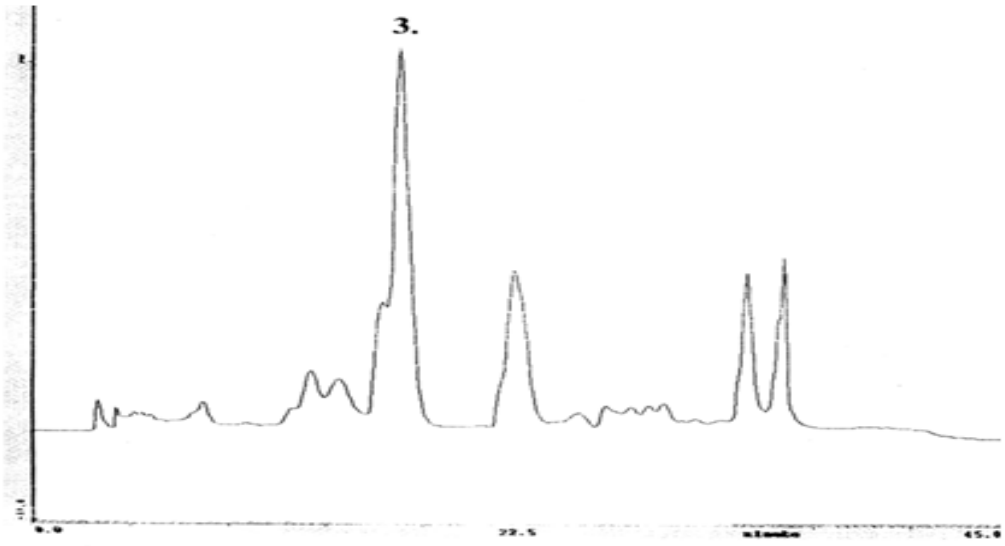


Figure :2 HPLC spectra of Fraction 'F'of CPC.
This yielded a new Curculigoside (F3 fraction)

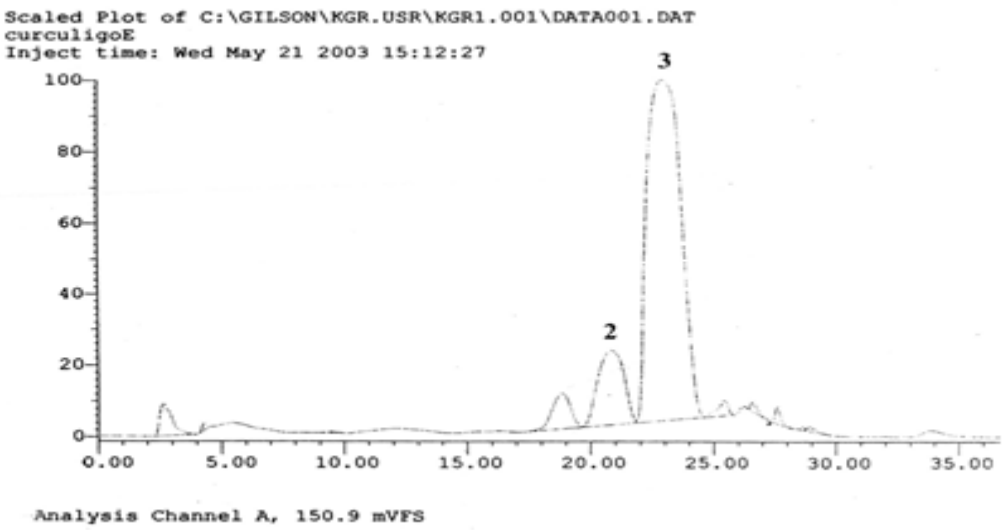


Figure 3: HPLC profile of fraction 'E' of CPC.
This yielded Curculigoside and Curculigoside-2.

MATERIAL AND METHODS

Plant material: Young leaves of *C. orchioides* from aseptically maintained plantlets, raised through leaf explants were used to initiate bulbils formation in MS liquid medium containing BA (0.1mg/l) and IBA (0.1mg/l). Four weeks old cultures containing bulbils were harvested, washed with distilled water and oven dried at 60ºC for 48 hours.

Extraction and isolation: Dried and powdered 42 g tissues were extracted with water-acetone (3:2, v/v) at 4ºC with maceration (3×1 L). Extract was filtered through filter paper and pooled solvent was evaporated at 40ºC under reduced pressure and then the residual aqueous phase was partitioned with ethyl acetate (300ml×3). The ethyl acetate fraction was concentrated under vacuum at 40ºC and redissolved in water to be freeze-dried.

Centrifugal Partition Chromatography (CPC): In CPC, either the heavier phase (lower phase) or the lighter phase (upper phase) of the biphasic solvent system can be used as mobile phase. In present work, the stationary phase was the lower aqueous phase (ascending mode). The column was first filled with the aqueous stationary phase without rotation and followed the procedure using hexane-ethyl acetate-ethanol-water as reported earlier[31], 300 mg freeze dried sample was dissolved in 50% aqueous methanol and passed through a 0.45µm Millipore filter and injected in the CPC injector. Fractions, 9ml each, were collected on a fraction collector (Gilson model FC-204, France) and grouped into eight

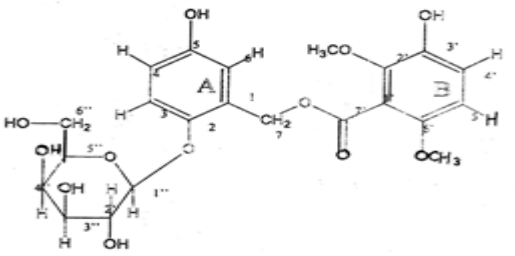


Figure 4: The novel compound Hydroxy Curculigoside (2-β-D-glucopyranosyloxy-5 -hydroxybenzyl- 2',6'-dimethoxy-3'hydroxybenzoate).

fractions on the basis of TLC. Plates were visualized under UV and by spraying anisaldehyde reagent. HPLC and Semi-Preparative HPLC: The final purification of the freeze-dried fraction F (12mg) was achieved by semi-preparative HPLC with an Ultrasep ES 100 RP reversed phase C_{18} column (250mmx8.0 mm I.D., 6µm) at room temperature. The mobile phase was composed of two solvents (A & B): A (H_2O with 0.0025% TFA, v/v) and B (80% ACN and 20% A). The gradient system at 2 ml min^{-1} was: 5% B (0-10 min), 5-30% B (10-40), 30-100% B (40-60 min). The chromatogram was monitored at 286 and 306 nm. The major peak of this fraction (Figure-2) yielded pure 2-β-D-glucopyranosyloxy-5-hydroxybenzyl-2',6'-dimethoxy-3'-hydroxybenzoate (2mg). The purification of the freeze-dried fraction E (72 mg) was achieved by semi-preparative HPLC using the same conditions. The major peaks of this fraction yielded curculigoside (10mg) and 2-β-D-glucopyranosyloxy-5-hydroxybenzyl-2'-methoxy-6'-hydroxybenzoate (6mg) (Figure-3).

NMR spectra: NMR spectra were recorded on Bruker Avance 300 MHz spectrometer. Chemical shift values are presented as d values with tetramethylsilane (TMS) as an internal reference. Peak multiplicities were quoted in Hz. Mass spectra were recorded with VG Autospec-Q in the positive FAB mode using glycerol as matrix. HPL purifications were carried out on a Gilson gradient system equipped with an UV-vis detector Varian Pro-Star 325.

TLC: All the fractions were monitored by thin layer chromatography (TLC) on plastic sheet coated by silica gel $60F_{254}$ using $CHCl_3$-MeOH-AcOH (85:15:3, v/v) as mobile phase.

RESULTS AND DISCUSSION

Polyphenolic extract of *C. orchioides* showed a large number of anisaldehyde positive spots. Therefore, using CPC, extract was fractioned as described in material and methods. Plant extract was separated in eight major fractions (A-H) by CPC. Following fractions were pooled:

Therefore, total crude extracts were grouped into eight fractions, making it less complicated for separation by semi-preparative HPLC. This enables the separation of compounds by HPLC. Pooled fraction of group E,F,G and H yielded six compounds. These compounds were compared with authentic polyphenolics present in the laboratory.

The novel compound recorded is a glucoside of substituted benzyl benzoate assigned to 2-β-D-glucopyranosyloxy-5-hydroxybenzyl-2', 6'-dimethoxy-3'-hydroxybenzoate (Figure-4). The molecular formula $C_{22}H_{26}O_{12}$ was deduced from HRFAB-MS m/z 505.1323 $[M+Na]^+$ (required 505.1322 for $C_{22}H_{26}O_{12}Na$). Assignments of proton and carbon resonances were deduced from analysis of ^1H-^1H COSY, heteronuclear HMQC, and HMBC 2D chemical shift correlations. The data reported in 1D and 2D NMR spectra are consistent with a benzyl benzoate structure. The examination of the data suggested the presence of two aromatic rings, of which one, ring A, is trisubtituted with three aromatic protons at δ 6.70 (1H, dd, J=8.8Hz and 3.0Hz, H-4), δ 7.08 (1H, d, J=8.8Hz, H-3) and δ 6.92 (1H, d, J=3.0 Hz, H-6). The other aromatic system, ring B, is tetrasubstituted.

With two aromatic protons in *ortho* relationship at δ 6.64 (1H, d, J=8.9Hz, H-4') and δ 6.86 (1H, d, J=8.9Hz, H-5'). In addition, the spectra exhibited signals for an ester (δ_{CO} 168.1), for a glucose unit (δ_{Canom} 104.4), for a oxymethylene protons (δ_C 65.1) and for two methoxy groups (δ_C 56.9 and 61.7). The HMBC spectrum showed that the methoxy groups were connected to the 2' and 6'-positions of the ring B and that the glucose unit was connected to the 2-position of ring A. The HMBC correlations between H-5'/C-7' indicated that the ring B was connected to the ester unit. The correlation between oxymethylene protons H-7a(H-7b) and ring A carbons C-1, C-2 and C-6 showed the connectivity between C-1 and C-7. The correlation between H-7/C-7' showed the linkage between the benzyl fragment and the benzoic acid fragment.

The two known compounds were identified by spectrometric methods as curculigoside[15] and 2-β-D-glucopyranosyloxy-5-hydroxybenzyl-2'-methoxy-6'hybroxybenzoate[9]. Three other compounds are yet to be identified. Therefore, more compounds are being isolated for verification.

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