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Neoverataline A and B, two antifungal alkaloids with a novel carbon skeleton from *Veratrum taliense*

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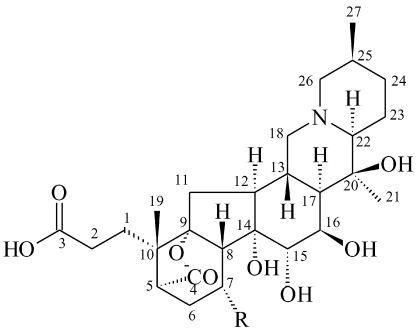
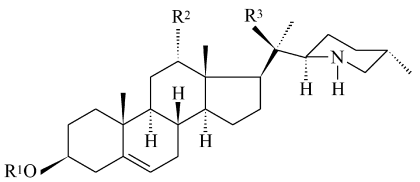
Abstract—Bioassay-guided fractionation of the ethanol extract of the roots and rhizomes of *Veratrum taliense* yielded two new and thirteen known steroidal alkaloids. The structures of the two new compounds, neoverataline A and B, were established by extensive spectroscopic analyses to be 3,4-secocevane-4,9-olid-14,15,16,20-tetra-ol-3-oic acid and 3,4-secocevane-4,9-olid-7,14,15,16,20-penta-ol-3-oic acid, respectively, and are a novel carbon skelton. All of the fifteen alkaloids were subjected to *in vitro* antifungal assays, which showed that the verazine- (veramitaline, stenophylline B, stenophylline B-3-*O*-β-D-glucopyranoside, veramiline-3-*O*-β-D-glucopyranoside) and jervera-trum-type (jervine, jervine-3-*O*-β-D-glucopyranoside) alkaloids exhibited strong antifungal activities against the phytopathogenic fungus *Phytophthora capisis* with MICs of 160, 120, 160, 80, 80 and 120 $\mu g \cdot L^{-1}$, respectively. Furthermore, the verazine-type alkaloids stenophylline B, stenophylline B 3-*O*-β-D-glucopyranoside and veramiline 3-*O*-β-D-glucopyranoside were shown to also inhibit the growth of another fungal phytopathogen *Rhizoctonia cerealis* with MICs of 160, 120 and 120 $\mu g\ mL^{-1}$. The MICs of triadimefon (an antifungal agrochemical used herein as a positive control) against *P. capisis* and *R. cerealis* were 120 and 80 $\mu g\ mL^{-1}$, respectively. A preliminary structure–activity relationship regarding these alkaloids has been formulated.

1. Introduction

The *Veratrum* species (Family: Liliaceae) have been found to be a rich source of new and/or bioactive steroidal alkaloids, some of which are well known for their pharmacological potential.[1,2] From *Veratrum taliense* Loes f., a folk medicine endemic to Yunnan Province, China,[3] we have previously characterized several new steroidal alkaloids and xanthine oxidase inhibitory stilbenoids.[4–6] In continuing our investigations of structurally novel and/or biologically active natural products from plants,[7] endophytes[8] and marine microorganisms,[9] we found that the alkaloidal fraction from the ethanol extract of *V. taliense* showed pronounced inhibitions on the growth of the phytopathogens *Phytophthora capisis* and *Rhizoctonia cerealis*, which are the causes of pepper blight and wheat sharp eyespot, respectively.[10] An antifungal bioassay-guided fractionation was therefore performed to identify the bioactive phytochemical(s) from the title species. We hereby wish to report the structure elucidation of two minor steroidal alkaloids named neoverataline A (**1**) and B (**2**), possessing a novel 3,4-secocevane-4,9-olid-3-oic acid skeleton, along with the thirteen known alkaloids (**3**–**15**), some of which were shown to be antifungal agents.



1 R=H; **2** R=OH



3 R^1=R^2=H, R^3=OH;
4 R^1=Glu, R^2=H, R^3=OH
5 R^1=Glu, R^2=R^3=H
6 R^1=R^3=H, R^2=OH

Keywords: *Veratrum taliense*; Liliaceae; secocevane; neoverataline A; neoverataline B; antifungal.

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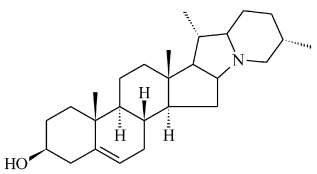

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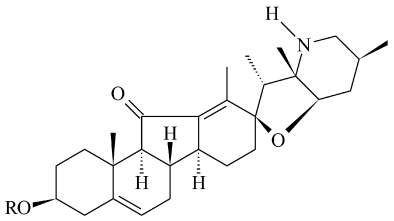
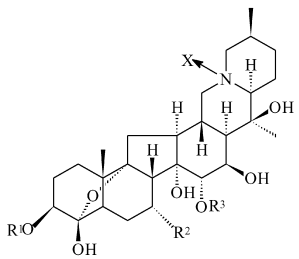


8 R=H; **9** R=Glu



10 R^1=veratroyl, R^2=R^3=H, X=:;
11 R^1=angeloyl, R^2=R^3=H, X=:;
12 R^1=R^3=H, R^2=OH, X=:;
13 R^1=H, R^2=OH,R^3=angeloyl, X=:;
14 R^1=H, R^2=OH, R^3=2-methylbutyryl, X=:;
15 R^1=angeloyl, R^2=R^3=H, X=O

2. Results and discussion

The isolates stenophylline B 3-*O*-β-D-glucopyranoside (**4**), veramiline-3-*O*-β-D-glucopyranoside (**5**), veramitaline (**6**), solanidine (**7**), jervine (**8**), 3-veratroylzygadenine (**10**), germine (**12**), 15-angeloylgermine (**13**), 15-(2-methylbutyroyl)germine (**14**) and 3-angeloylzygadenine-β-*N*-oxide (**15**) were obtained as well in our previous work,[4–6] and the identification of these constituents was accomplished readily by melting point, TLC and HPLC comparisons to authentic samples. Moreover, the present fractionation of the alkaloidal fraction gave, in addition to the new compounds **1** and **2**, other reported steroidal alkaloids **3**, **9** and **11**, which were identified spectroscopically (IR, MS, ^{1}H and ^{13}C NMR) as stenophylline B,[11] jervine-3-*O*-β-D-glucopyranoside[12] and 3-angeloylzygadenine,[13] respectively.

Neoverataline A (**1**) was obtained as white columnar crystals. Its molecular formula was determined to be $C_{27}H_{41}NO_8$ based on the protonated molecular ion at *m/z* 508.2908 in its HRESIMS (positive mode), which coincided with a total of 27 carbon resonance singals discerned in its ^{13}C NMR spectrum, edited by DEPT pulse sequences. The IR spectrum of compound **1** exhibited absorptions at 2744 cm^{-1} and 2772 cm^{-1} (Bohlmann bands), indicating the presence of the *trans*-quinolizidine moiety.[13] Compound **1** was confirmed as a ceveratrum-related alkaloid by the base peak at *m/z* 112 in its EIMS (characteristic of C-nor-D-homo steroidal alkaloids[12,14]), in addition to two tertiary and one secondary methyl signals at δ 1.09 (H-19), 1.35 (H-21) and 1.15 (*J*=7.6 Hz, H-27) in its ^{1}H NMR spectrum.[13] Futhermore, compound **1** was implied to be simlar to zygadenine in structure on the basis of two oxygenated methine proton signals at δ 3.57 (d, *J*=3.6 Hz, H-15) and 4.38 (br d, *J*=3.6 Hz, H-16).[13] Moreover, the three quaternary carbon signals at δ 181.2, 179.7 and 100.4, which did not appear in the ^{13}C NMR spectrum of any of the reported zygadenine-typed alkaloids, were attributed unambiguously to C-3, C-4 and C-9 (carbon numbering deduced from other zygadenine-type alkaloids[13]) respectively by extensive 2D NMR experiments (^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY and HMBC). The magnitude of the strikingly downfield chemical shifts for C-3, C-4 and C-9 could only be explained by assuming the presence of a 3,4-*seco*-4,9-olid-3-oic acid moiety. This assumption was also confirmed by both the IR absorption band at 1753 cm^{-1} ascribable to a γ-lactone,[15] and the carboxylic acid functional group which generated a positive response to bromocresol green reagent. Further comfirmation of the proposed structure was provided by extensive 2D NMR analyses allowing the unequivocal assignments of all ^{1}H and ^{13}C NMR data (Table 1). Especially, the quarternary carbon signal at δ 100.4 in the ^{13}C NMR spectrum assigned to C-9 according to the HMBC correlations of C-9 with H-C(5), H-C(8), H-C(11) and H-C(19). Obviously the C-9 signal of compound **1** was shifted downfield by 3–8 ppm relative to those of alkaloids **10**–**15**, which resonated between δ 92 and 97 ppm. This is due to the paramagnetic effect of γ-lactone moiety, which also induced a substantial downfield shift of the C-5 signal (approximately 3 ppm). Accordingly, compound **1** was established as 3,4-secocevane-4,9-olid-14,15,16,20-tetra-ol-3-oic acid, a hitherto unknown alkaloid with a novel carbon framework, presumably derived from zygadenine analog(s) biosynthetically.

Compared to the β-orientation of the electron pair on the nitrogen atom as observed with other biosynthetically related C-nor-D-homo steroidal alkaloids well defined stereochemically by organic synthesis,[16] an α- (viz., axially) oriented proton was thus assumed at C-22 according to Bohlmann bands in its IR spectrum (see above) indicative of *trans*-quinolizidine moiety.[13] This was also evidenced from the couplings between H-22 and H-23 [$J_{22,23\beta}$ (=J_{aa})=10.4 Hz, $J_{22,23\alpha}$ (=J_{ae})=4.8 Hz]. Similarly H-25 was deduced to be α- (viz., equatorially) by the magnitude of $J_{25,26\beta}$ (=J_{ae}=4.0 Hz) and $J_{25,26\alpha}$ (=J_{ee}≈0 Hz). In the NOESY spectrum of compound **1**, Me-21 showed NOE correlations with H-22, H-17 and H-16 leading to the assignments of HO-16β, H-17α and Me-21α, which was further supported by the triplet signal of H-16 at δ 4.38 ($J_{15,16}$=$J_{16,17}$=3.6 Hz). The proposed orientations of

Table 1. NMR (500 MHz for ^{1}H and 125 MHz for ^{13}C NMR) spectral data of **1** and **2**

Atom	**1** (in CD_3OD)			**2** (in C_5D_5N)	
	δ_C (DEPT)	δ_H (mult. *J*in Hz)	HMBC	δ_C (DEPT)	δ_H (mult. *J* in Hz)
1	32.8 t	1.76 (m), 1.92 (m)	HH-19	30.0 t	2.04 (m), 2.20 (m)
2	33.0 t	2.14 (t, 8.0)	*	30.6 t	2.57 (m)
3	181.2 s		*	178.1 s	
4	179.7 s		H-5	176.0 s	
5	49.6 d	2.49 (br d, 2.5)	H-19	45.5 d	2.93 (t, 3.0)
6	21.2 t	1.73 (m)	H-5, 7	32.5 t	2.02 (m), 2.36 (m)
7	18.6 t	1.90 (m), 1.61 (m)	H-5, 8	66.6 d	4.97 (br. t, 4.8)
8	44.5 d	2.74 (dd, 11.0, 6.0)	H-6, 12, 15	45.9 d	3.23 (d, 4.8)
9	100.4 s		H-5, 8, 11β, 19	98.0 s	
10	48.0 s		H-5, 8, 19	47.5 s	
11	33.6 t	1.82 (dd,15.0, 2.5) 2.26 (dd, 15.0, 7.2)	H-12, 13	34.0 t	2.03 (m), 2.56 (m)
12	47.4d	1.82 (ddd, 11.5, 7.2, 2.5)	H-13	46.4 d	2.19 (m)
13	33.2 d	2.14 (m)	H-11α, 11β, 12, 16, 17	34.0 d	1.96 (m)
14	80.6 s		H-8, 15	82.3 s	
15	71.7 d	3.57 (d, 3.6)	H-16	70.8 d	4.49 (d, 3.6)
16	73.0 d	4.38 (t, 3.6)	H-15	71.4 d	5.00 (br d, 3.6)
17	43.8 d	1.73 (dd, 13.2, 3.6)	H-15, 18β, 21	44.6 d	1.84 (dd, 11.8, 2.0)
18	61.0 t	2.59 (t, 12.0), 3.35 (br d, 12.0)	*	62.3 t	1.72 (t, 10.8) 3.01 (dd, 10.8, 2.0)
19	14.7 q	1.09 (s)	*	14.0 q	0.60 (s)
20	73.1 s		H-21	73.5 s	
21	22.7 q	1.35 (s)	*	22.3 q	1.38 (s)
22	71.6 d	2.89 (dd, 10.4, 4.8)	H-18β, 21, 26β	71.0 d	1.61 (dd, 11.2, 2.0)
23	18.7 t	1.90 (m)	*	19.2 t	1.52 (m)
24	28.6 t	1.79 (m)	H-26β, 27	30.0 t	1.45 (m)
25	27.8 d	2.13 (m)	H-26β, 27	28.3 d	1.78 (m)
26	60.4 t	3.05 (dd, 12.4, 4.0), 3.24 (br d, 12.4)	H-27	62.3 t	2.28 (dd, 11.2, 3.2), 2.80 (br d, 11.2)
27	16.6 q	1.15 (d, 7.6)	H-26β, 26α	17.9 q	1.05 (d, 7.2)

*Not observed.

HO-14α and HO-15α could be deduced from the similarity in the chemical shifts of H-8, H-12, H-15 and H-16 as well as C-14, C-15 and C-16 to those of 15-angeloygermine.[5] However no NOE correlation could be discerned between H-13 and H-17, or between H-12 and H-13, implying that H-13 was *trans*- to H-12 and H-17. This was confirmed by the splitting pattern of the three protons ($J_{12,13}$=J_{aa}=11.5 Hz; $J_{13,17}$=J_{aa}=13.2 Hz). Moreover, the NOE correlations of H-19 with H-5 at δ 2.49 (br d, *J*=2.5 Hz), H-8 at δ 2.74 (dd, *J*=11.0, 6.0 Hz) and H-11β at δ 2.26 (dd, *J*=15.0, 7.2 Hz) established the formulated stereochemistry of the stereocentres of the γ-lactone moiety and the five-membered ring.

Based on the above information, a computer-generated plot for the 3D structure of **1** (Fig. 1) was obtained with the molecular-modeling program CS CHEM 3D V5.0 by MM2 force-field calculations for energy minimization. The calculated distances between H–C(22)/Hα–C(21) (2.513 Å), H–C(22)/Hβ–C(21) (3.761 Å), H–C(22)/Hp–C(21) (3.253 Å), H–C(17)/H–C(16) (2.287 Å), H–C(17)/Hα–C(21) (2.577 Å), H–C(17)/Hβ–C(21) (2.973 Å), H–C(17)/Hp–C(21) (3.759 Å), Hβ–C(11)/Hα–C(19) (2.322 Å), Hβ–C(11)/Hβ–C(19) (2.993 Å), Hβ–C(11)/Hp–C(19) (3.867 Å), H–C(5)/Hα–C(19) (3.174 Å), H–C(5)/Hβ–C(19) (2.123 Å), H–C(5)/Hp–C(19) (2.447 Å), H–C(8)/Hα–C(19) (3.246 Å), H–C(8)/Hβ–C(19) (2.115 Å) and H–C(8)/Hp–C(19) (3.676 Å) are less than 4.00 Å, which are consistent with the well-defined NOEs observed for each of these proton pairs confirming the relative stereochemistry proposed above. We have named alkaloid **1** neoverataline A.

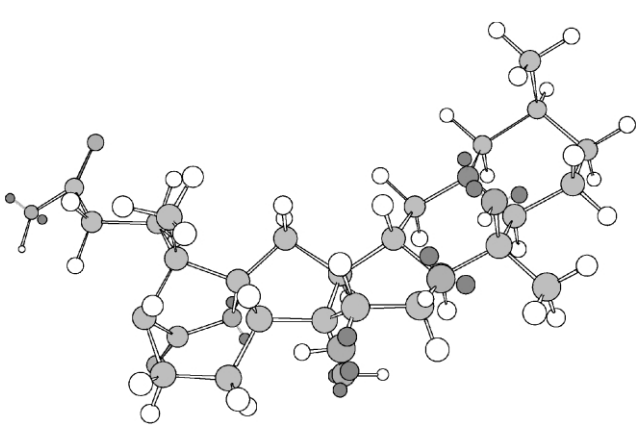


Figure 1. the 3D structure of **1**.

Neoverataline B (**2**) was obtained as colorless needles. Its molecular formula was determined to be $C_{27}H_{41}NO_9$ on the basis of the quasimolecular ion peak at *m/z* 524.2856 [(M+H)$^+$] in its HRESIMS (positive mode), which was in accord with a total of 27 discrete signals in its ^{13}C NMR spectrum edited with DEPT experiments. The molecular weight of base **2** was 16 amu higher than that of alkaloid **1**, suggesting that it could be a mono-hydroxylated derivative of alkaloid **1**. This deduction was confirmed by the close resemblance between the ^{1}H and ^{13}C NMR data of bases **1** and **2**, with their IR spectra being nearly identical. However, an additional broad triplet at δ 4.97 (*J*=4.8 Hz) in the ^{1}H NMR spectrum of **2** suggested that it possessed one more hydroxyl group in

the molecule. This was further confirmed by the ^{13}C NMR spectrum of compound **2** exhibiting a difference of an extra oxygenated methine carbon absorption at δ 66.6, which replaced the methylene carbon signal at δ 18.6 compared to that of **1**. This hydroxyl group was demonstrated to anchor on C-7, both by comparing the ^{1}H and ^{13}C NMR data of **2** with those of reported germine derivatives,[17] and by 2D NMR analyses (COSY, HMQC and HMBC) leading to the exact assignments of all ^{1}H and ^{13}C resonances of **2** (Table 1). Therefore, the structure of compound **2** was elucidated to be 3,4-secocevane-4,9-olid-7,14,15,16,20-penta-ol-3-oic acid, presumably derived from germine analog(s) biosynthetically. Concerning the relative configuration of compound **2**, except for C-7, the stereochemistry was established by the similarity of its ^{1}H and ^{13}C NMR spectra with those of compound **1**. The assignment of HO-7α was deduced from the coupling constant of H-7 and H-8 ($J_{7,8}=J_{ae}=4.8$ Hz). We have named alkaloid **2** neoverataline B.

All isolates were tested in vitro for antifungal activity against the phytopathogens *Phytophthora capisis* and *Rhizoctonia cerealis*. Neoveratalines A (**1**) and B (**2**) were moderately inhibitory to the two phytopathogens (MICs$\sim$200 $\mu g\ mL^{-1}$). The MICs of the positive control, triadimefon, against the two fungi were 120 and 80 $\mu g\ mL^{-1}$, respectively. The verazine- (**3**–**6**) and jerveratrum-type alkaloids (**8** and **9**) exhibited stronger antifungal activities against *P. capisis* with MICs at 120, 160, 80, 160, 80 and 120 $\mu g\ mL^{-1}$, respectively. Alkaloids **3**–**5** inhibited *R. cerealis* with MIC values of 160, 120 and 120 $\mu g\ mL^{-1}$, respectively. Based on the above observations, the preliminary structure–activity relationship regarding these alkaloids was tentatively summarized as follows. The potency of verazine-type alkaloids in inhibiting *P. capisis* could be decreased by the presence of 12- or 20-hydroxyl groups (e.g. **4** and **6**). Moreover, glycosylation at the 3-hydroxyl groups of both verazine (e.g. **4**) and jerveratrum (e.g. **9**) alkaloids lower substantially the antifungal action against *P. capisis*, whereas glycosylation at the 3-hydroxyl group (e.g. **4** and **5**) of verazine type alkaloids improve the inhibition of the growth of phytopathogenic fungus *R. cerealis*. In addition, the tethered piperidine as in compounds **3**–**6** could be essential for antifungal action.

3. Experimental

Melting points were measured on an XT-4 apparatus and were uncorrected. Optical rotations were determined in MeOH on a WXG-4 polarimeter and IR spectra on a Nicolet 170 SX FT-IR spectrometer. NMR spectra were recorded on a Bruker AMX-500 NMR spectrometer using TMS as internal standard with ^{1}H and ^{13}C nuclei observed at 500 and 125 MHz, respectively; MS were taken on a ZAB-HS mass spectrometer and HRESIMS on a Mariner Mass Biospectrometer. Silica gel (200–300 mesh) for CC and silica GF_{254} (10–20 μ) for TLC were products of Qingdao Marine Chemical Factory, China. Sephadex LH-20 was from Pharmacia Biotech, Sweden. ODS silica gel was from Nacalai Tesque, Kyoto, Japan. All chemicals used in the study were of analytical grade.

3.1. Plant material and test fungi

The roots and rhizomes of *V. taliense* were collected in July 1999 in Dali Prefecture, Yunnan Province, China. A voucher specimen (VT-99702), identified by Professor L. X. Zhang, was deposited in the Herbarium of Nanjing University, Nanjing, P. R. China. The test fungi were *Fusarium graminearum* Schw, *Bipolaris sorokiniana* (Sacc.) Shoem., *Phytophthora capsici* Leon and *Rhizoctonia cerealis* Vander Hoeven, respectively.

3.2. Extraction and isolation

The finely powdered roots and rhizomes of *V. taliense* (2.6 kg) were extracted thrice with ethanol under reflux for 3 h each time, yielding 145 g of a dark brown tarry mass after evaporation of the solvent. The mass was acidified (pH=4) with 5% AcOH followed by filtration, the acidic aq. filtrate was then basified with NH_4OH up to pH=11 and extracted successively with CH_2Cl_2, EtOAc and *n*-BuOH to give fractions A (12 g), B (26 g) and C (17 g). Fraction A contained pigments of no biological interest while fractions B and C were active against *P. capsici* and *R. cerealis*. Co-monitored by TLC and HPLC by comparing with samples obtained previously in our lab,[5,6] fraction B was chromatographed on silica gel (500 g, 200–300 mesh) eluting with a $CHCl_3$/MeOH/Et_2NH (95:5:1) mixture followed by repeated gel filtration over Sephadex LH-20 to give **3** (35 mg) and **11** (17 mg). CC of fraction C over silica gel (300 g, 200–300 mesh) using a step gradient of $CHCl_3$/MeOH/Et_2NH (90:10:1$\rightarrow$50:50:1) followed by repeated ODS silica gel column chromatography eluting with MeOH/H_2O (6:4) yielded **9** (45 mg), **1** (26 mg) and **2** (18 mg).

3.2.1. Neoverataline A (1). White columnar crystal [MeOH/EtOAc, 1:4]. Mp 220–222°C, $[\alpha]_D^{25}=+91.7$ (*c* 0.36, MeOH). IR (KBr): 3446 (OH), 2963, 2772, 2744 (Bohlmann bands), 1753, 1640 (C=O). EIMS: *m/z* (rel. int.)=112 (*trans*-quinolizidine moiety, 100), 98 (39), 44 (20). FABMS: 508 $[M+H]^+$. HRESIMS: *m/z* 508.2908 $[M+H]^+$, calcd for $C_{27}H_{42}NO_8$, 508.2910. ^{1}H- and ^{13}C NMR data: see Table 1.

3.2.2. Neoverataline B (2). Colorless needles. Mp 265–267°C; $[\alpha]_D^{25}=-46.8$ (*c* 0.32, MeOH). IR (KBr): 3428 (OH), 2965, 2768, 2742 (Bohlmann bands), 1754, 1636 (C=O). EIMS: *m/z* (rel. int.)=112 (*trans*-quinolizidine moiety, 59), 98 (53), 44 (100). FABMS: 524 $[M+H]^+$, HRESIMS: *m/z* 524.2856 $[M+H]^+$, calcd. for $C_{27}H_{42}NO_9$, 524.2860. ^{1}H and ^{13}C NMR data: see Table 1.

3.3. Antifungal bioassay

The toxicity of the isolates against the four phytopathogenic fungi was tested at 40, 80 and 160 $\mu g\ mL^{-1}$ as described in literature.[18] In treated plates, the requisite amount of the isolates, as well as the positive control triadimenfon (25% commercial product (WP) purchased from Bayer Company, Germany) was dissolved first in 0.1 mL MeOH and then

mixed thoroughly with 9.9 mL of PDA medium in pre-sterilized petri dishes to obtain the required concentrations of the samples. The negative control sets were prepared similarly using MeOH and sterilized water only. Fungal inoculum discs of 5 mm diameter cut from the periphery of a seven day old culture of pathogens were placed aseptically on the center of each petri dish separately, followed by incubation at 25±1°C for 4 days.

Acknowledgements

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