Bioorganic & Medicinal Chemistry Letters 22 (2012) 5819–5822

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

A new antifungal and cytotoxic C-methylated flavone glycoside from *Picea neoveitchii*

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ARTICLE INFO

Article history:
Received 25 April 2012
Revised 29 June 2012
Accepted 25 July 2012
Available online 2 August 2012

Keywords:
Picea neoveitchii
Pinaceae
C-Methylated flavone glycoside
Antifungal activity
Cytotoxicity

ABSTRACT

A new C-methylated flavone glycoside, 5,7-dihydroxy-3-methoxy-6-*C*-methylflavone 8,4′-di-*O*-β-D-glucopyranoside (**1**), was isolated from the twigs and leaves of *Picea neoveitchii* Mast, together with eight known compounds, 5,7,8,4′-tetrahydroxy-3-methoxy-6-methylflavone 8-*O*-β-D-glucopyranoside (**2**) kaempferol 3,4′-di-*O*-β-D-glucopyranoside (**3**), apigenin 7-*O*-β-D-glucopyranoside (**4**), tiliroside (**5**), massonianoside B (**6**), umbeliferone 7-*O*-β-D-glucopyranoside (**7**), dihydroconiferin (**8**) and gleditschiaside A (**9**). Their structures were elucidated on the basis of analyses of spectroscopic data. Compound **1** showed moderate antifungal activity against tested plant pathogens (*Pyricularia grisea* (Cooke) Sacc., *Sclerotium rocfsii* Sacc. and *Alternaria mali* Roberts), however, compounds **2** and **5** had obvious inhibitory effect against *S. rocfsii* and *A. mali*, respectively. Compounds **1**, **2**, **3** and **9** also exhibited potent cytotoxicity against *Spodoptera litura* Fabricius cells.

Food shortage is a major problem in certain remote locations or developing countries. High food prices have increased hunger and poverty, and produced rioting in some countries. As a result, how to raise crop yields is the urgent problem in the world. Plant fungal diseases reduce the yield and quality of crops, causing great losses in agriculture annually.[1] For a long period of time, fungal diseases were controlled with chemical fungicides such as carbendazim and triadimefon. Because of the abuse of chemical antimicrobial agents, many new problems have emerged, such as toxic reaction, drug tolerance and residue, and so on. Regarding the negative effects of chemical antimicrobial agents to non-target organisms and environment, as well as the high cost of them, recent years, much research emphasis has been focused on the more specific and environmentally friendly materials, which are generally of botanical origin.[2,3] Therefore, it is important to find antimicrobial agents from plants and further to exploit new plant-derived fungicides.

The genus *Picea* belonging to the Pinaceae family includes about 40 species around the world, which are distributed throughout the north temperate zone, 16 species and 9 varieties occur in China.[4] Previous chemical studies revealed that the plants of genus *Picea* contain the constituents of diterpenes,[5,6] triterpenoids,[7–11] flavonoids,[12–14] lignans,[15] phenolic compounds and alkaloids.[16,17] *Picea neoveitchii*, an endemic species native to China with needle leaves, and is mainly distributed at high altitudes of 1300–2000 m in Hubei, Gansu and Shanxi provinces, and scattered in ravines and mountain slopes.[18,19] So far its wood has been used for building materials, and its pine cones have been used in the folkloric medicine for the relief of coughs and the treatment of diabetes. Our previous study suggested that the methanol extract of the twigs and leaves of *P. neoveitchii* exhibited strong antifungal activities against some plant pathogens. To the best of our knowledge, the chemical constituent of *P. neoveitchii* has not been investigated, this situation prompted us to investigate the bioactive constituents of this plant. Our previous study led to the isolation of four new flavonoids and 15 known compounds from the EtOAc fraction of the twigs and leaves of *P. neoveitchii*, some of the compounds showed antifungal activities against *Fusarium oxysporum* and *Rhizoctonia solani*.[20] However, many constituents in other fractions were still not fully investigated. Therefore, further investigation was undertaken on the water soluble fraction, which resulted in the isolation and structure elucidation of a new C-methylated flavone glycoside (**1**), together with eight known compounds (**2–9**) (Fig. 1).[21] The present paper reports the isolation, structure characterization, antifungal activities and cytotoxicities of the isolated compounds.

The air-dried and pulverized twigs and leaves of *P. neoveitchii* (2.0 kg) were extracted three times with 90% aqueous EtOH at room temperature, and the EtOH extract of *P. neoveitchii* was extracted with petroleum ether and EtOAc to give the petroleum ether extract, EtOAc extract and water phase. The filtrated water phase (87.6 g) was subjected to D101 macroporous adsorption resin column and eluted with MeOH–H_2O (0%, 85%, MeOH) to give Fr. A–D. The Fr. B (35.6 g) was chromatographed over silica gel column

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http://dx.doi.org/10.1016/j.bmcl.2012.07.089

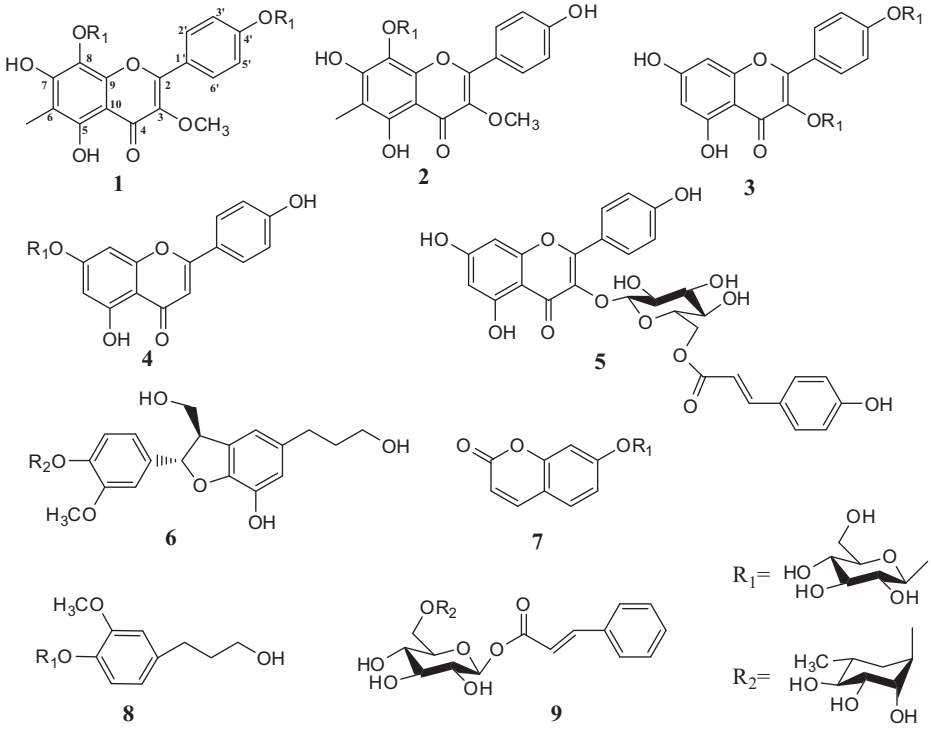

Figure 1. Structures of compounds **1–9**.

eluting with $CHCl_3$–MeOH–H_2O (10:10:1, 10:10:2) and MeOH to afford Fr. B_1–B_5 according to TLC analysis. The Fr. B_1 (12.8 g) was subjected to CC on polyamide and eluted with the mixture of $CHCl_3$–MeOH with increasing polarity ($CHCl_3$, 30:1, 20:1, 10:1, 7:1, 4:1, 2:1, 1:1, MeOH, v/v) to divide into subfr. I–VII. Fr. I (8.1 g) was fully purified by middle pressure liquid chromatography (MPLC) on ODS and eluted with MeOH–H_2O (10%, 20%, 30%, 40%, 50%, 60%, MeOH) to obtain Fr. I-1 to Fr. I-10 on the basis of TLC analysis. Compound **8** (23.4 mg) was isolated from Fr. I-1 by subjected silica gel column with a successive gradient mixture of $CHCl_3$–MeOH (10:1, 8:1, 6:1, 5:1, 4:1, v/v). Compound **7** (43.1 mg) was obtained from Fr. I-2, and compound **9** (14.4 mg) was isolated from Fr. I-6 through repeated crystallisation in MeOH–$CHCl_3$. Fr. I-9 was separated by CC on silica gel eluted with $CHCl_3$–MeOH (10:1, 8:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1, v/v), followed by Sephadex LH-20 CC, with acetone as eluent, to yield compound **4** (14.5 mg). Fr. II (1.7 g) was subjected to CC on polyamide and eluted with MeOH–H_2O (20%, 30%, 40%, 50%, 60%, 70%, MeOH) to obtained eight subfractions, and after recrystallization, compound **5** (8.8 mg) was isolated from Fr. II-3. The fourth Fr. IV (1.8 g) was also fully purified by MPLC on ODS and eluted with MeOH–H_2O (10%, 20%, 30%, 40%, 50%, 60%, 70%, MeOH) to afford Fr. IV-1 to IV-10. Compound **3** was precipitated from Fr. IV-2. Fr. IV-5 was subjected to Sephadex LH-20 CC with MeOH as eluent, gave a mixture, which were rechromatographed by PTLC ($CHCl_3$–MeOH 5:1, two times) to afford compound **6** (10.2 mg). Compound **2** (14.5 mg) was obtained from Fr. IV-9 by CC on Sephadex LH-20 eluting with acetone. After recrystallization in MeOH–$CHCl_3$, compound **1** (34.4 mg) was obtained from Fr. V.

Compound **1**, obtained as yellow amorphous powder.[22] It gave pink colour with vanillin–sulfuric acid and positive reaction to the magnesium hydrochloric acid and Molish tests suggested that **1** was a flavonoid. A molecular formula, $C_{29}H_{34}O_{17}$ was indicated by HR-ESI-MS at *m/z* 655.1861 $[M+H]^+$, (calculated 655.1869). The ^1H NMR spectrum (Table 1) indicated the presence of an aromatic methoxyl δ_H 3.82 (3H, s) and one *C*-methyl δ_H 2.03 (3H, s). Two doublets at δ_H 8.35 (2H, d, J = 9.0 Hz, H-2′,6′) and 7.24 (2H, d, J = 9.0 Hz, H-3′,5′) in ^1H NMR spectrum, typical of an AA′BB′ coupling system, indicated the presence of a *p*-disubstituted B ring. The ^{13}C NMR spectrum (Table 1) indicated a conjugated ketone carbonyl (δ_C, 178.0), characteristic of a flavone. In addition, two anomeric proton signals at δ_H 4.61 (d, J = 7.8 Hz, H-1″) and 4.99 (d, J = 7.2 Hz, H-1‴) in the ^1H NMR spectrum, as well as two anomeric carbon signals at δ_C 107.3 and 100.0 in ^{13}C NMR spectrum suggested the existence of two sugar moieties. Except one of the sugar moieties, the ^1H and ^{13}C NMR spectra of **1** were similar to those of 5,7,8,4′-tetrahydroxy-3-methoxy-6-metylflavone 8-*O*-β-D-glucopyranoside. Two sugar moieties were determined to be glucopyranose by ^{13}C NMR spectrum,[23] which were confirmed by acid hydrolysis and Co-TLC comparing with authentic samples. Their configurations were determined as β-orientation by coupling constants of two anomeric proton signals in the ^1H NMR spectrum, and the two glucopyranose moieties were determined as D-form by optical rotation ($[\alpha]_D^{20}$ +54.3°, *c* 0.094, H_2O).[24] The absence of any hydrogen signals of A ring indicated two hydroxys at A ring. These findings suggested **1** was a flavonoid with two hydroxyls, a methoxyl, a *C*-methyl and two β-D-glucopyranose moieties.

The absence of the singlet of H-3 signal at δ_H 6.70–6.90 and HMBC correlation of the methoxy hydrogens with C-3 (δ_C 138.0) suggested the methoxy group was at C-3 (Fig. 2). A downfield shifted signal at δ_H 12.77 indicated the existence of a 5-OH group, which was supported by long range correlations of the C-6 (δ_C 106.8), C-5 (δ_C 154.3), and C-10 (δ_C 103.4). In addition, the location of *C*-methyl group at C-6 was confirmed by HMBC correlations of 6-methyl hydrogens with C-5, C-6, and C-7 (δ_C 155.3). Moreover, the anomeric proton at δ_H 4.61 (d, J = 7.8 Hz, H-1″) was correlated with C-8 at δ_C 125.3, and another anomeric proton at δ_H 4.99 (d, J = 7.2 Hz, H-1‴) was correlated with C-4′ at δ_C 159.5 in HMBC spectra, suggesting that two of the β-D-glucopyranose moieties were located at C-8 and C-4′, respectively. Thus, the remaining hydroxyl group was located at C-7. Consequently, the structure of compound **1** was elucidated as 5,7-dihydroxy-3-methoxy-6-*C*-methylflavone 8,4′-di-*O*-β-D-glucopyranoside.

Table 1
^{1}H and ^{13}C NMR data of compound **1** (DMSO-d_6, δ in ppm, J in Hz)

Position	δ_H	δ_C	Position	δ_H	δ_C
2		154.6 s	Glc		
3		138.0 s	1″	4.61 (d, 1H, J = 7.2)	107.3 d
4		178.0 s	2″	3.43 (t, 1H, J = 8.4)	74.1 d
5		154.3 s	3″	3.29 (m, 1H)	76.6 d
6		106.8 s	4″	3.20 (m, 1H)	69.6 d
7		155.3 s	5″	3.28 (m, 1H)	77.4 d
8		125.3 s	6″	3.74 (m, 1H), 3.68 (m, 1H)	60.6 t
9		146.2 s	Glc′		
10		103.4 s	1‴	4.99 (d, 1H, J = 7.2)	100.0 d
1′		123.3 s	2‴	3.27 (m, 1H)	73.1 d
2′, 6′	8.35 (d, 2H, J = 9.0)	130.5 d	3‴	3.32 (m, 1H)	75.8 d
3′, 5′	7.24 (d, 2H, J = 9.0)	116.2 d	4‴	3.39 (m, 1H)	68.9 d
4′		159.5 s	5‴	3.40 (m, 1H)	77.1 d
6-Me	2.03 (s, 3H)	7.6 q	6‴	3.72 (m, 1H), 3.50 (m, 1H)	60.3 t
3-OMe	3.82 (s, 3H)	59.6 q			
5-OH	12.77 (s, 1H)				

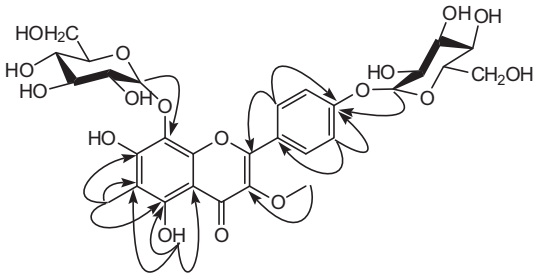

Figure 2. Key HMBC correlations of compound **1**.

The eight known compounds were identified as 5,7,8,4′-tetrahydroxy-3-methoxy-6-methylflavone 8-*O*-β-D-glucopyranoside (**2**),[13] kaempferol 3,4′-di-*O*-β-D-glucopyranoside (**3**),[25] apigenin 7-*O*-β-D-glucopyranoside (**4**),[26] tiliroside (**5**),[27] massonianoside B (**6**),[28] umbeliferone 7-*O*-β-D-glucopyranoside (**7**),[29] dihydroconiferin (**8**)[30] and gleditschiaside A (**9**),[31] respectively, by spectral analysis comparison with those already reported in the literature.

The isolated compounds were qualitatively evaluated for their antifungal potential using a modified disc diffusion method.[32] Compound **1** showed moderate antifungal activity against *Pyricularia grisea*, *Sclerotium rocfsii* and *Alternaria mali* with relative inhibitory percentages of 30.6%, 41.1% and 43.4%, respectively (Table 2). Compound **2** demonstrated strong activity against *S. rocfsii* with a relative inhibitory percentage of 81.0%, while compounds **3** and **4** showed moderate activity. Compound **6** displayed obvious inhibitory effect against *A. mali* (with 88.9% relative inhibitory percentage), and compounds **2**, **3**, **4**, **5** and **8** exhibited moderate antifungal activity. Meanwhile, compounds **2**, **6** and **8** showed moderate activity against *P. grisea*, and compounds **2**, **5** and **6** displayed weak antifungal activity against *Ceratocystis paradoxa*. However, all the tested compounds showed lower activities than carbendazim against the tested plant pathogens.

The cytotoxicities of all compounds against *Spodoptera litura* Fabricius cell (SL) were evaluated by MTT assay.[34] Compounds **1**, **2**, **5** and **9** exhibited potent cytotoxicity against SL cells with inhibition rates of 64.4%, 62.5%, 65.0% and 62.2%, respectively, at a concentration of 20 mg/L (Table 3). The other compounds showed weak cytotoxicity, while all of these compounds demonstrated lower cytotoxicity than that of the positive control, rotenone, at the same concentration.

In conclusion, a new C-methylated flavone glycoside, together with eight known compounds, were isolated from the twigs and leaves of *P. neoveitchii*. Their structures were established by spectroscopic analysis. All the isolated compounds were qualitatively evaluated for their antifungal activities by a modified disc diffusion

Table 2
In vitro antifungal activity of compounds **1–9** against tested plant pathogens

Compd	Mycelial growth[a] (mm)				Growth inhibition[b] (%)			
	P[c]	S[d]	A[e]	C[f]	P[c]	S[d]	A[e]	C[f]
1	5.7 ± 1.5	4.8 ± 0.6	4.3 ± 0.6	0.0	30.6 ± 8.0	41.4 ± 5.2	43.4 ± 6.1	0.0
2	7.6 ± 1.0	9.4 ± 0.5	7.2 ± 0.9	6.2 ± 1.0	40.9 ± 5.4	81.0 ± 4.3	72.7 ± 9.1	24.1 ± 3.9
3	0.0	3.7 ± 0.6	2.4 ± 0.8	0.0	0.0	31.9 ± 5.2	24.2 ± 8.0	0.0
4	3.3 ± 0.5	4.8 ± 0.9	5.8 ± 1.0	0.0	17.7 ± 2.7	41.4 ± 7.8	58.6 ± 10.1	0.0
5	0.0	2.6 ± 0.7	5.5 ± 0.5	7.1 ± 0.7	0.0	22.4 ± 6.0	55.6 ± 5.1	27.6 ± 2.7
6	7.0 ± 1.8	0.0	8.8 ± 1.0	5.6 ± 0.6	37.6 ± 9.7	0.0	88.9 ± 10.1	21.8 ± 2.3
7	0.0	2.8 ± 0.8	0.0	1.5 ± 0.5	0.0	24.1 ± 6.9	0.0	5.8 ± 1.9
8	8.6 ± 1.0	0.0	3.1 ± 0.6	0.0	46.2 ± 5.4	0.0	31.3 ± 6.1	0.0
9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C[g]	18.6 ± 1.4	11.6 ± 1.0	9.9 ± 0.6	25.7 ± 0.4	100.0	100.0	100.0	100.0

Data represent the mean ± S.D. of three separate experiments. Each filter paper disc was preloaded with 100 μg of the target compounds in MeOH, the same to the positive control.

[a] Mycelial growth inhibitory zone diameter (mm).
[b] Relative mycelial growth inhibitory percentage (%).
[c] P = *Pyricularia grisea*.
[d] S = *Sclerotium rocfsii* Sacc.
[e] A = *Alternaria mali*.
[f] C = *Ceratocystis paradoxa*.
[g] C = Carbendazim, positive control.

Table 3
Cytotoxicity of compounds **1–9**[a] against *Spodoptera litura* cells

Compound	Inhibitory rates (%)
1	64.4 ± 1.7
2	62.5 ± 3.8
3	23.1 ± 1.0
4	7.8 ± 2.4
5	65.0 ± 5.6
6	17.9 ± 1.2
7	15.3 ± 4.3
8	4.0 ± 0.9
9	62.2 ± 1.8
Rotenone[b]	74.4 ± 2.4

Data represent the mean ± S.D. of three separate experiments.
[a] Test concentration of each compound is 20 mg/L.
[b] Positive control (20 mg/L).

method, and their cytotoxicities all evaluated against *S. litura* cell by MTT assay. Compound **2** demonstrated strong activity against *S. rocfsii*, while compound **6** displayed obvious inhibitory effect against *A. mali*. The compounds **1**, **2**, and **6** are considered to be potential as antimicrobial agents, and would provide more potent derivative with a suitable modification. Moreover, compounds **1**, **2**, **5** and **9** exhibited potent cytotoxicity against SL cells at a concentration of 20 mg/L. These findings indicated that the twigs and leaves of *P. neoveitchii* is a promising source of valuable bioactive compounds and is worthy of further investigation. Ours is the first phytochemical analysis on the chemical constituents of *P. neoveitchii*, and so adds to the chemotaxonomic data of the Pinaceae family.

Acknowledgments

The work was supported by the grant from the National Department Public Benefit Research Foundation of China (No. 200903052) and China Postdoctoral Science Foundation (No. 20110490900).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.07.089.

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21. Plant material: The twigs and leaves of *P. neoveitchii* were collected from the Three Gorges, Hubei Province, PR China, in June 2008, and were identified by Professor Li PT (College Of Forestry, South China Agricultural University, PR China). A voucher specimen (No. 11614) was deposited in the Wuhan Botanical Garden, Chinese Academy of Sciences.
22. *Physical and spectroscopic data of compound* ***1***: 5,7-Dihydroxy-3-methoxy-6-*C*-methyl-flavone 8,4′-di-*O*-β-D-glucopyranoside, yellow amorphous powder, molecular formula: $C_{29}H_{34}O_{17}$; mp 180–182 °C; $[\alpha]_D^{20}$ −42.3° (*c* 0.086 MeOH); ^{1}H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150MHz, DMSO-d_6) data see Table 1; HR-ESI-MS *m*/*z*: 655.1861 $[M+H]^+$ (calculated for $C_{29}H_{35}O_{17}$, 655.1869).
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24. Acid hydrolysis and sugar analysis: Compound **1** (2 mg) was dissolved in 2 N HCl (H_2O–MeOH 1:1 2 mL) and refluxed with magnetic stirring in a water bath at 80 °C for 4 h. After cooling, the reaction mixture was diluted with H_2O (3 mL) and extracted three times with EtOAc. The aqueous layer were neutralized with 2 N NaOH, then concentrated and dried to furnish a monosaccharide residue. The residue was purified through Sephadex LH-20 column eluting with $CHCl_3$–MeOH (1:1) to afford sugar, which was identified as glucose by Co-TLC with authentic sample. The glucose was determined as D-form by optical rotation ($[\alpha]_D^{20}$ +54.3°, *c* 0.094, H_2O).
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32. The agricultural pathogenic fungi including *Pyricularia grisea* (Cooke) Sacc., *Sclerotium rocfsii* Sacc., *Alternaria mali* Roberts and *Ceratocystis paradoxa* (Dade) Moreau were obtained from the Key Laboratory of Natural Pesticides and Chemical Biology, South China Agricultural University. Antifungal activity assay was evaluated using previously reported method with a minor modification.[20,33] Briefly, sterile potato dextrose agar (PDA) medium was prepared and distributed into Petri plates of 90 mm diameter (each plate contained 15 mL of PDA medium). Sterilized filter paper discs (5 mm in diameter) were preloaded each with 10 μL of a solution of the tested compound containing 10 mg/mL in MeOH, and the discs were allowed to air dry, then the discs were placed at the centre of the PDA plates. Four plugs of fungal inoculums, 5 mm in diameter, were placed upside down at the quarter circle points 20 mm in radius around the drug-loaded disc in the Petri dishes. Blank control discs were treated with MeOH, and carbendazim was used as a positive control. The inoculated plates were incubated at 29 °C for 2–4 days, when the growth of fungi in the blank control would have reached the centre of the plates, the inhibitory zone was measured in mm. The growth inhibition of the tested compounds were calculated against carbendazim using the following formula: [inhibitory zone of treatment (mm)/inhibitory zone of positive control (mm)] × 100% (Table 2). Each treatment was run in triplicate.
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