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Article

LC-MS/MS Tandem Mass Spectrometry for Analysis of Phenolic Compounds and Pentacyclic Triterpenes in Antifungal Extracts of *Terminalia brownii* (Fresen)

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† With this paper we would like to honor our colleague, Dr. Hiba Ali, who passed away the 29.4.2016

Abstract: Decoctions, macerations and fumigations of the stem bark and wood of *Terminalia brownii* Fresen. are used in traditional medicine for fungal infections and as pesticides on field crops and in traditional granaries in Sudan. In addition, *T. brownii* is commonly used for protecting wooden houses and furniture. Therefore, using agar disc diffusion and macrodilution methods, eight extracts of various polarities from the stem wood and bark were screened for their growth inhibitory effects against filamentous fungi commonly causing fruit, vegetable and grain decay, as well as infections in the immunocompromised host. Ethyl acetate extracts of the stem wood and bark gave the best antifungal activities, with MIC values of 250 µg/ml against *Nattrassia mangiferae* and *Fusarium verticillioides*, and 500 µg/ml against *Aspergillus niger* and *Aspergillus flavus*. Aqueous extracts gave almost as potent effects as the ethyl acetate extracts against the *Aspergillus* and *Fusarium* strains, and were slightly more active than the ethyl acetate extracts against *Nattrassia mangiferae*. Thin layer chromatography, RP-HPLC-DAD and tandem mass spectrometry (LC-MS/MS), were employed to identify the chemical constituents in the ethyl acetate fractions of the stem bark and wood. The stem bark and wood were found to have a similar qualitative composition of polyphenols and triterpenoids, but differed quantitatively from each other. The stilbene derivatives, *cis*- **(3)** and *trans*- **(4)** resveratrol-3-*O*-β-galloylglucoside, were identified for the first time in *T. brownii*. Moreover, methyl-(*S*)-flavogallonate **(5)**, quercetin-7-β-*O*-di-glucoside **(8)**, quercetin-7-*O*-galloyl-glucoside **(10)**, naringenin-4`-methoxy-7-pyranoside **(7)**, 5,6-dihydroxy-3`,4`,7-tri-methoxy flavone **(12)**, gallagic acid dilactone (terminalin) **(6)**, a corilagin derivative **(9)** and two oleanane type triterpenoids **(1)** and **(2)** were characterized. Our results justify the traditional uses of macerations and decoctions of *T. brownii* stem wood and bark for crop and wood protection and demonstrate that standardized extracts could have uses for the eco-friendly control of plant pathogenic fungi in African agroforestry systems.

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Likewise, our results justify the traditional uses of these preparations for the treatment of skin infections caused by filamentous fungi.

Keywords: Africa; *Terminalia brownii*; antifungal extracts; *Aspergillus, Nattrassia, Fusarium*; triterpenoids; flavonoids; ellagitannins; stilbenes

1. Introduction

Fungal infections are both a pre- and a post-harvesting problem in crop production and pose a continuous and growing threat to global food crop production [1, 2]. Some of the fungal species generally considered to be phytopathogens, such as *Aspergillus* spp., are also known to be increasingly significant as human pathogens, especially in the immunocompromised host [3, 4, 5].

Aspergillus niger (van Tieghem, 1867) and *Aspergillus flavus* (Link, 1809) are both human [6, 7] and plant pathogens [8]. As human pathogens, especially *A. flavus*, but also *A. niger* cause aspergillosis in immunocompromised individuals [9,10]. Moreover, *A. flavus* causes grain crop infections in maize (*Zea mays* L.), leading to a substantial decrease in the commercial value of maize crop [11].

Nattrassia mangiferae [(Syd. & P. Syd.) B. Sutton & Dyko], previously known as *Hendersonula toruloi* Nattrass (HT) and *Dothiorella mangiferae* (Syd. & P. Syd), is a wound-invading dematiaceous (brown-pigmented) phytopathogenic fungus infecting hard wood species of *Citrus, Mangifera* and *Eucalyptus* and soft wood coniferous subtropical and tropical trees, causing dieback and vascular wilt diseases [12, 13]. *Nattrassia mangiferae* is also a human pathogenic fungus, especially in immunocompromised individuals [14], and is even known to cause community acquired infections in rural farmer societies worldwide [12].

Fusarium verticilloides and some other *Fusarium* species infect maize ears (husks) causing maize ear rot disease and contaminate maize grains with fumonisin mycotoxins leading to major pre- and post-harvest losses [15]. *Fusarium* spp. mycotoxins are toxic [16, 17], and fumonisin has been found to cause cancer in mammalians [18]. Another species of *Fusarium*, *F. oxysporum* is the causative agent of the "Panama disease" affecting the banana (*Musa paradisiaca*), the staple food of a large part of Africa. *F. oxysporum* is increasingly resistant to many crop protection agents [19].

Currently used fungicides are costly and toxic to the environment [20]. Besides, phytopathogenic and human pathogenic filamentous fungi have developed resistance to many conventional fungicides and to antibiotics [21, 22, 23, 24]. Thus, new effective and less toxic antifungals are needed [24]. Tropical and subtropical plants are known to contain a wide range of defense compounds due to their needs for constant defense compound production throughout the year as well as due to the high biodiversity in rain forests, woodlands and savannahs [25]. Thus, tropical plant species used for fungal infections in African traditional medicine, are expected to be good sources for new antifungal compounds [26, 27].

The pantropical genus of *Terminalia* contains a number of species known for their antifungal effects. Antifungal activities against *Aspergillus niger* and *A. flavus* have been reported for the Asian species, *Terminalia alata*, *T. arjuna*, *T. bellerica*, *T. catappa* and *T. chebula* [28, 29]. Extracts of the African species, *Terminalia nigrovenulosa* and *T. sericea*, gave considerable antifungal activity against *Fusarium*

spp. [30, 31]. Gallic acid from the bark of *Terminalia nigrovenulosa* was found to give growth inhibitory effects against *Fusarium solanii* [32].

Terminalia brownii (Fresen.) is a deciduous tree distributed throughout East African savannah regions in a wide range of temperature, rain fall and soil conditions [33]. In Sudan, *T. brownii* occurs in natural forested areas such as savannah woodlands (Figure 1). *T brownii* has been found to be exceptionally resistant against various pathogenic fungi that affect crops [34]. Therefore, decoctions of various parts of *T. brownii* have traditional applications as pesticides against fungal infections in crop plants (35, 34]. Moreover, fumigations produced from the burned stem wood and root are traditionally used as pesticides in grain silos as well as for the treatment of fungal infections on the skin.

A **B** **C** **D**

Figure 1. *Terminalia brownii*. (A) tree in savannah woodland; (B) stem bark; (C) flowers and leaves; (D) fruits. Photo: E. Y. A. Salih and Dr. H. H. Gibreel, 2006.

Investigations on the phytochemical constituents of *T. brownii* are scanty. The pentacyclic triterpenoids arjungenin and betulinic acid as well as β-sitosterol and ellagic acid derivatives have been characterized from the leaves and stem bark [36, 37, 38]. Of these compounds, arjungenin, betulinic acid and β-sitosterol showed good growth inhibitory activity against sweet potato fungal pathogens such as *Aspergillus niger, Fusarium solanii* and *Fusarium oxysporum* [38]. In addition, a number of known and unknown ellagitannins as well a chromone derivative have been characterized in *T. brownii* [36, 39, 40].

Although *T. brownii* extracts are used traditionally against fungal phytopathogens and for human fungal infections in Sudan, there is a limited number of reports on their *in vitro* antifungal activity against molds affecting crop production and human health. Moreover, only a small number of antifungal compounds in *T. brownii* have been characterized to date [37, 38]. Therefore, the current study was performed to verify the antifungal effects of decoctions and macerations and to compare the antifungal activity of *T. brownii* extracts of various polarities against significant phytopathogenic and human pathogenic opportunistic fungi of the genera *Aspergillus*, *Nattrassia* and *Fusarium*. Thin layer chromatography (TLC) and RP-HPLC/DAD were used to study the phytochemical composition of the ethyl acetate extracts of the stem wood and stem bark. Tandem mass spectrometry (LC-MS/MS) was used to elucidate the molecular masses of flavonoids, triterpenes and stilbenes in these extracts

2. Results

2.1. Extracts of Terminalia brownii Stem Bark and Wood exert Antifungal Effects

The results of the growth inhibition of various extracts of *Terminalia brownii* stem bark and wood against *Aspergillus*, *Nattrassia* and *Fusarium* strains are shown in Table 1. When compared to the other extracts, the ethyl acetate extracts of the stem wood and bark gave the highest antifungal activity. *Nattrassia mangiferae* and *Fusarium verticilliodies* were especially sensitive to these ethyl acetate extracts (MIC 250 µg/ml), whereas *Aspergillus niger* and *A. flavus* were more resistant (MIC 500 µg/ml). Moreover, we found that these MIC values correlated well with the sizes of the inhibition zones produced by these ethyl acetate extracts, so that small MIC values were coupled to large diameters of the inhibition zones (Table 1). To the best of our knowledge, this is the first report on antifungal effects of *T. brownii* against *Nattrassia mangiferae*. Besides, our results are in accordance with other authors [38], who also reported that especially ethyl acetate extracts of the stem bark of *T. brownii* give good antifungal effects against sweet potato infecting fungi, such as *Aspergillus niger* and *Fusarium solanii*.

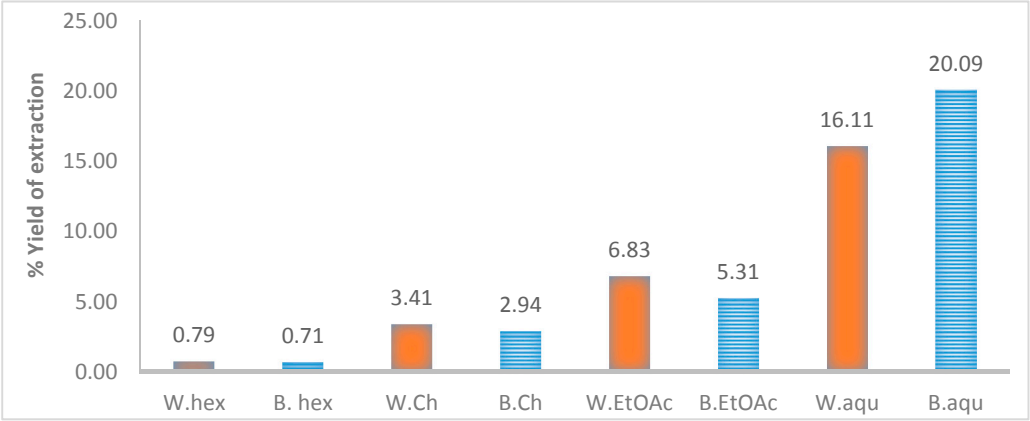

Figure 2. Percentage yield (% w/w) resulting from sequential extraction and liquid/liquid partition of the stem wood and stem bark of *Terminalia brownii*. W, stem wood; B, stem bark; hex, hexane extract; Ch, choloroform extract; EtOAc, ethyl acetate extract; aqu, aqueous extract.

Interestingly, we also found that aqueous extracts of the stem bark and wood of *T. brownii* gave good growth inhibitory effects (Table 1). Compared to the other extracts these aqueous extracts gave especially high extraction yields of 20 and 16 %, respectively for bark and wood (Figure 2). Thus, our results justify the traditional application of macerations (water extracts) of the stem wood and barks of *T. brownii* for the preservation of grains and for traditional medicinal treatment of fungal infections caused by *Aspergillus*, *Nattrassia* and *Fusarium*. Earlier studies have indicated that aqueous stem bark extracts of *T. brownii* are growth inhibitory also against yeast species, such as *Candida albicans* and *Cryprococcus neoformans* and in addition the aqueous extracts were found to be less toxic than other extracts against brine shrimps [41]. Thus, standardized aqueous extracts of *T. brownii* stem wood and bark could be used to treat fungal infections.

When compared to the more polar water and ethyl acetate extracts, we found that chloroform extracts of the stem wood and bark of *T. brownii* were slightly antifungal, while the petroleum ether

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extracts were devoid of antifungal activity (Table 1). In contrast to our results, in an earlier investigation, it was found that an n-hexane extract of the stem bark of *Terminali brownii* was active against another *Aspergillus* species, *A. fumigatus* [42]. Perhaps this result might indicate that different species of *Aspergillus* differ to their sensitivity to non-polar extracts of *T. brownii*, so that *A. fumigatus* is more sensitive than *A. niger* and *A. flavus*.

2.2. *Results from the Phytochemical Screening of Antifungal Ethyl Acetate Extracts of T. brownii stem wood and bark*

Owing to our promising antifungal results for the ethyl acetate extracts of the stem bark and wood of *T. brownii*, and to the few existing earlier records on phytochemical studies on this species, we investigated the secondary compound composition of these extracts with emphasis on the phenolic and triterpenoid composition.

2.2.1. TLC Results

RP-18 thin layer chromatograms of the ethyl acetate extracts of the stem wood and stem bark of *T. brownii* gave a negative reaction with Dragendorff reagent, suggesting that the extracts were devoid of alkaloids. Pink to purple colors were developed upon spraying with vanillin-H_2SO_4, which suggested the presence of triterpenoid and phenolic compounds. Spraying the TLC plates with aluminium trichloride ($AlCl_3$) and Natural Product reagent (NPR), revealed the presence of flavonoids, since color changes from quenching fluorescence to yellow, orange or blue color, typical for flavonoidal acids or other phenolic acids, could be observed at 366 nm [43]. When compared to the stem bark, the stem wood was richer in flavonoids.

2.2.2. HPLC-UV/DAD Results

HPLC-UV/DAD fingerprints of the ethyl acetate extracts of the stem bark and stem wood of *Terminalia brownii* are presented in Figure 3 (a) and (b). Altogether ten compounds with retention times between 6.8 and 25.5 minutes could be identified using internal standards and a computer library for standard compounds. At the wavelengths of 320 and 254nm, which were used for detection of stilbenes and flavonoids, the wood ethyl acetate extract displayed a higher diversity of flavonoidal and stilbenoid compounds. For example, the cis- and trans-isomers of resveratrol 3-*O*-β-galloyl-glucoside (**3** and **4)** at Rt 11.1 and 13.2 min, respectively, as well as naringenin-4'-methoxy-7-pyranoside **(7)** at 15.3 min, the corilagin derivative **(9)** at Rt 18.2 min, and quercetin 7-*O*-galloyl glucoside **(10)** at Rt 18.4 minutes, were present in the wood extract but absent from the stem bark extract as shown in Figure 3a and b.

Because of the high number of compounds present in the wood ethyl acetate extract and due to this extract being slightly more antifungal than the stem bark, this extract was subjected to LC-MS/MS advanced analysis for identification of the major compounds.

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Table 1. Antifungal activity of stem wood and bark extracts of *T. brownii*. Results obtained using cup well agar diffusion and agar dilution methods.

Fungal strain	Stem wood extracts		Stem bark extracts		Amphotericin-B
	IZ	MIC	IZ	MIC	MIC
Aspergillus niger					
Pt	NA		NA		
CHCl3	12 ± 0.9		13 ± 0.4		
EtOAc	17± 0.7	500	17 ± 0.8	500	31.25
aqueous	17± 0.5		16.5 ± 0.4		
Aspergillus flavus					
Pt	NA		NA		
CHCl3	14 ± 0.5		14 ± 0.9		
EtOAc	18.5 ± 0.4	500	18.5 ± 0.8	500	125
aqueous	18 ± 0.9		18 ± 0.5		
Nattrassia mangiferae					
Pt	NA		NA		
CHCl3	12 ± 0.5		12± 0.7		
EtOAc	19 ± 0.4	250	18.5 ± 0.4	250	62.5
aqueous	18.5 ± 0.4		19 ± 0.4		
Fusarium verticillioides					
Pt	NA		NA		
CHCl3	13 ± 0.6		11 ± 0.9		
EtOAc	20 ± 0.4	250	19 ± 0.2	250	62.5
aqueous	19 ± 0.3		18 ± 0.7		

For agar diffusion, extracts at the concentration 1 mg/ml were used. Diameter of inhibition zones (IZ) in mm: >18mm: sensitive; 14-18 mm: intermediate; < 14mm: resistant [44, 45]; Pt, petroleum ether extracts; CHCL3, chloroform extracts; EtOAc, ethyl acetate extracts; NA, Not active. IZ results as mean ± SEM of five measurements. MIC in µg/ml.

2.2. LC-MS and LC-MS/MS results

MS/MS using collision induced dissociation (CID), has been found to enable the accurate identification of stilbenes and flavonoids in complex extracts with co-eluting peaks [46]. Therefore MS/MS was employed as the method of choice for the identification of compounds in an ethyl acetate extract of *T. brownii* stem wood. A total of ten compounds were characterized by comparing the obtained molecular (precursor) ions and fragmentation patterns (i.e. product ions) from our LC-MS and LC-MS/MS data with data from the literature and with a computer library for the standard compounds (Table 2).

We found that the stem wood of *T. brownii* contains two oleanane triterpenoid acids which co-eluted at 6.8 min (Figure 3, Figure 4). For compound **(1)** a $[M-H]^-$ molecular ion at m/z 469 was

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detected, whereas compound **(2)** gave a molecular ion of m/z 491. In the MS^2 chromatograms, a fragment ion at m/z 425 was detected for compound **(1)** and at m/z 447 for compound **(2)** (Table 2). These fragment ions indicate the loss of a carboxylic acid (-COOH) group ($[M-H]^-$ for -COOH = 44) from both molecular ions. In agreement with our results, the loss of carboxylic acid at position 17 in pentacyclic triterpenoids has been observed when using atmospheric pressure chemical ionization (APCI)-MS [47, 48]. In addition, a fragment ion at m/z 407 indicated the loss of H_2O from m/z 425 in compound **1** [49]. This kind of mass spectral fragmentation pattern is typical for oleanane type triterpenes [50], therefore confirming that compounds **1** and **2** are oleanane type triterpenoids.

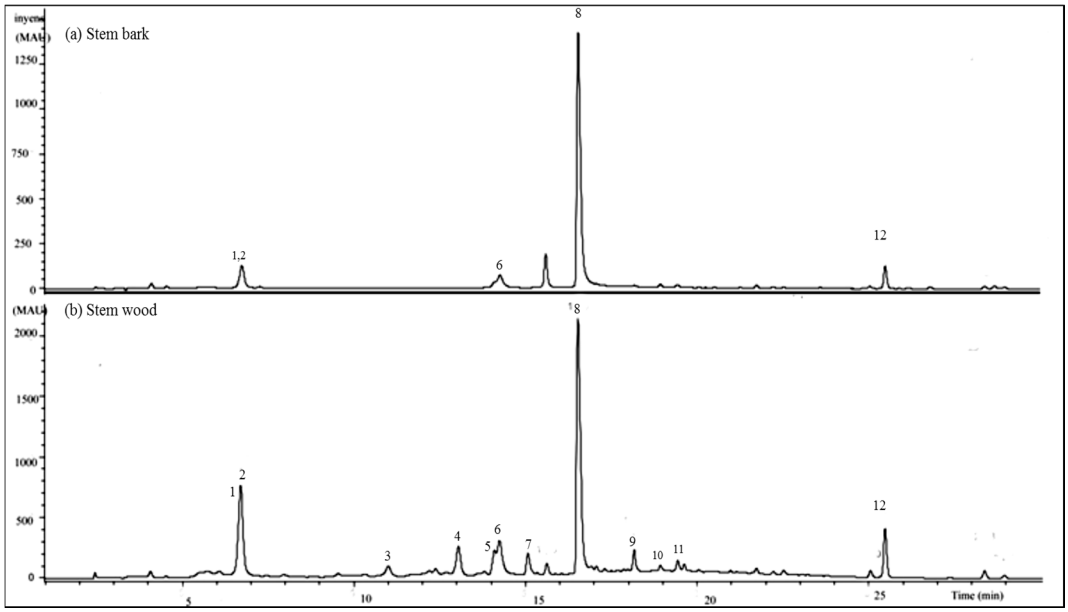

Figure 3. RP-HPLC/DAD chromatograms of ethyl acetate extracts of *T.brownii*. **(a)**, stem bark and **(b)**, stem wood extracts at 254nm. **(1) & (2)** Oleanane type triterpenoids; **(3)** cis-resveratrol-3-*O*-β-galloyl-glucoside; **(4)** trans-resveratrol-3-*O*-β-galloyl-glucoside; **(5)** Methyl-*(S)* flavogallonate; **(6)** Gallagic acid dilactone; **(7)** Naringenin 4'-methoxy-7-pyranoside; **(8)** Quercetin 7-ß-*O*-diglucoside; **(9)** Corilagin derivative; **(10)** Quercetin 7-*O*-galloyl-glucoside; **(11)** Unknown ellagitannin; **(12)** 5,6-dihydroxy-3',4',7-trimethoxy flavone.

In our HPLC-DAD system, compounds **(3)** and **(4)** eluted at Rt 11.1 and 13.2 min, respectively (Figure 3 **b**). Both compounds showed an identical $[M-H]^-$ molecular ion at m/z 541. Moreover, when subjected to MS^3, both compounds provided fragment ions of m/z 227 and 314 (Table 2). The later fragment ion indicates the presence of a galloylhexose fragment [51]. A comparison with the literature showed that the fragment ion at m/z 227 corresponds to the resveratrol unit [52]. Therefore, compounds **(3)** and **(4)** were tentatively assigned as resveratrol-3-*O*-β-galloyl-glucoside. Due to different retention times, the compounds were proposed to be cis- (3) and trans- (4) isomers of resveratrol-3-*O*-β-galloylglucoside (Figure 4).

When subjected to MS^2, compound **(5)** at Rt 14.1 min in HPLC-DAD, gave an $[M-H]^-$ molecular ion at m/z 483 (Table 2). The loss of the two oxygen molecules {483-451} at MS^3, gave a fragmentation ion at m/z 433. Also, the spectra was devoid of the fragment of $[M-H-CO2]^-$, which corresponds to a methyl ester molecule [53]. Therefore, and according to previous investigations [36, 54], compound **(5)** was tentatively assigned the structure of methyl-(*S*)-flavogallonate.

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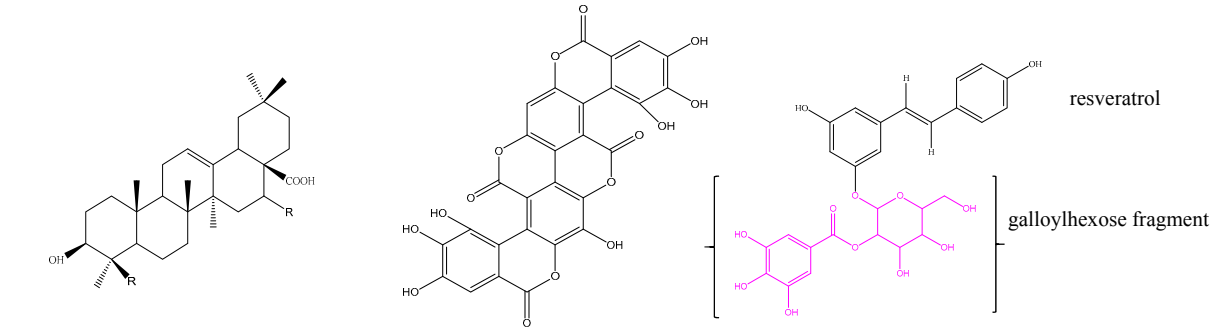
Compound **(6)** at Rt 14.4 min, gave a [M-H]$^-$ molecular ion at m/z 601 (Figure 3, Table 2). When subjected to MS^3, compound **(6)** yielded the fragment ions at m/z 271 and 301, the later corresponding to free ellagic acid. A molecular ion of m/z 601 and fragment ions at m/z 271 and 301 have been reported for gallagic acid [54]. Gallagic acid dilactone (syn. terminalin) has been reported in another species of *Terminalia, T. oblongata* [55]. Accordingly, compound **(6)** was tentatively assigned the structure of gallagic acid dilactone (terminalin) (Figure 4).



(1 & 2), Oleanolic acid triterpenoids **(6)** Terminalin (gallagic acid dilactone) **(4)** Resveratrol-3-*O*-β-galloyl-glucoside

Figure 4. Chemical structure of characterized compound **(1 & 2),** oleanane type triterpenoids; **(6),** Terminalin and **(3 & 4),** the stilbenes of resveratrol-3-*O*-β-galloyl-glucoside.

Compound **(7)** at Rt 15.3 min (Figure 3) gave an [M-H]$^-$ molecular ion at m/z 433 (Table 2). The deprotonation of [M-H]$^-$ at MS^2 resulted in a fragment ion at m/z 300, which indicates the loss of a pentose sugar [51, 56]. Moreover, MS^3 of this compound yielded the loss of an Y0 fragment at m/z 271, corresponding to the cleavage of the aglycone fragment ion of the flavanone naringenin [56, 57, 58]. The loss of 31 ions at MS^3, indicated the fragmentation of a methoxy group. In the MS^3, the [M-H]$^-$ yielded a fragment at m/z 284, therefore indicating that the methoxy group occurs at position 4' [56]. As flavonoids commonly occur as *O*-glycosides and *O*-glycosylation occurs at position 7 in flavanones [51, 59], compound **(7)** was tentatively assigned to naringenin-4'-methoxy-7-pyranoside.

The main compound **(8)** in the HPLC chromatogram at Rt 16.8 min (Figure 3) gave a [M-H]$^-$ molecular ion at m/z 625 (Table 2). The main fragmentation product ion at m/z 301 in the MS^2 and MS^3 chromatograms indicated the loss of two glucose molecules ([M-H]$^-$-2*162 Da) as well the presence of a quercetin aglycone moiety corresponding to m/z 301 [58]. Since glucose is usually β-glycosidically linked to the flavonoid aglycone and *O*-glycosidic linking is usually occurring at position 7 on the A ring of flavonoids [59], compound **(8)** was tentatively identified as quercetin-7-β-O-diglucoside.

Compound **(9)** at Rt 18.2 min (Figure 3), gave a [M-H]$^-$ molecular ion at m/z 633 (Table 2). MS/MS fragmentation resulted in a loss of a fragment product ion at m/z 481, corresponding to [M-galloyl-gallic acid]-. MS/MS resulted in the following product fragment ions; 463 [M-gallic acid]$^-$, 300 (hexahydroxydiphenoyl-H) and 169 corresponding to gallic acid [54, 60, 61]. Consequently, compound **(9)** is suggested to be a corilagin derivative.

Compound **(10)** at Rt 18.4 min gave a $[M-H]^-$ molecular ion at m/z 585 (Figure 3, Table 2). MS^2 fragmentation of this compound resulted in the loss of a pyranose sugar corresponding to the fragment ion at m/z 132 and a galloyl unit corresponding to a fragment ion of m/z 153. Moreover, a fragment product ion at m/z 301 {$[M-H]^-$-132-153} (Table 2), corresponding to the aglycone of quercetin, was present in the MS^2 chromatogram [58]. Consequently, compound **(10)** was tentatively assigned to be quercetin 7-*O*-galloyl-glucoside.

Table 2. HPLC-DAD and MS/MS data of phenolic compounds, ellagitannin and triterpenoids in an ethyl acetate extract of the stem wood of *T. brownii.*

Peak no	Rt (min)	$[M-H]^-$ *(m/z)*	CID M^n Main fragment ions *(m/z)*	Identified compound	Molecular formula	Exact mass (calc.)
1	6.8	469	425, 407, 379, 353, 300, 271	oleanane type triterpenoids	-	-
2	6.8	491	447, 429, 411, 401, 385, 301	oleanane type triterpenoids	-	-
3	11.1	541	532, 425, 397, 301, 273, 227, 199, 169	cis-resveratrol-3-*O*-β-galloyl-glucoside	$C_{27}H_{26}O_{12}$	542.1416
4	13.2	541	532, 424, 407, 300, 275, 227, 199, 169	trans-resveratrol-3-*O*-β-galloyl-glucoside	$C_{27}H_{26}O_{12}$	542.1416
5	14.1	483	451, 433, 407, 305, 405, 377	Methyl-(*S*)-flavogallonate	$C_{22}H_{12}O_{13}$	484.0273
6	14.4	601	583, 301, 299, 271, 243, 215	Gallagic acid dilactone	$C_{28}H_{10}O_{16}$	601.9964
7	15.3	433	314, 229, 271, 132	Naringenin-4'-methoxy-7-pyranoside	-	-
8	16.8	625	301, 284, 256, 229, 201,185, 129	Quercetin 7-β-*O*-diglucoside	$C_{27}H_{30}O_{17}$	626.1473
9	18.2	633	481, 463, 421, 387, 305, 275, 300, 169	Corilagin derivative	-	-
10	18.4	585	301, 284, 257, 229, 201, 185, 153, 132	Quercetin 7-*O*-galloyl-glucoside	-	-
11	19.1	725	665, 503, 409, 441, 379, 391	Unknown ellagitannin	-	-
12	25.5	343	328, 313, 298, 285, 270, 257	5,6-dihydroxy-3',4',7-trimethoxy flavone	-	-

Rt, retention time in HPLC-DAD; $[M-H]^-$ *(m/z)*, base ions at negative mode; CID M^n, Fragmentation ions resulting from collision induced dissociation; The Exact mass (calc.) according to molecular formula of identified compounds, as calculated from the mass of the hydrogen atom (1.0078), the oxygen atom (15.9949) and the carbon atom (12.0000); Aglycones are underlined. Peak numbers according to Figure. 3.

A polyphenol with a retention time of 19.1 min in HPLC-DAD (Compound **11,** Figure 3b) gave a molecular ion at m/z 725. The fragment ions of an hexose sugar was observed at m/z 665 and 503, indicating that the cleavage within this hexose sugar ring occurred at $_{0,3}X$ $[M-H-61]^-$ [51, 59]. More fragmentation was detected in $MS^{3,4}$ with m/z 441, 409, 379, 391 and 363 fragment ions. Thus compound **(11)** is suggested to be identical to an unknown ellagitannin on which we have reported in our recent publication [36].

Compound **(12)** at Rt 25.5 min (Figure 3b), gave a $[M-H]^-$ molecular ion at m/z 343 (Table 2). MS^3 and MS^4 fragmentation of this compound resulted in the loss of three methyl groups ($-CH_3$) corresponding to product fragment ions {[M-H]- 343-328-313} (Table 2). Moreover, in the fragment ion chromatogram resulting from MS^3, a high intensity of the product fragment ion at m/z 313 could be observed (Table 2). From this data, compound **(12)** was tentatively assigned as 5,6-dihydroxy-3',4',7-trimethoxyflavone.

3. Discussion

Terminalia brownii is a rich source of oleanane- and ursane-type pentacyclic triterpenoids, of which many might be prospective (new) antifungals. Several oleanane-based triterpenoid compounds have been identified in the genus of *Terminalia* [62, 63, 64, 65]. Arjunic acid, galloyl arjunic acid, tomentosic acid, sericic acid, arjungenin, sericoside, betulinic acid and arjunglucoside, have been characterized in the stem bark of *T. brownii*. In addition, a new oleanane type triterpenoid, designated as 3β,24-*O*-ethylidenyl-2α,19α-dihydroxyolean-12-en-28-oic acid, was identified in an ethyl extract of the bark of *T. brownii* [37, 38]. Of the mentioned compounds, betulinic acid and arjungenin, which were isolated from *T. brownii*, gave good antifungal effects against *Aspergillus niger*, *Fusarium solanii* and *Fusarium oxysporum* with MIC values ranging from 50-200 μg/ml [38]. Besides, arjunic acid has been shown to give good growth inhibition against *Cryptococcus neoformans* (IC_{50} 20 μg/ml) [66]. Moreover, combinations of arjunolic acid and asiatic acid, isolated from the leaves of *Combretum nelsonii*, showed promising antifungal effects against both filamentous fungi and *Candida spp*. [67]. Therefore, we suggest that the two oleanane-type triterpenes **(1)** and **(2)** would contribute significantly to the antifungal effects we have found for the ethyl acetate extract of the wood of *T. brownii* (Table 2, Figure 3). Pentacyclic triterpene saponins are known to complex with ergosterol and cholesterol in the fungal cell membrane, thus leading to loss of membrane integrity [68]**.** Besides, it has been found that triterpenoids decrease mycelial growth [69].

In the genus *Terminalia*, resveratrol and its glucoside and rutinoside derivatives have been reported in *Terminalia prunioides*, *T. sericea* and *T. ferdinandiana* [52, 70, 71]. We reported here for the first time on the occurrence of the resveratrol derivatives, *cis*- **(3)** and *trans*-resveratrol-3-*O*-β-galloylglucoside **(4)** in *Terminalia brownii* stem wood. Besides, galloylglucoside derivatives of resveratrol have not been reported before in the genus of *Terminalia*. Resveratrol and its derivatives are antifungal phytoalexins, protecting plants from pathogenic fungal and bacterial intrusion [72, 73]. Several investigations on *in vitro* antifungal activities of resveratrol and its derivatives indicate good antifungal potential of this compound class. Resveratrol and it is derivative, pterostilbene, were found to give substantial antifungal effects against *Botrytis cinerea*, a phytopathogenic fungi [74]. Moreover, resveratrol was found to be fungicidal against *C. albicans* [75]. Therefore, the good antifungal activity in the ethyl acetate extracts of *T. brownii* could be due to the resveratrol galloyl glucoside derivatives **(2)** and **(3).** To the best of our knowledge resveratrol-3-*O*-β-galloylglucoside has not been studied for its antifungal effects, which warrants further studies in this respect. The antifungal mechanisms of action of resveratrol and stilbenes are still not very well understood. However, there are some studies suggesting that resveratrol inhibits fungal cell respiration [74, 76]. It has also been suggested that hydroxystilbenes and pterostilbene cause membrane damage in fungi [77, 78, 79].

Ellagic acid derivatives, such as di-ellagic lactone, 3-*O*-methyl ellagic acid and 4-*O*-3",4"- di-*O*-galloyl-(α-L-rhamnopyranosyl) ellagic acid from *T. brownii* were found to give promising antifungal effects [37]. We reported here for the first time, of the occurrence of another ellagic acid derivative, gallagic acid dilactone **(6),** in the stem wood of *T. brownii*. Gallagic acid, is an analogue to ellagic acid, containing four gallic acid residues [80] and has restricted occurrence in plants. Gallagic acid and its derivatives have been found in various parts of some other *Terminalia* species such as in the leaves of

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T. catappa and *T. oblongata* [55, 81] and in the fruits of *Terminalia bellerica*, *Terminalia horrida* and *T. chebula* [54]. Gallagic acid is the fully lactonized from of the gallagyl moiety in the ellagitannin punicalagin, which is common in *Terminalia spp.* [54, 82]. Gallagic acid and its dilactone have also been characterized in the fruit peel of *Punica granatum*, which is distantly related to *Terminalia* as both genera are taxonomically classified to the order Myrtales [83]. Gallagic acid has been found to give concentration dependent growth inhibitory effects against *Fusarium* and *Alternaria* [84]. Thus, we suggest that gallagic acid dilactone **(6)** could be one of the main antifungal compounds in the stem wood of *Terminalia brownii*. However, to date there is no literature reported on the mechanism of action of gallagic acid dilactone growth inhibition against fungi. It has been found that tannins in general, including punicalagins, inhibit the growth of *Fusarium* and *Alternaria* dose-dependently [84]. Besides, punicalagin from the leaf of *T. brachystemma* was found to give a low MIC value of 6.25 µg/ml against *Candida* strains [49]. It remains to be investigated whether the ellagitannins we have found in *T. brownii* stem wood, such as methyl-*(S)*-flavogallonate **(5)**, the unknown ellagitannin **(11)** and other ellagitannins, including punicalagin, give low MIC values against *Aspergillus* and *Fusarium* spp. [36]. Recent studies [85], described good activity of the ellagitannins lambertianin C and sanguiin H-6 from raspberry against *Geotrichum candidum*, a fungus which causes deterioration in vegetable crops such as tomatoes, carrots and citrus fruits. Likewise, another ellagitannin, candelitannin, isolated from *Euphorbia antisyphilitica*, inhibited 20 % of the growth of *Fusarium oxyzporum* at 6mg/ml [86]. Therefore, we suggest that the ellagitannins might contribute to the growth inhibition of the stem wood extracts of *Terminalia brownii* against *Nattrassia*, *Fusarium* and *Aspergillus*. Standardized extracts of *T. brownii*, enriched with ellagitannins could be used as natural pesticides for protecting crops and as medicines to treat fungal infections. Also, purified ellagitannins, if found to be more active than extracts, could be used for ecological crop plant protection, while being relatively stable and possessing less toxicity than synthetic fungicides.

Our research resulted in the characterization of the flavonoids naringenin-4`-methoxy-7-pyranoside **(7)**, quercetin-7-β-*O*-diglucoside **(8)**, quercetin-7-*O*-galloylglycoside **(10)** and 5,6-dihydroxy-3,4,7-trimethoxy flavone **(12)** in the ethyl acetate extracts of stem wood and bark of *Terminalia brownii*. We suggest that quercetin-7-β-*O*-diglucoside **(8)**, which was the main peak in both stem bark and wood extracts of *T. brownii,* contributes significantly to the antifungal effects of these extracts. Accordingly, several authors have reported that quercetin and its derivatives give good antifungal effects against *Aspergillus* and *Fusarium* strains [87, 88, 89] and for quercetin as low MIC values as 15µg/ml were recorded against *Aspergillus niger*, *Fusarium moniliforme* and *F. sporotrichum* [90]. Furthermore, dihydroquercetin from barley suppressed the growth of *Fusarium spp.* [91]. A recent study [92], demonstrated that quercetin glycoside was not as antifungal as its aglycone. This study is in agreement with an earlier report according to which quercetin-3-*O*-rutinoside in the leaf extracts of *Ficus carica* gave moderate antifungal activity against *Aspergillus brasiliensis* [93]. In contrast to quercetin, some other flavonoids have demonstrated strong antifungal effects as glycosides. For example, naringenin pyranoside demonstrated some antifungal activity with MIC values of 16-32 mg/ml against *Candida albicans* and *C. krusei* [94]. Therefore, naringenin-4`-methoxy-7-pyranoside, which we have found in the stem wood of *T. brownii*, is suggested to give antifungal activity. Some SAR (Structure-activity relationship) studies on the antifungal potential of flavonoids have been performed. According to these studies, unsubstituted flavones and flavanones are highly active, whereas hydroxylated flavonoids possess weak activity against strains of *Aspergillus* [89]. Moreover,

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it was found that flavonoids possessing methoxy groups were more active [89]. This would apply to naringenin-4`-methoxy-7-pyranoside **(7)** and 5,6-dihydroxy-3,4,7-trimethoxy flavone **(12)** in the stem wood of *T. brownii*, possessing one and three methoxy groups, respectively and thus are suggested to give antifungal activities. Also 5,7-dihydro-3,4′,6-tri-methoxyflavone, isolated from the *Dodonaea angustifolia* gave promising growth inhibitory effects against *Saccharomyces cerevisiae* with the MIC value of 3.9 µg/ml [95]. Moreover, 5,6-dihydroxy-3',4′,7-trimethoxyflavone was found to affect the fungal development through disruption of the fungal metabolic pathway and of the fungal cell function [96]. Subsequently, we suggest that 5,6-dihydroxy-3,4,7-trimethoxy flavone **(12)** in *T. brownii* stem wood**,** might have a similar mechanism of action to inhibit fungal growth.

4. Materials and Methods

4.1. Collection of Plant Material

The stem wood and stem bark was collected from several individuals of *Terminalia brownii* growing in natural savannah woodland, in the Blue Nile Forest, in south-eastern Sudan (Figure 1). Voucher specimen were identified by the first author, Mr. Abdelazim Yassin Abdelgadir (Ph.D), Mr. Ashraf Mohamed Ahmed Abd Alla (Ph.D, Wood Sciences) and Mr. Haytham Hashim Gibreel (Ph.D, Taxonomy) at the Faculty of Forestry, University of Khartoum, Sudan and Mr. El Sheikh Abd alla Al Sheikh (Ph.D, Taxonomy) at Soba Forest Research Center, Khartoum, Sudan (PhD, Taxonomy). The specimens are deposited in the herbarium at the Department of Forest Products and Industries, Faculty of Forestry, University of Khartoum, Sudan.

4.2. Extraction

Hundred (100) grams of the dried and powdered stem wood and bark were used for the extractions. Extraction was initiated with sequential extraction, beginning with petroleum ether, followed by chloroform and finally the marc was extracted using 80% methanol. The 80% methanolic extract was subjected to liquid/liquid fractionation using ethyl acetate and this fractionation resulted in aqueous and ethyl acetate fractions.

4.3. Thin layer chromatography (TLC)

Using micro-capillary pipettes, 5 µl of ethyl acetate extracts (5mg/ml) of the stem bark and wood of *T. brownii* were applied on normal phase silica gel thin layer plates (Kieselgel 60 F254, aluminum backed, Merck, Darmstadt, Germany) and on reversed phase thin layer plates (RP-18 F254s, Merck, Darmstadt, Germany) to detect compounds of a wide range of polarities. Toluene: ethyl acetate: formic acid (4:5:1, v:v:v) was used as an eluent for NP-TLC, while methanol: water: acetic acid (6:2:2) was used for RP-TLC. The development distance was 8 cm. The plates were sprayed with Vanillin-H_2SO_4, Dragendorff reagent, Aluminium chloride and Natural Products reagents to detect various compound classes such as essential oils, terpenes, phenolic compounds, alkaloids and flavonoids [43]. The plates were observed in UV-light at 254 and 366 nm. A Camaq Video documentation system was used for photographing the plates.

4.4. Solid Phase Extraction (SPE)

LC-18 reversed phase cartridges (Supelco, Sigma-Aldrich, Germany) were used for solid phase extraction in order to purify and enrich flavonoids and for separation of sugars and other interfering

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matrix compounds. The columns were equilibrated with 100% water and elution was performed using a gradient from 100 to 50% water followed by 100% methanol.

4.5. Reversed Phase High Performance Liquid Chromatography coupled to diode array detection (HPLC-UV/DAD)

The Agilent 1100 series HPLC system was used for the HPLC runs. The system consisted of an Agilent 1100 autosampler connected to Agilent series 1200 binary pump system coupled to an Agilent series 1100 thermostatic column compartment and an Agilent series 1100 DAD detector. Separations were performed on a reversed phase column (Varian LC-18; 4.6 mm × 250 mm; ID 5 µm, USA) at 30°C and the flow rate was 0.5 ml/min. 5 µl of samples (5 mg/ml in 80% aqueous methanol) were injected. Gradient elution was performed using solvent (A) water + 1% of acetic acid to increase peak resolution. Solvent (B) 100% acetonitrile. The step gradient began with 90 % A and stopped while reaching 10 % B in 30 minutes. After this 100 % B was used for 5 minutes followed by 10 % B for 5 minutes. Wavelengths of 254, 320, 360 and 380 nm were used for detection. The data was compared to standard compounds and computer libraries of pure compounds.

4.6. LC-triple quadrupole mass spectrometric analysis (LC-MS and LC-MS/MS tandem mass spectrometry)

An HPLC apparatus (1100 series, Agilent, Waldbronn, Germany) connected to an electrospray ionization (ESI) triple quadrupole mass spectrometer (HTC Ultra-Bruker Daltonics-Advanced Mass Spectrometry Instrumentation, Germany) was used. Gradient elution was performed using acetonitrile (MeCN) and water containing 0.005% formic acid (Solvent A) and acetonitrile and glacial acetic acid (Solvent B). A linear gradient from 4% to 33% B was employed for 35 min and was increased to 100% B for 5 min. Then 4% B was used for 5 min to re-equilibrate. Mass analysis of compounds was performed using negative ion mode. The spray voltage was set to 5000 V and the capillary temperature to +280ºC. Nitrogen was used as sheathing gas and the flow was set to 40 U. Collision induced dissociation (CID-MSn) was applied to induce fragmentation of the molecular ions, and their fragments were analyzed using tandem mass spectrometry. Helium was used as collision gas at 0.8 mTorr. Collision energies of 15 and 30 eV were used to investigate neutral loss and product ions and scanning was performed using a mass range from 50 to 1000 m/z. Data from the literature, the Wiley Natural product library, and authentic samples were used for the structural identifications of phenolic compounds such as flavonoids, stilbenes and ellagic acid derivatives as well as triterpenes.

4.7. Antifungal assays

4.7.1. Fungal strains

Aspergillus niger ATCC 9763, *Nattrassia mangiferae* ATCC 96293, *Aspergillus flavus* ATCC 9763 and *Fusarium moniliforme* ATCC 24378 were obtained from National Research Center, Sudan. Before use, the strains were sub-cultured on Sabouraud dextrose agar (Oxoid™ CM0041B) slants, at + 35°C.

4.7.2. Agar well diffusion method

A cup well agar diffusion method [44, 45] with minor modifications was used. Before the test, the fungal strains were grown on petri dishes (∅ = 9 cm) containing Sabouraud dextrose agar at +35 °C overnight [97]. The resulting fungal growth was washed with 100 ml sterile normal saline to obtain fungal suspension containing conidia which were used for the tests. 200 µl ml of this fungal

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suspension was adjusted to 1.0 × 10^8 CFU/ml and mixed with 20 ml of sterile, molten Sabouraud dextrose agar which was poured into sterile petri dishes (∅=9 cm). The petri dishes were left to set at room temperature. Four holes were cut in the agar using a sterile cork borer (10mm in diameter) and each hole was filled with 100 µl of extracts (1 mg/ml in 50 % methanol) and amphotericin B (Sigma-Aldrich, 1 mg/ml in 50 % methanol). 100 µl of 50 % methanol was used as a negative control. The extracts/antibiotics/solvents were left to diffuse into the agar in the cold room (+4°C) for one hours. The plates were then incubated at + 35º C for 24 hours. For each experiment four replicates (n=4) were used. The diameters of the zones of inhibition (IZ) were measured in mm using a caliper and the mean of five diameters ± SD and SEM was calculated.

4.7.3. Agar dilution method

Minimum inhibitory concentrations were determined using a slightly modified agar dilution method [98]**.** Fungal conidial suspensions were grown for four days in Sabouraud dextrose broth at + 35 °C. For the test, 1 ml of these suspensions were diluted with 0.9 % (w/v) NaCl to contain 1.0 × 10^6 CFU/ml. 100 µl of these fungal suspensions were mixed with 10 ml molten Sabouraud dextrose agar which was pipetted into a petri dish (∅ = 9 mm). 10 ml of twofold dilutions of plant extracts (from 500 to 31.25 µg/ml) and amphotericin B (from 500-15.625 µg/ml) were added to the petri dishes. Each dilution contained 500 µl of 50 % methanol or hexane solutions of the plant extracts or antibiotics dissolved in 10 ml of molten Sabouraud dextrose agar. The petri dishes were incubated for 24 h at + 35 °C. The MIC was taken as those concentrations which resulted in clear petri dishes showing no visible fungal growth. All tests were performed in triplicates. The solvents used for the plant extractions, 50 % methanol or hexane, were used as negative controls. Hexane was used for dissolving those extracts which did not dissolve in 50 % MeOH, that is very nonpolar extracts, such as those originating from hexane and petroleum ether extractions.

5. Conclusions

Ethyl acetate and aqueous extracts of the stem wood and bark of *Terminalia brownii* give good antifungal effects against *Nattrassia mangiferae*, *Fusarium verticilliodes*, *Aspergillus flavus* and *Aspergillus niger*. Altogether twelve compounds were identified from the stem wood of *T. brownii.* Cis- and trans-isomers of resveratrol 3-*O*-β-galloyl-glucoside were characterized for the first time in this species of *Terminalia*. Likewise, gallagic acid dilactone has not been reported previously in the stem wood of *T. brownii.* Owing to its relative chemical stability and its reported antifungal efficiency against phytopathogenic molds, gallagic acid dilactone might be an especially interesting component in standardized antifungal extracts of *T. brownii*.

Our results justify the uses of water based extracts of *T. brownii* for the protection of crop plants and for wood preservation in traditional agroforestry in Sudan. Further studies are needed on the antifungal activities of separated compounds as well as on various controlled combinations of these compounds. Standardized extracts of *T. brownii* stem wood could be used as new, cheaper and eco-friendly pesticides for routine use in Sudanese agroforestry instead of toxic synthetic pesticides.

Supplementary Materials: Tandem mass spectral chromatograms of molecular ions and their fragment product ions resulting from selected compounds in *T. brownii* ethyl acetate extracts of the wood.

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