

DNA barcode analysis and antioxidant activity of *Desmodium molliculum* and *Desmodium adscendens*

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Abstract

In Ecuador, two species of *Desmodium* are used as traditional medicine. These are popularly known as “hierba del infante” and refer to *D. adscendens* and *D. molliculum*. The first species has a large base of information on its genetics and biological activity on which its traditional use is supported, while the second, *D. molliculum*, lacks this scientific information. This research aims to establish a base knowledge for the species *D. molliculum*: characterize the species genetically by obtaining the molecular DNA barcodes *trnh-psbA*, *rbcL*, *matK*, ITS1 and ITS2, evaluate its antioxidant effect and compare it with that of *D. adscendens* using the *in vitro* techniques of Folin-Ciocalteu, ABTS, DPPH and FRAP to assess its potential medicinal effect. De novo genetic DNA barcodes were obtained for the species *D. molliculum* and the phylogenetic analysis separated them from those obtained from *D. adscendens*, indicating that the analyzed species can be discriminated by DNA barcodes. In addition, the methanolic extracts of *D. molliculum* contain more than double the content of total polyphenols (30.1 ± 1.1 mg GAE/g dry plant) than those of *D. adscendens* (13.82 ± 0.74 mg AG/g dry plant) as well as better performance in all antioxidation assays. Additionally, this research established that the defatted extract of *D. molliculum* has the highest antioxidant activity in the ABTS (1.16 ± 0.001 mg TE/mg) and FRAP (0.39 ± 0.01 mg TE/mg) assays.

Introduction

The genus *Desmodium* comprises more than 300 species that can be found in subtropical and tropical climates (1). The species within this group are herbs, shrubs or sub-shrubs that can be recognized by the combination of trifoliate, unifoliate leaves or a mixture of both, stipel presence, hooked hairs and lomentaceous fruit (2). Many of these species are of great importance in the field of phytomedicine since they have reports of use as alternative treatment for various diseases around the world. Therefore, it is important to correctly establish the identity of each species through molecular barcodes to achieve a deeper knowledge about them and so that the investigation of their biological activities is accurate.

One of the most reported species within the genus is *D. adscendens*. This plant is used in Brazil, France, Belgium, Africa and China to combat a number of ailments such as infections, body pains, liver problems, fever, cough, dyspnea, muscle spasms, among others (3). In Ecuador, *D. adscendens*, in conjunction with *D. molliculum* are popularly known as “hierba del infante” and are used to treat wounds and inflammatory processes (4, 5). Thus, its wide traditional use has led to scientific interest in revealing its biological activity. Consequently, it is now known that the extracts and preparations of the plant have a high content of flavonoids, phenolic acids, terpenes, essential oils (6, 7) and alkaloids (8), an hypocholesterolemic and hepatoprotective effect (9), antimicrobial activity against *Staphylococcus aureus*, *Candida albicans* (10) and *Escherichia coli* (11) an ability to regulate bronchospasms (12) and a great antioxidant power (6, 13) that could be linked to an anti-inflammatory capacity due to the interdependence between plant-derived antioxidant and anti-inflammatory activities (14). Beyond its biological activity, there are also studies carried out on the genetics of the plant. *D. adscendens* has been molecularly characterized and accessions of the species molecular DNA barcodes obtained from samples from New Caledonia (15),

Tanzania (16) and Mexico (17) for the markers *trnH-psbA*, *rbcL* and ITS1 can be found in the NCBI database.

This information serves as the basis for the traditional uses of *D. adscendens*; however, as mentioned before, in Ecuador the traditional medicine derived from “hierba del infante” also includes *D. molliculum*, a species with little information about its potential biological activities and, to the date of this research, without an elucidated molecular DNA barcode. Hence, this research focused on creating a knowledge base for *D. molliculum* by obtaining the molecular DNA barcodes *trnH-psbA*, *rbcL*, *matK*, ITS1 and ITS2 of the plant, comparing them with the same DNA barcodes obtained from *D. adscendens* and in obtaining the methanolic extracts of both plants to compare their antioxidant effect in the assays: Folin-Ciocalteu, ABTS, DPPH and FRAP.

Results

DNA barcode analysis

In most DNA barcodes, the similarity with GenBank accessions was with species of the genus *Desmodium*. Blast analysis, DNA barcode markers, sequences and replicas obtained from *D. molliculum* (DM) and *D. adscendens* (DA) for all markers can be observed in the Supplementary Table 1. DNA barcode and phylogenetic analysis of all markers used showed that *D. molliculum* and *D. adscendens* sequences arranged in distinct and separate clades in their original trees (Fig. 1, Fig. 2 and Supplementary Figures). It is important to note that the accessions obtained for the DNA barcodes *trnH-psbA*, *rbcL*, *matK*, ITS1 and ITS2 for *D. molliculum* are *de novo* and were elucidated for the first time in this research. Consequently, no accessions for any *D. molliculum* DNA barcode were found in the NCBI for comparison with the accessions obtained in this investigation.

Chromatographic analysis

TLC assays showed that the *D. molliculum* crude extract (DMC) and the *D. molliculum* defatted extract (DMD) maintain the same qualitative composition sharing spots with the same R_f values in both, the phenolic acids and flavonoids plates (Fig. 3). The standard used, caffeic acid, was found in both extracts with a R_f: 0.79 in the plates developed with DPPH.

In vitro antioxidant activity

Total polyphenol content and antioxidant activity of DMC, DMD and the *D. adscendens* crude extract (DAC) were analyzed. The results of each extract can be observed in Table 1.

Table 1
Total polyphenol content and antioxidant activity of the *Desmodium* extracts.

Extract	Polyphenol content	ABTS (TEAC)	DPPH	FRAP
	(mg AG/g dry plant)	(mg TE/mg)	% RSA	(mg TE/mg)
DMC	30.80 ± 1.05 ^a	0.26 ± 0.004 ^a	72.66 ± 0.72 ^a	0.30 ± 0.02 ^a
DMD	30.09 ± 0.53 ^a	1.16 ± 0.001 ^b	74.93 ± 0.65 ^a	0.39 ± 0.01 ^b
DAC	13.82 ± 0.74 ^b	0.11 ± 0.008 ^c	31.97 ± 4.74 ^b	0.08 ± 0.01 ^c
Note: Values with different letters in the same column have a statistically significant difference between them (p > 0.001).				

Polyphenol content

No significant difference between the polyphenol content of DMC and DMD was observed. Both extracts had almost double the content of polyphenols (± 30 mg GAE/g dry plant) than DAC (13.82 ± 0.005 mg GAE/g dry plant). The total polyphenol content found in *D. molliculum* extracts is also higher than other *D. adscendens* extracts reported with 11.1 mg GAE/g dry plant (6) and 10 mg/g dry plant (13).

Antioxidant capacity

Overall, antioxidant capacity from the extracts followed the order: DMD > DMC > DAC (Table 1). DMD excels in performance in the ABTS assay with a TEAC value of 1.16 mg TE/mg, over 4 times greater than the obtained by DMC (0.26 mg TE/mg) and more than 10 times the amount of TEAC value obtained by DAC (0.11 mg TE/mg). All ABTS results showed a statistically significant difference ($p < 0.001$). In the DPPH assay, both *D. molliculum* extracts performed similarly (DMC: $72.66 \pm 0.72\%$ RSA; DMD: $74.93 \pm 0.65\%$ RSA) with no significant difference between them while DAC had a result with statistically significant difference ($p < 0.001$) with a lower % RSA value ($31.97 \pm 4.74\%$). FRAP results also showed DMD as the most active extract followed closely by DMC with 0.39 ± 0.01 and 0.30 ± 0.02 mg TE/mg, respectively and places DAC as the least active (0.08 ± 0.01 mg TE/mg). All FRAP results had a statistically significant difference ($p < 0.001$).

Discussion

The distribution of the *D. molliculum* and *D. adscendens* sequences in the trees of all markers, clearly indicates that the collected species are different. *D. adscendens* sequences obtained in this study were compared with other accessions available in the NCBI for the same species. Included accessions were: MF084237 (*trnH-psbA*), KY702612 (*rbcl*) and KY702672 (ITS1) from New Caledonia (15), MN166747 (*rbcl*) from Tanzania (16) and LC377390 (ITS1) from Mexico (17). The *trnH-psbA* tree (Fig. 1) shows the *D. adscendens* sequences separated from the MF084237 accession while the *rbcl* topology (Supplementary Figures) shows all *D. adscendens* sequences sharing the same node. The *D. adscendens*

sequences of this study are closer to the Tanzania (MN166747) accession than the New Caledonia (KY702612) which is in the consequent closest branch. Similarly, the ITS1 tree (Fig. 2) showed *D. adscendens* sequences in the same clade as the accession LC377390 (17) but, separated from KY702672 (15). Finally, the ITS2 tree (Supplementary Figures) separates *D. adscendens* consensus sequences (CS) from all the others samples.

Regarding the *matK* tree (Supplementary Figures), it is important to mention that the sequences obtained for this locus were not of optimal quality due to drawbacks in the recovery and amplification process. Therefore, the first *D. adscendens* sequence (DA1) had to be removed from the analysis. This locus has been reported as problematic by other authors due to its low amplification and recovery success (18, 19). This phenomenon can be roughly reflected in the topology of the tree where different branch distance can be observed from the two *D. adscendens* sequences obtained from the same sample to the node where they originate.

In addition, bootstrap support in all of the phylogenetic trees was analyzed. The bootstrap value serves as an aid to evaluate the branching pattern of the tree. If it exceeds 95%, it indicates that clades are correctly formed and that their topology is correct (20). However, it has been suggested that values over 70% also represents true clades and correct clade formation (21). With these considerations, we found that the markers *trnH-psbA*, *matK* and ITS1 had the highest support percentage being the most apt for *Desmodium* species discrimination.

All three markers had a bootstrap support higher than 95% in all the clades formed for DA CS while clades formed for DM CS nodes had a support of 91% (*trnH-psbA*), 79% (*matK*) and 93% (ITS1). These findings agree with the literature. According to Hollingsworth et al. (22) the *trnH-psbA* and *matK* loci have a good discrimination power and offer higher resolution when discriminating angiosperms at the specie level. Moreover, Costion et al. (18) describes *trnH-psbA* as the most efficient marker to use in species discrimination in poorly known floras. ITS1 has also shown to be useful for species discrimination within the Sapotoideae family in Ecuadorian specimens (23). On the other hand, the bootstrap support for the *rbcL* tree, was low for both *D. molliculum* CS (59%) and *D. adscendens* CS (61%) clades, making this marker the least suitable for discrimination between the presented species.

Concerning the ITS2 tree, the bootstrap analysis showed mixed results. The clade for *D. adscendens* CS had a 99% support while the *D. molliculum* CS clade was supported by only 61%, one of the lowest percentages obtained in this research. Despite the fact that the ITS2 marker has been reported to be successful in identifying specimens to the species level by up to 92.7% (24) and other studies on medicinal plants highly recommend the use of the ITS markers for species identification and differentiation (23, 25–28), we found that is not the best marker for *D. molliculum* identification.

Overall, *D. molliculum* clades where not highly supported by bootstrap analysis indicating that the topology of its branches may not be correct. These misassignments at species level may occur because of a high genetic similarity between the specimens studied (19) which supports the idea that *D.*

adscendens is the farthest relative specie from the other *Desmodium* samples. Nevertheless, these results may also occur due to morphological misidentifications in species with similar phenotype (19).

In regard of the antioxidant potential of the plants, both *D. molliculum* extracts had more total polyphenols (± 30 mg GAE/g) than the *D. adscendens* crude extract (13.82 ± 0.005 mg GAE/g) which was a significantly different from *D. molliculum* crude extract and *D. molliculum* defatted extract ($p < 0.05$). Other studies on *D. adscendens* have reported a polyphenol content similar to that found in this research. Muanda et al. (6) and Ayoola et al. (13) reported a value of 11.1 mg GAE/g and 10 mg/g dry plant, respectively. Both authors agreed that *D. adscendens* extracts have a great potential as antioxidants agents due to their high content of these phytochemicals. Thus, considering that DMC and DMD almost doubles the amount of gallic acid equivalents of DAC, *D. molliculum* extracts have a higher potential to interact with oxidants within the ABTS, DPPH and FRAP assays.

In the ABTS assay, the highest antioxidant activity was found for DMD with 1.16 ± 0.001 mg TE/mg followed by DMC with 0.26 ± 0.004 mg TE/mg and, similarly to the polyphenol content, DAC had the lowest performance. Other authors have reported the TEAC value of different *Desmodium* species. Tsai et al. (29) analyzed 10 different species and concluded that *D. sequax* had the highest TEAC value at 0.3754 mg TE/mg, a lower value than the one found on DMD. On the other hand, DAC TEAC value of 0.11 ± 0.008 mg TE/mg is similar to the one obtained from *D. triflorum* (0.1285 mg TE/mg), another species with high polyphenol content and great antioxidant capacity as reported by Lai et al. (29). Thus, it is relevant to suggest that DMC and DMD have a greater antioxidant capacity than similar extracts from other *Desmodium* species when addressed in the ABTS assay.

D. molliculum extracts also showed a better performance in the FRAP assay when compared with DAC and in Table 1 it can be appreciated that DMD had the best antioxidant results. This could indicate that the removal of lipophilic material may cause an improvement of the ability to delocalize electrons from the defatted extract components. Likewise, the DPPH results showed that DMC and DMD had a better antioxidant potential than DAC. But, contrary to what was observed in the FRAP assay, both *D. molliculum* extracts performed similarly with no significant difference between them. The Table 1 shows that DMC and DMD had more than twice the radical scavenging activity (% RSA) of DAC ($31.97 \pm 4.74\%$) with 72.66 ± 0.72 and $74.93 \pm 0.65\%$, respectively. The reason why an increase in activity was not observed in this test when addressing DMD may be because the solvent is forming hydrogen bonds with the polyphenols, which would prevent its interaction with the DPPH radical due to steric hindrance (30). Moreover, the percentages obtained for DMC and DMD are higher than the reported for the antioxidant standard Trolox ($53.4 \pm 1.9\%$) and other antioxidants used in the food industry like butylated hydroxyanisole ($22.3 \pm 0.6\%$) and butylated hydroxytoluene ($8.0 \pm 0.4\%$) (31) while DAC had an RSA percentage lower than Trolox but higher than the synthetic antioxidants mentioned.

Overall, DM extracts had a better antioxidant activity than DA extracts in the performed assays. These results indicate that *D. molliculum* extracts could be more beneficial when used as remedies in alternative medicine than the obtained from *D. adscendens*. Furthermore, between both *D. molliculum* extracts, the

one defatted with n-hexane had the best performance despite the fact that its polyphenol content is indistinguishable from the crude extract polyphenol content. Thus, the removal of apolar compounds from the extract may have allowed polyphenols to have a better medium to interact with oxidants and themselves promoting synergism and consequently improving the defatted extract antioxidant power (32). A possible explanation may be due to the presence of plant cuticle components in crude extracts. Plant cuticle is rich on epicuticular and intra cuticular waxes that can interact directly with phenolic compounds via ether or ester bonds and via covalent bonds with low-molecular weight phenolics (33). Thus, free plant cuticle components may hinder phenolic compounds antioxidant ability by keeping them secluded.

Methods

Plant collection and taxonomic identification

Aerial herbaceous parts (leaves, stems, flowers) of *D. molliculum* were collected in San Joaquín, Azuay. Decimal degree coordinates for its location are - 2.886450, -79.07892. In addition, *D. adscendens* was collected as a reference specie in Santa Rosa, El Oro (-3.52500000, -79.71833333). Both species were taxonomically identified in the Herbarium Azuay (HA). The vouchers rest in the HA digital repository with the numbers 13673 and 13676, respectively.

DNA extraction and PCR

Leaves from *D. molliculum* and *D. adscendens* were pulverized with liquid nitrogen and stored at -80°C until DNA extraction. DNA extraction was performed in three biological replicates for each species. The extraction was performed with the CTAB protocol (34) with some modifications. PCR was performed as described by Bustamante et al. (23) using 0.5 µM of each primer (Table 2) with a reaction volume of 30 µL. GoTaq® master mix was used at a final concentration of 1x according to the manufacturer instructions. PCR conditions were as follows: 95°C for initial denaturation; 35 cycles of 95°C for 30 s, 60°C (for *trnH-psbA* and *rbcl*) or 56°C (for *matK*, ITS1 and ITS2) for 30 s, 72°C for 90 s; and final extension of 72°C for 5 minutes. For the gel electrophoresis, 5 µL of PCR reaction was loaded onto a 1.5% agarose gel to check for amplicons. Once expected amplicons were detected, the remaining 25 µL of the PCR reaction was sent for PCR purification and commercial sequencing (Psomagen, USA).

Table 2
Primers for markers *trnH-psbA*, *rbcL*, *matK*, ITS1 and ITS2.

<i>Locus</i>	<i>Primer pairs</i>	<i>Sequence</i>	<i>Reference</i>
<i>trnH-psbA</i>	<i>trnHf</i>	GTTATGCATGAACGTAATGCTC	Basak et al., 2019
	<i>psbA3</i>	CGCGCATGGTGGATTACAATCC	
<i>rbcL</i>	<i>rbcLA_F/</i>	ATGTCACCACAAACAGAGACTAAAGC	Costion et al., 2011
	<i>rbcLA_R</i>	GTAAAATCAAGTCCACCRCG	
<i>matK</i>	<i>matK_3FKIMf</i>	CGTACAGTACTTTTGTGTTTACGAG	
	<i>matK_1R_KIMR</i>	ACCCAGTCCATCTGGAAATCTTGGTTC	
ITS1	ITS 5a F/	CCTTATCATTTAGAGGAAGGAG	Chen et al., 2010
	ITS 4 R	TCCTCCGCTTATTGATATGC	
ITS2	S2F/	ATGCGATACTTGGTGTGAAT	
	S3R	GACGCTTCTCCAGACTACAAT	
Note: Adapted from “Morphological and molecular barcode analysis of the medicinal tree <i>Mimusops coriacea</i> (A.DC.) Miq. collected in Ecuador” by Bustamante et al. (2019). <i>PeerJ</i> , 7: e7789 https://doi.org/10.7717/peerj.7789 .			

Bioinformatic analysis

MEGA11 (35) was used for processing the sequences. Forward and reverse sequences for each amplicon were used to form a consensus sequence. Sequences were used for Blast analysis (36) in the GenBank non-redundant nucleotide database. Sequences with similarities found in the analysis were selected to develop a phylogenetic tree for each DNA barcode. All sequences were trimmed at the ends to contain the same regions and then aligned by the multiple alignments generating program MUSCLE. Promptly, the recommended model was used for each tree. A total of 100 replicates were performed using the bootstrap test. Alignment sequence analysis was performed for variant detections including insertion/deletions (InDels), nucleotides substitutions, and/or single nucleotide polymorphisms (SNPs) with MUSCLE and trimming the sequences at the edges for the six sequences to contain the same region (three biological replicates for *D. molliculum* and three biological replicates for *D. adscendens* to search for differences.

Plant extraction

Crude extract preparation

Plant material (aerial parts) of both species were selected, rinsed, dried and pulverized to proceed with extraction via percolation under United States Pharmacopeia specifications with methanol as solvent. The obtained DMC and DAC were lyophilized and stored at -20°C until use.

Defatted extract obtention

In order to have an extract rich in polar polyphenols, a second *D. molliculum* extract was obtained by removing lipids, waxes, paraffins, and pigments via liquid fractionation with n-hexane according to the group of Medicinal Plants and Natural Products of the University of Cuenca SOP (Standard Operating Procedure). Briefly, lyophilized extracts were redissolved in methanol; water was added until a ratio of 80:20 methanol-water was achieved. Solutions were placed in separatory funnels with an equal amount of n-hexane and shaken vigorously for 5 minutes. Finally, n-hexane phase was discarded. The last two steps were performed three times adding 50% of the n-hexane volume each time. The resulting DMD was concentrated, lyophilized, weighed and stored at -20°C until use.

Chromatographic analysis of the extracts

To assess the possible loss of bioactive compounds due to liquid fractionation, thin layer chromatograms were performed to qualitatively compare the composition of DMC and DMD. Phenolic acids, flavonoid and alkaloid content were evaluated. Mobile phases, and standards preparations were done according to Wagner & Bladt (37). All extracts were redissolved in methanol at 5 mg/mL while the standards were prepared at a concentration of 1 mg/mL.

Total polyphenol content and in vitro antioxidant activity of the extracts

Polyphenol content was evaluated with the Folin-Ciocalteu assay with gallic acid as standard. The antioxidant activity was determined with the ABTS (38), DPPH (39) and FRAP (40) assays. For each assay the vitamin E analogue Trolox was used as a standard for the construction of the calibration curves. Values were expressed as mg of Trolox equivalent per mg of dry extract (mg TE/mg). Additionally, values for the DPPH assay were expressed as the percentage of radical scavenging activity (% RSA) calculated with the equation:

$$\%RSA = 100 \times (1 - AE/AD)$$

Where *AE* is the absorbance of the solution with the extract added at a particular concentration, and *AD* is the absorbance of the DPPH solution without test sample (41).

Plant guidelines statement

We, Josué Sarmiento-Pacurucu, Efren Santos-Ordoñez, Fabian León-Tamariz, Liliana Villao-Uzho, Jessica Calle-Lopez and Eugenia Peñaherrera, acknowledge and affirm that the collection of plants in the course of activities carried out in the development of the project "DNA barcode analysis and antioxidant activity of *Desmodium molliculum* and *Desmodium adscendens*" was in strict compliance with all local, national, and international laws and regulations related to plant collection, as well as adherence to guidelines established by international bodies and organizations dedicated to the conservation of flora.

We understand the importance of preserving biodiversity and respecting the natural ecosystems in which we operate. We recognize that responsible management of natural resources is essential to ensure their

availability for future generations.

This declaration represents our firm commitment to operate ethically and responsibly in all activities related to plant collection, thereby contributing to the conservation of biodiversity and the protection of the environment.

Declarations

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Author contributions statement

E.P, F.L. and E.S. conceived the experiments, J.S., L.V. and J.C. conducted the experiments, J.S., E.S. and E.P. analyzed the results. All authors reviewed the manuscript.

Additional information

Competing interests

The authors declare no competing interests.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files). Accession DNA barcodes can be found in Supplementary Data 1.

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Figures

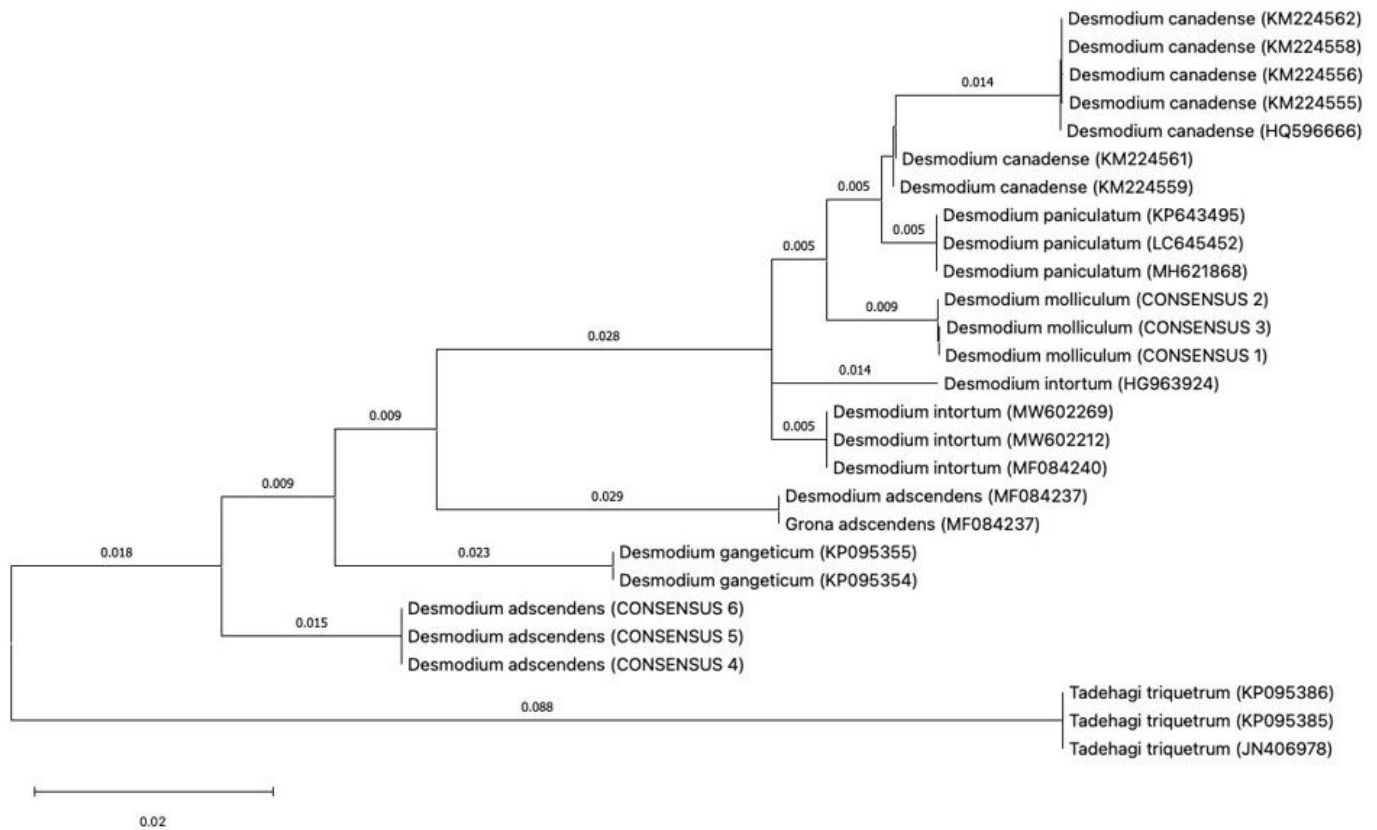


Figure 1

Topology of the *trnH-psbA* original tree.

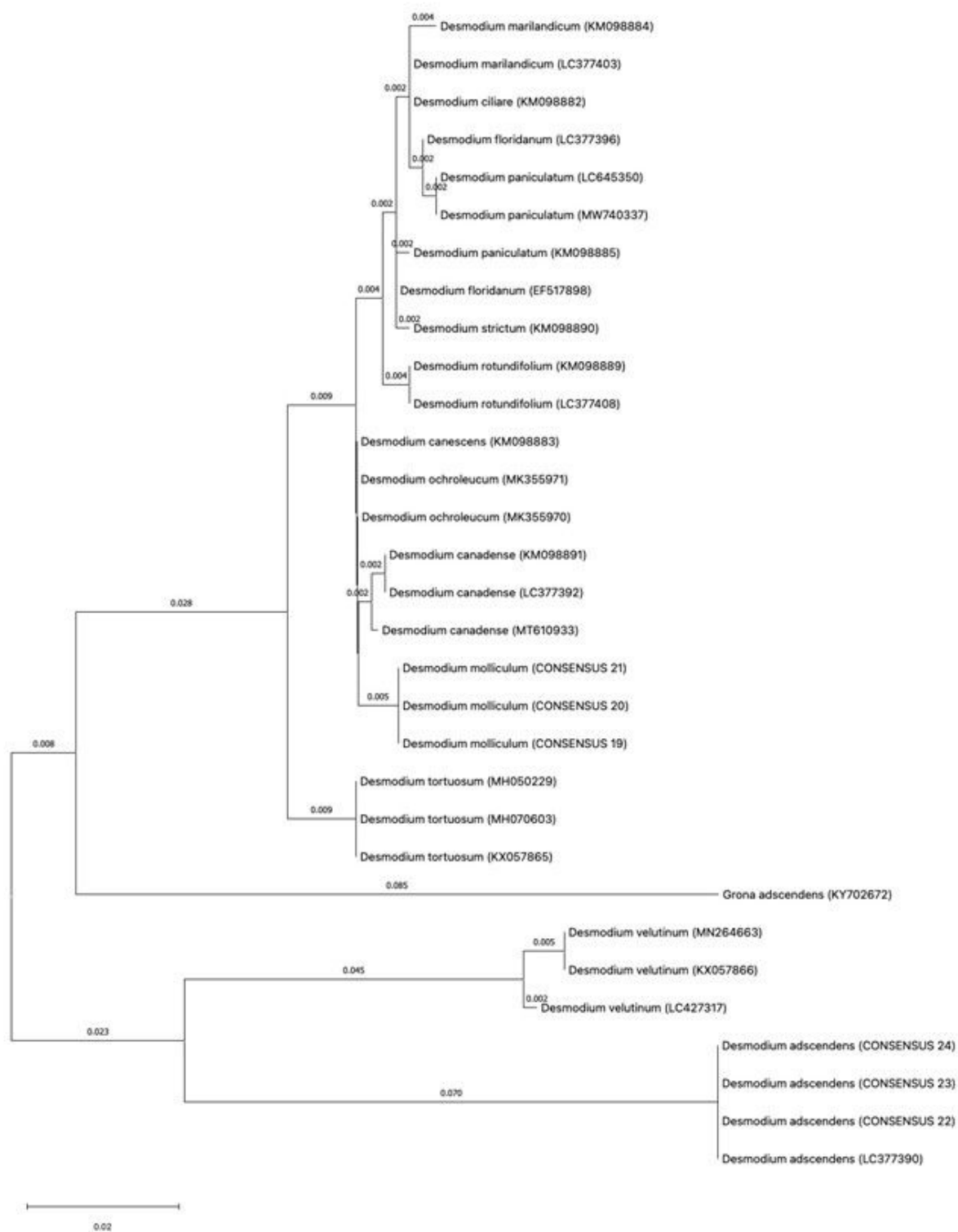


Figure 2

Topology of the ITS1 original tree.

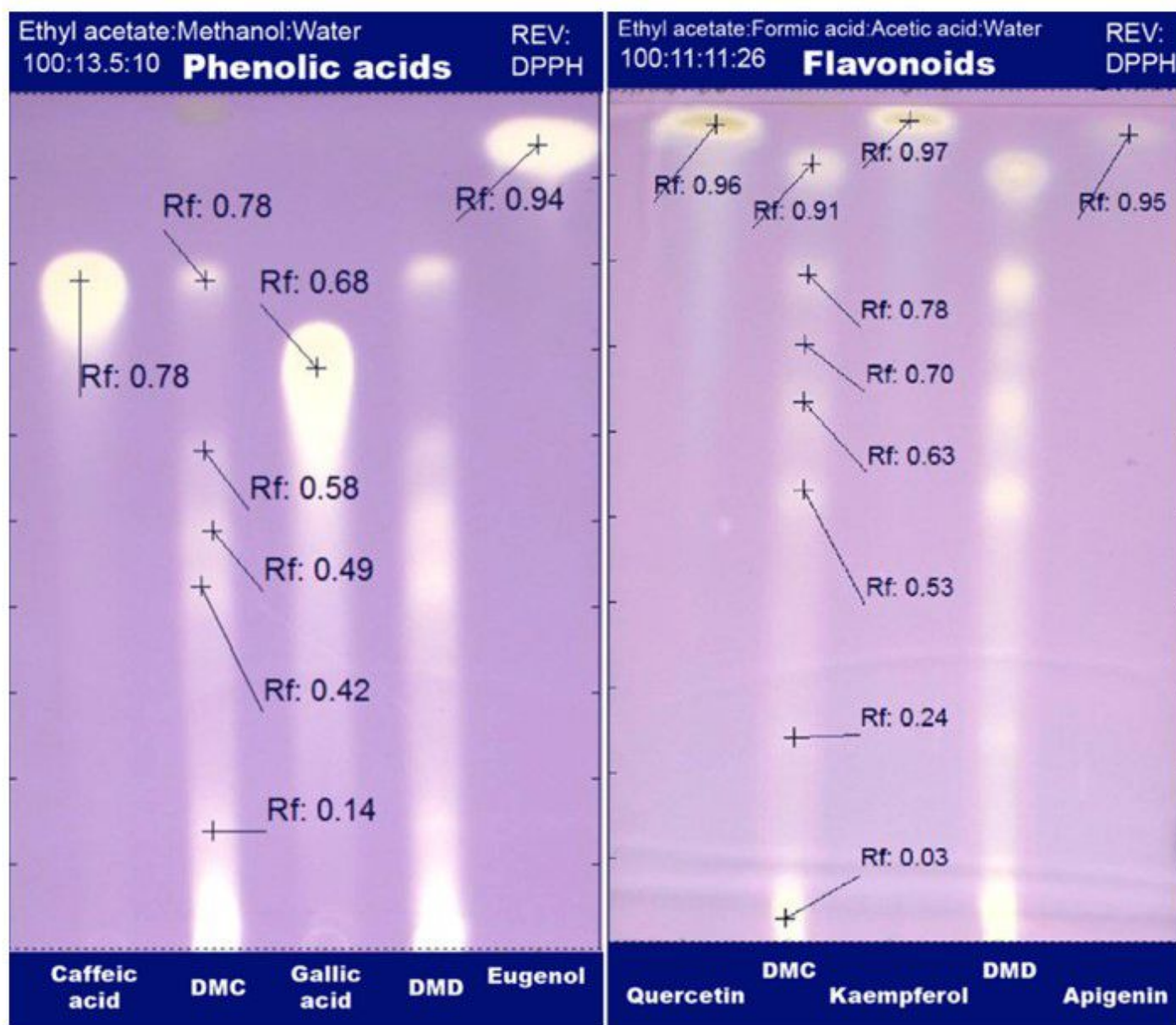


Figure 3

Qualitative comparison of the content of phenolic acids and flavonoids of the extracts of *D. molliculum* in chromatographic plates developed with DPPH. Antioxidant activity can be seen qualitatively as yellow-white spots over the violet background. Brighter spots can be appreciated from DMD components in both plates.

Supplementary Files

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- [SupplementaryData1.xlsx](#)
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- [SupplementaryfigureBOOSTRAPITS1.png](#)
- [SupplementaryfigureBOOSTRAPtrnHpsbA.png](#)
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- [SupplementaryfigureBOOTSTRAPmatK.png](#)
- [SupplementaryfigureBOOTSTRAPrbcL.png](#)
- [SupplementaryfigureITS2.png](#)
- [SupplementaryfigurematK.png](#)
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