IJPS INTERNATIONAL JOURNAL OF PLANT SCIENCES
ISSN-0973-1547 Volume 16 | Issue 1 | January, 2021 | 39-45

DOI: 10.15740/HAS/IJPS/16.1/39-45
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RESEARCH ARTICLE

Variation in arjunolic acid in bark of *Terminalia arjuna* from different geographical locations of India using high performance liquid chromatography

■ **Sonu Bharti, Sandeep Kumar, Neelu Singh and Shamim Akhtar Ansari**

SUMMARY

Major active ingredient of *Terminalia arjuna* bark, a triterpenoid saponins -arjunolic acid was determined in fifty-one accessions of different geographical locations from seven different states *i.e.* Madhya Pradesh, Orissa, Uttar Pradesh, Maharashtra, Jharkhand, Uttrakhand and Andhra Pradesh of India.Arjunolic acid was extracted from bark samples with the help of microwave assisted method and quantified by High Performance Liquid Chromatography (HPLC). A significant variation in arjunolic acid content was observed in bark from different regions.The quantity of arjunolic acid varied 0.01-0.29 µg/mg, maximum quantity was observed in Accession- UKDDLP belongs to Uttrakhand, Western Himalayan region. The eastern plateau and hill region of the state produced the lowest amount, whereas the western Himalayan regions zone of the state had higher yields. The populations with high arjunolic acid content can be utilized for mass multiplication and genetic improvement.

Key Words : *Terminalia arjuna*, Bark, Arjunolic acid, Geographical regions, HPLC

How to cite this article : Bharti, Sonu, Kumar, Sandeep, Singh, Neelu and Ansari, Shamim Akhtar (2021). Variation in arjunolic acid in bark of *terminalia arjuna* from different geographical locations of India using high performance liquid chromatography. *Internat. J. Plant Sci.*, **16** (1): 39-45, **DOI: 10.15740/HAS/IJPS/16.1/39-45,** Copyright@ 2021: Hind Agri-Horticultural Society.

Article chronicle : Received : 11.10.2020; **Revised :** 10.11.2020; **Accepted :** 11.12.2020

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Terminalia arjuna L. (Roxb. Ex DC) Wight and Arnot: 2n=24, is a large tree of the flowering plant family Combretaceae, distributed in tropical regions of the world. The tree is common throughout the greater part of the Indian peninsula along rivers, streams, ravines and dry watercourses, found in sub-Himalayan tract. Arjun bark is used as anti-oxidant, antibiotic (Perumal *et al.,* 1998), astringent, cooling, aphrodisiac

cardio tonic (Karthikeyan *et al.,* 2003), in fractures, ulcers, spermatorrhae, leucorrhea, diabetes, cough, tumor, excessive perspiration, asthma, inflammation, hypocholesteremia (Miller, 1989) and skin disorders (Dwivedi and Agarwal, 1994 and Raghavan and Kumari *et al.,* 2006) also shows anti-mutagenic activity (Scassellati-Sforzolini *et al.,* 1999).

The active constituents in *T. arjuna* include tannins, triterpenoid saponins (arjunic acid, arjunolic acid, arjungenin, arjunglycosides), flavonoids (arjunone, arjunolone, luteolin), oxalic acid, gallic acid, ellagic acid, oligomeric proanthocyanidins (OPCs), phytosterols, calcium, magnesium, zinc, and copper (Dwivedi and Udupa, 1989). The arjunolic acid isolated by King *et al.* (1954) is pentacyclic triterpenoid saponins. It is the major extractable bio-active compound (1.5-2%) present in the heavy wood and bark. Arjunolic acid is used as a cardiac tonic in Ayurvedic medicine for centuries. It is a promising compound in nano-chemistry and its rigid molecular framework is suited for the construction of nanostructures (Romson *et al.,* 1983). Increasing demand of bark for the preparation of ayurvedic formulations and overexploitation of species from natural sources make this plant important for further multiplication for future need. Keeping these facts in mind, the present investigation was conducted to study variation in arjunolic acidin 51 different genotypes of *Terminalia arjuna.*

MATERIAL AND METHODS

Collection and processing of bark samples:

The bark samples were collected from All India germplasm bank of arjun (*Terminalia arjuna*) established at Central Tasar Research and Training Institute (CTRTI), Nagri, Ranchi that maintains superior arjun accessions from six states (Fig. A), *viz.,* Andhra Pradesh, Chhattisgarh, Jharkhand, Maharashtra, Uttrakhand and Uttar Pradesh (Fig. A) altogether representing five agro-climatic zones - Eastern plateau and hill (EPH) regions, Southern plateau and hills (SPH) region, Eastern Himalayan (EH) Region, Western Himalayan (WH) region and Central plateau and hills (CPH) region (Table 1). A total of 52 accessions were sampled for bark at about 1.34m height of tree for estimation of arjunolic acid content in the month of April, 2015. A bark patch (10cm x 10cm) was sustainably removed from each of accessions of *T. arjuna.* The thickness of sampled bark patch was measured at several places with the help of Vernier caliper. The bark was washed with distilled water and fresh weight was determined. Subsequently, the bark patch was cut into small pieces and dried at room temperature in the shade. The dried bark was ground to fine powder and sieved though a 25 mm fine mesh and divided into three parts representing as replicates for extraction and estimation of arjunolic acid.

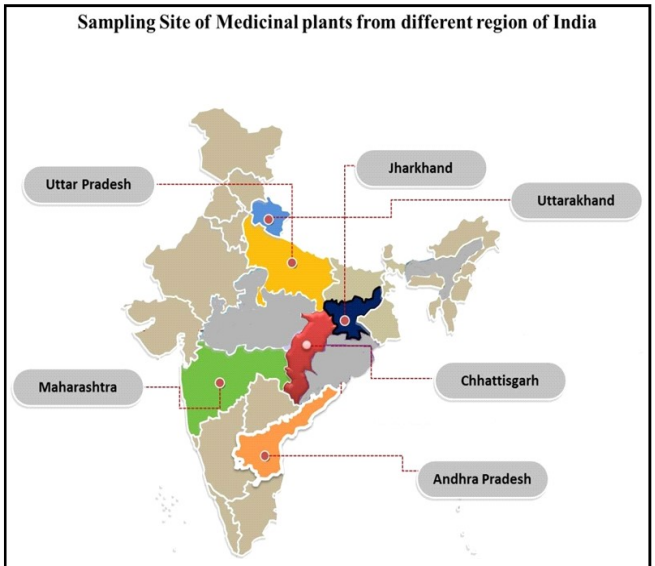

Fig. A: Map showing state-wise location of the sampled mapping population

Extraction of arjunolic acid from bark powder:

200 mg bark powder was accurately weighed and placed in a capped conical flask (100 ml) and then mixed with 20 ml of ethyl acetate. All conical flasks were kept for 10 min for pre-soaking as pre-leaching. It was followed by extraction process that was performed by warming the content at 65°C for five min, *i.e.* below boiling point (71° C) of the ethyl acetate in a 600W microwave oven. The sample was treated under microwave irradiation in an intermittent way, *i.e.* irradiation cooling- irradiation. The irradiation time was kept for 1 min and 1 min was taken to cool the sample solution between two irradiations. The suspension obtained so was filtered through Whatman filter paper no. 1. The residue was washed twice with ethyl acetate. The washings were pooled with the filtrate and the solvent was evaporated at 40° C under reduced pressure and lyophilized. The solvent free residue was dissolved in 1 ml HPLC grade methanol and transferred to 1.5 ml Eppendorf® tubes that were stored at -20°C in deep

freezer until estimation of arjunolic acid.

Estimation of arjunolic acid with the help of High-Performance Liquid Chromatography (HPLC):

Preparation of arjunolic acid standard solution:

A pure sample of arjunolic acid (MW 488.70, Melting point 296°C, purity >99%) one mg powder (vial) was purchased from Sigma-Aldrich, India. The content was dissolved in 1ml HPLC grade methanol to make a standard stock solution, *i.e.* 1000 µg/ ml. The working standard solution of 100 µg /ml was prepared from the standard sock solution by drawing known volume of the latter and diluting in ratio of 1:10 by HPLC grade methanol. A volume of 20µl of working standard (arjunolic acid) solution was injected. The peak area of the standard arjunolic acid was used for computation of arjunolic acid in the bark sample extract.

HPLC operating conditions:

HPLC analysis of arjunolic acid was carried out on a WATER'S 600 HPLC equipped with C_{18} HPLC column (250 mm × 4.6 mm × 7 mm) column using System LC 2010-CHT with LC solution software, equipped with a degasser, an auto-sampler, a diode array detector (PDA) and 20-L injector loop. Gradient mixture of buffer solution (solvent A) prepared by dissolving 0.136 g of potassium dihydrogen orthophosphate in 900 mL Milli-Q-water and added 0.5 mL of ortho-phosphoric acid and made up it to 1000 mL with Milli-Q-water.

Acetonitrile (solvent B, pH 2.5) was used as the mobile phase with a gradient elution as follows: 0.01–18 min, 50–50% A; 18–20 min, 60–40% A; 20–22 min, 85-15% A; 22–25 min, 530–70% A; 25–30 min, 30- 70% A; 30-35 min, 30-70% A; at a flow rate of1 ml/min to elute out arjunolicacid. Detection wavelength, column temperature, and injection volume were set 205 nm, 28±3°C and 20 µL, respectively. HPLC system equilibrated underthe starting conditions for 5 min before starting the analysis. Chromatograms weremonitored at 205 nm wavelengths and analyzed using with LC solution software.

Statistical analysis:

Data were analyzed using ANOVA test to observe variation in arjunolic acid in bark of *Terminalia arjuna* from different geographical locations of India using SPSS version 14.

RESULTS AND DISCUSSION

Secondary metabolites are organic compounds that are synthesized by the plant in its metabolic pathways and do not involved in the growth, development, or reproduction of an organism. Unlike primary metabolites, the scarcity of secondary metabolites does not result in immediate death, but rather a long-term deficiency of the organism's survivability, fecundity, or aesthetics, or perhaps in no significant change at all. The major active constituents in *T. arjuna* is arjunolic acid, known for biological activities of this plant. Arjunolic acid along with other secondary metabolites like phenols and tannins accumulates in the bark and possibly confer resistance against natural vagaries and insect pest attack. Interestingly, the compound has been extensively investigated for pharmaceutical purposes. Arjunolic acid was estimated in the bark samples collected from fifty-one accessions, belonging to seven states and five agro-climatic zones (Fig. 2). Table 1 summarizes details of geographical locations, bark moisture per cent and variation in arjunolic acid content as µg/mg. HPLC analysis revealed remarkable significant ($p<0.05$) variation in arjunolic acidcontent in different accessions.

The quantity of arjunolic acid varied 0.01-0.29 µg/mg, maximum quantity was observed in Accession-UKDDLP, Uttrakhand(Western Himalayan region).On the other hand, the accessionsAPMD03 (Southern Plateau and Hill region) showed significantly lowest value of arjunolic acid (0.01ug/mg). The mean value of arjunolic acid ug/100mg in different accessions of different states, agro-climatic zones of India is presented in Fig. 3.

Very few investigations incorporate tropical forest trees that, however, exclude *T. arjuna*, a versatile medicinal tree miraculously valued for cardiac ailments. Consequently, *T. arjuna* accessions, established at All India germplasm bank of arjun (*T. arjuna*), Central Tasar Research and Training Institute (CTRTI), Nagri, Ranchi, collected from country-wide natural habitats, representing different states and five agro-climatic zones.

The content showed significant ($p<0.05$) difference among agro-climatic zones and accessions. However, there was no significant difference in the arjunolic acid content across accessions belonging to different states. Among different states, accessions from Uttrakhand (UK) state showed the highest value for arjunolic acid on bark dry weight basis. On the other hand, accessions from Andhra Pradesh (AS) state contained the lowest

Table 1 : Variation in moisture % and arjunolic acid content as µg/100mg in bark samples in different accessions from different agro-climatic zone

Sr. No.	Accession No.	State Code	Agroclimatic zone	Latitude	Longitude	Moisture % in bark samples	Argunolic acid ug/mg weight*
1.	Acc101	JHRNRN08	Eastern Plateau and Hills region	23.18N	85.18E	62.63	0.02
2.	Acc103	MHCPCP02	Eastern Plateau and Hills region	20.18N	79.99E	71.84	0.09
3.	Acc104	JHRNSH01	Eastern Plateau and Hills region	23.18N	85.18E	61.87	0.33
4.	Acc106	UPJNJN01	Central Plateau and Hills region	25.44N	78.57E	67.13	0.11
5.	Acc112	MHCPCP06	Eastern Plateau and Hills region	19.97N	79.30E	61.9	0.06
6.	Acc113	UKUSTD	Western Himalayan region	28.94N	79.45E	66.99	0.21
7.	Acc117	CGDT01	Eastern Plateau and Hills region	20.61N	81.78E	68.02	0.11
8.	Acc122	MHBDAL	Eastern Plateau and Hills region	21.17N	79.65E	59.51	0.08
9.	Acc133	CGDT04	Eastern Plateau and Hills region	20.61N	81.78E	56.93	0.09
10.	Acc134	JHRNSH03	Eastern Plateau and Hills region	23.18N	85.18E	64.92	0.07
11.	Acc201	CGSGBN	Eastern Plateau and Hills region	20.74N	82.21E	63.1	0.15
12.	Ac 206	CGSGWN3	Eastern Plateau and Hills region	21.70N	82.52E	66.69	0.11
13.	Acc209	JHRNSL02	Eastern Plateau and Hills region	23.34N	85.83E	67.1	0.06
14.	Acc210	JHRNSL03	Eastern Plateau and Hills region	23.34N	85.83E	63.6	0.04
15.	Acc212	JHRNSL05	Eastern Plateau and Hills region	23.34N	85.83E	68.89	0.07
16.	Acc213	JHRNRN01	Eastern Plateau and Hills region	23.20N	85.70E	64.07	0.22
17.	Acc214	JHRNRN02	Eastern Plateau and Hills region	23.20N	85.70E	65.56	0.07
18.	Acc215	JHRNRN03	Eastern Plateau and Hills region	23.19N	85.70E	66.03	0.11
19.	Acc217	JHRNRN04	Eastern Plateau and Hills region	23.20N	85.7E	66.43	0.16
20.	Acc218	MHBDBD8	Eastern Plateau and Hills region	23.20N	79.66E	63.58	0.07
21.	Acc220	MHBDGV	Eastern Plateau and Hills region	21.25N	80.02E	68.71	0.09
22.	Acc222	JHRNRN05	Eastern Plateau and Hills region	23.20N	85.71E	64.62	0.07
23.	Acc224	CGDTSG	Eastern Plateau and Hills region	21.36N	81.81E	56.74	0.08
24.	Acc225	JHRNRN06	Eastern Plateau and Hills region	23.20N	85.71E	64.09	0.11
25.	Acc227	MHCPMD3	Eastern Plateau and Hills region	20.47N	79.80E	56.63	0.19
26.	Acc228	JHRNRN07	Eastern Plateau and Hills region	23.20N	85.71E	64.7	0.13
27.	Acc230	CGSJ04	Eastern Plateau and Hills region	23.29N	82.54E	56.65	0.09
28.	Acc337	CGSGOD	Eastern Plateau and Hills region	23.29N	82.54E	62.32	0.05
29.	Acc338	MHCPCP07	Eastern Plateau and Hills region	19.98N	79.30E	64.31	0.06
30.	Acc407	CGSGKH	Eastern Plateau and Hills region	22.84N	83.31E	64.25	0.02
31.	Acc414	MHBDNJ	Eastern Plateau and Hills region	20.80N	79.64E	65.09	0.37
32.	Acc417	MHBDPH	Eastern Plateau and Hills region	20.80N	79.64E	65.28	0.19
33.	Acc424	UKDDLP	Western Himalayan region	29.94N	78.16E	64.78	0.29
34.	Acc425	UPBDBD01	Central Plateau and Hills region	25.48N	80.34E	64.57	0.08
35.	Acc431	JHWSHJ	Eastern Plateau and Hills region	23.18N	85.18E	67.84	0.18
36.	Acc432	APRR01	Southern Plateau and Hills region	17.39N	77.84E	65.52	0.0
37.	Acc435	UKCMKP	Western Himalayan region	30.26N	79.22E	63.44	0.12
38.	Acc438	APAB02	Southern Plateau and Hills region	19.08N	79.56E	61.12	0.01
39.	Acc439	JHGWGW	Eastern Plateau and Hills region	24.11N	83.68E	62.74	0.30
40.	Acc441	JHWSGS02	Eastern Plateau and Hills region	22.59N	86.47E	61.92	0.07
41.	Acc504	APRR02	Southern Plateau and Hills region	17.39N	77.84E	61.52	0.14
42.	Acc505	APKN01	Southern Plateau and Hills region	18.44N	79.13E	59.31	0.04
43.	Acc506	APMD03	Southern Plateau and Hills region	17.87N	78.11E	56.07	0.01
44.	Acc507	JHGWRK01	Eastern Plateau and Hills region	23.99N	83.79E	62.64	0.14
45.	Acc508	JHLTLT	Eastern Plateau and Hills region	23.75N	84.35E	64.44	0.17
46.	Acc509	APRRVB01	Southern Plateau and Hills region	18.26N	78.58E	66.84	0.19
47.	Acc511	UPBDBD02	Central Plateau and Hills region	25.48N	80.34E	65.26	0.21
48.	Acc512	JHWSGS03	Eastern Plateau and Hills region	22.58N	86.47E	60.2	0.09
49.	Acc513	JHWSAD02	Eastern Plateau and Hills region	22.36N	85.44E	66.97	0.11
50.	Acc515	UPBDBD03	Central Plateau and Hills region	25.48N	80.34E	68.75	0.09
51.	Acc516	JHWSBG	Eastern Plateau and Hills region	22.36N	85.44E	55.88	0.27
52.	Acc517	APRRVB02	Southern Plateau and Hills region	17.34N	77.90E	61.1	0.09
	CD (P=0.05)						0.024

Values are the mean of three replications

*First two initials represent to the states: AP= Andhra Pradesh, CG=Chhattisgarh, JH=Jharkhand, MH=Maharashtra, UK=Uttrakhand, UP= Uttar Pradesh

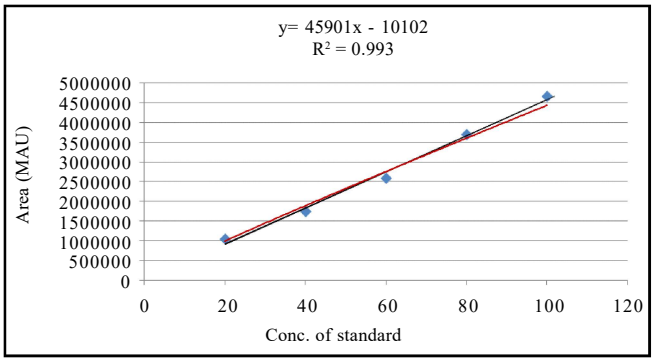

Fig. 1: Calibration curve for the standard of Arjunolic acid

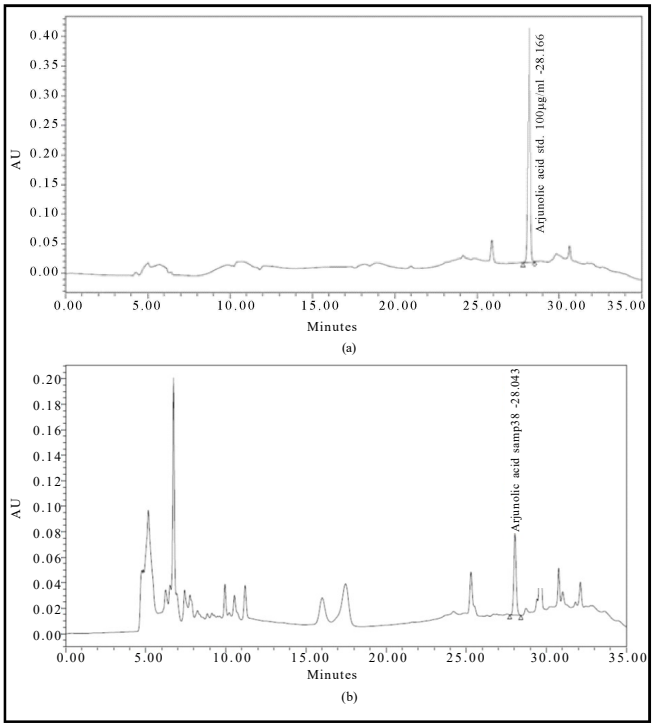

Fig. 2: Representative HPLC-PDA chromatogram of sample track of *T. arjuna* measured at λ 205 nm (a) and chromatogram of standard track measured at λ 205nm (b)

quantity of arjunolic acid.

Arjunolic acid has also been reported from other plants such as *Cochlospermum tinctorium*, *Cornus capitata*, *Leandra chaetodon*, *Combretum leprosum*, *Campsis grandiflora*, *Syzygium guineense*, *Combretum nelsonii* (Hemlatha *et al.,* 2010). Like morphometric traits, arjunolic acid content in bark also exhibits a great variation across accessions, locations and agro-climatic zones. Consequently, the observed variability of its content across accessions, locations and

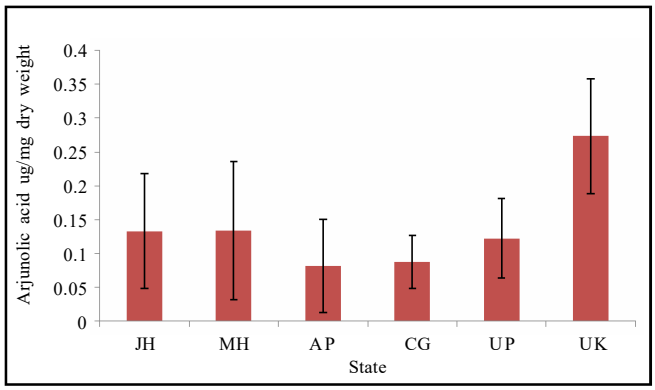

Fig. 3 : Arjunolic acid content (ug/mg dry weight) in the bark samples obtained from accessions of six states. Vertical lines represent standard deviation (SD). Data are mean of three replicates and significant at p<0.05. Average arjunolic acid content in accessions of different states

agro-climatic regions may be attributed to interaction between genotype and geo-climatic condition. In consonant, accession UKDDLP from Uttrakhand and Western Himalayan agro-climatic region produces the highest amount of arjunolic acid and accession APMD03 from Andhra Pradesh of Southern plateau and Hill agro-climatic region, the lowest amount of arjunolic acid. The plausible reason appears to be the temperate region and high annual rainfall of 1500mm in Uttrakhand in comparison to arid condition with 50-100mm average annual rainfall in Andhra Pradesh. The present study endorses that geo-climatic variables like environment, habitats, geographical conditions, altitude etc. have reflective and reproducible effects on the quantitative content of arjunolic acid in *T. arjuna*.

In the literature, the investigations have been only devoted to extraction and purification of arjunolic acid from the bark of *T. arjuna* employing HPLC or HPTLC systems (Verma *et al*., 2012; Ramesh and Dhanaraj, 2015 and Sariga *et al*., 2015). Our results bring out a great opportunity for field selection of superior accessions of *T. arjuna* for obtaining high yield of arjunolic acid. The accession may be introduced for large scale plantation for commercial extraction of arjunolic acid on a sustainable basis.

Conclusion:

Terminalia arjuna (Arjuna) is a well-known medicinal plant whose bark has been used in India's native Ayurvedic medicine for centuries, primarily as a cardiac tonic. Demand for Arjuna bark, both in India and abroad

has been growing rapidly for over a decade. The bark is collected entirely from the wild sources. Raw, chipped, or powdered bark is sold to pharmaceutical industries, where various formulations e.g. arista, capsules, and ashava are made. Presently the bark of Arjuna is being extracted through unscientific and destructive harvesting practices and population of species are declining rapidly.

Bark samples sourced from various geographical locations evidently prove that there are significant variations observed in arjunolic acid. While taking a close look at these wide variations, Uttrakhand can be identified as a spot of well flourished geographical conditions favoring the rich constituency in the natural sources. The climatic conditions and topography are really suitable for the quantitative production of herbal sources of drugs.

Acknowledgments:

I acknowledge with thanks, Director of Tropical Forest Research Institute, Jabalpur, to provide me necessary facilities during the present work. I am thankful to Dr. S. A. Ansari, my Ph.D. supervisor for his unconditional support and encouragement during this work.

Funding:

This study was not funded by grant.

Conflict of interest:

Author (Sonu Bharti) declares that he has no conflict of interest.

Ethical approval:

This article does not contain any studies with human participants or animals performed by any of the authors.

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