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Macroscopic and Microscopic Evaluation of *Triclisia subcordata* Oliv. (Menispermaceae) towards its Standardization

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: *Triclisia subcordata* Oliv. from the family Menispermaceae has been reported for anti-inflammatory, internal wound healing and anticancer activities in ethnomedicine.

Objectives: The study was designed to carry out pharmacognostical investigation on the fresh, powdered and anatomical sections of the leaf and petiole of *Triclisia subcordata* to determine its macro and microscopic characteristics.

Materials and Methods: Macroscopic and organoleptic evaluations were carried out on the plant using standard procedures. Microscopic evaluation was done using a light microscope to study the epidermis and transverse sections of the leaf and petiole. Chemo-microscopy of plant sample was done for cell inclusions. The solvent fractions were analysed by the use of thin layer chromatoghraphy for the constituents. Powdered sample and leaf extract were subjected to phytochemical screening and to fluorescence analysis using different organic solvents.

Results: Macroscopically, the leaves have a cordate base, an entire margin, caudate apex and a glossy surface. The microscopic evaluation shows the presence of paracytic stomata, unicellular, uniseriated-covering trichomes with an acute apex. The chemo-microscopy revealed the presence of lignin, fats and oils. Phytochemical screening of the powdered leaves revealed the presence of tannins, alkaloids, cardiac glycosides, while flavonoids were absent. The thin layer chromatography showed spots of different retardation factors indicating the presence of different classes of secondary metabolites. The fluorescence analysis also revealed the presence of different colours, which could be employed for the identification of the classes of compounds present in the plant.

Conclusion: The results of the study could be useful in setting some diagnostic indices for the identification and preparation of a monograph of the plant.

Keywords: *Triclisia subcordata*, Standardization, Microscopy, Phyto-constituents

INTRODUCTION

Triclisia subcordata Oliv. is a climbing shrub in the family Menispermaceae. It is a flowering plant of about 1.6 m long. *Triclisia subcordata* is commonly used in the preparation of remedies for diverse disease conditions such as inflammation, gout and internal wound healing (Abo *et al.*, 2011; Odukoya *et al.*, 2012). It is locally known as Kanranjongbon and Alugboran (Yoruba, southwestern Nigeria). Abo *et al.* (2011), Odukoya *et al.* (2012) and Uche *et al.* (2016) reported that the plant also possesses anti-inflammatory, internal wound healing and anticancer activities. The reported pharmacological activities of *T. subcordata* include cytotoxic effects and apoptosis induction by Bisbenzylisoquinoline alkaloids from *T. subcordata* (Uche *et al.*, 2016). Both isochondodendrine and 2'-norcocsuline exhibited potent *in vitro* cytotoxicity in four ovarian cancer cell lines (A2780, Igrov-1, Ovcar-8, and Ovcar-4) with an IC_{50} range of 3.5–17 μM and 0.8–2.9 μM (assessed via sulforhodamine B dye assay), respectively.

Also, Abo *et al.* (2011) reported the antimicrobial potential of extracts of roots of *T. subcordata* and

whole plant of *Heinsia crinita* (Afz) G. Taylor used as components of various herbal portions in southwestern Nigerian ethnomedine to treat acute urinogenital infections and infertility. Extracts of *T. subcordata* and *H. crinita* were tested against strains of *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and fungi including four species of *Candida*. The study showed that extracts of *H. crinita* and *T. subcordata* exhibited profound antibacterial activity against the type and clinical isolates obtained from patients with STD and meningitis.

However, the major setback in promoting the use of medicinal plants is the lack of standardization as well as the confusion in the identification of the plant and their substitutes or adulterants (Annan *et al.,* 2013; Deoda *et al.,* 2013).

Botanical identification of medicinal plants, which are the starting material used in herbal medicines production, is an inevitable step towards their standardization. Pharmacognostical techniques used in standardization of plants include; morphological characteristics involving the test of the taste, odour, shape, texture, size and anatomical evaluation of morphological parts of plants. Parameters such as epidermal characters, vein-islet number, palisade ratio are studied. The stomata are evaluated for size, type, density and index. Phytochemical characters are also assessed to check for the presence of phytochemicals in the plants that are either responsible for their activity or used as chemotaxonomic markers (Sonibare *et al.,* 2005; Hu *et al.,* 2014; Venditti *et al.,* 2016).

The many medicinal applications of *T. subcordata* necessitate its correct botanical identity. The present study was therefore designed to evaluate the morphological parts of *T. subcordata* for diagnostic microscopical and pharmacognostical characters that may be employed for its correct identification

METHODOLOGY

Plant collection

A dirt free sample of *T. subcordata* was collected from the wild forest in Idi Awewe, Oke Ila Orangun, Osun State Nigeria in June, 2017. The plant was identified and authenticated by Mr. Adeyemo and Mr Odewo at Forest Herbarium Ibadan (FHI), where voucher specimen was deposited with voucher number FHI 110974. A copy of the authenticated plant was also deposited at the Department of Pharmacognosy Herbarium, University of Ibadan (DPHUI).

Macroscopic evaluation

For macroscopic evaluation, organoleptic parameters like Size, Length, Width, Shape, Apex, Margin, Base, Petiole, Surface, Colour, Odour and Taste of *Triclisia subcordata* were studied according to methods described by Sowjanya *et al.* (2013).

Microscopic evaluation

Microscopic examination was carried out by taking transverse hand sections of the fresh leaves and stems of *Triclisia subcordata*. Description of the tissues was supplemented with photomicrographs. The anatomical sectioning and microscopy were done according to the methods of Disha and Sharma, (2012).

The fresh leaves of *Triclisia subcordata* which were preserved in 70% ethanol for microscopic evaluation were rinsed severally using distilled water before being sectioned into tiny strands for microscopy using sharp new razor blades.

The sectioned leaf parts were cleared and bleached to remove chlorophyll using sodium hypochlorite. The bleached sectioned leaves were then rinsed severally in distilled water to remove excess sodium hypochlorite. Thereafter, the leaves were stained in Safranin O and counter-stained in Methylene blue for few minutes for clearer and better features.

This was followed by rinsing of the sectioned leaves in gradient dilutions of ethanol from 95%, 70%, 50% to 30% and distilled water for 2 min in each cases.

The sectioned leaves were mounted on a clean slide and glycerin was applied as the mountant, covered with a cover slip with the edges of the coverslip sealed with nail vanish to prevent dehydration and then viewed under the microscope (XSP-103A).

Plant extraction

Air-dried leaves of *T. subcordata* were pulverized and macerated in methanol for 72 h. The procedure was repeated using the same solvent for 48 and 24 h, respectively for effective extraction. The mixture was stirred intermittently using a glass rod to facilitate extraction followed by filtration using Whatman's No. 1 filter paper. The filtrate was evaporated *in vacuo* using a Rotary evaporator (Buchi, rotavapour R-210, Switzerland). The crude extract was stored in a sterile air-tight bottle.

Phytochemical screening

Phytochemical screening of the crude extract was done for the presence of various secondary metabolites (alkaloids, glycosides, tannins, terpenoids *etc*) was performed following standard methods (IJARCS, 2015).

Chemo-microscopy

Chemo-microscopy of *Triclisia subcordata* was done according to the procedure described by International

Journal of Advances in Pharmacy, Biology and Chemistry, (2013).

Triclisia subcordata powdered leaves were treated with Phloroglucinol, Hydrochloric acid, Iodine, Sudan IV reagent to determine the presence of lignified fibres, calcium oxalate crystals, starch grains and fats and oils.

Thin Layer Chromatography

Thin layer chromatographic analysis was carried out using pre-coated TLC plates (Silica gel F_{254} 20×20) cm (Merck Damstadt, Germany). The plates were activated for 1 h before use.

Several mobile phases were tried for the development of the spotted TLC plates, until a suitable mobile phase was obtained.

The developed chromatoplates were allowed to dry, viewed under the UV lamp at 254 and 365 nm and sprayed with chromogenic reagents to check for the presence or absence of phytochemicals as reported in this study.

Fluorescence analysis

For fluorescence analysis the plant powder and methanolic extract of plant were treated with different acids and alkali (50% H_2SO_4, 50% HCl, 50% HNO_3, 1N NaOH, 5% KOH, Acetic acid) separately and then these extracts were observed in visible/day light and UV light at 254 and 365 nm (Kumar *et al.,* 2013, Neyanila *et al.,* 2013).

RESULTS AND DISCUSSION

Systematic approach and well-designed methodologies for the standardization of herbal raw materials and herbal formulations are developed in response to the rapid growing interest in herbal medicine in the world today (Bele and Khale, 2011; Kunle *et al.,* 2012). The availability of many commercial herbal products in the market emphasizes the need for continued effort towards more meaningful and effective standardization of herbal medicines so as to prevent counterfeiting of those medicines (Kunle, 2012). Macroscopic evaluation of vegetable drugs has been the first point of standardizing herbal drugs over the years. This is because of its reproducibility and reliability of its methodology and outcomes. This tool was also employed in this study to set a morphological standard for *T. subcordata* which can be included in herbal pharmacopoeia (Musharaf *et al.*, 2011; Kumari, 2016).

The macroscopic evaluation of *T. subcordata* shows that the plant is a climber found in the southwestern part of Nigeria. It has a caudate leaf apex with a cordate leaf base. It has an entire leaf margin with a simple leaf shape. The adaxial surface is glossy with a little hairy abaxial surface with a pronounced midrib at the back (Fig. 1). The plant has a hard leaf texture and an alternate leaf arrangement on the stem. It has a leaf width of 4.99 cm ± 0.94 with the leaf, petiole and total leaf length of 8.91 cm ± 1.34, 3.61 cm ± 1.17 and 12.52 cm ± 1.75, respectively (Table 1). Organoleptic evaluation of the leaf showed that the leaf colour was army green, hard to touch with an aromatic smell and a bitter taste.

The use of microscopic procedure to check for distinguishing features which differentiates plants from other species and also as a tool for standardization has gained ground over the years. Plant leaves are known to have stomata for respiration and other features such as trichomes, palisade cells and epidermal cells as reported in this work (Patweker *et al.,* 2015). The epidermal features observed on the adaxial and abaxial surfaces of *T. subcordata* include unicellular trichomes, elliptic paracytic stomata, palisade cells and polygonal epidermal cells. The stomata were found to be predominantly present on the abaxial surface of the epidermis, while none was found on the adaxial surface (Fig. 2 A & B). The epidermal cell shape is polygonal on both adaxial and abaxial surfaces with straight anticlinal walls. The epidermal cell length size ranged from 30 -60 µm. The vein termination number also ranged from 6-10. The palisade cells, which were present only on the adaxial surface of the epidermis ranged from 7.0 – 4.5 (Table 2).

The transverse section (TS) of *T. subcordata* leaf passing through the midrib is concave on the abaxial and straight on the adaxial side (Fig. 2 C). It consists of vascular bundles, which are oval-shaped and protected by the bundle sheath. The epidermis is rectangular and trichomes were also observed at the abaxial layer. The transverse section of the petiole of *T. subcordata* shows the presence of vascular bundles and well-developed xylem and phloem vessels. Mucilage canals were also observed at the pith of the petiole (Fig. 2 D).

The result of the chemomicroscopy carried out on powdered sample of the leaf showed the presence of calcium oxalate crystals as well as oils and lignins. However, no free starch was observed. The presence of lignified tissues in the powdered sample of this plant is an evidence of their supportive and protective roles they play in plants.

Herbal medicine is such that consists of several chemical components, which are responsible for their bioactivities, hence phytochemical and TLC analyses are often employed to screen plant extracts and fractions for the evaluation of their constituents. The

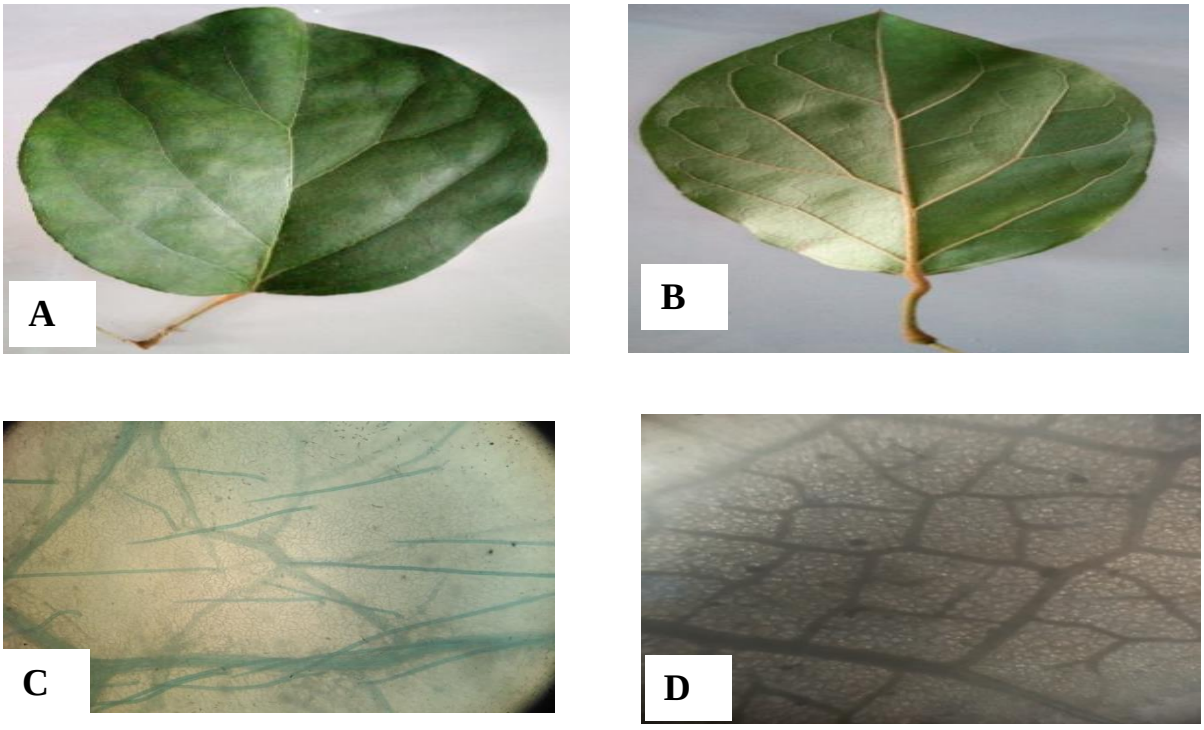

Fig. 1. A & B: Adaxial and Abaxial surfaces of *Triclisia subcordata* leaf showing macroscopic features*;* C: Adaxial epidermis of *T. subcordata* showing covering trichomes (at x100); D: Adaxial epidermis of *T. subcordata* showing venation pattern (at x100)

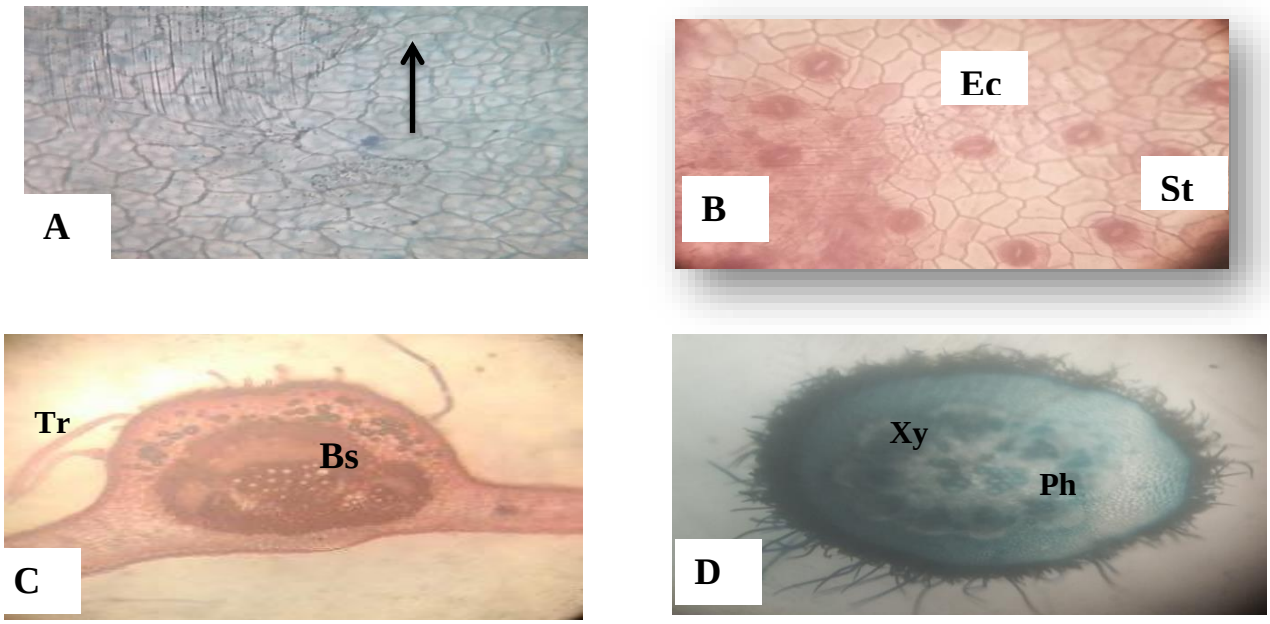

Fig. 2. A & B: Adaxial and Abaxial epidermal surfaces of *Triclisia subcordata* (at x400, arrow showing palisade cells in A); C: Transverse section of *T. subcordata* leaf passing through the mid-rib (at x100); D: Transverse section of *T. subcordata* petiole (at x100). Ec: Epidermal cell, St: stomata, Tr: Trichome, Bs: Bundle sheath, Xy: Xylem, Ph: phloem.

Table 1: Macromorphological Characters of *Triclisia subcordata*

Characters	*Triclisia subcordata*
Habit	Climber
Leaf apex	Caudate
Leaf base	Cordate
Leaf margin	Entire
Leaf shape	Simple
Leaf colour	Army green
Leaf surface	Glossy
Leaf texture	Hard
Leaf arrangement on petiole	Marginal
Leaf arrangement on stem	Alternate
Venation pattern	Fimbrial vein
Petiole	Present; Pulvinate
Midrib	Pronounced at the back
Flower colour	Grey
Fruit shape	Circular
Fruit colour	Grey
Stem colour	Greenish grey
Leaf width (cm)	4.99 ± 0.94
Leaf length (cm)	8.91 ± 1.34
Petiole length (cm)	3.61 ± 1.17
Total leaf length (cm)	12.52 ± 1.75

Table 2: Micromorphological characters on the adaxial and abaxial layers of *Triclisia subcordata*

Characters	*Triclisia subcordata* Adaxial layer	*Triclisia subcordata* Abaxial layer
Stomata	Absent	Present
Stomata type	Absent	Paracytic
Stomata shape	Absent	Elliptic
Epidermal cell shape	Polygonal	Polygonal
Anticlinal walls	Straight	Straight
Crystals (Solitary)	Absent	Present
Epidermal cell size range (µm)	30-60	30-60
Vein termination number	-	6-10
Palisade ratio range	4.50-7.00	-
Trichomes	Present	Absent
Trichomes type	Unicellular, Stellate	-

preliminary phytochemical analysis of the powdered leaf sample and extract of *T. subcordata* showed the presence of different secondary metabolites in varying amounts. The presence of alkaloids was confirmed by the three phytochemical test reagents- Dragendorff that gave orange brown precipitate with the sample, Meyer that produced cream colour precipitate and Wagner that gave reddish brown colouration when added to the sample. Saponin was indicated by frothing that disappeared on standing. Also, tannins and phenolics were confirmed with the appearance of the intense dark brown colouration, while the presence of free anthraquinones was indicated by the delicate rose pink colour. The presence of these metabolites can be used as chemical fingerprint profile of this plant extract (Choudhary and Sekhon, 2011).

The TLC analysis of the crude extract and fractions revealed different spots when viewed in the daylight and under UV at 254 nm and 365 nm, respectively (Fig. 3). This was suggestive of the presence of several compounds in the extract and fractions of *T. subcordata*. Also, the analysis of the extract showed the presence of flavonoids in the fractions and the presence of phenolics and tanins when sprayed with $FeCl_3$ reagent (Fig. 4). Alkaloids were also suspected to be present with coloured spots observed in the ethyl acetate fraction when sprayed with Dragendorff reagent. The fluorescence analysis of the powdered sample of *T. subcordata* showed different colours when treated with the different reagents (**Table 3**). Also, this could be employed as a useful tool for the identification of the classes of compounds in the plant. The macroscopic characters of *Triclisia subcordata* include hard leaf texture, alternate leaf arrangement, pronounced midrib at the lower surface and a circular fruit shape. Microscopically, the leaf is hypostomatic having elliptic paracytic stomata on the abaxial epidermis only. Polygonal epidermal cells on both adaxial and abaxial surfaces had straight anticlinal walls. The sizes of the palisade cells found only on the adaxial surface of the plant ranged from 4.5-7.0. Also, stellate trichomes were observed on the adaxial epidermis.

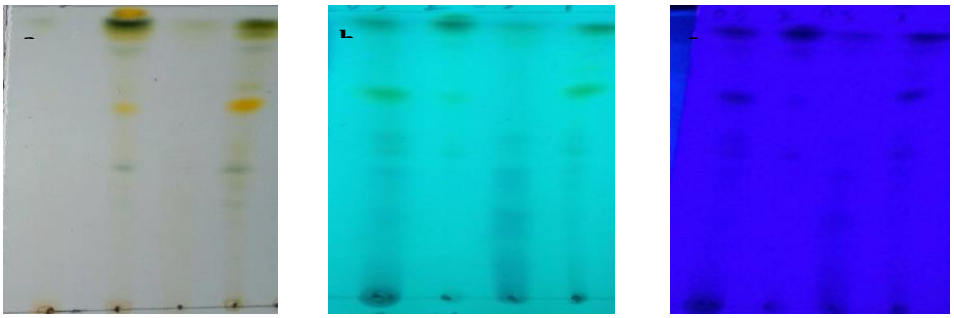

Fig.3. Thin layer chromatographic plates of *Triclisia subcordata* extract and fractions Solvent system: MeOH: *n*-Hex: EtOAc: DCM (0.5:2.0:0.5:1.0)
(a) Visualization at daylight **(b)** under UV lamp at 254 nm **(c)** under UV lamp at 365 nm after development. C: Crude extract, H: *n*-hexane fraction, E: Ethyl acetate fraction, D: Dichloromethane fraction

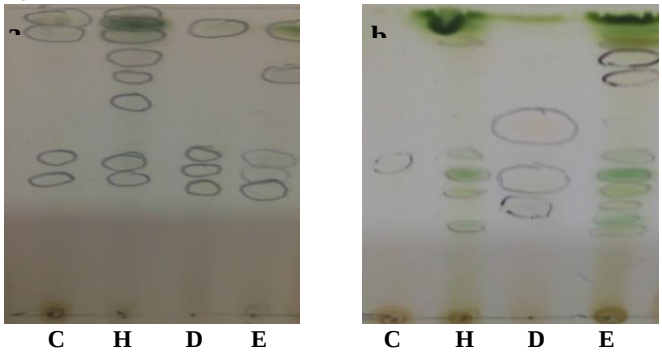

Fig. 4. Thin layer chromatographic plates of *Triclisia subcordata extract* Solvent system: MeOH: *n*-Hex: EtOAc: DCM (0.5:2.0:0.5:1.0)
(a) Visualization at daylight after spraying with $FeCl_3$ **(b)** Visualization at daylight after spraying with Dragendorff reagent. C: Crude extract, H: *n*-hexane fraction, E: Ethyl acetate fraction, D: Dichloromethane fraction

Table 3: Fluorescence characters of powdered leaf sample of *Triclisia subcordata*

Drug + Reagent	Daylight	254 nm	365 nm
TS + Picric Acid	Brownish green	Lemon green	Deep green
TS + Acetic Acid	Brown	Black	Brown
TS + 50% HNO_3	Light brown	Green	Brown
TS + $FeCl_3$	Black	Greenish Black	Black
TS + 50% HCl	Dark brown	Dark green	Dark green
TS + 50% H_2SO_4	Green	Green	Brown

TS: *Triclisia subcordata*

CONCLUSION

The thin layer chromatographic analysis provides a baseline standard regarding the likely classes of compounds in the plant extract. The macroscopic, microscopic and pharmacognostic characters presented in this study could serve as unique diagnostic features for the identification of *Triclisia subcordata*

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