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Research Article

EVALUATION OF *IN VITRO* ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF *AEGLE MARMELOS* LEAF EXTRACTS

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ABSTRACT

Objective: The present study aims to investigate preliminary phytochemicals to determine the total phenolic and flavonoid contents and to undergo *in vitro* antioxidant and anti-inflammatory activities of leaf extracts of *Aegle marmelos*.

Methods: Estimation of total phenols and flavonoids and *in vitro* antioxidant and anti-inflammatory activities of leaf extracts of *A. marmelos was carried out using standard protocols.*

Results: The highest amount of phenolic and flavonoid content was present in aqueous extract (92.08±0.91 mg/g gallic acid equivalent) and (52.73±0.51 mg/g QE), it also proved to have the most potent antioxidant activity at a concentration of 10 µg/ml (45.57±0.27%) with IC_{50} values 190 µg/ml. Ethanol extract exhibited the highest anti-inflammatory activity (88.16±0.25) at 100 µg/ml with IC_{50} values 34.59 µg/ml.

Conclusion: It can be concluded that the tested extracts hold significant antioxidant and anti-inflammatory activities. However, further investigation of purified compounds of *A. marmelos* will enable its medicinal use in the treatment of various disorders.

Keywords: *Aegle marmelos*, Phytochemicals, Antioxidant, Anti-inflammatory.

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INTRODUCTION

Free radical or ions present in biological systems through reactive oxygen species (ROS) leads to many human diseases. The development and existence of an organism in the presence of O_2 are associated with the generation of ROS, even under various physiological conditions [1]. In modern medicinal practice, it is believed to be a difficult role to maintain balance between antioxidant defense system and ROS formation in a healthy living system. Antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene which are synthetically derived are being used as antioxidants since the beginning of this century. Consequently, there is need to find new sources of safe and cost-effective antioxidants from natural origin to make their use in food and pharmaceutical formulations. Inflammation is believed to be a prime physiological defense mechanism that helps the body to prevent itself against chronic infection, burn, dreadful chemicals, allergens, or other noxious stimuli. During an inflammatory response, mediator such as cytokines, interleukin-1, tumor necrosis factor, and interferons is released [2,3]. Use of current inflammatory drugs corresponds to various side effects. Therefore, the development of a strong anti-inflammatory drug with least side effects is essential to improve the quality of life. Medicinal plants for a long time have been a therapeutic source and plant products played an indispensable part in ancient medicine [4].

As estimated by the World Health Organization, about 80% of the world population depends on traditional system of medicine [5]. Since ages, the use of phytoconstituents and secondary metabolites due to the richness of Indian floral biodiversity and the medicinal potential of their extracts are well documented in ancient scriptures for the treatment of various ailments [6]. *Aegle marmelos* is a medium-sized, armed deciduous tree, commonly known as Bael, which belongs to the family Rutaceae. *A. marmelos* is widely distributed in India and is still growing in most of the countries of Southeast Asia. All the different parts of *A. marmelos* tree, namely, stem, bark, root, leaves, and fruits at their maturity stage are medicinally important and widely used as medicine since long time [7,8]. In this connection, the present study focuses on screening of phytochemicals for determination of the total phenolic and flavonoid contents of the plant and to study *in vitro* antioxidant and anti-inflammatory activities of *A. marmelos* leaf extracts.

METHODS

Collection of plant material

A. marmelos was collected from the Dharwad region early morning between 7 am and 8.30 am in the month of February 2016. Dr. Kotresha K, Department of Botany, Karnatak Science College, Dharwad, Karnataka, identified and authenticated the leaves of the plant. The fresh leaves were then washed with tap water; air-dried completely and was then integrated to fine powder. For the further use powder was maintained at −20°C in an airtight container.

Preparation of extracts

About 100 g dried leaves were coarsely powdered and subjected to extraction by Soxhlet extractor. The extraction was done with different solvents in their increasing order of polarity such as petroleum ether, chloroform, methanol, ethanol, and aqueous. For each solvent, fresh plant material was used for the extraction. All extracts obtained were concentrated under reduced pressure at 40°C using a rotary vacuum evaporator and evaporated to dryness.

Preliminary phytochemical analysis

The crude powder of *A. marmelos* leaf extract was qualitatively and quantitatively tested for the presence of numerous secondary metabolites following the standard procedure of Deepti *et al.* [9], namely, alkaloids, flavonoids, glycosides, phenols, lignins, saponins, sterols, tannins, anthraquinone, and reducing sugar.

Estimation of total phenolic content

Folin–Ciocalteu method of Singleton *et al.* with slight modification was used to estimate total phenolic content of the leaf extract [10]. Gallic

acid was used as the standard reference. Approximately 0.5 ml of plant extract was mixed thoroughly with 2 ml of the Folin–Ciocalteu reagent (10 fold) and sodium carbonate solution (8% w/v) was added 4 ml to neutralize the reaction. The reaction mixture was incubated at room temperature for 30 min for color development. The absorbance was measured at 765 nm using ultraviolet–visible spectrophotometer. The total phenolic contents were calculated from the linear equation of standard curve plotted against gallic acid. The total phenolic content was expressed in terms of mg/g gallic acid equivalent (GAE).

Estimation of total flavonoid contents

The total flavonoid content in the leaf extract of *A. marmelos* plant was determined as the method described by Chang *et al*. [11]. A stock solution of quercetin of 1000 µg/ml was prepared by dissolving 100 mg of quercetin in 100 ml of 80% ethanol (v/v); further, various dilutions were made to prepare 6.25, 12.5, 25.0, 50.0, 80.0, and 100 µg/ml solution of quercetin; absorbance was measured at 415 nm; standard curve was plotted with concentrations and absorbance. The dried extracts were again dissolved in 80% ethanol (v/v). 100 mg of each dried extracts was dissolved in 10 ml of 80% ethanol; 1 ml of each sample (separately) was mixed with 3 ml 95% ethanol (v/v), 0.2 ml 10% aluminum chloride, 0.2 ml of 1 mol/liter potassium acetate, and 5.6 ml of double-distilled water to make final volume up to 10 ml, and incubated for 30 min, the absorbance of the reaction mixture was recorded at 415 nm. Distilled water was used as blank solution.

Antioxidant activity using *in vitro* methods

Ferric ion reducing antioxidant power (FRAP) assay

FRAP assay was measured according to the method of Oyaizu with a slight modification [12]. Methanol, ethanol, and aqueous extracts of *A. marmelos* in different concentrations ranging from 100 to 500 µL were mixed with 2.5 ml of 20 mmol/L phosphate buffer and 2.5 ml (1% w/v) potassium ferricyanide, the mixture was allowed to incubate at 50°C for 30 min. Nearly 2.5 ml of (10% w/v) trichloroacetic acid and 0.5 ml of (0.1% w/v) ferric chloride was added to the reaction mixture, incubated again for 10 min. The absorbance was read at 700 nm. Positive standard reference used was ascorbic acid.

Phosphomolybdenum (PM) assay

PM assay for total antioxidant activity was estimated by using standard procedures of [13]. To tubes containing 3 ml of distilled water and 1 ml of molybdate reagent solution, various concentrations of methanol, aqueous and ethanol extract of *A. marmelos* plant ranging from 100 to 500 µl were added. The reaction mixture was incubated at 95°C for 90 min. After the incubation period, tubes were maintained at room temperature for approximately 20–30 min and the absorbance of the reaction mixture was recorded at 695 nm. Ascorbic acid was used as standard reference for the comparison of results.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability assay

The free radical scavenging effect of methanol, ethanol, and aqueous leaf extracts of *A. marmelos* of plant was determined using the free radical DPPH as a reagent, as described by the methods of Rice-Evans *et al*. [14]. Roughly, 1 g of a DPPH radical reagent was completely mixed with 100 ml of sample solution in ethanol or methanol. The tubes containing reaction mixture was incubated in the dark at room temperature for 30 min and the absorbance of the ascorbic acid (standard) and the plant extracts (working) was measured at 517 nm. The scavenging activity of DPPH for each sample was calculated using the following formula:

$$\text{Scavenging activity of DPPH } (\%) = \frac{Ac - At}{Ac} \times 100$$

Where, Ac is considered to be the absorbance of the control reaction (100 µl of ethanol with 100 µg of the DPPH solution) and At as the absorbance of the test sample. The results of the experiment were noted in triplicate readings. The concentration of sample required to inhibit 50% of the DPPH free radical solution is studied as IC_{50} values. Lower the absorbance, the higher will be the free radical activity of the reaction mixture.

Evaluation of in vitro anti-inflammatory activity

The protein denaturation method as described by Padmanabhan and Jangle [15] with slight modifications was used to determine the anti-inflammatory activity of methanol, ethanol, and aqueous extracts of plant *A. marmelos* and a nonsteroidal anti-inflammatory drug diclofenac sodium was used as a standard reference drug. Around 2 ml of reaction mixture consisting of different concentrations of extracts (100–500 µg/ml) and standard diclofenac sodium (100–500 µg/ml), and phosphate-buffered saline (pH6.4) of 2.8 ml was mixed roughly with 2 ml of egg albumin which was retained from fresh hen's egg and incubated for 15 min at (27±1)°C. Protein denaturation was developed by incubating the reaction mixture at 70°C in a hot water bath for 10 min. The absorbance was measured at 660 nm and double-distilled water was used as blank. An experiment was performed in three sets and mean with standard deviation was calculated. The inhibition of protein denaturation was calculated in percentage using the below given formula:

$$\text{Inhibition } (\%) = \frac{At - Ac}{Ac} \times 100$$

Where, At=Absorbance of test sample and Ac=Absorbance of control.

Statistical analysis

Results obtained from the present study were calculated using SPSS software (SPSS Statistics Version 20.0). One-way analysis of variance was performed to evaluate the significant differences between sample means, with significant level being considered at $p<0.05$. Mean comparisons were assessed by Duncan's test, with values expressed as mean±standard deviations. All the data reported are the mean values of triplicates (n=3), obtained from the different runs.

RESULTS

Total yield of crude extract

The total yield of crude leaf extracts of *A. marmelos* plant using various solvents, namely, petroleum ether, chloroform, methanol, ethanol, and aqueous was 5.6 g, 13.5 g, 15.0 g, 19.2 g, and 24.4 g w/w, respectively (Table 1).

Phytochemical analysis

Leaf extracts of *A. marmelos* plants showed the presence of a wide range of rich secondary metabolites. Alkaloids, phenols, and flavonoids were highly present in chloroform; methanol, ethanol, and aqueous extracts, glycosides, sterols, and tannins were moderately present in chloroform; methanol, ethanol, and aqueous solvents, and lignins were absent in all the five solvents, saponins in chloroform; and methanol, ethanol, and aqueous extracts, sterols, and tannins were present in ethanol and

Table 1: Total yield of crude extract of *Aegle marmelos* leaves

Leaf material (g)	Solvent (1000 ml)	No. of cycles	Rota evaporation (ml)	Drying days	Total yield (g)
100	Petroleum ether	35 min/12 cycles	100	1	5.6
100	Chloroform	40 min/12 cycles	120	4	13.5
100	Methanol	45 min/12 cycles	115	2	15.0
100	Ethanol	47 min/12 cycles	120	3	19.2
100	Aqueous	92 min/12 cycles	135	4	24.4

Table 2: Phytochemical screening of *Aegle marmelos* leaves extracts

Constituents	Test	Pet-ether	Chloroform	Methanol	Ethanol	Aqueous
Alkaloids	Iodine's	–	–	–	–	–
	Wagener's	–	+	+	+	++
	Dragendroff's	+	+	+	+	++
Flavonoids	Pew's	–	+	+	++	++
	Shinoda	+	+	++	++	++
	NaOH	+	+	++	++	++
Glycosides	Keller-Killani	–	+	+	+	+
	Glycosides	–	+	+	+	+
	Conc. H_2SO_4	–	+	++	++	+
	Molisch's	–	+	++	++	++
Phenols	Ellagic acid	–	+	++	++	++
	Phenols	–	+	++	++	++
Lignins	Lignin	–	–	–	–	–
	Labat	–	–	–	–	–
Saponins	Foam	–	+	+	+	+
	Hemolysis	–	+	+	+	+
Sterols	Liebermann–Burchard	–	–	–	–	–
	Salkowski's	+	–	–	+	+
Tannins	Gelatin	–	+	+	+	+
	Lead acetate	+	–	+	++	+
Anthraquinone	Bomtrager's	–	–	–	–	–
Phlobatannin		–	–	–	–	–
Reducing sugar		–	–	–	–	–
Volatile oil		–	–	–	–	–

–: Absent, +: Moderate, ++: High presence

Table 3: Total phenolic content of *Aegle marmelos* leaf extracts

Leaf extracts	TPC1	Units equivalent	R^2 values
Methanol extract	72.58±0.58	mg/g GAE	0.977
Ethanol extract	89.64±0.71	mg/g GAE	0.986
Aqueous extract	92.08±0.91	mg/g GAE	0.991
Gallic acid	98.43±0.67	mg/g GAE	0.995

GAE: Gallic acid equivalent

Table 4: Total flavonoid content of *Aegle marmelos* leaf extracts

Leaf extracts	TFC	Units equivalent	R^2 values
Methanol extract	46.32±0.94	mg/g QE	0.981
Ethanol extract	48.54±0.84	mg/g QE	0.988
Aqueous extract	52.73±0.51	mg/g QE	0.991
Quercetin	59.64±0.74	mg/g QE	0.997

aqueous extracts. Anthraquinone, phlobatannins, and reducing sugar were absent in all extracts and volatile oil was present in chloroform and ethanol extract (Table 2).

Total phenolic content

Total phenolic content estimated in methanol, ethanol, and aqueous extracts of *A. marmelos* is shown in Table 3. The amount of total phenolic content was calculated using the R^2 values from the equation (y=mx+c), however, y is absorbance of unknown, m is value from the graph, x is concentration, and a is value from the graph and the results were expressed as mg/g GAE, respectively.

Total flavonoid content

Total flavonoids present in methanol, ethanol, and aqueous extracts of *A. marmelos* are shown in Table 4. The R^2 values from the equation (y=mx+c), however, y – absorbance of unknown, m – value from the graph, x – concentration, and a – value from the graph was used to calculate total flavonoid content and the results were expressed in terms of mg/g QE, respectively.

FRAP assay

In the present study, methanol, ethanol, and aqueous extracts were subjected to FRAP assay with gallic acid (standard). The results reveal that aqueous extract showed higher activity than the ethanol and methanol extract followed by ethanol extract (Fig. 1) which was comparable to standard ascorbic acid.

PM assay

Methanol, ethanol, and aqueous extracts were determined by PM assay along with standard ascorbic acid. The comparatively aqueous extract showed more activity than ethanol and methanol extract when compared with standard ascorbic acid (Fig. 2).

DPPH assay

The antioxidant activity of leaves of *A. marmelos* plant extract methanol, ethanol, and aqueous was studied using DPPH radical scavenging assay. The results in the leaf extracts concentration are shown in Table 5. The antioxidant capacity of the aqueous extract showed promising results when compared with ascorbic acid a standard antioxidant.

***In vitro* anti-inflammatory assay**

Various concentrations of aqueous, ethanol, and methanol extracts of *A. marmelos* were subjected to anti-inflammatory activity of protein denaturation. Aqueous, ethanol, and methanol extracts showed a significant difference when compared with the standard drug at a concentration of 100 µg/ml. The ethanol extract showed promising results, then methanol followed by aqueous extract when compared with diclofenac sodium (Table 6).

DISCUSSION

Plants and plant-based drugs as herbal medicine in the treatment of various diseases and infections are known since long back. Earlier studies on *A. marmelos* report that the preliminary phytochemical investigations revealed the presence of carbohydrates, phenols, flavonoids, tannins, alkaloids, betacyanins, quinones, coumarins, steroids, and glycosides in large quantity in the ethanolic extract than any other solvent [16], with this connection the present study reports to have presence of phenols, flavonoids, and tannins in all solvent extracts except petroleum ether extract, whereas the presence of oils and fats was absent in all solvent extracts. Substantially, phenols and phenolic compounds are more capable in donating a hydrogen atom to free radicals, which help in retarding the lipid peroxidation process. Medicinal plants containing polyphenolic compounds have a lead role in biological activities such as antioxidant, antitumor,

Table 5: Percentage inhibition of DPPH free radical scavenging activity of *Aegle marmelos* leaf extracts

Concentration percentage Inhibition of plant extracts	10 µg/ml	20 µg/ml	30 µg/ml	40 µg/ml	50 µg/ml	IC_{50} value µg/ml
Methanol extract	38.10±0.77[a]	49.08±0.19[b]	54.59±0.15[c]	74.76±0.25[d]	83.50±0.07[e]	273
Ethanol extract	42.16±0.29[a]	52.37±0.27[b]	60.27±0.20[c]	78.30±0.28[d]	88.04±0.17[e]	252
Aqueous extract	45.57±0.27[a]	58.44±0.41[b]	69.17±0.31[c]	80.18±0.17[d]	91.21±0.21[e]	190
Ascorbic acid	51.27±0.42[a]	62.27±0.19[b]	75.14±0.13[c]	90.40±0.36[d]	94.99±0.30[e]	150

All results expressed are mean of three individual replicates (n=3±standard deviations) on dry weight basis. Mean values followed by different letters in a row are significantly different (p<0.05) from each other

Table 6: *In vitro* anti-inflammatory effect of *Aegle marmelos* leaf extracts

Treatment	Concentration (µg/ml)					IC_{50} value (µg/ml)
	20	40	60	80	100	
Methanol extract	36.13±0.15[a]	48.29±0.25[b]	58.26±0.14[c]	65.21±0.14[d]	79.14±0.90[e]	46.03
Ethanol extract	42.58±0.46[a]	51.32±0.26[b]	65.10±0.25[c]	79.20±0.12[d]	88.16±0.25[e]	34.59
Aqueous extract	33.10±0.98[a]	37.31±0.33[b]	51.39±0.16[c]	59.15±0.90[d]	66.47±0.39[e]	61.56
Diclofenac sodium	48.78±0.21[a]	59.11±0.11[b]	70.42±0.18[c]	82.14±0.52[d]	96.11±0.55[e]	24.05

All results expressed are mean of three individual replicates (n=3±standard deviations) on dry weight basis. Mean values followed by different letters in a row are significantly different (p<0.05) from each other

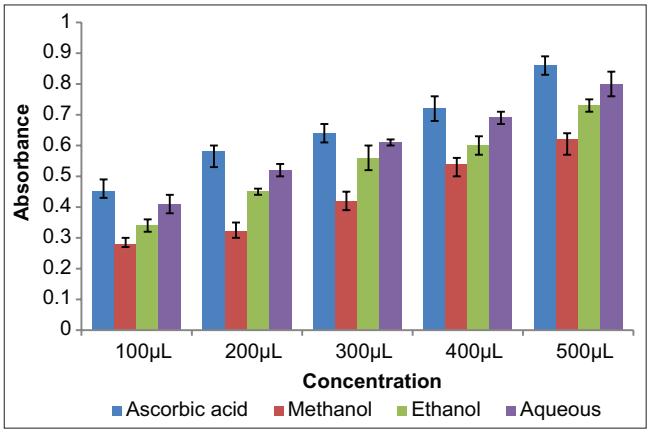

Fig. 1: Ferric ion reducing antioxidant power assay for extracts of *Aegle marmelos*

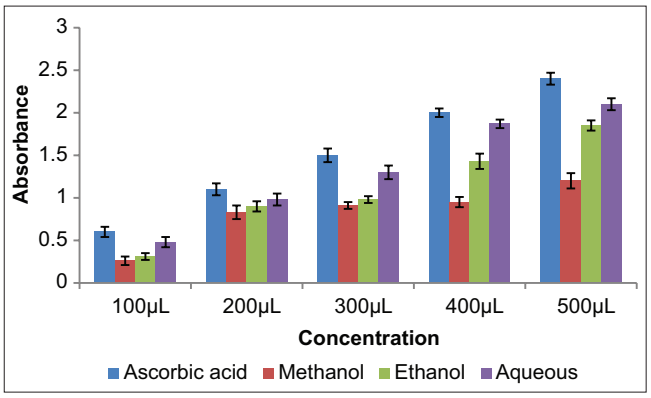

Fig. 2: Phosphomolybdenum assay for leaf extract of *Aegle marmelos*

anti-inflammatory, and anticancer [17,18]. The previous study reports that the total phenolic contents of *A. marmelos* seeds (GAEs, mg/g) in methanol extract and aqueous extract were estimated to be 65.20±0.2 and 27.12±0.6 mg/g, respectively [19], whereas in the present study the total phenolic contents of A. marmelos leaf extract showed good quantity of phenolic compounds in methanol, ethanol, and aqueous extracts. Aqueous extract exhibited the highest amount of phenolic content (92.08±0.91 mg/g GAE) as compared to ethanol (89.64±0.74 mg/g GAE) and methanol (72.58±0.58 mg/g GAE) extracts. Estimation of total flavonoid content in earlier study on *A. marmelos* ethanolic leaf extract has been performed using a colorimetric method and the flavonoid content of *A. marmelos* ethanolic leaf extract was 82.486±1.45 mg/g of extract [16]. However, the current state of work reports that estimation of total flavonoids in the *A. marmelos* leaves of aqueous extract showed the good amount of flavonoid content (52.73±0.51 mg/g QE).

To measure the reducing potential of the extracts and standard, FRAP assay is studied. It is estimated when a reduction reaction occurs, where Fe (III) gets reduced to form Fe (II) forming a color complex at 510 nm of absorption maxima due to the action of phenols, polyphenols, and flavonoids present in the plant. Absorption rate is directly proportional to the rate of reducing potential. Higher the absorbance more will be the reducing capacity of the antioxidants [13]. In comparison with standard ascorbic acid, the present work shows that aqueous extract exhibited higher antioxidant activity than ethanol and methanol extract. PM method is used to estimate the reduction rate among antioxidant and molybdenum ligand. Absorbance obtained is directly proportional to the antioxidant activity, thus giving reducing potential of the various plant extracts [14]. In the present study, aqueous extract exhibited good results when compared to ethanol and methanol extracts. Earlier reports on the DPPH radical scavenging activity of the leaf extract of *A. marmelos* (AME) showed that the IC_{50} values were found to be 160.47±8.51 µg/ml at 10 µg/ml [20]. Aqueous leaf extract in the current study showed better results with (45.57±0.27) radical scavenging activity for a minimum concentration of 10 µg/ml with IC_{50} values 190 µg/ml as compared to ethanol and methanol extracts. Aqueous leaf extract of *A. marmelos* proved to be more potent in the DPPH radical scavenging activity, in FRAP and PM assay when compared with ethanol and methanol.

Nonsteroidal anti-inflammatory drugs available in the market are basically used to treat inflammatory diseases, but several side effects such as gastric irritation and stomach ulcer are related to the use of these drugs [21]. Different extracts were seen effective in inhibiting heat-induced albumin denaturation in a previous study in *A. marmelos* plant with IC_{50} values 95.64 µg/ml at 100 µg/ml concentration in cold aqueous extraction [22]. Hence, in the present study, aqueous, ethanol, and methanol extracts of *A. marmelos* leaves were subjected to anti-inflammatory assay with diclofenac sodium as a reference drug. Comparing with reference drug, ethanol extract showed the highest inhibitory activity (79.14±0.90) at 100 µg/ml with IC_{50} values 34.59 µg/ml, whereas the aqueous and the methanol extract exhibited moderate inhibitory activity.

CONCLUSION

The present work is a comparative study of qualitative and quantitative estimation of secondary metabolites along with antioxidant and anti-inflammatory activities of *A. marmelos* leaf extract. Aqueous extract of leaves of *A. marmelos* has shown significant antioxidant activities compared to ethanol and methanol extract. In anti-inflammatory activity, ethanol extract exhibited good results than the other two extracts. The present study describes that methanol, ethanol, and aqueous extracts are more effective against antioxidant and anti-inflammatory activities, respectively. The current study supports that *A. marmelos* can be used as one of the plants in formulation of herbal medicine. Thus, a further study on screening, isolation and purification of secondary metabolites is needed to validate for their pharmacological applications.

AUTHOURS' CONTRIBUTIONS

The author declares that all the named authors have contributed equally to this article.

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CONFLICTS OF INTEREST

Nil.

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