

Lonicera quinquelocularis: A rich source of antioxidant for protection against chronic diseases

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Abstract: The purpose of the research work was to examine the *in vitro* antioxidant activity of the different aqueous and organic fractions of *Lonicera quinquelocularis* Hardwicke. The methanol extract was dissolved in distilled water and fractionated with *n*-hexane, chloroform, ethyl acetate and *n*-butanol, successively. The antioxidant potential of the remaining aqueous and organic fractions was determined by using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity, total antioxidant activity, ferric reducing antioxidant power (FRAP) assay, ferric thiocyanate assay and total phenolics method. Among these fractions ethyl acetate fraction displayed the maximum antioxidant activity with IC₅₀ of (11.13±0.12µg/ml). It also exhibited the highest total antioxidant activity (0.595±0.00), FRAP value (128.2±4.54µg/mL) and total phenolic contents (66.89±7.73µg/g) as compared to other organic fractions. Phytochemical investigation of the above mentioned fractions showed the presence of flavanoids, phenolics, terpenoids, sugars, alkaloids, tannins, saponins and cardiac glycosides in appreciable amounts, which have major contribution towards antioxidant activity.

Keywords: *Lonicera quinquelocularis*, DPPH assay, total antioxidant activity, FRAP value, total phenolics, inhibition of lipid per oxidation (%).

INTRODUCTION

Since ancient times, the medicinal importance of plants has been known, due to their effective antioxidant activities. Antioxidants prevent oxidative damage caused by free radicals by reacting with them and also act as chelators and oxygen scavengers (Patel *et al.*, 2010).

A lot of work has been done on oxidative stress study which is due to reactive oxygen species that are the root cause of many unrelieved diseases, such as atherosclerosis, cardiovascular diseases, cancer, diabetes mellitus, neurodegenerative diseases and ageing (Azizova, 2002). Free radicals emerge constantly in every cell as it is a part of normal cellular function. Excess of free radicals plays a role in many diseases. Antioxidants avoid free radical damage by reducing the formation of radicals, scavenging them, or by decomposing them (Young and Woodside, 2001). Non-enzymatic antioxidant defense system of the body comprises of antioxidants, such as vitamins (C, E, K) and glutathione (Chae *et al.*, 2004). The antioxidants can be synthetic or natural. Food industry utilizes synthetic antioxidants in protecting food from oxidation and spoilage. However, some of synthetic antioxidants, e.g. butylated hydroxyanisole and butylated hydroxytoluene, have been found to be injurious for health due to their potential carcinogenic activity (Kahl and Sies, 1991 and Botterweck *et al.*, 2000). Natural antioxidants in fruits and vegetables are the least key

factors associated with chronic diseases, such as heart and cancer syndromes (Duthie *et al.*, 2000).

Phytochemicals mainly include phenolic compounds, which are major dietary constituents and are widely distributed in plants and have high antioxidant, free radical scavenging capacity, anti-inflammatory and anticarcinogenic effects (Miller, 1996). Flavanoids are a group of phenolic compounds, which have gained importance because they are potential agents for improving nutritional significance and retarding oxidative degradation of lipids (Franker, 1995).

The redox property, hydrogen donating capacity and quenching ability of singlet oxygen species are major key factors for the antioxidant capacity of phenolics. Apart from antioxidant capacity, phenolics exhibit an extensive range of medicinal properties e.g. anti-inflammatory, anti-microbial, anti-thrombotic, cardio-protective effects (Balasundram *et al.*, 2006).

Recently, researches on antioxidant activities of medicinal plants have extraordinarily increased by virtue of their potential high antioxidant capacity and positive health benefits (Katalinic *et al.*, 2006 and Liu *et al.*, 2008). Therefore, screening of plants possessing strong antioxidant activities is very important. The crude extract if nontoxic, further isolation of components is not necessary because its activity might be due to synergic effects of Phytochemicals in the extract (Liu, 2003). Plants mainly consist of components like flavanoids,

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phenolic acids, anthocyanin, etheral oils and tannins, which are mostly biologically active (Korotkova *et al.*, 2003).

Lonicera quinquelocularis Hardwicke is locally known as Bakhur. Translucent Honeysuckle belongs to Caprifoliaceae (Honeysuckle family). It is a small family comprising of about 12 genera and 450 species (Akhtar, 1986). It occurs mainly in the temperate regions of the northern hemisphere. In Pakistan it is represented by 4 genera and 27 species. *Lonicera quinquelocularis* is a large hairy shrub, which grows up to 6 meters tall with hollow pith and pubescent to willow branches. Berries occur in pairs, ovoid and white in color. It is widely distributed in Afghanistan, Pakistan, India, Nepal, Bhutan and Tibet. It is one of the commonest and largest species, which grows in dry lands between 750-3000m in North West Himalaya (Ajaib *et al.*, 2010). Powdered leaves of the plant are used for healing wounds and plant material is also used as fuel (Kumar *et al.*, 2000 and Ahmed *et al.*, 2013).

The above data motivated us to explore new resources of natural antioxidants by evaluating various aqueous and organic fractions of selected plant.

MATERIALS AND METHODS

Plant material

The plant, *Lonicera quinquelocularis* Voucher Specimen No. GC. Herb. Bot. 820 was collected from Azad Jammu Kashmir in December 2010. It was recognized by M. Ajaib, a taxonomist in the Department of Botany GC University, Lahore.

Extraction and partitioning

The whole plant weighing 1 kg was dried under shade and grinded in powder form. The powdery material was extracted at room temperature with methanol (3L×5). The extract was evaporated on rotary evaporator to obtain residue weighing 690g, which was further dissolved in distilled water (1L) and fractionated with *n*-hexane (1L×4), chloroform (1L×4), ethyl acetate (1L×4) and *n*-butanol (1L×4), respectively. These organic fractions and remaining water fraction was concentrated separately on rotary evaporator and the residues obtained after evaporation were used for evaluation of their *in vitro* antioxidant potential.

Chemicals and standards

DPPH[•] (1,1-Diphenyl-2-picrylhydrazyl radical), TPTZ (2,4,6-Tripyridyl-s-triazine), Trolox, Gallic acid, Follin-Ciocalteu reagent and BHT (Butylated hydroxytoluene) were obtained from Sigma Chemical Company Ltd. (USA). *n*-hexane, chloroform, ethyl acetate, *n*-butanol, sulphuric acid, sodium phosphate, ammonium molybdate, ferric chloride and ferrous chloride from Merck (Pvt.) Ltd. (Germany).

Phytochemical screening

The phytochemical screening of various components of plant extracts were identified by using standard procedures as illustrated by (Aylooa *et al.*, 2008 and Trease and Evans, 1989).

Test for reducing sugars (Fehling's test)

The plant extracts (0.5g) solubilized in 5ml of water was boiled with Fehling's solution A and B in a test tube. Formation of red precipitates indicated the presence of sugars.

Test for terpenoids

Presence of terpenoids was confirmed using two different methods. First, 3ml concentrated H₂SO₄ was added carefully in 0.5g of each of extracts in 2ml chloroform in a test tube. Reddish brown coloration at interface indicated terpenoids. Second, sample solutions were spotted on TLC card and then sprayed with ceric sulphate solution and further heated on TLC heater. Brown color spots appeared on TLC card, which indicated the presence of terpenoids.

Test for flavonoids

Flavonoids can be detected in plant extracts by four methods. First, to a portion of aqueous extracts 5ml ammonia and concentrated sulphuric acid was added. Disappearance of yellow coloration on standing confirmed flavanoids. Second, in a sample solution few drops of 1% Aluminium solution were added. The presence of flavonoids was indicated by the appearance of yellow coloration. Third, a Benedict's reagent was sprayed on TLC card having sample spots of extracts. Green fluorescence in UV light confirmed flavanoids. Fourth, lead acetate was sprayed on TLC card having spots of sample solution. Flavonoids presence was detected by green fluorescence in UV light.

Test for saponins

In a test tube 0.5g of extract was dissolved in 5ml distilled water. Few drops of olive oil were added further. Vigorous shaking of solution till the formations of emulsion indicated the presence of saponins.

Test for tannins

2ml volume of sample solution was warmed with 5ml of *n*-butanol and HCl. Red precipitates indicated the presence of tannins.

Test for alkaloids

Dragendroff's reagent was sprayed on TLC card having spots of the sample. Orange coloration indicated the presence of alkaloids.

Test for cardiac glycosides (Keller-Killiyani test)

5ml of water was added in 0.5g of extract 2mL of glacial acetic acid having 1 drop of ferric chloride solution was

added drop wise in the diluted extract. This was underplayed 1ml of concentrated sulphuric acid. At interface the formation of brown ring indicated the presence of deoxy sugar. Below the brown ring, a violet ring may appear, while in acetic acid layer above brown ring, a greenish ring may form and slowly spread throughout this layer.

DPPH Radical scavenging activity

The DPPH assay for various fractions of plant were examined by comparing with the known antioxidant, butylated hydroxytoluene (BHT) using the method reported by (Lee and Shibamoto, 2001). Different concentrations (1000, 500, 250, 125, 60, 30, 15, 8µg/mL) of different extracts of the plant was dissolved in 3 ml of methanolic solution of 0.1mM DPPH. The contents were shaken vigorously and were allowed to stand for one hour at room temperature. After 1h the absorbance was measured at 517nm. Methanol was used as a blank. Lowering in the absorbance indicated by spectrophotometer higher the free radical scavenging activity. The percent of DPPH de-coloration of different extracts was calculated using following:

$$\text{Antiradical activity} = \frac{A_{\text{control}} - A_{\text{extracts}}}{A_{\text{control}}} \times 100$$

Every extract was assayed thrice and mean values for each were calculated.

Total antioxidant activity by phosphomolybdenum method

The total antioxidant activities of various fractions of plant were evaluated by method reported by (Prieto and Pineda, 1999). 500µg/mL of each extract were added in 4mL of reagent solution (0.6 M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) in capped test tubes. The blank solution consists of 4mL of reagent solution. The test tubes were incubated for 90 min at 95°C in water bath. After incubation the test tubes were cooled down to room temperature and absorbance was measured at 695nm on spectrophotometer. The antioxidant activity was expressed relative to that of butylated hydroxytoluene (BHT). All the extracts were assayed in triplicate and their mean values were calculated.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was done according to the reported method of (Benzie and Strain, 1996) with some variation. The stock solutions constitutes 300mM acetate buffer (3.1 g CH₃COONa.3H₂O and 16mL CH₃COOH) maintained at pH 3.6, 10mM TPTZ (2,4,6-Tripyridyl-s-triazine) solution was taken in 40mM HCl and 20mM FeCl₃.6H₂O solution. The fresh solution was prepared by mixing 25mL acetate buffer, 2.5mL TPTZ solution and 2.5mL FeCl₃.6H₂O solution, which was warmed at 37°C before use. The solutions of plant extracts and trolox were formed in methanol (250µg/mL). 10µL of each of sample solution and BHT solution were taken in test tubes and

2990µL of FRAP solution was added in each test tube. The test tubes were kept in the dark for 30 min. After 30min the readings of the colored ferrous tripyridyltriazine complex was taken at 595nm. The FRAP values were calculated as µmoles of trolox eq/mL of each extract by computing with standard calibration curve build for different concentrations of trolox. Results were expressed in TE µg/mL.

Total phenolic contents

Total phenolics content of various fractions of plant under study were determined by the method used by (Makkar *et al.*, 1993). 0.1mL (0.5mg/mL) of sample was added in 2.8mL of 10% Na₂CO₃ and 0.1mL of 2 N Folin-Ciocalteu reagent. After 40 min absorbance was measured at 725 nm on spectrophotometer. Total phenolics were determined as mg of gallic acid eq/g. of sample by computing with standard calibration curve constructed for different concentrations of gallic acid. The standard curve was linear between 50mg/mL to 400mg/mL of gallic acid. Results were expressed in GAE mg/mL.

STATISTICAL ANALYSIS

All the calculations were made in triplicate and statistical analysis was performed using MS excel 2007. Results are shown as average ± SEM.

RESULTS

The phytochemical screening was performed on all the fractions of the plant under study and results are tabulated in (table 1). It was observed from the results that chloroform, ethylacetate and *n*-butanol fractions carried flavanoids and phenolics, as well as alkaloids, which were absent in *n*-hexane fraction. Cardiac glycosides were present in all fractions but chloroform, ethyl acetate and *n*-butanol soluble fraction showed them in higher concentration and *n*-hexane fraction had least in them. Tannins and sugars were present in all fractions except *n*-hexane soluble fraction whereas *n*-butanol contained sugars in appreciable amount. Most of the terpenes were present in *n*-hexane soluble fraction and lesser in all other fractions. Saponins were also present in all fractions except *n*-hexane but in smaller amount. The remaining aqueous layer indicates the presence of phenolics, flavanoids, cardiac glycosides, tannins, saponins and alkaloids, which are significantly biologically active components. The study also revealed that the plant extracts were found to possess potent antioxidant activity.

DISCUSSION

Free radicals e.g. super oxides, hydroxyl radicals and other reactive species e.g. H₂O₂ and hypochloric acids are produced during aerobic metabolism in body. Over production of ROS or inadequate antioxidant defense can

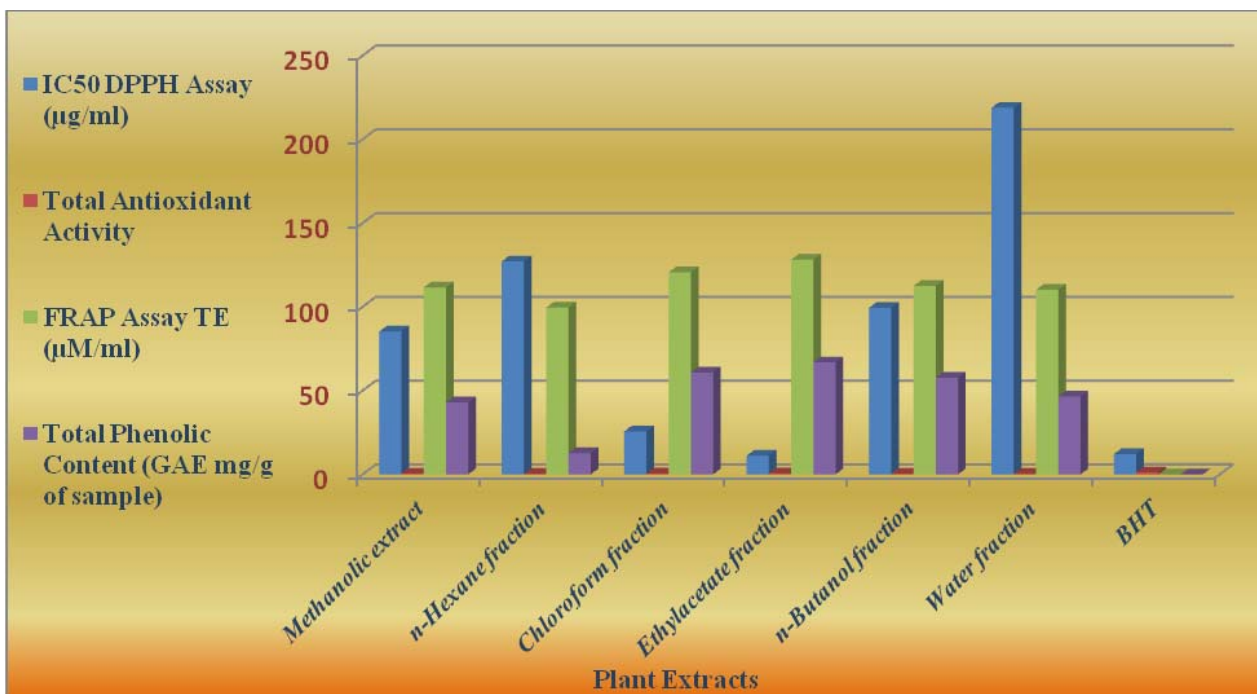


Fig. 1: IC_{50} , total antioxidant activity, FRAP values and total phenolics content's inhibition values of *L. quinquelocularis* extract and fractions.

cause oxidative damage to various biomolecules. Free radicals have the potential to detoxify the Reactive Oxygen Species (Halliwell and Gutteridge, 1998). DPPH assay was performed to investigate the scavenging activities of the crude extracts of natural products. DPPH forms a stable diamagnetic molecule by accepting an electron of H-radical.

In various plant fractions antioxidants scavengers DPPH radical and *in vitro* transforms it to reduced DPPH, the color changes from purple to yellow after reduction and this also results in decrease of absorbance, which is observed at wavelength 517nm. The tendency of scavenging free radical increased with increase in %age of free radical inhibition (Haung *et al.*, 2005).

The present research utilizes DPPH assay for the determination of free radical scavenging activity of various extracts from *Linocera quinquelocularis*. The various fractions of the plant under study reduced DPPH radicals appreciably. Values of percent scavenging of DPPH radicals have been shown in (table 2). Ethyl acetate fraction showed highest value of % inhibition of DPPH radical (98.00%), The IC_{50} value for every sample is the concentration of sample causing fifty per cent inhibition of absorbance. It was determined and shown in (table 2, fig. 1). Lower value of IC_{50} reflects increased antioxidant activity of the various extracts (Habla *et al.*, 2010 and Abbasi *et al.*, 2011).

Ethyl acetate fraction showed the highest antioxidant activity, IC_{50} (11.13±0.12µg/ml), followed by chloroform fraction (IC_{50} 25.6±1.66µg/ml), crude methanolic extract (IC_{50} 85.31±0.25µg/ml), *n*-butanol fraction (IC_{50} 99.43±2.91µg/ml), *n*-hexane fraction (IC_{50} 127.14±1.65µg/ml), and aqueous fraction (IC_{50} 218.9±7.21µg/ml) respectively, relative to butylated hydroxytoluene (BHT), which have IC_{50} 12.1±0.92µg/ml which clearly depicts that chloroform fraction possess maximum antioxidant potential which may be attributed to the synergic effects of various natural products i.e. alkaloids, phenolics and flavanoids which also coincides with the phytochemical screening data as illustrated in (table 1).

It is a known fact that phenolic compounds contribute in quality and nutritional value of food by modifying color, taste, smell, and flavor and also provides health benefits. Antioxidants are plant's defense system, which acts against reactive oxygen species (Sengul *et al.*, 2009). Constituents of medicinal plants have been credited in control of various diseases e.g. polyphenolic compounds (Ivanova *et al.*, 2005).

These compounds exhibit anti-allergic, anti-inflammatory, anti-microbial, anti-thrombotic, cardio-protective and vasodilatory properties. Polyphenolic compounds plays vital role in stabilizing lipid oxidation, the phenolic content of plant materials is associated with antioxidant activity (Demairy *et al.*, 2009). It is determined in different fractions, expressed as mg of Gallic acid equivalent (GAES) mg/g of fraction.

The total phenolic content is of various extracts of selected plant was measured at 725nm spectrophotometrically against Gallic acid which was taken as standard. All the determinations were performed in triplicate. Amongst different fractions (table 3) ethyl acetate soluble fraction showed the highest phenolic

content (66.89 ± 7.73), followed by chloroform soluble fraction (60.80 ± 4.12), *n*-butanol fraction (57.76 ± 8.02), aqueous fraction (46.45 ± 15.63), methanolic extract (42.97 ± 1.6) and lowest phenolic contents were found in *n*-hexane soluble fraction (12.7 ± 3.01).

Table 1: Phytochemical screening of *Lonicera quinquelocularis*

S No.	Test	<i>n</i> -Hexane fraction	Chloroform fraction	Ethylacetate fraction	<i>n</i> -Butanol fraction	Methanol fraction	Remaining aqueous fraction
1.	Alkaloids	-	++	++	++	+	+
2.	Terpenoids	+++	++	++	++	+	++
3.	Saponins	-	+	+	+	+	++
4.	Tannins	-	+	+	+	+	+
5.	Sugars	-	++	+++	++++	+++	+++
6.	Phenolics	-	+++	+++	++	+	++
7.	Flavanoids	-	++	++++	+++	+	+++
8.	Cardiac Glycosides	+	+	++	+++	+	++

Note: '+' represents presence and '-' represents absence.

Table 2: Free radical scavenging activity of methanolic extract of *Lonicera quinquelocularis*, its organic fractions and aqueous fraction using 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH).

S. No.	Sample	Concentration in Assay ($\mu\text{g/ml}$)	% age Scavenging of DPPH radical \pm S.E.M ^{a)}
1	Methanolic extract	500	83.10 \pm 0.10
		250	84.20 \pm 0.34
		125	58.80 \pm 0.23
		60	44.00 \pm 0.11
		30	39.00 \pm 0.11
2	<i>n</i> -Hexane soluble fraction	1000	85.70 \pm 0.08
		500	69.83 \pm 0.41
		250	57.43 \pm 0.37
		125	45.67 \pm 0.26
3	<i>n</i> -Butanol soluble fraction	500	84.37 \pm 0.10
		250	65.83 \pm 0.55
		125	53.14 \pm 1.15
		60	44.50 \pm 0.57
		30	32.00 \pm 0.29
4	Ethyl acetate soluble fraction	500	98.00 \pm 0.13
		250	85.20 \pm 0.07
		125	60.67 \pm 0.04
		60	50.89 \pm 0.31
5	Chloroform soluble fraction	500	90.80 \pm 0.16
		250	70.29 \pm 0.71
		125	60.70 \pm 0.37
		60	54.15 \pm 0.36
6	Water fraction	1000	86.50 \pm 0.25
		500	53.60 \pm 0.46
		250	57.80 \pm 1.07
		125	45.60 \pm 0.43
7	BHT ^{b)}	60	91.25 \pm 0.13
		30	75.56 \pm 0.07
		15	42.67 \pm 0.04
		08	23.57 \pm 0.31

a) Standard mean error of three assays b) Standard antioxidant.

In the present study, ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) assays were performed to evaluate the antioxidant capacities of selected medicinal plant extracts relative to BHT, a reference standard. The FRAP assay is based on capacity of antioxidants to reduce colorless oxidized Fe^{3+} formed by electron donation through antioxidants to Ferrous (II) ions (intense blue Fe^{2+} tripyridyltriazine complex) (Hodzic *et al.*, 2009). The present work showed that the various fractions of *Lonicera quinquelocularis* showed potent antioxidant power with FRAP values in $\mu\text{M}/\text{mL}$ (table 3) at 595nm. The ethyl acetate soluble fraction exhibited highest FRAP value of (128.2 \pm 4.54), chloroform soluble fraction (120.6 \pm 6.57), followed by *n*-butanol soluble fraction (112.5 \pm 7.88), crude methanolic extract (111.6 \pm 7.99), aqueous fraction (110.2 \pm 2.61) while the lowest value was shown by *n*-hexane fraction (99.6 \pm 7.86).

Total Antioxidant Activity of various extracts of *L. quinquelocularis* was determined by phosphomolybdenum method. The method involved the reduction of molybdenum (VI) to molybdenum (V) by the antioxidants indicated by the formation of a green phosphate Mo (V) complex in acidic conditions with maximum absorption at 695nm (Prieto and Pineda 1999). The antioxidant activities of various extracts of *L. quinquelocularis* were compared with the reference standard antioxidant BHT.

The highest total antioxidant capacity (fig. 1) is in the order of ethyl acetate soluble fraction (0.595 \pm 0.00), chloroform soluble fraction (0.483 \pm 0.03), crude methanolic extract (0.431 \pm 0.02), water fraction (0.294 \pm 0.01), *n*-butanol soluble fraction (0.364 \pm 0.01), and lowest by *n*-hexane fraction (0.280 \pm 0.02).

CONCLUSION

All the assays confirmed the appreciable amount of phytochemical constituents in *L. quinquelocularis* and in particularly in ethyl acetate, chloroform and *n*-butanol soluble fraction and least components were present in *n*-hexane layer. The most efficient activity was exhibited by ethyl acetate extract, which may be due to a high content of polyphenols. It also showed good scavenging activity on DPPH free radical. This can be concluded that antioxidant activity is shown by flavones and polyphenols while *n*-hexane has low polyphenolic content and consequently, proved weaker antioxidant activity. The high antioxidant potential of some extracts of *L. quinquelocularis* can enhance their applications towards the prevention of various degenerative diseases of organs. The research needs to be continued to further analyze the role of various classes of vegetal compounds, other than polyphenols, in the antioxidant defense.

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