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Phytochemical investigation and evaluation of anti-oxidant potential of *Berberis lycium* roots from Himachal Pradesh

Punita Sharma, Abhishek Awasthi and Ishita Gupta

Abstract
Berberis lycium, a thorny evergreen shrub, commonly known as kashmal, found in subtropical and temperate regions of the world and mentioned in Indian traditional system of medicine. The present study deals with the preliminary phytochemical evaluation of different extracts of roots of *B. lycium*, isolation and identification of bioactive constituents from root extracts, its fractions and evaluation of their anti-oxidant potential by using hydrogen peroxide radical scavenging assay and reducing power assay. Extracts were fractionated and screened for their phytochemicals which resulted in isolation and identification of Stigmasterol (steroid), berberine, palmatine and jatrorrhizine (alkaloids), 4-methyl-7-hydroxycoumarin (coumarin) and 4,4-dimethylhexadeca-3-ol, responsible for the good anti-oxidant potential of the roots of *B. lycium*.

Keywords: *Berberis lycium,* phytochemical screening, isolation, phytoconstituents, anti-oxidation activity

Introduction
Since the time immemorial, human beings depend completely upon nature for their livelihood. Ancient civilizations valued plants the most as they supply humans with food, shelter and medical treatment. Studies show that maximum population of the world depends upon plant based medicine systems for their healthcare [1]. Different ethnic groups have their own traditional medical knowledge and experiences, on the basis of which they have developed different system of medicines [2, 3]. India is known for its diversity, where a number of traditional systems of medicine were invented (Ayurveda, Yoga, Naturopathy, Homeopathy, Siddha and Unani), flourished and practiced till date. India has varied climatic conditions and different composition of soil in different geographical regions that have caused a wide distribution of medicinal plant species which is why the people living over here have relied on plants for treatment of their medical implications. *Berberis lycium* Royle is a shrub that grows in the Himalayas from Kashmir to Kumaun at altitudes ranging between 900-2700m, belongs to family Berberidaceae [4]. Ancient manuscripts based on traditional medicinal system of India, i.e. Charka Samhitta, *B. lycium* is referred as “lekhaniya” for reducing obesity and scarifying, “arsaghna” for curing piles and haemorrhoids, and “kandughna”, curative intense itching sensation that can have various causes whereas according to Sushruta Samhitta, it is used for the treatment of uterine disorders, dysentery, indigestion and quick healing of wounds [5]. Dried decoction of roots is known as ‘Rasount’ which is reported to cure many disease such as boils, conjunctivitis, piles, leprosy, oral ulcers, liver disorders, kidney, chest and throat troubles, chronic diarrhoea, ophthalmia and skin diseases [6]. Previous studies shows that *B. lycium* plant has a wide range of biological activities including anti-hyperglycemic [7-8], anti-hyperlipidemic [9-10], anti-cancer and anti-tumor [11-12], wound and bone healing [13-14], anti-microbial [15-16], anti-coccidial [17], anti-oxidant [18-19], immunity enhancing [20-21], hepatoprotective [21], anti-urolithic and anthelmintic [22]. As mentioned in the earlier reported literature, *B. lycium* is found to be an important herb which shows numerous biological activities and used widely as folk medicine in Himachal Pradesh, India. In Himachal Pradesh, it is found in abundance in forest area and roadside. These has prompted us to carry out research to explore *B. lycium* found in Himachal Pradesh chemically and determine its anti-oxidant potential using *in-vitro* assays like hydrogen peroxide scavenging assay and reducing power assay.

Material and Methods

Collection, extraction and fractionation of plant material

The roots of *Berberis lycium* have been collected from Haripurdhar, Himachal Pradesh, India. Collected roots were dried under shade and kept free from foreign matter like soil, dust, insect, fungal and other extrinsic contamination. Dried plant material was grounded to coarse powder. Coarsely grounded plant material (1kg) was extracted with 95% ethanol in soxhlet for 48 hours. Extract after removal of ethanol under reduced pressure yielded hot ethanolic extract (190g) and subjected to fractionation by dissolving the extract in water and the solution was successively extracted with petroleum ether (three times), chloroform (three times) and n-Butanol (three times) in a separating funnel. The petroleum ether, chloroform and n-butanol fractions were distilled under reduced pressure to yield the residues petroleum ether (5g), chloroform (26g) and n-butanol (20g) fractions. The hot ethanolic extract and its fractions were taken for phytochemical screening.

Phytochemical Screening

The hot *ethanolic* extract and its fractions i.e. Petroleum ether, Chloroform and n-butanol fractions, were subjected to qualitative phytochemical analysis for the identification of phytoconstituents using standard tests for alkaloids (Dragendorff's reagent), tannins (Ferric Chloride test), saponins (Froth test), anthraquinones (Borntrager's reaction), flavanoids (NaOH test), carbohydrates (Benedict's reagent and Molisch's reagent test), protein (Biuret test), steroids (Liebermann-Burchard's and Salkowski's test), terpenoids (Salkowski's test) and glycosides (Keller-killani test) [23-27].

Isolation of Phytoconstituents

Isolation of phytoconstituents from the roots of *B. lycium* was done according to the method of Miana AG, (1973) with a little modification [28]. The hot ethanolic extract (70g) was dissolved in 1L of water and acidified with dilute HCl upto pH 4-5. Acidified extract was kept for precipitation under low temperature overnight. Yellow colored precipitates were formed which were subjected to filtration. Thin layer chromatography of the precipitates was carried out using Dragendorff's reagent as visualizing agent showing three major spots indicating it to be a mixture of alkaloids which were separated by repeated column chromatography resulting in isolation of Compound 1 (eluted in Chloroform:Methanol:: 2:23), compound 2 (eluted in $CHCl_3$-MeOH:: 87:13) and compound 3 (eluted in $CHCl_3$:MeOH:: 4:1). The petroleum ether fraction of hot ethanolic extract yielded compound 4 when subjected to column chromatography using Petroleum ether: Ethyl acetate as eluting solvent while compound 5 and 6 were isolated from Chloroform fraction of hot ethanolic extract. The All the precipitated compounds were subjected to physical and spectroscopic analysis using melting point, UV-VIS spectrophotometer and ^1H and ^{13}C- NMR for identification.

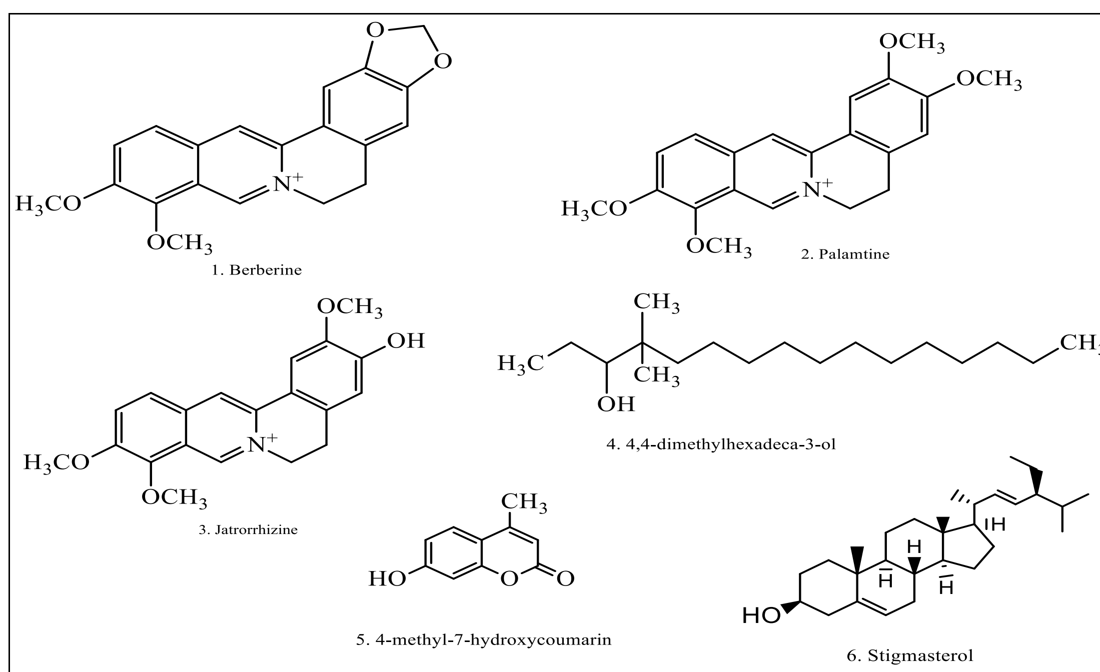

Fig 1: Structures of compounds isolated from different fractions of roots of *B. lycium*

Biological Activities

In-vitro anti-oxidant activity

Hydrogen peroxide scavenging (H_2O_2) assay

Earlier reports reveals that humans consume about 0.28mg/Kg/day hydrogen peroxide directly or indirectly from environment, that becomes toxic due to formation of hydroxyl radicals that can cause DNA damage in human body and initiates lipid peroxidation resulting into redox imbalance and oxidative stress. Thus, removing H_2O_2 or to terminate the long chain reactions of free radicals formed by it is an essential step [29-30]. Antioxidants have capability to terminate the production of free radicals by donating electrons to free radicals and stabilizing them that causes balance in the environment for proper cell functioning. The most important and common source of antioxidants is plants and fruits that are enriched which antioxidants and regulates proper functioning of body by resisting our body against various diseases [30-31]. Plants can scavenge Hydrogen peroxide due to the presence of secondary metabolites that acts as antioxidants. The H_2O_2 scavenging potential of the different extracts of roots of *B. lycium* was estimated according to the method mentioned by Alam MN *et al.*, (2013) [30-31].

Hydrogen Peroxide (40mM) solution was prepared in phosphate buffer (50mM, pH 7.4) whose concentration was

determined from Hydrogen peroxide calibration curve by measuring absorbance at 230nm using UV-VIS spectrophotometer. Plant extract of different concentrations (0.2-1.0 mg/ml) were prepared in distilled water and hydrogen peroxide containing phosphate buffer was added to these. Absorbance of the reaction mixtures at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of Hydrogen Peroxide Scavenging by the plant extract was calculated as follows:

% scavenged (H_2O_2) = [(A_c– A_t)/A_s] x 100

Where A_c is the absorbance of control and A_s is the absorbance of the test.

Determination of the reducing power

Substances having reduction potential can react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}) which reacts with $FeCl_3$ on addition and forms a ferric-ferrous complex (Perls's Prussian blue) which can be spectrophotometrically measured at 700nm. The free-radical scavenging activity of the reducing power of ethanolic extract and its fractions of roots of *B. lycium* was determined according to the method of Oyaizu (1986) described by Alam *et al*., (2013) [30, 32]. Various concentrations (2.5 mg-12.5mg) of root extracts of *B. lycium* were prepared in methanol and 0.2 M Phosphate Buffer (5ml, pH 6.6) was added to it followed by addition of 1% potassium ferricyanide (5ml). The resulting mixture was incubated at 50°C for 20min and 10% trichloroacetic acid (5ml) was added to it. The incubated reaction mixture was centrifuged at 3000g for 10 min and the supernatant (5ml) was mixed with distilled water (5ml) and 1% ferric chloride (1ml). The absorbance of the final mixture was measured at 700nm using UV-VIS spectrophotometer. L-ascorbic acid at different concentrations was used as standard. All the tests were carried out in triplicates.

Results and Discussions

Qualitative analysis of phytoconstituent of *Berberis lycium* roots:

Hot ethanolic extract of roots of *Berberis lycium* collected from Himachal Pradesh were found to be rich in secondary metabolites like alkaloids, flavonoids, steroids, terpenoids and their glycosides whereas tannins, saponins, anthraquinones and anthocyanides are completely absent. Ethanolic extract was fractionated successively with Petrol, Chloroform and n-butanol to yield respective fractions. Phytochemical screening results of different fractions reveal that petrol fraction contains only alkaloids whereas chloroform fraction is rich in alkaloids, flavonoids, terpenoids and steroids while n-butanol fraction shows the presense of alkaloids and terpenoids. Detailed results of qualitative analysis of phytoconstituents of *B. lycium* roots are shown in Table 1.

Table 1: Preliminary phytochemical screening of ethanolic extracts and its fractions of roots of *B. lyceum*

Phytochemicals	Test performed	Ethanol Extract	Fractions of ethanol extract of *B. lycium*		
			Petrol	$CHCl_3$	n-BuOH
Alkaloids	Dragendorff's reagent	+	+	+	+
Tannins	Ferric Chloride test	-	-	-	-
Saponins	Froth test	-	-	-	-
Anthraquinones	Borntrager's reaction	-	-	-	-
Flavonoids	NaOH test	+	-	+	-
Carbohydrates	Benedict's reagent	+	+	+	+
Proteins	Biuret test	+	-	-	+
Steroids	Liebermann-Burchard test	+	-	+	-
Terpenoids	Salkowski's test	+	-	+	+
Glycosides	Keller-killani test	+	-	+	-

Isolation and characterization of Phytoconstituent

Berberine, palmatine and jatrorrhizine were isolated from the ethanolic extract of roots of *B. lycium* whereas 4,4-dimethylhexadeca-3-ol was isolated from petrol fraction and 4-methyl-7-hydroxycoumarin and stigmasterol isolated from chloroform fraction. The isolated compounds were identified and characterized by using different chromatographic, spectroscopic techniques and comparing the results with existing literature [19, 33]. The experimental data of the isolated compounds is as under.

1. **Berberine:** Yellow crystals, soluble in methanol. Molecular formula [$C_{20}H_{18}NO_4^+$], Melting Point- 198°C. **UV** (MeOH), λ_{max} (nm) = 236, 357, 469. **^{1}H-NMR** (DMSO, 400MHz), δ (ppm):- 3.20 (t, 2H, J = 6.1 Hz, H-5), 4.05 (s, 3H, -OCH_3), 4.09 (s, 3H, -OCH_3), 4.94 (t, 2H, J = 6.2 Hz, H-6), 6.15 (s, 2H, -OCH_2O-), 7.06 (s, 1H, H-4), 7.75 (s, 1H, H-1), 7.99 (d, 1H, J = 9.12 Hz, H-11), 8.16 (d, 1H, J = 9.16 Hz, H-12), 8.93 (s, 1H, H-13), 9.89 (s, 1H, H-8).
2. **Palmatine:** Yellow crystals, soluble in methanol**.** Molecular formula [$C_{21}H_{22}NO4^+$], Melting Point- 217°C. **UV** (MeOH), λ_{max} (nm) = 231, 351, 441. **^{1}H-NMR** (DMSO, 400MHz), δ (ppm):- 3.20 (t, 2H, J = 6.40 Hz, H-5), 4.04 (m, 6H, 2-OCH_3), 4.08 (m, 6H, 2-OCH_3), 4.95 (t, 2H, J = 6.0 Hz, H-6), 7.05 (s, 1H, H-4), 7.74 (s, 1H, H-1), 7.99 (d, 1H, J = 9.08 Hz, H-11), 8.15 (d, 1H, J = 9.12 Hz, H-12), 8.93 (s, 1H, H-13), 9.89 (s, 1H, H-8).
3. **Jatrorrhizine:** Yellow crystals, soluble in methanol. Molecular formula [$C_{20}H_{20}NO_4^+$], Melting point- 236°C. UV (MeOH), λ_{max} (nm) = 229, 307, 436. ^{1}H-NMR (DMSO, 400MHz), δ (ppm):- 3.16 (s, 3H, -OCH_3), 3.20 (t, 2H, J = 6.1 Hz, H-5), 4.06 (s, 3H, -OCH_3), 4.09 (s, 3H, -OCH_3), 4.94 (t, 2H, J = 6.04 Hz, H-6), 6.16 (s, 1H, C3-OH), 7.07 (s, 1H, H-4), 7.76 (s, 1H, H-1), 7.96 (d, 1H, J = 9.12 Hz, H-11), 8.18 (d, 1H, J = 9.16 Hz, H-12), 8.92 (s,1H, H-13), 9.88 (s, 1H, H-8).
4. **4,4-dimethylhexadeca-3-ol:** White sticky mass, soluble in ethyl acetate. Molecular formula [$C_{18}H_{38}O$], Melting point- 162°C.^{1}H-NMR ($CDCl_3$, 400MHz), δ (ppm):- 0.46 (s, 3H, -CH_3), 1.02 (t, 3H, J = 7.16 Hz, -CH_3), 1.96 (m, 3H, -CH_3), 2.36 (t, 2H, J = 5.18 Hz, -CH_2), 2.56 (m, 2H, -CH_2), 4.19 (t, 3H, J = 5.14 Hz, -CH_3). ^{13}C-NMR ($CDCl_3$, 300MHz), δ (ppm): 15.36 (CH_3, C-16), 19.16 (CH_3, C-1), 22.91 (CH_3, C-17 and C-18), 31.15 (CH_2, C-2), 33.61 and 33.63 (CH_2, C-5 and C-15), 37.14 (C, C-4), 75.23 (CH, C-3).

5. **4-methyl-7-hydroxycoumarin:** White crystals, soluble in chloroform. Molecular formula [$C_{10}H_8O_3$], Melting Point- 189°C. ^{1}H-NMR ($CDCl_3$, 400MHz), δ (ppm):- 3.28 (s, 3H, -CH_3), 3.59 (s, 1H, H-3), 6.19 (s, 1H, H-8), 6.61 (d, J = 6.63 Hz, 1H, H-5), 7.51 (d, J = 6.69 Hz, 1H, H-6). ^{13}C-NMR ($CDCl_3$, 400MHz), δ (ppm): 19.07 (CH_3, C-8), 102.35 (CH, C-8), 114.09 (C, C-4a), 115.61 (CH, C-3), 117.31 (CH, C-6), 129.06 (CH, C-5), 153.95 (C, C-8a), 156.87 (C, C-4), 166.78 (C, C-7), 167 (C, C-2).
6. **Stigmasterol:** White solid, soluble in chloroform. Molecular formula [$C_{29}H_{48}O$], Melting point- 186°C. ^{1}H-NMR ($CDCl_3$, 400MHz): 0.66 (s, 3H, H-18), 0.72 (d, 3H, J = 6.32 Hz, H-27), 0.80 (d, 3H, J = 6.33 Hz, H-26), 0.83 (t, 3H, J = 7.26 Hz, H-29), 1.06 (s, 3H, H-19), 1.13 (d, 3H, J = 6.3 Hz, H-21), 3.52 (m, 1H, H-3), 5.36 (s, 1H, H-6), 5.09 (dd, 1H, J = 15.19 and 8.36 Hz, H-23), 5.11 (dd, 1H, J = 15.17 and 8.38 Hz, H-22).

Biological Activities

In-vitro anti-oxidant activity

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging potential of plant extracts is due to the secondary metabolites present in them which can donate electrons to the free radicals generated by hydrogen peroxide, thus neutralizing free radicals to water. Current study reveals that hot ethanolic extract of roots of *B. lycium* and its fractions were capable of scavenging hydrogen peroxide in a concentration-dependent manner. The scavenging activity of ethanolic extract and its fractions at 0.8mg/ml concentration is above 40%. At 1.0 mg/ml concentration all the fractions except petroleum ether fraction exhibit more than 50% scavenging activity indicating the roots of *B. lycium* to be a good antioxidant. The inhibiting percentage of various extracts is in the following order: ethanol extract> Chloroform fraction> n-butanol fraction> petrol fraction (Fig 2.).

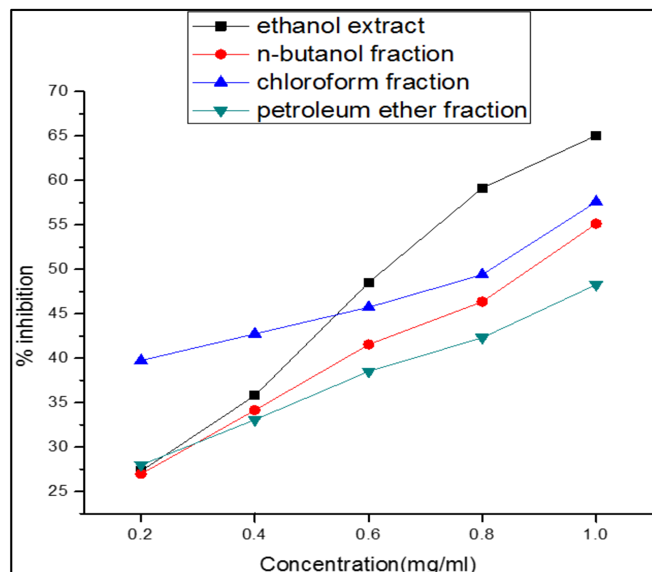

Fig 2: Percentage of H_2O_2 scavenging by alcoholic extract and its fractions of roots of *B. lycium* at different concentrations

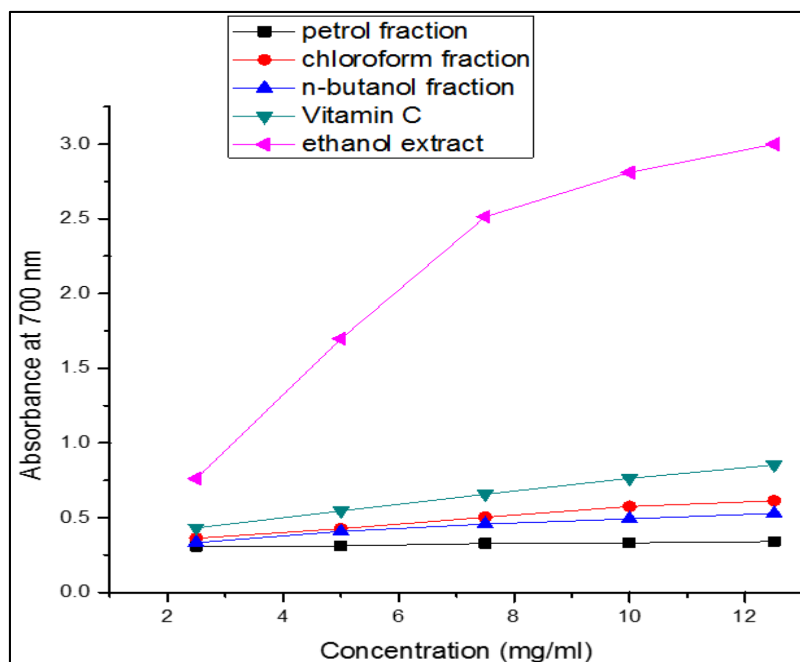

Fig 3: Reducing powers of alcoholic extract and its fractions of roots of *B. lycium* at different concentrations

Reducing power activity

The reducing capacity of the ethanolic extract and its fractions of roots of *B. lycium* were compared with Vitamin C (L-ascorbic acid). A higher value of absorbance indicates a stronger reducing power of the extracts/fractions. The reduction potential of ethanolic extract and its fractions, i.e. petroleum ether, chloroform and n-butanol fractions, of the roots of *B. lycium* at various concentrations is illustrated in Fig 3. All extracts have shown good reducing powers that are comparable with L. The reducing powers of the samples are in the following order: Ethanol extract > $CHCl_3$ fraction> n-butanol fraction > petroleum ether fraction. The ethanolic extract of the roots of *B. Lycium* had shown better reducing power than Vitamin C. On the basis of present investigation it was found that the reducing powers of ethanolic extract and its fractions also increased with the increase of their concentrations. So, it can be concluded that extract and its fractions exhibited increase in their reducing potential in concentration dependent manner.

Conclusion

Berberis lycium is a significant herb mentioned in Indian Pharmacopeia and has great ethno medicinal values. In Himachal Pradesh, *Berberis lycium* is a main source of primary healthcare in the rural area, its ripened fruits are edible and its tender shoots are chewed for curing skin diseases and a blood purifier[34]. In this study, phytochemical investigation of roots of *B. lycium* was carried out showing that the roots are rich in secondary metabolites such as alkaloids, flavonoids, steroids and Terpenoids which resulted in the isolation of stigmasterol (steroid), berberine (alkaloid), palmatine (alkaloid), jatrorrhizine (alkaloid), 4-methyl-7-hydroxycoumarin (coumarin) and 4,4-dimethylhexadeca-3-ol. Hot ethanolic extract was also used for determination of free radical scavenging or its antioxidant profile (Hydrogen peroxide scavenging assay) and reducing power that resulted in good anti-oxidant potential of this plant indicating ethanolic extract to display maximum potential among ethanolic extract and its fractions. Our study on its active constituents and anti-oxidant potential made us to conclude that *B. lycium* form Himachal Pradesh can be explored to great extent to be developed and used commercially for its medicinal values.

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