Free radical scavenging activity, total phenolic content, total flavonoid content and ferric reducing antioxidant power assay of '*Maha Agad*'- A research article

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Abstract

Introduction: The present study was undertaken to explore the antioxidant properties of *Maha Agad*. *Maha agad* is one such formulation mentioned in classics which is used in acute toxicity conditions. The ingredients present in *Maha agad* are known for their rasayana karma which can be correlated with the antioxidant activity of the modern era. The primary objective of this study was to evaluate the antioxidant effect of *Maha agad*.
Materials and methods: In this study, free radical scavenging activity, total phenolic content, total flavonoid content, ferric reducing power assay of ethanol and aqueous extracts of *Maha Agad* were measured spectroscopically. Free radical scavenging activity was determined according to the elimination of DPPH radicals, total phenol content was determined by the Folin–Ciocalteu method, total flavonoid content was determined by aluminum chloride colorimetric method, ferric reducing power assay was determined by using ascorbic acid as standard.
Results: Both ethanol and aqueous extracts of *Maha Agad* had radical scavenging effects at all concentrations. The percentage inhibition of 200mg of ethanol extract exhibited the highest DPPH radical scavenging activity of 85% followed by aqueous extract of 81%. For ethanol extract, the Ic50 value ranged from 20 to 40 mg, whereas for aqueous extract, it was 40 to 80 mg. TPC values varies from 7.9 to 8.1mgGAE/g in ethanol extract and 7.8 to 7.9 mg GAE/g in aqueous extract. TFC values ranged from 13.57 to 13.79 mg QE/g in ethanol extract and 13.61 to 13.92 mg QE/g in aqueous extract. The range of FRAP values in ethanol extract was 3.3-26.7 µ mol Fe (II)/g, whereas in aqueous extract it was 8.4-20.5 µ mol Fe (II)/g.
Conclusion: Results clearly showed that both the extracts of *Maha Agad* have showed good antioxidant activity. Ethanol extract of *Maha Agad* had strong DPPH radical scavenging, FRAP activity, and also have higher phenolic content whereas aqueous extract had high flavonoid content. As a result, *Maha Agad* can be used in pharmaceutical products as a source of natural antioxidants.
Keywords: Antioxidant, Ethanol and Aqueous extract, *Maha Agad*, DPPH, Phenolic content, Flavonoid content, FRAP, *Rasayana.*

1. Introduction

Free radicals are chemical entities which contains one or more unpaired electrons due to which
they are extremely unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. The human body constantly produces free radicals as it is needed for immune system functioning, chemical signaling, detoxification, and energy provision [1].
Undoubtedly, oxygen is a necessary component of aerobic life. However, under certain circumstances, it can seriously affect our wellbeing through the formation of reactive oxygen species (ROS) representing both non-free radical and

free radical species, and their potential deleterious effects such as atherosclerosis, ageing, inflammation, ischemic heart disease, diabetes, immunosuppression, neurodegenerative diseases, cancer and others[1, 2] The most frequently encountered free radicals are the hydroxyl radical, the nitric oxide radical, the superoxide radical and the lipid peroxyl radical while non-free radical species principally being H_2O_2 and singled oxygen [3] Fortunately, almost all organisms are protected from free radical attack by defense mechanisms such as preventive antioxidant system that reduces the rate of free radical formation, and another is a system to produce chain-breaking antioxidants that scavenge and stabilize free radicals. But significant tissue damage arises when the pace of creation of free radicals surpasses the ability of antioxidant defence mechanisms [4]. Currently available synthetic antioxidants like butylated hydroxy anisole (BHA), tertiary butylated hydroquinone, butylated hydroxy toluene (BHT), and gallic acid esters, have been suspected to cause or prompt negative health effects. As a result, there are severe limitations on their use, and replacing them with antioxidants that occur naturally is becoming more popular. Additionally, these artificial antioxidants have limited antioxidant activity and poor solubility. [5] Recently there has been an upswing of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury. [6] Medicinal plants comprise bioactive chemicals such as tannins, alkaloids, carbohydrates, terpenoids, steroids, and flavonoids that have specific physiological effects on human bodies [7, 8].

The endeavour of present study is focused on determination of antioxidant activity of a polyherbal drug *Maha Agad* mentioned in classical text *Ashtang sangrah Uttarsthan. Trivrit, Vishalya, Haridra, Daruharidra, Yashtimadhu, Manjishtha, Pippali, Maricha, Shunthi, Panch lavana,* and *bhavana dravya of Basta mutra*/Goat urine are among its 14 constituents. In *Ayurveda acharyas* had mentioned that *Pippali, Daruharidra, Manjishtha, Yashtimadhu* contains *rasayana* property which can be correlated with the antioxidant activity of the modern era. *Maha Agad* contains these drugs by virtue of which it could act as an antioxidant. This information encouraged us to systematically study *Maha Agad* for its effective antioxidant activity.

2. Materials and methods

2.1 Collection of Drugs

The ingredients of *Maha Agad* were collected in the quantity of 100gm each in pure form from different sources. *Langali, Yashtimadhu, Manjishtha, Pippali, Maricha, Sauvarchala Lavana, Saindhava Lavana, Vida Lavana, Samudra Lavana* were collected form Pannalal store, Haridwar in the month of July 2022. *Haridra* and *Shunthi* were collected from Dehradun in the month of June 2022. *Audbhida lavana(reh)* was collected from Nidco Herbal Heritage, Dehradun in the month of August 2022, *Daruharidra* was collected from Nainital district in the month of August 2022. *Trivrit* was collected from Rishikul campus in the month of July 2022. Lastly, about 5 liter of *Basta mutra*/ goat urine was collected from a meat vendor in Haridwar.

2.2. Drug identification and authentication

All the ingredients of *Maha Agad* were identified and verified by imminent experts of Dravyaguna Department, Rishikul Campus, Haridwar (UAU) with reference no- DG-RC-UAU- 154 dated on 22-06-2023.

2.3. Preparation of Churna and Bhavana process

The ingredients of *Maha Agad* were taken in dried form and these were made into coarse powder one by one with the help of iron mortar and pestle. Finally, they were made into fine powder form by grinding and passed through sieve no. 80 separately. The process of *bhavana* was done in Ras shastra department, Rishikul campus. *Basta mutra* was collected in sterile bottles in the morning. 300gm of drug was taken for *bhavana* process. It took 2-3 days to complete one *bhavana*. In one day, it took 5-6 hour for *bhavana.* So, 7 *bhavana* were given to *Maha Agad*. Lastly the weight of the drug was increased with *bhavana* and it was 311gm. It was then kept in an air tight container for further use.

2.4. Preparation of plant extract

The dried powder of *Maha Agad* was taken for the extraction process. It was performed in the hot continuous process through Soxhlet apparatus. Two types of extractions were performed-

1. Ethanol extraction
2. Aqueous extraction

For Ethanol extraction- 100gm of drug was taken and placed in the Soxhlet chamber, then 250ml of ethanol was poured into it, 100ml of ethanol was poured in the round bottom flask with some glass beads then the whole setup was attached to a heating mantle, the temperature was maintained at a temperature below the boiling temperature of ethanol at 60°C and allowed to run for 6 consecutive days, content of round bottom flask was collected in a petri dish and was allowed to evaporate in water bath for 1 to 2 days and then dried in room temperature.

For Aqueous extraction- 100gm of drug was taken and placed in the Soxhlet chamber, then 250ml of distilled water was poured into it, 200ml of distilled water was poured in the round bottom flask, then the whole setup was attached to a heating mantle, the temperature was maintained at temperature below the boiling temperature of water at 80°C and allowed to run for 5 consecutive days, content of round bottom flask was collected in a petri dish and allowed to evaporate in water bath for 1 to 2 days and then dried in room temperature.

Both the extracts were preserved aseptically in air tight containers for further research work.

2.5 *In vitro* Antioxidant activity

2.5.1. DPPH free radical Scavenging activity

The antioxidant activity of plant samples was estimated using modified DPPH (2,2-diphenyl-1-picrylhydrazyl method [9]. Sample (ethanol and aqueous extracts) with different concentration (20,40,80,120,160,200 mg) was taken and made up to 3ml by adding ethanol. To this 1ml of 0.004% DPPH solution (4 mg of DPPH dissolved in 100 ml of ethanol) was added, mixed well and incubated in dark for 30 min. Later the absorbance was measured at 517 nm using the spectrophotometer. The experiment was carried under dark condition as DPPH is light sensitive. Ascorbic acid was taken as standard (20 µL/4 µg, 40 µL/8 µg, 80 µL/16 µg,120 µL/24 µg ,160 µL/32 µg, 200 µL/40 µg). Percentage of antioxidant activity was calculated using the formula:

$$\text{Radical scavenging activity (\%)} = \frac{\text{A control- A sample}}{\text{A control}} * 100$$

where, control was 3ml ethanol + 1ml DPPH solution. Sample was plant extract made up to 300 µL using ethanol + 1mL DPPH solution.

2.5.2 Estimation of total phenolic contents

Total phenolics in the sample were estimated by modified Folin-Ciocalteu's (FC) method [10]. To the concentrations of (20,40,80,120,160,200 mg) of ethanol and aqueous extracts, 2ml of distilled water was added to dilute the extract and 150 µL of FC reagent (diluted with distilled water in the ratio 1:1 v/v) was added and vortexed. To this 500 µL of 20% (w/v) Na2CO3 was added and incubated in dark for 1 h. The absorbance of greenish-blue color developed was measured at 650 nm. In 2ml of ethanol/distilled water, all the reagents were added except plant extract and considered as blank. Gallic acid (20,40,80,120,160,200 µg) was taken as a standard to determine the phenolic content of the samples.

2.5.3. Estimation of total flavonoid contents

Total flavonoids in the plant sample were estimated by the aluminium chloride method [11]. To the concentrations of (20,40,80,120,160,200 mg) of ethanol and aqueous extracts, 1ml distilled water was added to dilute its concentration. To this 100 µL of 10% aluminum chloride solution was added and mixed well. Later 100 µL of 1M sodium acetate was added and incubated in dark at room temperature for 45 min. The absorbance of the developed golden-yellow color was measured at 510nm. Ethanol/distilled water (1ml) and all the reagents were added except plant extract and taken as blank. Quercetin was taken as a standard (20,40,80,120,160,200 µg) to calculate the concentration of flavonoids in the plant sample.

2.5.4. Estimation of Ferric reducing antioxidant power assay [FRAP]

The reducing potential of the sample was estimated by according to Jung *et al.* method [12]. To the concentrations of (20,40,80,120,160,200 mg) of ethanol and aqueous extracts, 1ml of distilled water was added to dilute the extract. To this 250 µL of phosphate buffer (0.2 M, 6.6 pH) was added and mixed well. Then 250 µL of 1% potassium ferrocyanide was added and incubated in the water bath at 50 ºC for 20 min. Later to this 250 µL of 10% trichloroacetic acid was added and shaken well using a vortex. From this 250 µL of the solution was taken into new tubes and 250 µL of distilled water was added. Now 100 µL of 0.1% FeCl3 was added and vortexed. The absorbance of the blue-colored product was measured at 700 nm using the spectrophotometer.1 ml distilled water was taken as blank, while ascorbic acid was used as standard (20 µL/4 µg, 40 µL/8 µg, 80 µL/16 µg,120 µL/24 µg ,160 µL/32 µg, 200 µL/40 µg).

3. Results and Discussion

Since the antioxidant compounds found in plants have different polarities, different solvents are used to isolate antioxidants. Water, methanol, ethanol, and acetone are solvents commonly used in extraction processes. The antioxidant activity of the extract and the yield depends on the selected solvent [13]. In this study, ethanol and water were preferred as solvents for the extracts to be prepared. In this study, free radical scavenging effect, reducing power assay, the amounts of phenolic content as well as the flavonoid content were determined.

3.1 In *vitro* antioxidant activity

3.1.1- DPPH free radical Scavenging activity

The DPPH method is recommended since it doesn't require a particular reaction or device and is quick, simple, and reliable. DPPH is a stable, synthetic radical that does not disintegrate in water, ethanol, or methanol. The capacity of antioxidant compounds to lose hydrogen and the structural conformation of these elements determine the extracts' capacity to scavenge free radicals. [14,15]. The DPPH free radical, which is at its maximum wavelength at 517 nm, can easily receive a hydrogen or electron from antioxidant molecules to become a stable diamagnetic molecule [16]. It is thought to possess a radical scavenging capability because of its capacity to bind hydrogen. In Table no. 1 the radical scavenging effects of different concentrations of ethanol and aqueous extracts of *Maha Agad* are demonstrated. Both ethanol and aqueous extracts had radical scavenging effects at all concentrations. The percentage inhibition of 200mg of ethanol extract of *Maha Agad* exhibited the highest DPPH radical scavenging activity of 85% followed by aqueous extract of 81%. The standard antioxidant activity of ascorbic acid was 100% at 40 µg. The minimum percentage inhibition of ethanolic extract was 47% and aqueous extract was 43% at 20mg concentration while standard had shown percentage inhibition of 89% at 4 µg. The Ic50 value of ethanolic extract was shown between 20 to 40mg concentration whereas of aqueous extract it was in between 40 to 80mg concentration. Ethanol extract had lower Ic50 value thus showing good antioxidant activity than aqueous extract.

3.1.2 Estimation of total phenolic content

Phenolic compounds and polyphenols are the amplest structures in plants. Antioxidant compounds are usually in the phenolic form. The antioxidant properties of phenolic compounds originate from their properties of proton loss, dismutation of radicals, chelate formation. Phenols have the ability to destroy radicals because they contain hydroxyl groups. These plant components give up hydrogen atoms from their hydroxyl groups to radicals and then form stable phenoxyl radicals. Hence, they play an important role in antioxidant activity. Therefore, determination of the quantity of phenolic compounds is necessary in order to determine the antioxidant capacity of plant extracts [17,18.19]. The total phenolic content for ethanol and aqueous extracts of *Maha Agad* were estimated by Folin Ciocalteu's method using gallic acid as standard. The absorbance values obtained at different concentrations of gallic acid(20-200µg) were used for plotting the calibration curve conformed to Beer's Law at 650 nm as shown in figure no. 1. Total phenolic content of the extracts was calculated from the regression equation of calibration curve ($y = 0.005x - 0.038$, $(R2) = 0.999$), where the plot has a slope (m) = 0.005 and intercept = 0.038 and expressed as mg gallic acid equivalents (GAE)

per gram of sample in dry weight (mg/g). Different concentrations of ethanol and aqueous extract from 20-200mg were used to determine their phenolic content. In table no. 2 TPC values are shown, which varies from 7.9 to 8.1mgGAE/g in ethanol extract and 7.8 to 7.9 mg GAE/g in aqueous extract. Result showed that ethanol extract had the highest total phenolic content (8.1mgGAE/g) followed by aqueous extract (7.9 mg GAE/g).

3.1.3. Estimation of total flavonoid content

Plant phenolic compounds are classified into a number of categories, the most important of which are flavonoids, which have strong antioxidant properties. [20]. Flavonoids occurs naturally in plants and are thought to have positive effects on human health. Studies on flavonoid derivatives have shown a wide range of antibacterial, anti-inflammatory, antiviral, anticancer, and anti-allergic activities [21,22]. They are one of the highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals implicated in several diseases [23]. So, in comparison with the findings in the literature for other extracts of plant products [24] Our results suggested that phenolic compounds and flavonoids may be the major contributors for the antioxidant activity. The total flavonoid content of ethanol and aqueous extracts of *Maha Agad* were measured with the aluminum chloride colorimetric assay using quercetin as standard. The absorbance values obtained at different concentrations of quercetin (20-200µg) were used for the construction of calibration curve conformed to Beer's Law at 510 nm as shown in figure no. 2. Total flavonoid content of the extracts was calculated from the regression equation of calibration curve (y =0.002x - 0.027, (R2) = 0.980), where the plot has a slope (m) = 0.002 and intercept = 0.027 and expressed as mg quercetin equivalents (QE) per gram of sample in dry weight (mg/g). Different concentrations of ethanol and aqueous extract from 20-200mg were used to determine their flavonoid content. In table no. 3 TFC values are shown, which varies from 13.57 to 13.79 mg QE/g in ethanol extract and 13.61 to 13.92 mg QE/g in aqueous extract. Result showed that aqueous extract had the highest total flavonoid content (13.92 mg QE/g) than ethanol extract (13.79 mg QE/g).

3.1.4. Estimation of ferric reducing antioxidant power assay (FRAP)

Reducing power can be a key indicator of antioxidant activity. In reducing power assay, the yellow color of the test solution changes to green color depending on the reducing power of the test specimen. The presence of the reductants in the solution causes the reduction of the ferricyanide complex/ Fe^{3+} to the ferrous form. Therefore, Fe^{2+} can be monitored by absorbance measurement at 700 nm. Prior studies have indicated that the reducing characteristics have demonstrated an antioxidant effect by the donation of a hydrogen atom, which breaks the chain of free radicals [25]. Increasing absorbance at 700 nm indicates an increase in reducing ability. In our study ethanol and aqueous extracts of *Maha Agad* were estimated by ferric reducing antioxidant power method using ascorbic acid as standard. The absorbance values obtained at different concentrations of ascorbic acid (4-40µg) were used for the construction of calibration curve conformed to Beer's Law at 700 nm as shown in figure no. 3. The reducing power of the extracts were calculated from the regression equation of calibration curve (y =0.009x + 0.110, (R2) = 0.981), where the plot has a slope (m) = 0.009 and intercept = 0.110. Different concentrations of ethanol and aqueous extract from 20-200mg were used to determine their reducing property. In table no. 4 FRAP values are shown, which varies from 3.3 to 26.7 µ mol Fe (II)/g in ethanol extract and 8.4 to 20.5 µ mol Fe (II)/g in aqueous extract. The results indicated that ethanol extract exhibited significantly higher antioxidant activity (26.7 µ mol Fe (II)/g) than aqueous extract (20.5 µ mol Fe (II)/g). The antioxidants present in the fractions of *Maha Agad* caused their reduction of Fe^{3+}/ ferricyanide complex to the ferrous form, and thus proved its reducing power.

Table 1: Showing DPPH free radical scavenging assay of *Maha Agad* at different concentrations: -

S.No.	Conc. of sample (in mg)	% inhibition of ethanol extract	% inhibition of aqueous extract
1.	20	47%	43%
2.	40	56%	44%
3.	80	64%	53%
4.	120	73%	56%
5.	160	79%	69%
6.	200	85%	81%

Table 2: Total Phenolic content of extracts of *Maha Agad*: -

S. No.	Concentration of sample(mg)	TPC content mg GAE/g	
		Ethanol extract	Aqueous extract
1.	20	7.919	7.825
2.	40	7.959	7.856
3.	80	7.983	7.858
4.	120	7.994	7.919
5.	160	7.996	7.928
6.	200	8.183	7.973

Table 3: Total Flavonoid content of extracts of *Maha Agad*: -

S. No.	Concentration of sample(mg)	TFC content mg QE/g	
		Ethanol extract	Aqueous extract
1.	20	13.575	13.615
2.	40	13.606	13.623
3.	80	13.609	13.636
4.	120	13.613	13.664
5.	160	13.739	13.703
6.	200	13.796	13.923

Table 4: Reducing power of extracts of *Maha Agad*: -

S. No.	Concentration of sample(mg)	Reducing power µ mol Fe (II)/g	
		Ethanol extract	Aqueous extract
1.	20	3.3	8.4
2.	40	5.6	8.6
3.	80	12.7	9.7
4.	120	18.3	10.4
5.	160	21.1	19.3
6.	200	26.7	20.5

Figure 1. Graph showing calibration curve of Gallic acid:

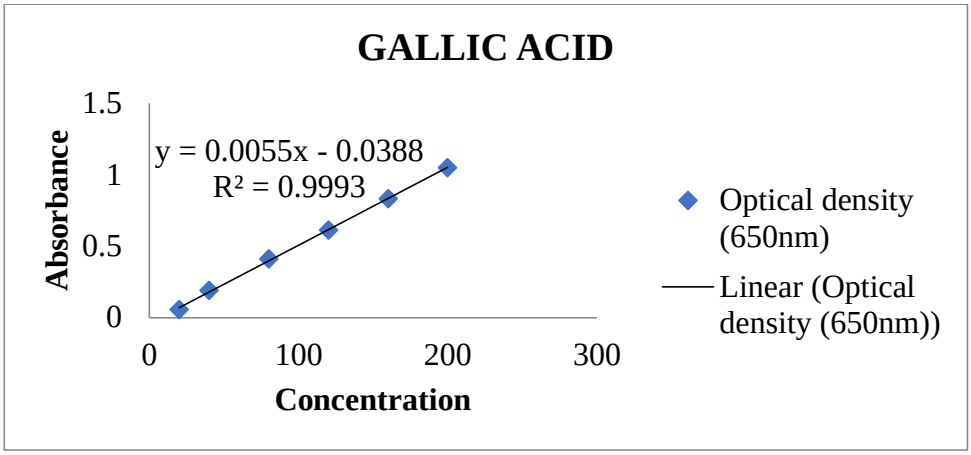


Figure 2. Graph showing calibration curve of Quercetin:

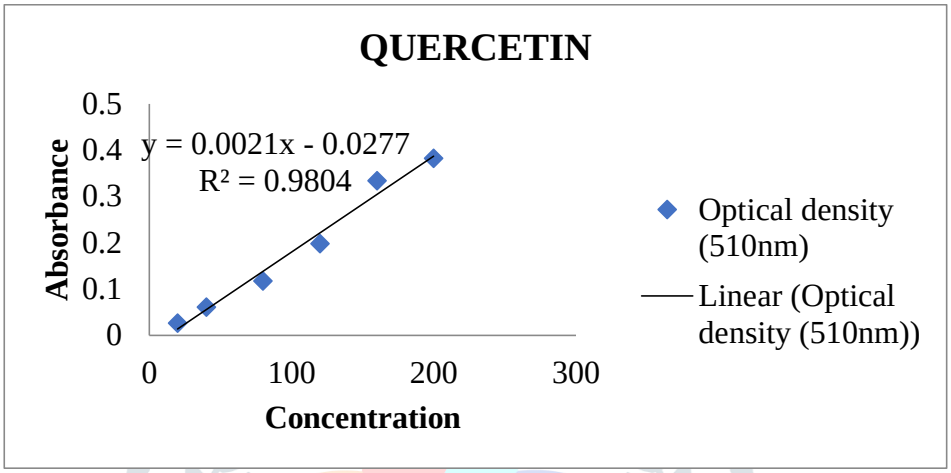
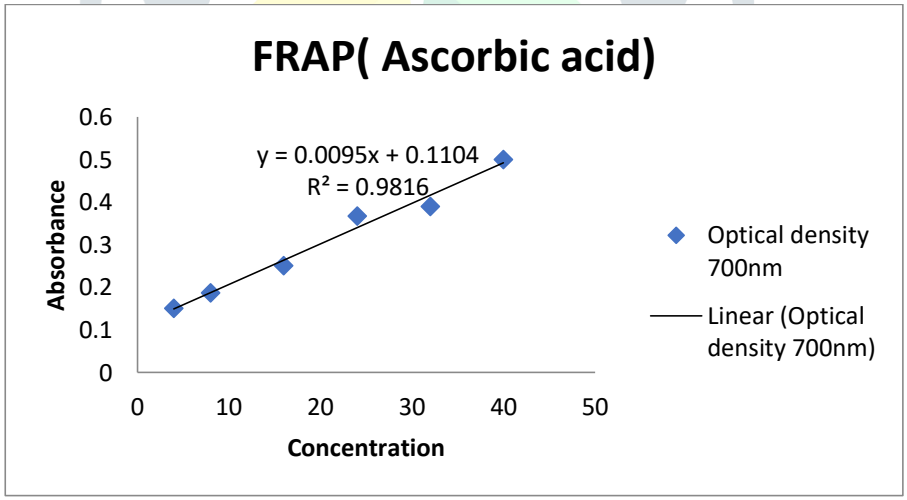


Figure 3. Graph showing calibration curve of Ascorbic acid:



4. Conclusion

In this study, free radical scavenging activity, total phenolic content, total flavonoid content, and ferric reducing power assay of ethanol and aqueous extracts of *Maha Agad* were determined. Results clearly showed that both the extracts of *Maha Agad* have showed good antioxidant activity. Ethanol extract of *Maha Agad* had strong DPPH radical

scavenging, FRAP activity, and also have higher phenolic content whereas aqueous extract had high flavonoid content. As a result, *Maha Agad* can be used in pharmaceutical products as a source of natural antioxidants.

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