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Original Article

# EVALUATION OF *IN VITRO* ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF VARIOUS SPICES OF INDIAN ORIGIN

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**ABSTRACT**

**Objective**: The present study was carried out to examine the *in vitro* antioxidant activity of the methanolic extracts of fifteen spices of Indian origin. The antimicrobial activity and constitution of spices of high antioxidant activity were also examined.

**Methods**: The activity was assessed using various *in vitro* assay models viz., Phenolic and flavonoid content, ferric reducing antioxidant power, DPPH•, ABTS•+scavenging activity using standard protocols. Antibacterial (against gram-positive bacteria *Staphylococcus aureus* and gram-negative bacteria *Escherichia coli)* and antifungal activity (against *Aspergillus niger* and *Fusarium* species) of two spices having significantly high antioxidant potential was assessed using agar well method. The results were interpreted with natural antioxidants and known antibiotics and fungicides.

**Results**: Among the samples analyzed, Clove and Cinnamon showed the highest antioxidant potential, reducing power and antimicrobial activity. Antifungal activity for clove was found to be highest observed up to the period of seven days.

**Conclusion**: Based on these findings, Clove and Cinnamon can be considered as a potent source of nutraceutics which could offer protection and oxidative stress-induced physiological malfunctions.

**Keywords**: Antioxidant, Spices, FRAP, Phenolic, Antibacterial and antifungal.

## INTRODUCTION

Spices and herbs of Indian origin are known for their pungent aroma and delightful flavor and used indispensably in culinary preparations. They are derived from the different parts of the plants like bud (clove), bark (cinnamon), fruit (pepper), leaves (bay leaf) or rhizome (ginger). They are reputed to possess several medicinal and pharmacological properties and used in the number of Ayurveda medicines. These properties are due to various substances which include flavonoids, carotenoids, terpenoid, some vitamins and minerals [1].

Many of the spices with well-versed activities are turmeric (anti-inflammatory, antibacterial, anti-tumor, anti-allergic, stimulant, anti-oxidant and antiseptic agent), cardamom (anti-inflammatory, carminative, stimulant and flavoring agent), fennel (antispasmodic, antimicrobial, anticancer and reduce oxidative stress), carom seeds (contains thymol, carvacrol, terpilene, dipentene and pinene having healing properties), cinnamon (anti-bacterial, anti-diabetic, antiseptic and anti-inflammatory), ginger (carminative, anti-carcinogenic and antioxidant), black pepper (anti-inflammatory and anti-arthritic), clove (stimulant, antiviral, antibacterial, antifungal, local anesthetic, anti-inflammatory and incredible antioxidant). Clove oil is used to clean wounds, scratches and to treat toothache [2]. It is identified to have the highest capacity to give off hydrogen and thereby reducing lipid peroxidation [3].

Fenugreek seeds are little bitter but are aromatic, carminative, astringent and thermogenic. Cumin seeds have antiseptic, carminative and stimulant effect. Coriander seeds are carminative, digestive, analgesic and antidiabetic agent. It is gifted with the lot of medicinal, antibacterial, antidiabetic, anti-cancerous and antioxidant properties. It's essential oil inhibit mainly against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Aspergillus niger*. It relieves arthiritis, reduces cholesterol and blood sugar level, improves digestion, heals oral ulcer and keeps the skin healthy [4-5]. *Illiciumverum* is rich in linalool which is chiefly responsible for its antioxidant, antibacterial, antifungal, and analgesic properties. It is used to treat asthma, dry cough and bronchitis [6]. Black pepper the king of spices mainly contains piperine, an anti-inflammatory and anti-arthritic agent. It was used therapeutically in dentistry as an antiseptic for tooth-decay and gum swellings, as an antioxidant in tea form for relieving arthritis, fever, migraine headaches, poor digestion and even coma. It increases the flow of saliva and stimulates appetite. It is a powerhouse of antioxidant which works against hypertension, diabetes, cancer and cardiovascular disease [7-8].

The objective of this study is (a) to evaluate and compare total phenolic content, flavanoid content, antioxidant potential of methanolic extracts of the spices from India and also evaluate their antibacterial and antifungal potential (b) To establish the relationship between antioxidant activity and phenolic compounds of the spice extracts to confirm that the phenolic constituents are responsible for their antioxidant activity.

## MATERIALS AND METHODS

### Materials

All solvents used were of HPLC grade. Deionzied water was used. Ascorbic acid, DPPH, quercetin, gallic acid, ferrous sulphate and TPTZ (2, 4, 6-tripyridyl-s-triazine) were procured from Sigma Aldrich. Sodium acetate, glacial acetic acid, sodium nitrate, Folinciocalteu's reagent, sodium hydroxide, aluminium chloride, hydrochloric acid and sodium nitrite used were of analytical grades. The UV-Visible spectrophotometer used was the Shimadzu UV-1800 model.

### Preparation of extracts of spices

Each spice chosen namely turmeric, bay leaf, nutmeg, green cardamom, fennel seeds, carom seeds, cinnamon, fenugreek, ginger (dried), clove, red chilli, cumin seeds, coriander, star anise and black pepper were powdered properly and 0.1 g of each were extracted with 5 ml of methanol by ultra-sonication for 30 min at 20 ° C and then centrifuged at 9000 rpm for 10 min. Supernatant was collected in different tubes. The process was repeated again and the supernatants were mixed to get fixed volume of 10 ml. This extract was stored below 4 °C till it was used. All the determinations were done in triplicates.

**Total phenolic content (TPC) determination**

Total phenolic content of all the extracts was evaluated with Folin-Ciocalteu method [9-10]. This method relies upon the reduction of a mixture of phosphotungstic ($WO_4^{2-}$) and phosphomolybdic ($MoO_4^{2-}$) reagent by the phenolic hydroxyl group resulting in the blue solution with λmax at 765 nm. The standard curve is prepared by using Gallic acid (1 mg/ml) and results are reported as mg/ml of gallic acid equivalent (GAE).

**Total flavonoid content determination**

Aluminum chloride method was followed to determine the total flavonoid content in the selected spice extracts. The absorbance was measured at 316 nm. The total flavonoid content was expressed in terms of mmol of quercetin equivalent [11].

**Ferric reducing antioxidant power (FRAP) assay**

This method used was suggested by Benzie and Strain, 1996; [12-13]. It is based on the reduction of the complex of Fe (III)$(TPTZ)_2Cl_3$ or ferric 2,4,6-tripyridyl-s-triazine to the ferrous form (intense blue coloured) at low pH by the reductant. This reduction is monitored by the change in absorption at 595 nm using mmol of ferrous sulphate as standard.

**Reducing power assay**

This assay is based on the principle that substances that have high reduction potential react with potassium ferricyanide ($Fe^{3+}$) and forms potassium ferrocyanide ($Fe^{2+}$) which then reacts with ferric chloride and forms ferric ferrous complex showing absorbance at 700 nm at pH6.6. Ascorbic acid was used for the standard curve. Spice extract (0.5 ml) and 1 ml of potassium ferricyanide was mixed and incubated at 50 °C for 20 min in a water bath. After cooling it to room temperature 1 ml of trichloroacetic acid was added to it and it was then centrifuged at 3000 rpm for 10 min. 1 ml of the supernatant was taken out carefully into separate tubes and 1.5 ml of de ionized water was added to it. After this, 100 µl of freshly prepared ferric chloride solution was added and was shaken well and incubated again for 10 min at 50 °C. Absorbance was measured after shaking the mixture properly and taking water as the reference. The increase in reducing power was shown by an increase in absorbance of the reaction mixture [14].

**DPPH Assay**

This assay was done according to Brand–Williams *et al.*, 1995 [15]. This method is based on the reduction of alcoholic solution of DPPH radical (0.6 mmol stock) in the presence of an antioxidant which is a hydrogen donor. Solutions of DPPH (1,1-Diphenyl-1-picrylhydrazyl) with variable deep violet color showed the strong absorption band at 515 nm. The absorbance was measured at 0 min for blank determination using 1 ml DPPH and 2 ml methanol without adding any sample. For sample analysis, 25 µl of each spice extract, 2 ml of methanol and 1 ml of DPPH was taken. The solutions were incubated for 1 h to let the reduction complete. Absorbance was measured at 515 nm taking methanol as the reference [16]. Results were expressed as % inhibition in radical scavenging activity after 1 h and 24 h.

**ABTS Assay**

For ABTS assay the method used was described by Arnao *et al.*, 2001 [17]. For preparation of active reagent 7.4 mmol ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] and 2.6 mmol potassium thio sulphate were mixed in equal quantities. The solution was allowed to stand for 12 h at room temperature (28 °C) in the dark to generate free radicals. The blue-green colour of the solution also turns darker. The solution was diluted 10 times with ethanol and used as working reagent. The wavelength of maximum absorption was found to be 750 nm. Fresh $ABTS^{\cdot+}$ solution was used for each assay. 20 µl of sample was mixed with 3 ml of working $ABTS^{\cdot+}$ solution and measured immediately after 3-4 min and after 1 h incubation at 750 nm. Results were expressed as % inhibition in radical scavenging activity after 1 h and 3 h.

**Anti-microbial activity**

Antibacterial activity of spice extracts was measured against gram positive bacteria-*Staphylococcous aureus* (ATCC 25923) and gram negative bacteria-*Escherichia coli* (ATCC 25922). Fungal strain-*Aspergillus niger* (with white mycelia and black spores) (ATCC 16404), *Fusarium* species (with white mycelia and spores) were selected for antifungal studies. The method used was agar well method. Bacterial inoculum was prepared up to 0.5 McFarland turbidity standards. Fungal spore culture was adjusted to$10^5$ CFU/ml. Nutrient Agar medium and Czapek Dox Agar were used for antibacterial and antifungal activity measurement respectively.

Antifungal activity of two of the spice extracts (Clove and Cinnamon) was checked against *A. niger* and *Fusarium* species. For checking the MIC, 6 concentrations of spice extracts (0.01, 0.03, 0.05, 0.1, 0.2 and 0.5 mg/ml) were poured (20 µl) one in each well and a mycelium punch of fungus was put at the center of petri plate. The plates were incubated at 32 °C and the growth pattern of fungal mycelium was observed after 48 h, 96 h and 1 week [18].

For antibacterial activity, inoculation of agar plates was done by pouring 20 µl of the culture medium and spreading uniformly by the edge of sterilized glass slide. 20 µl of the prepared dilutions (0.01-0.6 mg/ml) of the spice extracts were poured in the center of well vertically by a micropipette. One well of positive control (antibiotic chloramphenicol 20µg/ml concentrations) and one of negative control (MeOH) was used [19]. After loading the samples, the plates were incubated at 37 °C for 18-24 h for antibacterial study. ZOI were observed by measuring the diameter of the clear zone around the wells using zone scale reader. The plates were studied in triplicates.

**RESULTS**

**Total Phenolic and total flavonoid content**

We have determined the total phenolic content (TPC) and flavonoid content of methanolic extracts of 15 spices samples under study.

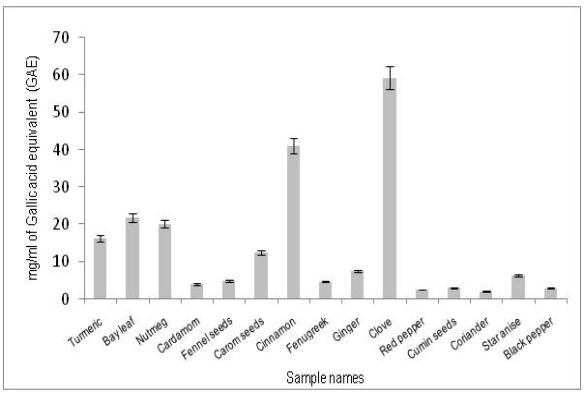


**Fig. 1(a): TPC in mg/ml of Gallic acid equivalent (GAE) for different spice extracts**

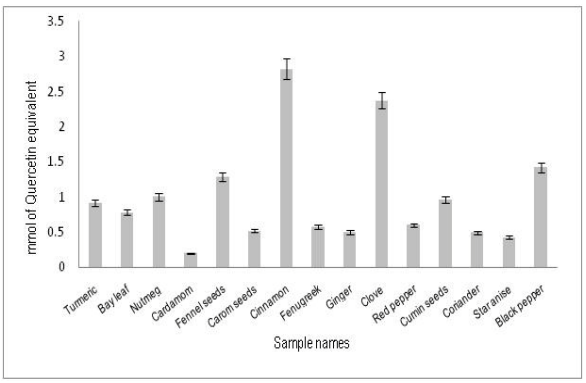


**Fig. 1 (b): Total Flavonoid content in mmol of Quercetin equivalent of spice extracts**

The standard used was gallic acid for TPC and quercetin for flavonoid content measured at 756 nm and 316 nm respectively under testing condition. Among them *Syzygiumaromaticum* showed highest phenolic content (59.278 mg/ml of Gallic Acid Equivalent GAE) while *Cinnamamumverum* showed highest flavonoid content (2.823 mmol of quercetin equivalent). The order of performance for TPC observed was highest in Cloves>Cinnamon>Bay leaf>Nutmeg> Turmeric> Carom seeds> Ginger> Star anise> Fennel seeds> Fenugreek> Cardamom> Cumin seeds> Black pepper> Red pepper> Coriander as depicted in fig. 1(a).

The order of performance for Flavonoid content observed as shown in fig. 1(b)was highest in Cinnamon> Clove> Black pepper> Fennel seeds> Nutmeg> Cumin seeds> Turmeric> Bay leaf> Red pepper> Fenugreek> Carom seeds> Ginger = Coriander> Star anise> Cardamom.

**Reducing power assay and FRAP assay**

Reducing power method reflects antioxidant activity. Reductant in a redox reaction has the power of antioxidant as referred to as its reducing ability [20]. The reducers converts $Fe^{3+}$/ferricyanide complex to ferrous form which then reacts with ferric chloride and forms ferric ferrous complex (blue complex) with λ max at700 nm. Higher absorbance of the mixture gives higher reduction potential. Reducing power was expressed as mM of ascorbic acid equivalent per ml of extract. fig. 2 shows highest % reduction for Clove (1.107 mmol of AA equivalent/ml of extract). The order of performance for reducing power observed was highest in Clove>Nutmeg>Bay leaf> Cinnamon> Turmeric> Star anise> Ginger> Carom seeds> Black pepper> Coriander> Red pepper> Cumin seeds> Fennel seeds> Fenugreek> Cardamom.

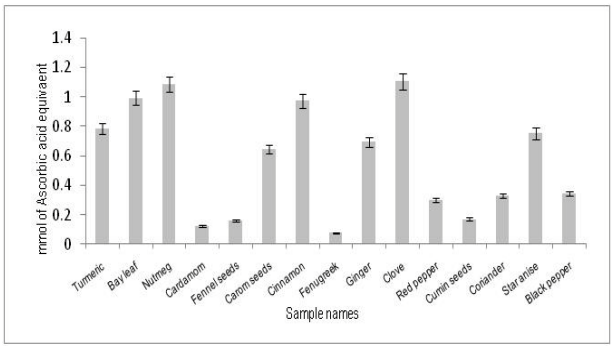


**Fig. 2: Bar graph showing reducing power of spice extracts at 700 nm**

In FRAP assay, an easily reducible oxidant ($Fe^{3+}$) is used in excess. This method is based on the reduction of the complex of ferric ions and TPTZ (2,4,6-tripyridyl-s-triazine) to the ferrous form at low pH [21]. The latter forms a complex which is blue in color with absorbance measured at 595 nm in terms of ferrous sulphate (mmol) equivalent concentrations. fig. 3(a)showed response of increasing concentration of $FeSO_4$ (mmol) in FRAP assay at 0, 4 and 10 min time intervals. The curve showed high linearity index with R value nearing 0.974. Fig. 5 depicts that highest % reduction is shown by *Syzygiumaromaticum* (Clove) followed by cinnamon (*Cinnamomumverum*). Absorbance was observed at 596 nm immediately at 0, 4 and 10 min intervals against the reagent blank.

The order of performance for FRAP assay was highest in Clove> Cinnamon> Nutmeg> Bay leaf> Ginger> Carom seeds> Star anise> Turmeric> Red pepper> Black pepper> Coriander>Cumin seeds> Fennel seeds> Cardamom> Fenugreek as shown in fig. 3(b).

**ABTS and DPPH Assays**

ABTS assay involves a radical cation while DPPH assay make use of stable free radical. Addition of antioxidants to the pre-formed radical cation reduces it based on activity/concentration of antioxidant or duration of the reaction. In both the assay the % inhibition activity increased with time of measurement.fig. 4 shows maximum reduction in radical scavenging activity as observed in case of clove (94%) followed by cinnamon (89%).

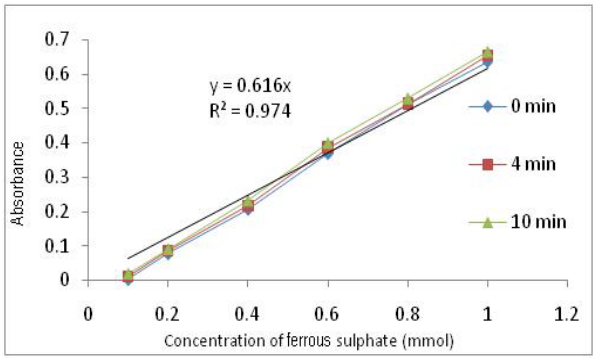


**Fig. 3 (a): Standard curve of ferrous sulphate of known concentration at 595 nm**

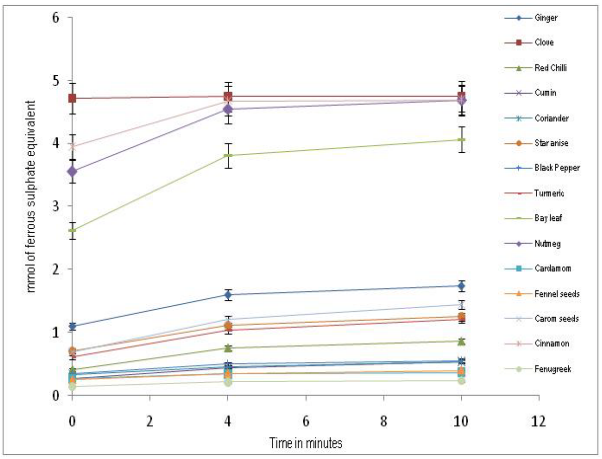


**Fig. 3 (b): Ferric Reducing Antioxidant Power (FRAP) curves of spice extracts at 595 nm**

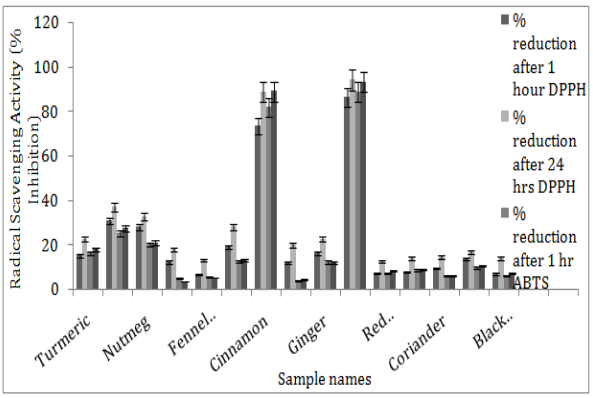


**Fig. 4: Bar graph showing % inhibition caused by spice extracts after 1 hour and 24 hours at 515 nm in DPPH assay and after 1 hour and 3 hours at 750 nm in ABTS assay**

Fig. 5 gives the correlation curve between TPC and antioxidant potential (as % reduction in DPPH assay). The value of $R^2$= 0.979 is near to 1 and the curve is linear thus we can conclude that there is a direct relationship between the phenolic content and DPPH radical scavenging tendency of a spice with an increase in phenolic content.

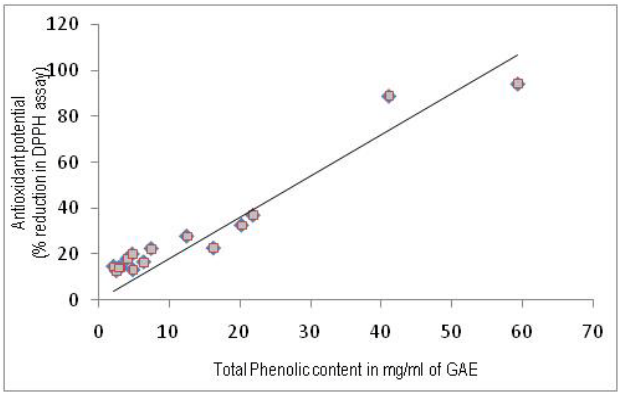


**Fig. 5: Correlation diagrams of Total phenolic content and Antioxidant Potential**

### Antimicrobial activity

Cinnamon, Carom seeds and Clove showed remarkable antibacterial properties against both gram positive (*S. aureus*) and gram negative (*E. coli*) bacteria. The results of antibacterial activity are tabulated in table 1. Clove and Black pepper showed good antibacterial activity against gram positive bacteria *S. aureus* at MIC of 0.05 mg/ml followed by Cinnamon, Carom seeds and Turmeric showing MIC of 0.1 mg/ml. MIC against *E. coli* was found to be 0.1 mg/ml for Clove, Cinnamon, Carom seeds and Turmeric.

Antifungal activity of different concentrations of clove extracts (concentrations 0.01, 0.03, 0.05, 0.1, 0.2 and 0.5 mg/ml) were checked against *A. niger* and *Fusarium* up to 7 days. Zone of Inhibition (ZOI) of 2 cm diameter was obtained at 0.1 mg/ml concentration indicating clear antifungal activity against *A. niger*.

Activity against *Fusarium* showed obstruction in fungal growth showing mycelium mat span of 2.8 cm as compared to control showing mycelium mat span of 7.5 cm under similar conditions up to 7 days of study at a minimum spice extract concentration of 0.07 mg/ml. Under similar conditions, Cinnamon extract showed 0.2 mg/ml concentration as MIC against *A. niger* and 0.01 mg/ml concentration against *Fusarium* species.

## DISCUSSION

The above studies showed that Clove, cinnamon, bay leaf and nutmeg showed higher antioxidant potential, higher phenolic content and good reducing potential. The FRAP and reducing potential assay both indicated higher reducing capacity of cinnamon and clove over other spices. The radical scavenging DPPH and ABTS assays also show similar results. Also the phenolic content has a direct correlation with antioxidant activity showing good order of linearity ($R^2$=0.98). According to a survey published on modern survival blog, clove and cinnamon are rated at the highest position among top 100 high ORAC value antioxidant foods [22]. The high concentration of eugenol, β-Caryophyllene and eugenyl acetate may be responsible for high antioxidant potential, antibacterial and antifungal activities of clove extract [23-24]. In cinnamon, cinnamaldehyde, 4-methoxy cinnamaldehyde, eugenol, d-cadinene and coumarin may be attributed for its greater activity [25].

**Table 1: Antibacterial activity of various spice extracts against *E. coli* and *S. aureus* indicating Zone of Inhibition and MIC**

| Name of spice | MIC (S. aureus) in mg/ml | ZOI (S. aureus)in mm | MIC (E. coli) in mg/ml | ZOI (E. coli) in mm |
|---|---|---|---|---|
| *Clove* | 0.05 | 10.0±0.1 | 0.1 | 10.0±0.1 |
| *Cinnamon* | 0.1 | 9.0±0.0 | 0.1 | 11.0±0.1 |
| *Ajwain or Carom seeds* | 0.1 | 13.0±0.2 | 0.1 | 12.0±0.0 |
| *Black pepper* | 0.05 | 12.0±0.1 | - | - |
| *Turmeric* | 0.1 | 12.0±0.0 | 0.1 | 12.0±0.1 |
| *Ginger* | 0.5 | 13.0±0.2 | 0.5 | 12.0±0.1 |
| *Chloramphenicol (positive control)* | 0.02 | 16.0±0.1 | 0.02 | 22.0±0.2 |
| *Methanol(negative control)* | - | - | - | - |

MIC: Minimum Inhibitory Concentration; ZOI: Zone of Inhibition; -No inhibition found

## CONCLUSION

This study indicates that these spices may be used as natural medicine for combating stress and reducing the ill effects of free radicals related disorders. Further investigations are required to check their activities *in vivo* to assess the health implications of these wonderful, aromatic plant produce.

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## CONFLICT OF INTERESTS

Declared None

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