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Antioxidant Properties and In-Vitro Radical Scavenging Activities of Tannin-Rich and Flavonoid-Rich Fraction of *Annona senegelensis* and *Vernonia amygdalina* leaves

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ABSTRACT: Medicinal Plants have demonstrated history of managing some ailments caused by free radicals as a result of some chemical constituents they possess. This study was aimed at assessing antioxidant and free radical scavenging properties of Tannin-rich and Flavoniod-rich fraction of *Annona senegelensis* and *Vernonia amygdalina* leaves via in vitro assays such as; reducing power, nitric oxide scavenging activity, Hydrogen peroxide scavenging activity and Lipid peroxidation scavenging activity. The results obtained indicated that both medicinal plants are antioxidant reservoir. The values for Tannin rich fraction of *Annona senegelensis* (TRFAS) and Tannin rich fraction of *Vernonia amygdalina* (TRFVA) are Reducing power absorbance TRFAS (0.077-0.187), TRFVA (0.168-0239). % Nitric oxide scavenging: TRFAS (4.46-30.40), TRFVA (5.23-42.24). % H_2O_2 scavenging: TRFAS (7.30-20.35), TRFVA (8.12-22.32). % Lipid Perioxidation: TRFAS (6.81-32.76), TRFVA (5.16-26.16). Also the values for Flavonoid rich fraction of *Annona senegelensis* (FRFAS) and Flavonoid rich fraction of *Vernonia amygdalina* (FRFVA) are reducing power absorbance: FRFAS (0.109-0.342), FRFVA (0.124-0.388). % Nitric oxide scavenging: FRFAS (33.55-43.57), FRFVA (21.10-47.46). % H_2O_2 scavenging: FRFAS (39.01-74.96), FRFVA (45.80-75.20). % Lipid Perioxidation: FRFAS (24.81-59.69), FRFVA (41.43-59.98). The Tannin-rich and Flavonoid-rich fraction of both plants exhibited good antioxidant activity on all models employed at increasing concentrations but Flavoniod-rich fraction of *Vernonia amygdalina* had the highest inhibitory effect than the Tannin fraction. The findings from this study validated the pharmacological potency of the two plants and their potential use in combating free radical-related diseases, which are often triggered by oxidative stress.

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Free radicals are reactive oxygen species produced by the body during normal metabolism and during the immune response to pathological cell metabolism. They can also be triggered by exogenous chemicals and a variety of environmental factors such as smoke and dust. Free radicals can oxidize biomolecules including RNA, DNA, proteins, and lipids, causing cell membrane lysis, cell death, and tissue damage (Idris *et al*., 2020). However, Medicinal Plants have shown some demonstrated history of managing some ailments caused by free radicals as a result of some chemical constituents they possess. The identification of medicinal plants with antioxidant potential has become a realistic and powerful tool in ethnomedicine for reducing the complication associated with environmental pollution in humans. Plants contain chemical substances/compounds that allow them to fulfill their responsibilities of preserving human health and curing disease, and phytochemicals are the names given to these compounds (Altemimi *et al*., 2017). Malaria, cancer, dysentery, filariasis, convulsions, diarrhea, impotency, inflammations, pains, snake bites, and sexually transmitted diseases are among the diseases and symptoms for which *A. senegalensis* is used in ethnomedicine by various peoples of tropical Africa (Udodeme *et al*., 2019). According to Udodeme *et al* (2019), the plant's (A. senegalensis) decoction is used in folkloric medicine to treat kwashiorkor, marasmus, eyelid swelling, and body ache, while the stem bark is used to treat hepatitis, gastroenteritis, guinea worms, toothache, pneumonia, and respiratory infections in Northern Nigeria. *Vernonia amygdalina* (Astereacea) is a tropical African shrub or small tree that grows to a height of 1 to 5 meters. Bitter leaf is, as its name implies, bitter in taste but shockingly tasty in meals. In Nigeria, bitter leaf is known as, Onugbo in Igbo-Eastern Nigeria, Ewuro (Yoruba), and Shuwaka in Hausa (Bashir *et al*., 2020). Bitter leaf is a well-cultivated plant with common market merchandise in some African countries such as Nigeria, Cameroon, Ethiopia, and Zimbabwe due to the bitterness of its leaves (Adesonoye and Farombi, 2014). The leaves are

eaten as an appetizer, and the leaves' extract is used to facilitate digestion in Nigeria. Hausa women in Northern Nigeria eat it with the belief that it improves sexual attractiveness (Oyeyemi *et al*., 2018). Leaf extracts of *V. amygdalina* in Nigeria are used in ethnomedicines for treatment of diabetes (Akwasi, 2018). Therefore, it is clear that these plants contains chemical constituents responsible for its pharmacological actions. This study was designed to evaluate the antioxidant and radical scavenging activities of Tannin and Flavoniod-rich fraction of *Annona sengalensis* and *Vernonia amygdalina* leaves in vitro.

MATERIALS AND METHODS

Plant Collection: Fresh leaves of *Annona senegalensis* and *Vernonia amygdalina* were collected from Nekede Owerri, Imo State Nigeria. The plants were identified and authenticated by a Plant Taxonomist at Michael Okpara University of Agriculture Umudike.

Preparation of Plant Sample: The fresh leaves of *Annona senegalensis* and *Vernonia amygdalina* were washed and allowed to dry to a constant weight and pulverized into powder using Pulverizer (5126 TP).

Extraction of tannin rich fraction: The powdered sample of *Annona sengalensis* and *Vernonia amygdalina* leaves (100g) were weighed and each of the sample was defatted with petroleum ether in a mechanical shaker for 48hrs at room temperature. Then it was extracted with aqueous acetone and water at 420ml and 180ml respectively for 60min at 60^{o}c in water bath with constant stirring. The mixture was then filtered and centrifuged at 300rpm for 10mins. The filtrate was allowed to evaporate at room temperature. The acetone free extract was lyophilized and the powdered sample was collected, weighed and stored in sterile bottle at 4^{o}c in a refrigerator for further study.

Flavonoid Extraction: Five hundred gram (500g) of powdered leaves was macerated in 80% methanol at room temperature for 72h. It was continuously mixed and then filtered using a filter paper (Whatman size No.1). The filtrate was dried in a water bath at 45^{o}C and concentrate was kept in air tight bottle at 4°C until used. The concentrate obtained from each sample was subsequently extracted in petroleum ether, diethyl ether and ethyl acetate following the method of Subramanian and Nagarajan (1969). Petroleum ether fraction was discarded due to its being rich in fatty substances. Ether fraction was used for free flavonoids whereas ethyl acetate fraction for bound flavonoids. Ethyl acetate fraction of each sample was hydrolysed further with 7% Sulphuric acid for 24hours and was then re-extracted with ethyl acetate. The fraction obtained was repeatedly washed with distilled water to neutrality, dried and weighed.

In-vitro antioxidant Analysis

Determination of total phenol content: Total phenolic content was determined by Folin Ciocalteu's method as described by Bhalodia *et al*. (2011) and Patel *et al*. (2010) with slight modification.

Determination of total flavonoid content: Total flavonoid content was determined using aluminium chloride colorimetric assay as used by Patel *et al.* (2010); Pallab *et al.* (2013) and Satish *et al*. (2008).

Reducing power Assay: Following the report of Kowsalya and Namasivayam (2014), the Fe^{3+} reducing power of the plants was determined by the method of (Oyaizu, 1986) with a slight modification. Different concentrations (200-800 µg mL^{-}1) of the extracts were mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium hexa cyanoferrate (1%), followed by incubation at 50°C in a water bath for 20 min. After incubation, 2.5 mL of TCA (10%) was added to terminate the reaction. The upper portion of the solution (2.5 mL) was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ solution (0.1%) was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. A higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as a reference standard.

Nitric oxide radical scavenging activity: Nitric oxide radical scavenging activity was assayed according to the method reported by Kowsalya and Namasivayam (2014). Nitric oxide was generated from sodium nitro prusside in aqueous solution at physiological pH, which interacts with oxygen to produce nitric ions, which may be determined by the Griess Illosvoy reaction. The 2.0mL of 10mM sodium nitro prusside, 0.5mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of sample at different concentrations and the mixture was incubated at 25°C for 150 min. From the incubated mixture 1.5mL was taken out and added into 1.0 mL of griess reagent (1% sulphanilamide, 2% O-phosphoric acid, 1% napthyl ethylene diamine di HCl) and incubated at room temperature for 5 min. The absorbance of the mixture was measured at 546nm.

Hydrogen peroxide scavenging assay: This activity was determined according to a previously described method by Floriano-Sanchez *et al*. (2006) with minor changes in the report of Kowsalya and Namasivayam

(2014). An aliquot of 50mM H_2O_2 and various concentrations (200-800µg mL^{-1}) of samples were mixed (1:1 v/v) and incubated for 30 min at room temperature. After incubation, 90µL of the H_2O_2 sample solution was mixed with 10 µL HPLC-grade methanol and 0.9 mL FOX reagent was added (4.4 mM BHT added in 9 volumes of Methanol and 1 volume of 1mM xylenol Orange, 2.56mM Ammonium ferrous sulphate in 0.25 M H2SO4). The reaction mixture was then vortexed and incubated at 37°C for 30 min. The absorbance of the ferric-xylenol orange complex was measured at 560nm.

Lipid Peroxidation Assay: Lipid peroxidation assay was carried out by measuring lipid peroxide content and the conjugated diene formation and the method of Salawu *et al.* (2006) was employed.

Lipid Peroxide Formation: A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed using egg yolk homogenates as lipid rich media. Egg yolk homogenate was prepared according to a standard method. Malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid (TBA) yielding a pinkish red chromogen with absorbance maximum at 532 nm. Egg homogenate (0.5 mL of 10% v/v) and 0.1 mL of each extract were added to a test tube and made up to 1 mL with distilled water. 0.05 mL of FeSO4 (0.07 M) was added to induce lipid peroxidation and incubated for 30 min. Then 1.5 mL of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 mL of 0.8% (w/v) TBA in 1.1% sodium deodecyl sulphate and 20% TCA were added and the resulting mixtures were vortexed and then heated at 95°C for 60 min. After cooling, 5.0 m of butan-1-ol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic layer was measured at 532 nm. Inhibition of lipid peroxide formation (%) by the extract was calculated according to [(1-E/C) x 100] where C is the absorbance value of the fully oxidized control and E is (Abs 532+TBA-Abs532-TBA).

RESULTS AND DISCUSSION

The present study indicates the in-vitro antioxidant activity of Isolated Tannins and Flavoniods from *Annona senegalensis* and *Vernonia amygdalina* leaves. Phytochemicals studies have confirmed and justified the use of these folkoric herbs. The results (Tables 1 to 10) from the current study clearly justified the use of both medicinal plants in the management various pathologic conditions. It gives protection against various free radicals. There was significant ($p<0.05$) increase in a dose dependent manner of the reducing power ability of the two plants with *Vernonia amygdalina* having more potency compared to *Annona senegalensis*. There was significant ($p<0.05$) increase in a dose dependent manner of the percentage nitric oxide scavenging ability of the two plants with *Vernonia amygdalina* having more potency compared to *Annona senegalensis*. There was significant ($p<0.05$) increase in a dose dependent manner of the percentage Hydrogen peroxide inhibition of the two plants with 400µg/m and 600µg/m of *Annona senegalensis* having more potency compared to 400µg/m and 600µg/m of *Vernonia amygdalina*. Although the ascorbic acid has more percentage Hydrogen peroxide inhibition.

There was significant ($p<0.05$) increase in a dose dependent manner of the Lipid Peroxidation scavenging ability of the two plants with 800µg/m of *Annona senegalensis* having more potency than *Vernonia amygdalina*. Although the ascorbic acid has more Lipid Peroxidation scavenging ability. For the tannin fraction of both plants, there was significant ($p<0.05$) increase in a dose dependent manner of the reducing power ability of the two plants with *Vernonia amygdalina* having slightly more potency compared to *Annona senegalensis*. The flavonoid fraction from both plant have more reducing power than the Tannin fraction.

There was significant ($p<0.05$) increase in a dose dependent manner of the percentage nitric oxide scavenging ability of the two plants with *Vernonia amygdalina* having more potency compared to *Annona senegalensis*. Although the 200µg/m of *Annona senegalensis* has higher % ihnibition of Nitric oxide than the 200µg/m of *Vernonia amygdalina*. There was also significant ($p<0.05$) increase in a dose dependent manner of the Lipid Peroxidation scavenging ability of the two plants.

Different models used to evaluate the antioxidant activity suggests that the fractions from *V. amygdalina* and *A. senegalensis* showed good source of natural antioxidants. Based on the result of analysis for the Tannin-rich fraction, increasing concentration showed some significant increase in absorbance. *V. amygdalina* tannin-rich fraction exhibited strong reducing power effect at 400µg/ml but had little reducing power effect as the concentration increased (Table 3). TRFAS and TRFVA exhibited scavenging effect on nitric oxide radical (NO^+) at increasingly high concentration while the Ascorbic acid exhibited the highest effect (Table 4). However, the H_2O_2 scavenging activity of Tannin-rich fraction of both plants was relatively low on comparison to Ascorbic acid standard (Table 5). Although TRFAS and TRFVA showed some scavenging ability to donate electrons to H_2O_2 and neutralize it to water at

increasing concentrations. The percentage inhibition of lipid peroxidation by TRFAS and TRFVA were relatively low when compared with Ascorbic acid. However, TRFVA had more lipid peroxidation activity than TRFAS at concentrations of 400µg/ml and 600µg/ml (Table 6).

Table 1: Total Phenolics and Total Flavonoids of Tannin- Rich Fraction of *Annona senegalensis* (TRFAS) and Vernonia amygdalina (TRFVA) Leave

Samples	Total Phenolics (mgGAE/g)	Total flavonoids (mgQCE/g)
Tannin Rich Fraction of *Annona senegalensis* (TRFAS)	32.96±2.80	28.46±1.16
Tannin Rich Fraction of *Vernonia amygdalina* TRFVA	38.07±5.94	36.25±2.56
Flavonoid Rich Fraction of *Annona senegalensis* (FRFAS)	56.15±4.63	126.04±7.28
Flavonoid Rich Fraction of *Vernonia amygdalina* (FRFVA)	52.49±3.67	164.18±4.32

The results are mean±SD of triplicate determination

The Flavoniods-rich fraction of both plants revealed that they had a good scavenging activity to reduce Fe $^{3+}$→ Fe $^{2+}$ at increasing concentration when compared to the standard (table 7). At Concentration of 200µg/ml, FRFAS had high Nitric oxide scavenging activity than FRFVA while at 600µg/ml, the both fractions had the same activity. FRFVA showed more activity at 400µg/m and 800µg/ml respectively than FRFAS (Table 8). However, ascorbic acid exhibited the greatest effect. FRFVA showed high H_2O_2 scavenging activity at 200µg/ml than FRFAS. At 400µg/ml FRFAS demonstrated a higher scavenging activity than FRFVA. But at 600µg/ml and 800µg/ml FRFVA exhibited greater activity over FRFAS. Nevertheless, both fractions exhibited increasing antioxidant activity on comparison to standard (Table 9). However, at increasing concentrations, both FRFAS and FRFVA had higher lipid peroxidation activity but had almost same activity at concentration of 800µg/ml (Table 8). At 200µg/ml and 400µg/ml concentrations, the activity of the FRFAS and FRFVA was relatively same as the standard Ascorbic acid (Table 8).

Table 2: IC50 of the various Scavenging activities of Tannin- Rich Fraction of *Annona senegalensis* (TRFAS) and *Vernonia amygdalina* (TRFVA) Leave

Samples	Reducing Power (µg/m)	Nitric Oxide Scavenging (µg/m)	H_2O_2 Scavenging (µg/m)	Lipid Peroxidation (µg/m)
Tannin Rich Fraction of *Annona senegalensis* (TRFAS)	1399.59±95.54	6.11±1.06	10.65±2.18	5.19±1.03
Tannin Rich Fraction of *Vernonia amygdalina* TRFVA	2920.61±125.18	4.23±0.85	10.28±1.63	6.89±1.64
Flavonoid Rich Fraction of *Annona senegalensis* (FRFAS)	647..53±62.74	5.75±1.65	1.45±0.08	2.86±0.25
Flavonoid Rich Fraction of *Vernonia amygdalina* (FRFVA)	540.48±34.98	3.94±0.72	1.54±0.82	2.39±0.61
Ascorbic acid	249.73±35.85	2.18±0.54	0.24±0.04	1.59±0.34

The results are mean±SD. The lower the IC50 the higher the antioxidant activity.

Table 3: Reducing power ability of Tannin- Rich Fraction of *Annona senegalensis* (TRFAS) and Vernonia amygdalina (TRFVA) Leave

Concentration (µg/m)	Absorbance of TRFAS	Absorbance of TRFVA	Absorbance of Standard Ascorbic Acid
200	0.077±0.01	0.168±0.03	0.976±0.06
400	0.102±0.03	0.602±0.05	1.193±0.05
600	0.129±0.01	0.219±0.02	1.325±0.11
800	0.187±0.04	0.239±0.01	1.589±0.08

The results are mean±SD of triplicate determination.

Table 4: Percentage Nitric oxide Scavenging of Tannin- Rich Fraction of *Annona senegalensis* (TRFAS) and *Vernonia amygdalina* (TRFVA) Leave

Concentration (µg/m)	% inhibition of TRFAS	% inhibition of TRFVA	% inhibition of Standard Ascorbic Acid
200	4.46±0.16	5.23±0.82	62.20±3.14
400	22.99±1.42	31.21±1.78	71.70±1.92
600	25.77±1.08	37.68±1.24	74.90±2.86
800	30.40±2.17	42.24±5.02	76.30±4.10

The results are mean±SD of triplicate determination.

Medicinal plants are significant source of efficient chemotherapeutics, which are critical for human health maintenance. In terms of mechanism of action, biological properties, and chemical structures, these phyto-compounds are special (Arika *et al*., 2019). Antioxidants counteract the effects of free radicals by either reacting with, neutralizing, or fighting for substrates with molecular oxygen (O_2) as their terminal electron acceptor (Arika *et al.,* 2019; Lallianrawna, 2013). Antioxidant compounds and phytochemicals abundant in medicinal plants are thought to be responsible for these plants' therapeutic properties (Ekaluo *et al.*, 2015).

Table 5: Hydrogen Peroxide Scavenging of Tannin- Rich Fraction of *Annona senegalensis* (TRFAS) and *Vernonia amygdalina* (TRFVA) Leave

Concentration (µg/m)	% inhibition of TRFAS	% inhibition of TRFVA	%inhibition of Standard Ascorbic Acid
200	7.30±1.05	8.12±0.74	57.50±4.12
400	13.88±1.42	11.48±1.34	73.40±1.54
600	17.84±2.04	14.85±2.05	86.65±3.02
800	20.35±0.98	22.32±1.18	94.42±5.46

The results are mean±SD of triplicate determination.

Table 6: Lipid Peroxidation scavenging of Tannin-Rich Fraction of *Annona senegalensis* (TRFAS) and *Vernonia amygdalina* (TRFVA) Leave

Concentration (µg/m)	% inhibition of TRFAS	% inhibition of TRFVA	% inhibition of Standard Ascorbic Acid
200	6.81±0.58	5.16±0.82	41.84±2.16
400	8.66±0.72	18.32±1.65	51.63±1.34
600	16.99±1.03	26.08±3.14	78.60±6.83
800	32.76±1.68	26.16±1.26	91.15±5.17

The results are mean±SD of triplicate determination.

Table 7: Reducing power ability of Flavonoid- Rich Fraction of *Annona senegalensis* (FRFAS) and *Vernonia amygdalina* (FRFVA) Leave

Concentration (µg/m)	Absorbance of flavonoid-Rich Fraction	Absorbance of flavonoid- rich Fraction	Absorbance of Standard Ascorbic Acid
200	0.109±0.03	0.124±0.04	0.977±0.05
400	0.145±0.01	0.151±0.02	1.198±0.12
600	0.218±0.05	0.284±0.07	1.324±0.08
800	0.342±0.05	0.388±0.02	1.588±0.04

The results are mean ± SD of triplicate determination

Table 8: Percentage Nitric Oxide Scavenging of flavonoid - Rich Fraction of *Annona senegalensis* (FRFAS) and *Vernonia amygdalina* (FRFVA) Leave

Concentration (µg/m)	% inhibition of FRFAS	% inhibition of FRFVA	% inhibition of Standard Ascorbic Acid
200	33.55±4.13	21.10±2.64	62.20±5.18
400	40.19±2.15	48.43±1.78	71.70±5.83
600	41.70±5.81	41.38±3.25	74.90±3.98
800	43.57±3.92	47.46±2.84	76.30±4.73

The results are mean±SD of triplicate determination.

Table 9: Hydrogen Peroxide Scavenging of flavonoid - Rich Fraction of *Annona senegalensis* (FRFAS) and *Vernonia amygdalina* (FRFVA) Leave

Concentration (µg/m)	% inhibition of FRFAS	% inhibition of FRFVA	% inhibition of Standard Ascorbic Acid
200	39.01±2.62	45.80±4.65	57.50±2.18
400	65.50±4.17	51.63±6.12	73.40±7,24
600	66.08±2.41	67.52±2.43	86.65±5.31
800	74.96±3.86	75.20±1.94	94.42±4.93

The results are mean±SD of triplicate determination. There was significant ($p<0.05$) increase in a dose dependent manner of the percentage Hydrogen peroxide inhibition of the two plants. Although the ascorbic acid has more percentage Hydrogen peroxide inhibition.

Table 10: Lipid Peroxidation Scavenging of flavonoid - Rich Fraction of *Annona senegalensis* (FRFAS) and *Vernonia amygdalina* (FRFVA) Leave

Concentration (µg/m)	% inhibition of FRFAS	% inhibition of FRFVA	% inhibition of Standard Ascorbic Acid
200	24.81±2.74	41.43±2.17	41.84±3.16
400	47.71±4.12	48.22±3.15	51.63±4.23
600	51.91±5.31	52.91±2.83	78.60±3.86
800	59.69±3.62	59.98±1.95	91.15±7.13

The results are mean±SD of triplicate determination.

Both fractions showed some does-dependent free radicals scavenging activity. Furthermore, Ryszard (2007) in his review highlighted that Tannins are good and a new natural antioxidants but do not function solely as primary antioxidants but also as a secondary antioxidant. The antioxidant properties of plants are related to the extent of Proanthocyanidins content because proanthocyanidins (Condensed Tannins) are oligomeric and polymeric products of flavonoid biosynthetic pathway (Lutigen, 2018), which was

confirmed in an aforementioned study of Ryszard (2007), and was also confirmed in the study of Lingxi *et al.* (2020) in which condensed tannin gave a strong antioxidant effect but the combination of hydrolyszable and condensed tannin was more potent. Additionally, most phenolic compounds have been associated with antioxidant properties. The antioxidant activity of *V. amygdalina* due to the flavonoid fraction was in line with the report of Igile *et al.* (1994). Flavoniods are known for their widespread health benefits in respect to managing oxidative stress due to their hydroxyl group (OH^-) directly bonded to the benzene ring, thus allowing them to easily donate electrons to electron-deficient radicals (Ekaluo *et al*., 2015). Going further, *A. senegelensis* in a previous study conducted by Fahun *et al.* (2018) demonstrated some antioxidative effect which could be attributed to the presence of Flavoniods. This study justifies the claims for the use of *Annona senegelensis* and *Vernonia amygdalina* for treating ailments.

Conclusion: The tannin-rich fraction and flavoniod fraction of *Vernonia amygdalina* and *Annona senegalensis* leaves in this study was found to possess potent antioxidants. The different models employed in evaluating the antioxidant activity suggested that both plants are good source of natural antioxidants and hence has lend credence to the use of both plants in the management of oxidative stress associated diseases.

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