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COMPARATIVE STUDIES ON PHYTOCHEMICAL SCREENING, CYTOTOXICITY AND ANTIOXIDANT ACTIVITIES OF STEM EXTRACTS OF FOUR *AMARANTHUS* SPP.

Md. Omar Faruq*, Md. Latifuzzaman and Gour Pada Ghosh
Professor MI Zuberi Ethnobotany Laboratory, Department of Botany, University of Rajshahi, Bangladesh

Abstract

Amaranthus spp. are widely consumed as vegetables for their nutritious qualities and also have applications in traditional medicine. The current research attempts to identify the phytochemicals, cytotoxicity effects, and antioxidant activities of the stem of *A. tricolor*, *A. blitum*, *A. viridis*, and *A. spinosus*. The stem powder was extracted by solvent using methanol, ethanol, and aqueous. The methods were used for phytochemical screening (standard protocol), total phenol content (Folin-ciocalteau assay), total flavonoid content (aluminum colorimetric assay), cytotoxicity (brine shrimp lethality assay) and antioxidants (phosphomolybdate and DPPH assay). The phytochemical analysis revealed the existence of alkaloids, flavonoids, terpenoids, carbohydrate, glycosides, c. glycosides, amino acids, xanthoproteins, phenols, saponins, steroids, and coumarins. Among the four species, the methanolic extracts of *A. blitum* showed maximum quantities of phenol (20.02 ± 0.32 mg GAE/g), flavonoid (27.76 ± 0.29 mg QE/g) and high antioxidant activity (IC_{50} = 103.25 ± 1.52 µg/ml). The highest total antioxidant capacity was observed in the ethanolic extract of *A. viridis* (48.53 ± 2.98 mg AA/g). The stem of *A. blitum* showed little cytotoxic impact (LC_{50} <1000) and the rest of the species were non-cytotoxic (LC_{50}>1000). The findings demonstrated that the selected *Amaranthus* spp. are a valuable source of phytochemicals and natural antioxidants. This research suggests conducting further investigation to detect novel compounds.

Key words: *Amaranthus*, Antioxidants, Cytotoxicity, Phytochemicals, Total Phenol and Flavonoids.

Introduction

A balanced diet must include a variety of vegetables since they provide a wealth of nutrients and bioactive substances that are vital to human health (Papastavropoulou and Proestos 2023). Many Asian and African nations, such as Bangladesh, India, China, Zimbabwe, and South Africa, commonly use aerial parts of *Amaranthus* species as vegetables (Yanga et al. 2020). Because they are rich in essential nutrients, phytochemicals, and proteins (Maroyi 2013). The most common *Amaranthus* species in Bangladesh are *A. tricolor* (known as lalshak), *A. blitum* (known as datashak), *A. viridis* (known as shaknotey), and *A. spinosus* (known as katanotey). *A. tricolor* and *A. blitum* are cultivated commercially in several locations, whereas *A. viridis* and *A. spinosus* are grown wild on fallow ground. Extracts from Amaranth are used as traditional therapeutic herbs; they are particularly effective as antiviral, antimalarial, anti-diabetic, antibacterial, anti-helminthic, and anti-snake remedies (Sarker et al. 2020a, Sarker et al. 2020b). *A. tricolor* is traditionally used to cure ailments like coughs, throat infections, skin diseases, toothache, eczema, piles, hemorrhages, diarrhea, gonorrhea, leucorrhea, diabetes, and anemia (Kumar et al. 2019). *A. blitum* is treated against diseases like lung disorders, mouth and throat ulcers, fever, bleeding, inflammations, diarrhea, dysentery, cough, skin disease, and anemia (Nehal et al. 2016, Gavit and Patel 2019). Pulipati et al. (2015) reported that *A. viridis* traditionally used as diuretic, analgesic, antipyretic, vermifuge, antiulcer, antidiabetics, laxative,

*Author for correspondence: omrfrq39@gmail.com

asthma and venereal diseases. *A. spinosus* used in traditional medicines such as malaria, antipyretic, laxative, stomachic, febrifuge, gonorrhea, snake-bit and poultice for broken bones (Asha et al. 2016). Drug formulations based on antioxidants are used to treat and prevent diseases such as cancer, diabetes, stroke, atherosclerosis, and Alzheimer's disease (Devasagayam 2004).

Antioxidants are compounds that give free radicals electrons, preventing the damage these radicals cause to cells. As a result, the chemical is stabilized and harm to neighboring cells is avoided. Antioxidants also convert free radicals into waste byproducts that the body finally removes (Islam et al. 2013). Phenolic substances, including flavonoids, phenolic acids, alkaloids, tannins, coumarins, terpenoids, and vitamins, are frequently abundant in medicinal plant components. These substances exhibit a variety of biological activities, such as antioxidant activity (Packer et al. 1999, Ho et al. 2012). The cytotoxic activity of plants regulates the progress of various therapeutic drugs such as tumors, cancer, and microbial disease (Akter et al. 2013). The brine shrimp lethality test is applied to estimate plants cytotoxicity and pesticidal properties (Pisthanan et al. 2004). A low LC_{50} (<1000) suggests the use of anticancer or cytotoxic medications, whereas a large LC_{50} (>1000) can be utilized to design non-toxic drugs (Kamanja et al. 2018). The toxic effect of medicinal plants is responsible for their phytochemical content such as alkaloids, heavy metal contamination, and substitution with toxic herbs (Zahir et al. 2021).

Studies on *Amaranthus* species have revealed that their leaves have greater levels of minerals, phytochemicals, and antioxidant activity (Tharun et al. 2012, Sadia et al. 2016, Sharma et al. 2021, Jayarajan et al. 2022). These species also play a big part in food security and health benefits. Nevertheless, the phytochemical screening, cytotoxicity, and antioxidant activity of *A. tricolor*, *A. blitum*, *A. viridis*, and *A. spinosus* stem extracts are not well documented in the scientific literature. This study was the first attempt to identify the phytochemical content of stems from four *Amaranthus* spp. and to determine their potential for cytotoxicity and antioxidant activity.

A. tricolor *A. blitum* *A. viridis* *A. spinosus*

Fig. 1: The whole plant of four *Amaranthus* spp.

Materials and Methods

Chemicals

DPPH (2,2-diphenyl1-picrylhydrazyl), folin-ciocalteu reagent, aluminium chloride, ascorbic acid, gallic acid, quercetin, sodium carbonate, potassium acetate, sodium bicarbonate, etc. have been used these experiments. All chemicals and reagents were of analytical grade and were purchased from Tokyo Chemical

Industry Co. Ltd. China; Loba Cheme Pvt. Ltd. India; Sisco Research Lab., Pvt. Ltd. India; Merck Life Science Pvt. Ltd., India.

Plant Materials

The fresh *A. tricolor* and *A. blitum* plants were purchased from the local market in Rajshahi. *A. viridis* and *A. spinosus* were collected from the University of Rajshahi campus areas. The plants were identified by the Plant Taxonomy Laboratory, Department of Botany, University of Rajshahi, Rajshahi.

Preparation of crude extracts

The stem parts were separated from the plants and dried at room temperature (26±2ºC). Stem parts were powdered using an electric grinder and extracted with absolute ethanol, methanol, and distilled water in a ratio of 1:10 (w/v). The extracts were filtered through the Whatman paper No. 1 filter paper and allowed to evaporate at 35±2°C in a water bath. The crude extracts were transferred into airtight vials and kept at 4°C for further investigations.

Phytochemical analysis

Phytochemical screening for the existence of alkaloids, flavonoids, terpenoids, carbohydrates, glycosides, c. glycosides, amino acids, xanthoproteins, phenols, saponins, tannins, steroids, and coumarins were carried out using established techniques as outlined by Balamurugan et al. 2019.

Cytotoxicity analysis

The cytotoxicity activity was determined by the brine shrimp lethality assay described by Bhatt et al. (2016) with slight modifications. Brine shrimp eggs (*Artemia salina*) were hatched in an artificial seawater. Ten nauplii were treated with different concentrations (10, 20, 40, 80, and 100µg/ml) of plant extracts. The data were recorded for 24 hours and calculated LC_{50} values. The percentage of mortality was calculated using the following formula:

$$\text{Mortality (\%)} = \frac{\text{No. of nauplii taken} - \text{No. of nauplii lived}}{\text{No. of nauplii taken}} \times 100$$

Estimation of total phenol content (TPC)

The Folin-Ciocalteu Reagent (FCR) technique was used to calculate the total phenolic content described by Alhakmani et al. (2013) with some modifications. 1 ml of each extract (1 mg/ml concentration) was diluted in 3 ml of distilled water. Then 1 ml of FCR was mixed with the solution and incubated for 5 min in the dark. After that 2 ml of 7.5% sodium carbonate (Na_2CO_3) was added and placed in the dark for 30 min. and the absorbance was measured at 765 nm with a UV-vis spectrophotometer. The gallic acid equivalent (mg/g) of the dry weight is used to represent the total phenol content.

Estimation of total flavonoids content (TFC)

With some changes, the aluminum chloride colorimetric technique described by Aruna and Sharma (2015) was used to assess the total flavonoid content (TFC). For this experiment, 1 ml of each extract (1 mg/ml concentration) was dissolved with 100 µl of 10% aluminum chloride solution and 1M potassium acetate solution, and 2.8 ml of distilled water for 30 minutes rest at room temperature (26±2ºC). The absorbance was measured at 415 nm with a UV-vis spectrophotometer. The quercetin equivalent (mg/g) of the dry weight is used to represent the total flavonoid content.

Determination of antioxidant activity

Total antioxidant capacity (TAC)

The phosphomolybdate method was employed to evaluate the total antioxidant capacity following a procedure outlined by Prieto et al. (1999). 100 µl of each extract was combined with 1 ml of phosphomolybdate reagent, and the mixture was incubated for 90 minutes at 95ºC. A UV-vis spectrophotometer was used to measure the absorbance at 695 nm after the solution was cool.

DPPH radical scavenging activity

The free radical scavenging activity of methanol, ethanol and aqueous extracts of stem was measured by a modified DPPH assay (Alvarez-Jubete et al. 2010). Mixed with 3 ml of DPPH solution and 2 ml of each concentration (25, 50, 100, 200, and 400 µg/ml) in a test tube. After 30 minutes in the dark, these solution combinations were measured at 517 nm with a UV-vis spectrophotometer. The percentage of DPPH scavenging activity was measured as follows:

$$\text{Scavenging activity (\%)} = \left(\frac{A_b \times A_s}{A_b} \right) \times 100$$

Where, A_b = Blank absorbance

A_s = Sample absorbance

Data analysis

The results were calculated as the mean ± SE of three separate replications. Statistical analyses and graphical presentations were performed using Origin Pro 9 software. The means were also compared by Duncan's multiple range (DMRT) using the Statistical Package for the Social Sciences (IBM SPSS.23). The significance level was $p<0.05$.

Results

Phytochemicals screening

The results of phytochemicals identification of four *Amaranthus* spp. are presented in Table 1. Among these species, the highest 12 (alkaloids, flavonoids, terpenoids, carbohydrates, glycosides, c. glycosides, amino acids, xanthoproteins, phenols, saponins, steroids, and coumarins) were detected in *A. tricolor* and *A. spinosus* stem extracts. Conversely, the stem extracts of *A. blitum* and *A. viridis* contained 11 phytochemical components, respectively. Only coumarins were absent in *A. blitum* and *A. viridis*, and tannins were not detected in any of the extracts. Compared to methanol and aqueous solvents, the ethanolic solvent was the most effective in identifying the phytochemical components.

Cytotoxicity evaluation

The percentage of probit kills at various log concentrations and the LC_{50} values of selected *Amaranthus* spp. stem extracts are displayed in Figs. 2 and 3. The maximum range (3.66 to 4.23%) of probit kill was found in *A. tricolor*, and the minimum range (3.12 to 3.87%) of probit kills was in *A. viridis*, respectively. The low LC_{50} value (722.63 µg/ml) was shown in *A. blitum*, which was lower than the standard LC_{50} value (1000 µg/ml). On the other hand, the highest LC_{50} value (42757.66 µg/ml) was observed in *A. viridis* which was significantly higher than 1000 µg/ml.

Table 1: Phytochemical analysis of methanol, ethanol, and aqueous stem extracts of four *Amaranthus* spp.

Phytochemicals	*A. tricolor*			*A. blitum*			*A. viridis*			*A. spinosus*		
	M	E	Aq	M	E	Aq	M	E	Aq	M	E	Aq
Alkaloids	-	-	+	-	-	+	-	-	+	-	-	+
Flavonoids	+	+	-	+	+	-	+	+	+	-	-	+
Terpenoids	+	+	-	-	+	-	+	+	-	-	+	+
Carbohydrate	+	+	-	+	+	+	+	+	-	+	+	-
Glycosides	-	+	-	-	+	-	+	+	-	+	+	+
C. glycosides	+	+	-	+	+	-	+	+	-	-	+	-
Amino acid	+	+	+	+	+	+	+	+	+	+	-	+
Xanthoprotein	-	-	+	-	+	-	-	+	-	-	+	-
Phenol	+	-	+	+	+	+	+	+	+	-	-	+
Saponins	+	+	+	+	-	+	-	-	+	-	-	+
Tannins	-	-	-	-	-	-	-	-	-	-	-	-
Steroids	+	+	-	+	+	-	+	+	+	+	+	-
Coumarins	+	-	+	+	-	-	-	-	-	-	-	+

"+" means Presence and "-" means Absence, M = Methanol, E = Ethanol, Aq = Aqueous.

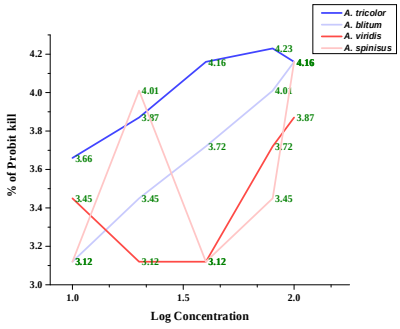

Fig. 2: Percentage of probit kills at different log concentration in four *Amaranthus* spp.

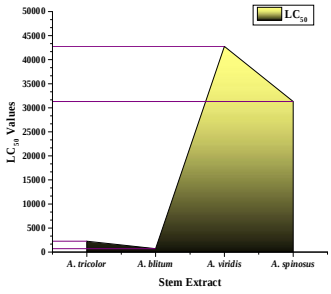

Fig. 3: LC_{50} values of four *Amaranthus* spp.

Total phenol content

Fig. 4 is shows the results of the total phenol content of *Amaranthus* spp. at various solvents, which was measured by a folin-ciocaltue reagent assay and calculated with the gallic acid equivalent standard curve equation (y = 0.01831*x + 0.1454). The maximum phenol content was found in the methanolic extract of *A. blitum* (20.02 ± 0.32 mg GAE/g). This value was significantly difference at p<0.05% level with other all extracts. Whereas, aqueous extract of *A. spinosus* was obtained the minimum total phenol content (1.16 ± 0.09 mg GAE/g), respectively.

Total flavonoid content

Using the aluminum chloride colorimetric technique, the total flavonoid content (TFC) of the methanolic, ethanolic, and aqueous extracts of *Amaranthus* spp. was calculated and compared with the quercetin equivalent standard curve equation (y = 0.01120*x + 1.124). The results are presented in Fig. 5. Among these species, the methanolic extracts of *A. blitum* contained the highest amount (27.76±0.29 mg QE/g) of total flavonoids, which was significantly different at the p<0.05% level from other species. The lowest amount (1.73±0.10 mg QE/g) of total flavonoids was observed in the aqueous extract of *A. viridis*.

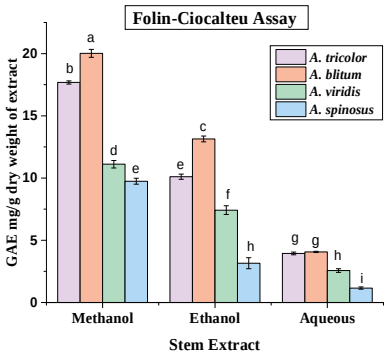

Fig. 4: Total phenol content of four *Amaranthus* spp.

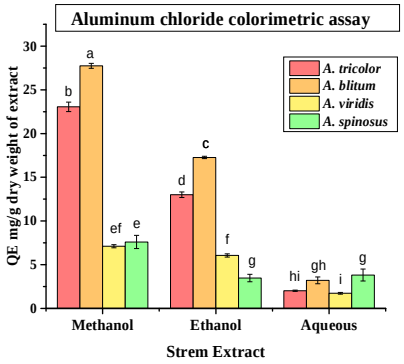

Fig. 5: Total flavonoids content of four *Amaranthus* spp.

Antioxidant activity

Total antioxidant capacity: The total antioxidant capacity of four different species of *Amaranthus* is shown in Fig. 6. The results showed that the ethanolic stem extract of *A. viridis* had the highest antioxidant capacity (48.53±2.98 mg AA/g), which differed significantly from the other extracts at the p<0.05% level. The methanolic stem extract of *A. tricolor* had the lowest antioxidant capacity (1.82±0.33 mg AA/g), but this difference was not significant compared to the methanolic extract of *A. spinosus*.

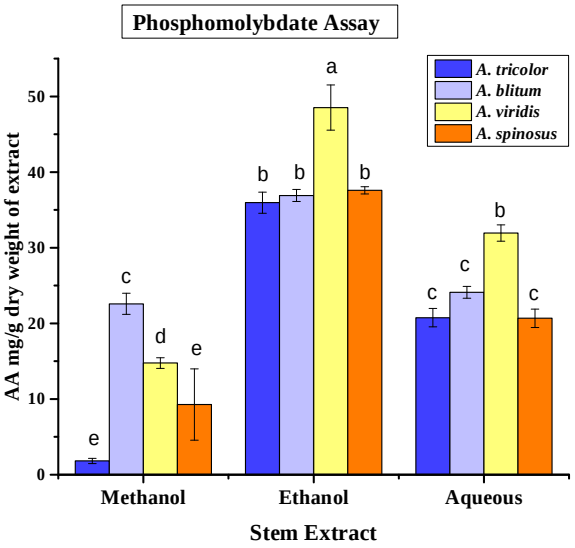

Fig. 6: Total antioxidant capacity of four *Amaranthus* spp.

DPPH scavenging activity

The antioxidant activity of four *Amaranthus* spp. stem extracts was measured as the percentage of DPPH free radical scavenging activity and IC_{50} values displayed in Figs. 7 and 8. According to the result, *A. blitum* stem showed a high percentage of scavenging activity (range: 33.41±0.10 to 65.37±0.10) at 25-400 µg/ml concentration and low IC_{50}-103.25±1.52 µg/ml indicates it contains high antioxidant activity, which was a significant difference at the $p<0.05\%$ level. The lowest antioxidant activity was found in the aqueous stem extract of *A. spinosus* with a height IC_{50} value of 394.74±4.31 µg/ml

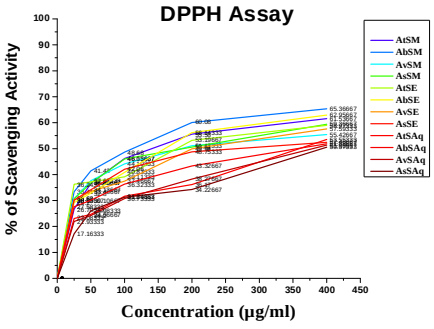

Fig. 7: Percentage of scavenging activity of four *Amaranthus* spp.

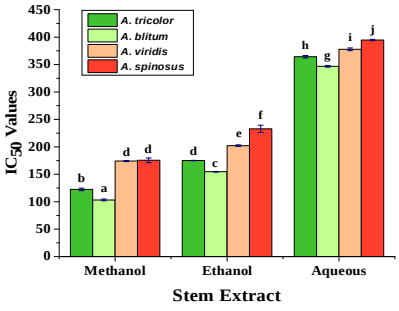

Fig. 8: IC_{50} values of four *Amaranthus* spp.

Discussion

Comparative studies were carried out on four species of *Amaranthus* spp. (*A. tricolor, A. blitum*, *A. viridis* and *A. spinosus*) for their phytochemicals, cytotoxicity and potential antioxidant activities. The present study revealed that selected four *Amaranthus* spp. methanolic, ethanolic and aqueous stem extracts were rich in phytochemicals including alkaloids, flavonoids, terpenoids, carbohydrate, glycosides, c. glycosides, amino acids, xanthoproteins, phenols, saponins, steroids, and coumarins. Sharma et al. (2021) reports that *A. spinosus* stem contains alkaloids, saponins, flavonoids, and polyphenols in different solvents. The current findings of stem extracts are nearly identical to some earlier research on the phytochemicals found in the leaves of *A. tricolor* (Sable and Saswade 2017), *A. blitum* (Gavit and Patel 2019), *A. viridis* (Sunday et al. 2021), and *A. spinosus* (Sable and Saswade 2017). According to some studies, flavonoids primarily reduce the risk of cancer and cardiovascular illnesses (Ballard and Marostica 2019). Alkaloids are useful in the synthesis of strong analgesics for human health (Kam and Liew 2012). It is well recognized that glycosides reduce blood pressure (Ullah et al. 2019). The applications of saponins include weight reduction, antioxidants, anticancer, anti-inflammatory, hypercholesterolemia, and hyperglycemia (Murugan and Parimelazhagan 2014). These findings suggest that *Amaranthus* spp. stems may be responsible for many of the therapeutic properties.

In the present study, *Amaranthus* spp. stem extracts LC_{50} values sequences are *A. viridis*>A. *spinosus*>*A. tricolor*>*A. blitum*. The stems of *A. viridis*, *A. spinosus*, and *A. tricolor* have LC_{50} values of more than 1000 µg/ml, indicating that they are safe to consume and can be applied as non-toxic medications. *A. blitum* stems contain LC_{50} values less than 1000 µg/ml, indicating that they are moderately toxic and might be used as cytotoxic drugs. Phytochemical groups such as flavonoids, alkaloids, and tannins have been shown to exhibit cytotoxic effects (Chowdhury et al. 2017). Khanal et al. (2015) reported that aqueous, hexane, and chloroform leaf extracts of *A. spinosus* are safe to use, and ethanolic extracts are slightly toxic. Some cytotoxicity studies of other species, like *Amaranthus retroflexus* are toxic in renal cells (Amoli et al. 2009), while *Amaranthus caudatus* has toxic effects (Shafie et al. 2023). The extracts with low LC_{50} values (>1000) have potential candidates for cytotoxic or chemotherapeutic medicines and may require more investigation.

A. tricolor, A. blitum, *A. viridis* and *A. spinosus* are frequently eaten as vegetables and also utilized as herbal treatments in rural regions. Antioxidants present in plants have led to evaluations of their therapeutic qualities. The antioxidant activity of medicinal plants depends on quantities of phenolic and flavonoid compounds (Krings and Berger 2001). According to the current research, there was a significant rise in antioxidant activity (DPPH) and total antioxidant capacity when total phenol and flavonoid content increased. Because phenolic compounds function as reducing agents, and hydrogen donors, they can scavenge free radicals (Wojdylo et al. 2007). It has also been demonstrated in earlier research that TPC and TFC accelerate the antioxidant activities of plants (Wong-Paz et al. 2015, Yang et al. 2023). The greatest antioxidant activity in this context was found in the methanolic stem extract of *A. blitum*, which is also connected with higher TPC and TFC values. Lastly, the order of the four samples examined for antioxidant activity, TPC, and TFC was *A. blitum* >*A. tricolor* >*A. viridis* >*A. spinosus*. The order of total antioxidant capacity was *A. viridis* >*A. blitum* >*A. spinosus* >*A. tricolor*. Kavita et al. (2020) reported that the *A. tricolor* hexene extracts of stem contained TPC (0.11 ± 0.001 mg GAE/g), TFC (0.28 ± 0.08 mg QE/g) and IC_{50} value (59.3 µg/mL) of DPPH which are lower values than current studies. Compared to some earlier research, the TPC, TFC, and antioxidant activity of *Amaranthus* spp. leaves are almost similar to our study values for stem (Khanam and Oba 2012, Menon and Thakker 2020, Jahan et al. 2022).

Conclusion

The stems of the four *Amaranthus* species were found an excellent source of several phytochemicals, including phenols, saponins, steroids, coumarins, alkaloids, flavonoids, terpenoids, carbohydrates, glycosides, and c. glycosides. These species exhibited notable levels of antioxidant, flavonoid, and phenolic activity. The cytotoxicity statistics confirm that the stems of *A. blitum* are a little bit toxic and *A. tricolor*, *A. viridis*, and *A. spinosus* are considered non-toxic. These findings suggest that *Amaranthus* species are safe to consume as a vegetable and may have some therapeutic uses for human health.

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Conflict of interest

The authors declare that there are no conflicting interests.

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