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Article

Formulation of novel polyherbal compound MAT20 with phytochemicals found in amla, tulsi, and moringa

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SUMMARY

Herbal products play an important role in mitigating the harmful effects of oxidative stress caused by free radicals. The purpose of this study was to prepare a novel antioxidant polyherbal formulation as a supplemental treatment to reduce oxidative stress. We hypothesized that the formulation "MAT20", a polyherbal mixture containing three herbal plants, *Phyllanthus emblica* (amla), *Ocimum sanctum* (tulsi), and *Moringa oleifera* (moringa), possesses maximum antioxidant properties compared to each individual herb. These herbs are known in Ayurveda for their medicinal use. After obtaining extracts by using five different solvents (water, methanol, ethanol, chloroform, and acetone), we tested each for polyphenols, flavonoids, alkaloids, tannins, and saponins, which are known to have antioxidant properties and free radical scavenging activity against a free radical which reduces in the presence of antioxidants. After extensive analysis, we formulated a combination of the herbs by extracting moringa with chloroform, amla with acetone, and tulsi with methanol, which we named "MAT20." Our results show that "MAT20", a polyherbal formulation, is an excellent source of antioxidants.

INTRODUCTION

An imbalance in the production of free radicals and reactive metabolites, so-called oxidants or reactive oxygen species (ROS), is described as oxidative stress (1). This imbalance leads to damage of cells, as well as vital biomolecules, with potential impact on the whole organism (2). Through normal cellular metabolism, ROS are produced and play essential roles in stimulation of signaling pathways in plant and animal cells that respond to modifications in intra- and extracellular environmental conditions (3). In cells, ROS are usually generated by the mitochondrial respiratory chain. The electron transport chain is located in the mitochondrial membrane and the electron transfer to molecular oxygen occurs at the level of the respiratory chain. During endogenous metabolic reactions, aerobic cells produce ROS, such as hydroxyl radical (OH), superoxide anion (O2-), hydrogen peroxide (H2O2), and organic peroxides as normal products of the biological reduction of molecular oxygen (4). Lipids and proteins are also significant targets for oxidative attack, cancer and mutagenesis risk (due to chemical or physical agents permanent changes happens in genetic material) can also increase due to these molecular modifications. Antioxidants are agents that scavenge the free radicals and reduce oxidative stress (5). Antioxidants can be used to prevent some chronic diseases such as cardiovascular diseases, cataracts, etc. (6).

Conventional therapies for cancer are radiotherapy, chemotherapy and surgeries which cause some side effects. Due to these conventional therapies cancer cells go under resistance to the chemo drugs, which can cause reverse effects. Integrative oncology is an advanced research where complementary treatment can be given to the patients along with conventional treatments. A different option for treatment is the utilization of these conventional therapies in conjunction with herbal adjuvants as a complementary medicine. In this study, we performed an experiments of qualitative and quantitative analysis of the phytochemicals obtained from the extracts of three medicinal plants. We tested if a blend of three herbs could be useful as a supplemental treatment to conventional medicine to reduce oxidative stress (7). The plants *Phyllanthus emblica* (amla), *Ocimum sanctum* (tulsi) and *Moringa oleifera* (moringa) are commonly used both in Ayurveda and in the Indian culture. The phytochemicals we tested were polyphenols, flavonoids, alkaloids, tannins, and saponins. Secondary metabolites or phytochemicals are useful in defense mechanism and protection. These phytochemicals show great importance in scavenging free radicals. They have many biological properties such as antimicrobial, antifungal, antioxidant and anticancer, etc. Due to the presence of these properties from ancient times herbal products have been used for the treatment of many diseases.

Amla is cultivated throughout India and close-by countries, and it has gained a reputation throughout the world as a "superfruit" (8). Amla has flourished as an excellent nutritional source, being a rich in polyphenols and ascorbic acid, which are considered to be responsible for their antioxidant properties. Amla is believed to increase protection against various ailments, including diabetes, ulcers, heart troubles, and gastrointestinal disorders (9). Additionally, it is beneficial in improving memory, reducing cholesterol, and combatting ophthalmic disorders and antimicrobial agents (10). Fresh or dry fruit is commonly used in ayurvedic medicines for the remedy of jaundice, diarrhea, and inflammation (11).

Holy basil, commonly called tulsi, is a herbaceous plant belonging to the family Lamiaceae. Its slightly hairy, light green leaves are widely used as a flavoring agent in

Southeast Asian cuisine, mainly in Thai stir-fries. Tulsi leaves are spicy and have lemony notes, and many Indians consume small quantities of the younger leaves either as an offering after divine worship in temples or as a food additive (12, 13). Traditional use has attributed a number of properties to holy basil, such as rejuvenating, tonic, and vitalizing properties that would contribute to longevity and a healthy life, as well as antiseptic, antiallergic, and anticancer effects (14, 15). These antioxidant properties have been observed previously in ethanol extracts (16).

M. oleifera is cultivated mostly in India. It has common names, such as drumstick tree or horseradish tree. Moringa leaves are rich in vitamins such as Vitamins A, B, C, D and E as well as some minerals such as calcium, potassium, zinc and magnesium (17). Moringa also has antioxidant properties and can help reduce inflammation (18, 19).

Our hypothesis for this study was that it is possible to create an anti-oxidant agent out of a polyherbal formulation from extracts of tulsi, amla, and moringa. We wanted to create a polyherbal formulation instead of the single herb formulation because it gave us more control over the levels of phytochemicals present in the optimal formulation. Furthermore, a polyherbal formulation could show better antioxidant properties compared to individual herbal extracts.

RESULTS

Our study focused on three herbs, namely amla, tulsi, and moringa, which were chosen based on ayurvedic properties. We performed a phytochemical analysis of each plant extract to create a formulation that has abundant antioxidants as compared to each individual herb. This study mainly focused on the formulation of a potential polyherbal compound called MAT20.

Firstly, we used different solvents for the extraction of the three plants. We obtained dry leaf powders of each plant from a local organic store in Bangalore. We then used different solvents for the extraction of each plant material, including acetone, chloroform, ethanol, methanol, and water. Secondly, we performed qualitative tests on the 15 extracts to assess the presence of the phytochemicals and their rough estimates (**Table 1**).

S.No.	Plant	Solvent	Polyphenol	Flavonoid	Alkaloid	Tannin	Saponin
1	Amla	Acetone	-	++	-	-	-
2		Chloroform	-	-	-	-	-
3		Ethanol	+++	++	-	+++	+++
4		Methanol	+++	+++	+++	+++	+++
5		Water	-	-	-	+++	+++
6	Tulsi	Acetone	+	+	++	-	+++
7		Chloroform	+	+	+++	+	-
8		Ethanol	+	+	-	+	+++
9		Methanol	++	-	+++	-	+++
10		Water	+++	-	+++	+++	+++
11	Moringa	Acetone	+	-	+++	+	+++
12		Moringa	+	+	-	-	-
13		Ethanol	++	-	+++	-	+++
14		Methanol	+++	+	+++	+++	+++
15		Water	+++	+++	+++	+++	+++

Table 1. Qualitative and quantitative analysis for the presence of phytochemicals in various extracts of amla, tulsi, and moringa. The intensity of the color is represented using '+', '++' and '+++', which indicates the presence of phytochemicals in the range 0-500, 501-1000 and 1001-2000 µg/ml, respectively. '-' represents absence of the phytochemical.

Presence of phytochemicals in different extracts

Qualitative analysis of the different extracts was performed and showed the presence or absence of the phytochemicals in the individual plant extract. Plus sign is an indication of the presence of phytochemicals and based on the intensity of the color as well as the quantified ranges of phytochemicals, the different number of plus signs were assigned, such as '+,' '++,' '+++' indicates 0-500 µg/ml, 501-1000 µg/ml, and 1001-2000 µg/ml, respectively (**Table 1**).

Amla extracts

All the phytochemicals were present in the methanol extract from amla, with ranges from 1001-2000 µg/ml. Furthermore, flavonoids were comparatively low (501-1000 µg/ml) with all solvents other than methanol. The water extract showed tannins and saponins at the highest range. Acetone extract showed flavonoids in the medium range (501-1000 µg/ml) and no other phytochemical. All the phytochemicals were absent in the chloroform extract (**Table 1**).

Tulsi extracts

Both acetone and ethanol extracts showed saponins in the highest range (1001–2000 µg/ml). Water extract showed polyphenols, alkaloids, tannins, and saponins at the highest range (1001–2000 µg/ml) whereas flavonoids were absent. Moreover, all solvents except chloroform were able to extract saponins in tulsi. Methanol extracted alkaloids and saponins at the highest range (1001–2000 µg/ml) and polyphenols at the medium range (501-1000 µg/ml).

Moringa extracts

Water extract showed all phytochemicals in the highest range (1001–2000 µg/ml). The methanol extract showed the highest range for all phytochemicals except flavonoids (0-500 µg/ml). Ethanol and acetone extract were similar in their phytochemical analysis and ranges. There was a noticeable high range in alkaloids and saponins, low to medium in polyphenols, and no trace of flavonoids and tannins. Chloroform extract showed a small range (0-500 µg/ml) of both polyphenols and flavonoids, and other phytochemicals were completely absent.

Estimation of different phytochemicals in various extracts

For each phytochemical, we performed the quantitative tests on the extracts that showed a positive result during the corresponding qualitative analysis (**Table 1**). Quantitative analysis of phytochemicals is a method to quantify the concentration of phytochemicals in the individual plant extract. This quantitative analysis of each of the phytochemicals showed highest concentrations in some plant extracts which has been used further for formulating MAT20.

Polyphenol content

The extracts with the three highest concentrations of polyphenols were methanol extract of amla, tulsi, and moringa, having a level of 1415.4 µg/ml, 1107.2 µg/ml, and 986.3 µg/ml, respectively (**Figure 1**).

Flavonoid content

The extracts with the three highest concentrations of flavonoids were methanol extract of amla having a level of

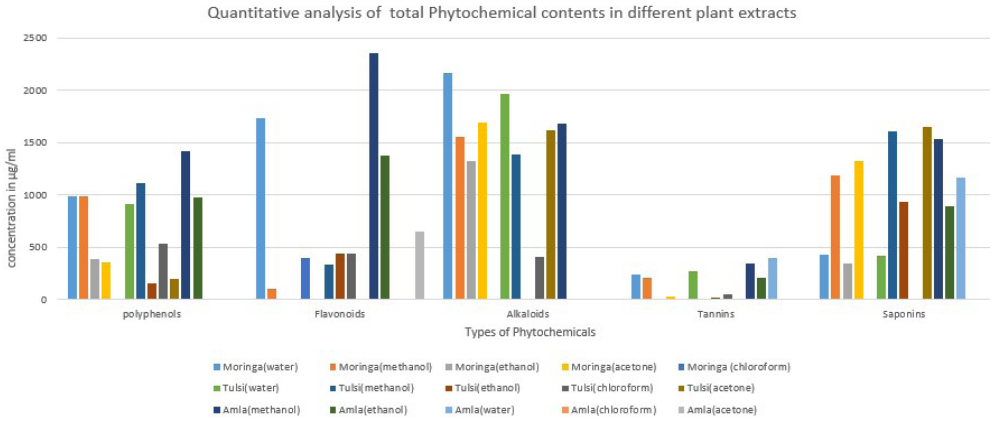

Figure 1. Quantitative analysis of phytochemicals. Bar graph showing the quantitative analyses of different phytochemicals in various plant extracts using different solvents (acetone, chloroform, ethanol, methanol and water). These phytochemicals included: polyphenol, flavonoid, alkaloid, tannins, and saponins, with concentrations measured in µg/mL.

2354.3 µg/ml, water extract of moringa having a concentration of 1731.75 µg/ml, and ethanol extract of amla having a concentration of 1379.3 µg/ml (**Figure 1**).

Alkaloid content

The extracts with the three highest concentrations of alkaloids were water extract of moringa and tulsi, having a concentration of 2168.3 µg/ml and 1967.3 µg/ml, respectively, and acetone extract of moringa having a concentration of 1694.6 µg/ml (**Figure 1**).

Tannin content

The extracts with the three highest tannin concentrations were water and methanol extract of amla having a concentration of 391.7 µg/ml and 345.3 µg/ml, respectively, and tulsi with water having a concentration of 271.7 µg/ml (**Figure 1**).

Saponin content

The extracts with the three highest saponin concentrations were methanol extract of tulsi and amla having a concentration of 1602.8 µg/ml and 1536.4 µg/ml, respectively (**Figure 1**). Acetone extract of moringa had a concentration of 1323.81 µg/ml (**Figure 1**).

In summary, the amount of polyphenols was the highest in methanol extract of tulsi, but there was none in the chloroform extract of moringa and acetone extract of amla. Subsequently, it brings the total amount of polyphenols to the desired '+++' range. The amount of flavonoids was 644.25 µg/ml in acetone extract of amla and 399.5 µg/ml in chloroform extract of moringa, and none in methanol extract of tulsi, which brings the total amount of flavonoids to the desired '+++' range. The amount of alkaloids was 1389.3 µg/ml in methanol extract of tulsi and none in the other extracts, which contributes to the total amount of alkaloids to the desired '+++' range. None of the extracts had tannins. The methanol extract of tulsi has 1602.8 µg/ml of saponins and none in acetone extract of amla and chloroform extract of moringa and keeps tannins to nil. The extracts were chosen in such a way that it fulfills the desired range for total polyphenols, saponins, alkaloids and flavonoids to formulate MAT20. Three different plant extracts with desired ranges of the phytochemicals were dissolved in one best solvent i.e. Dimethyl sulfoxide (DMSO).

Anti-oxidative activity of MAT20 formulation

The optimum extract of each plant was chosen based on qualitative and quantitative analyses of different phytochemicals. MAT20 was composed of methanol extract of tulsi, chloroform extract of moringa, and acetone extract of amla. This combination has the greatest amount of polyphenols, flavonoids, alkaloids in the '+++' range (1000-2001 µg/ml), and only one of either tannins or saponins in the highest amount possible because tannins and saponin belong to the same family. The combination of the three extracts was formulated by determining the weight before and after air-drying the extracts. Subsequently, we weighed 0.01 g of each dried extract and dissolved in 1 ml of dimethyl sulfoxide (DMSO). 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to determine the antioxidant activity of the final solution MAT20.

DPPH is a colorimetric assay to determine the free radical scavenging activity of a compound. DPPH assay was performed with individual plant extracts as well as with MAT20 formulation to determine its free radical scavenging potential. We found that the 50% free radicals scavenging activity of tulsi methanol occurred at 3.1mg/mL, amla acetone extract occurred at 4.1mg/mL and moringa chloroform occurred at 6.32mg/mL concentration (**Figure 2**). The MAT20 formulation showed 50% free radicals scavenging activity at 0.278 mg/mL concentration DPPH was used as a positive control (**Figure 2**).

DISCUSSION

An excess of free radicals in the body causes oxidative stress, which can harm cells and tissue. Oxidative stress may cause chronic conditions such as cancer, diabetes, and heart disease (18). Antioxidants are substances that counteract or scavenge free radicals by donating an electron. The scavenging effect of antioxidants helps to protect the body from oxidative stress.

This study focused on three herbs, amla, tulsi, and moringa, which were chosen based on ancient ayurvedic properties, such as antioxidant properties. We performed a phytochemical analysis of the extracts of each plant to create a formulation for reducing oxidative stress. This study focused

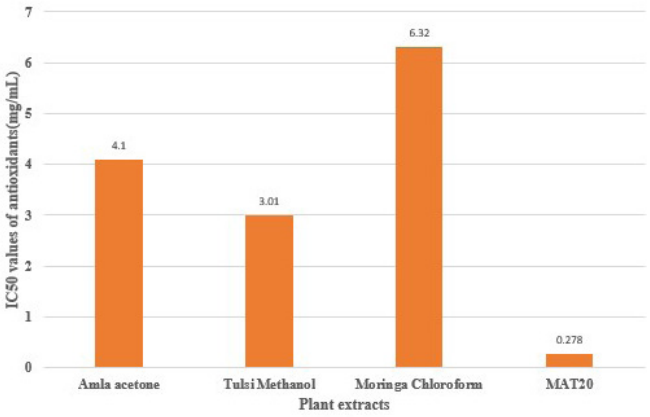

Figure 2. Antioxidant activity of MAT20 using DPPH assay. Amla extracted by acetone, tulsi extracted by methanol, moringa extracted by chloroform and MAT20 showing 50% inhibition (IC_{50}) of free radicals at the concentrations in mg/mL. DPPH reagent was used as a positive control for normalization.

on the formulation of a potential polyherbal compound called MAT20.

We decided to use the combination of three plants because each individually had medicinal properties. The goal of this study was to determine if an herbal adjuvant could have anti-oxidant properties. MAT20 could also act as anti-cancer agent due to its anti-oxidative property, as anti-oxidants can reduce the chance of mutagenesis (20). In future experiments, we could test the MAT20 formulation for its anti-cancer properties and the mechanism of action.

The limitation of our study is that we conducted the qualitative and quantitative phytochemical studies once for each extract because this is a preliminary study. Future studies could increase the number of sets and calculate the average phytochemicals of each extract to improve the reliability of the results. Additionally, we could explore employing other antioxidant assays, such as the Total Antioxidant Capacity (TAC) Assay or Phosphomolybdate assay. Also, it is very important to know the components in respective plant extracts to avoid dangerous effects because few phytochemicals show side effects (21). Saponin or tannin is considered to be highly toxic and has less therapeutic potential if used at a high concentration. As we know saponins and tannins belong to the same family, they could be toxic if used together (22).

Future explorations could examine this formulation for other pharmacological activities, such as anticancer, antimicrobial, and antifungal activities. This will allow the assessment of other properties which may be beneficial in the treatment of various ailments. The efficacy of crude extracts from other species can be compared to the MAT20 formulation. Furthermore, inter-species combinations may also be explored for enhanced biological activities.

In the overall summary, MAT20, a polyherbal formulation has high antioxidant properties as compared to the individual extract. This can potentially be used as an antioxidant drug and further study can be done to check its cytotoxic effect on cell lines.

METHODS

Extraction

Dry leaf powders of moringa, tulsi, and amla were obtained from a local organic store in Bangalore. The following solvents were used for the extraction of each plant product: Acetone (Qualigens), chloroform (Qualigens), ethanol, methanol (Qualigens), and water. Dry leaf powder (1 g) was added to 10 ml of each solvent in respective tubes. The mixtures were placed on a rocker for 24 hours and then centrifuged at 3000 rpm for 20 minutes. The supernatants were collected in fresh collection tubes and stored at 4˚C for further use.

Qualitative analyses

The qualitative tests enabled us to assess the presence of the phytochemicals and their rough estimates in the extracts. The following procedures were followed for the presence of different phytochemicals in each extract. The presence of polyphenol was confirmed by the formation of blue color, whereas a colorless solution after adding 2M HCl was an indication of the presence of flavonoids. The observation of an orange-brown precipitate indicated the presence of alkaloids. Furthermore, the presence of tannin was confirmed by the formation of a blue-green color after the addition of ferric chloride solution. A soluble emulsion indicated the presence of saponin in the extract (23, 24).

Folin-Ciocalteu's (FC) reagent test

In a test tube, 0.2 mL of the extract was added, 4 drops of FC reagent (SDFCL) and $NaCO_3$ solution was added. The solution was incubated for 20 min in the dark at room temperature. The color change was recorded for the presence of polyphenol in the extract (23, 24).

Alkaline reagent test

In a test tube, 0.2 mL of the extract was added to 5 drops of 5% NaOH and 3 drops of 2 M HCl. The color change was recorded for the presence of flavonoids (23, 24).

Olive oil test

In this test, 0.2 mL of olive oil was added to 0.5 mL of the extract and left for 5 min after vigorous shaking. A soluble emulsion of the solution was recorded to confirm the presence of saponin (23, 24).

Dragendorff's test

In a test tube, 0.2 mL of the extract was added to 0.2 mL dilute HCl (1:1), and 1 mL of Dragendorff's reagent (Nice chemicals) and the observations were recorded for the presence of alkaloid (23, 24).

Ferric chloride test

In a test tube, 5 drops of 5% $FeCl_3$ (Qualigens) were added to 0.2 mL of the extract and recorded the color change for the presence of tannin (23, 24).

Quantitative analysis

The quantitative tests enable quantifying the concentration of phytochemicals present in the extracts. The quantitative tests were performed with each extract that had a positive result during the corresponding qualitative analysis. The quantitative test was not performed for the extracts with a negligible amount of phytochemicals. The protocols for determining each phytochemical are as follows:

Estimation of alkaloid content

The standard curve was obtained with the bismuth nitrate pentahydrate stock solution (50mg/L). Five ml of 1M thiourea (Qualigens) solution was added to serially diluted bismuth nitrate pentahydrate (Himedia) stock by distilled water. Absorbance was recorded at 435 nm.

For measuring the concentration of alkaloids in the extracts, the pH was maintained at 2-2.5 using 1M HCl, and then 2 mL of Dragendorff's solution was added. After centrifugation, 2 mL of 5% disodium sulphide (Qualigens) solution was added to the pellet to form a brown-black precipitate. Two mL of concentrated nitric acid was added and made the volume up to 10 mL with distilled water. One ml of the solution was discarded, and 5 ml of thiourea was added. The absorbance was recorded at 435 nm (23).

Estimation of polyphenol content

The concentration of polyphenols in the extract was determined using FC reagent. Standard curve was made using Gallic acid (SDFCL) as standard. 0.2 ml of the extract was diluted with 0.6 mL of distilled water, and 0.2 mL of FC reagent was added to it. One mL of 8% sodium carbonate was added to the solution and volume was made up to 3 mL with distilled water. The solution was incubated for 30 minutes and centrifuged at 1500 rpm for 10 minutes. The optical density of the clear supernatant was measured at 765 nm using the spectrophotometer against a blank (25).

Estimation of flavonoid content

The quantity of flavonoids in the extracts was measured using aluminum chloride ($AlCl_3$) (Qualigens) colorimetric assay. We added 0.2 mL of the extract and 1 mL of 100mg/50mL standard Quercetin (Sigma) solution into separate test tubes, then 4 mL of distilled water and 0.3 mL of 5% $NaNO_3$ solution was added in each. After 5 minutes, 0.3 mL of 10% $AlCl_3$ was added. At the sixth minute, 2 mL of 1 M NaOH was added, volume was brought up to 10 mL with distilled water and mixed well. The absorbance of the orange-colored solution was recorded at 630 nm, and the concentrations were determined against quercetin as standard (25).

Estimation of tannin content

The concentration of tannins in the plant extracts was measured using the FC method (Folin-Ciocalteu's method). A standard curve was made using tannic acid (Nice chemicals) along with blank. We added 0.2 mL of the extract in 7.5 ml of distilled water. One ml of 35% saturated sodium carbonate and 0.5 mL of FC reagent was added to the mixture and volume was made up to 10 ml with distilled water. The mixture was vortexed and incubated for 30 minutes at room temperature, and absorbance was read at 700 nm (25).

Estimation of saponin content

Diosgenin (Himedia) was prepared in methanol and was used for a standard curve. We added 0.25 mL of Vanillin reagent (8%) (SDFCL) and 2.5 mL sulphuric acid (72% v/v) to the tubes. The test tubes were vortexed and incubated at 60°C in a water bath for 10 minutes. After the incubation absorbance was measured at 545 nm against the blank. Individual solvent was used as a blank. Similar procedure was followed using 0.25mL volume for each plant extract (26).

Formulation of MAT20

The optimum extract of each plant was selected based on qualitative and quantitative analyses of different phytochemicals. A combination of the three extracts was formulated by determining the weight before and after air-drying the extracts. Subsequently, we weighed 0.01 g of each dried extract and dissolved in 1 ml of dimethyl sulfoxide (DMSO) (Qualigens). This final solution was named MAT20 and used to determine its antioxidant activity using DPPH assay.

Determination of the antioxidant activity of MAT20 using DPPH assay

Serially diluted samples were added to test tubes, and volume was made up to 1 mL by methanol. The tubes were immediately incubated in the dark for 15 min at room temperature after adding 3 ml of DPPH solution. DPPH reagent (Himedia) was used as a positive control and individual solvent as a negative control for blanking out to calculate percentage inhibition. Absorbance was measured at 520 nm, and the graph was plotted using ascorbic acid as a standard. Using the following formula, the percentage inhibition of free radicals by MAT20 was obtained (27, 28):

% inhibition = Absorbance of control - Absorbance of sample/ Absorbance of control * 100

The IC_{50} value was found using the following straight-line equation:

$IC_{50} = mx + c$

where, x: concentration, m: slope of the line, c: y-intercept

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