Research Article

Antioxidant protection of *Cannabis sativa* extract against wild-type strains BY-4743 and deletion yeast strains (Δ trx2, Δ ctt, Δ cta) for H₂O₂ induced oxidative stress

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Abstract

Objective: This study was aimed to evaluate antioxidant activity of the *Cannabis sativa* extract (aqueous) using eukaryotic model *Sachharomyces cerevisiae* (both wild BY 4743 and knockout Δ trx2 Δ ctt Δ cta) against H₂O₂ induced oxidative stress. **Material and Methods:** The antioxidant activity was investigated using viability assay, growth curve, spot assay and level of mitochondrial ROS determined by MitoSOX staining method. **Results:** The study showed the significant (**P<0.01) antioxidant activity of ACS. The treated yeast cells were able to maintain a higher level of growth as compared to the control. In viability assay the H₂O₂ stressed group showed 35% (wild) and 33% (Δ trx2) % viability of yeast cell while treated ACS 400µg/ml+H₂O₂ group showed improved % viability i.e. 50% (wild) and 57% (Δ trx2) after incubating for 30 minutes. In spot assay also ACS (400 µg/ml) showed a protective effect against challenge which is indicated by the growth in extract treated group. Compared to the negative control group the extract-treated group showed less fluorescence indicating the ability of the extract to scavenge mitochondrial superoxide and thereby protecting the cells. **Conclusion:** In this study, we found that aqueous extract of *C. sativa* exhibited marked free radical scavenging and antioxidant activity against hydrogen peroxide radicals in vivo. This extract can be used for the management of diseases like cancer, diabetes, neurodegenerative disease etc.

Keywords: Antioxidant, Hemp, *Saccharomyces cerevisiae*, ROS, oxidative stress

Introduction

Oxidative stress is an imbalance between antioxidant and pro-oxidant resulting in irreversible cellular damage. ROS (reactive oxygen species) produced by a living organism as a result of normal cellular metabolism, they function in physiological cell process at low to moderate concentrations, but they produce adverse reaction at high concentration to cell components such as lipids, proteins, and DNA. RNS (reactive nitrogen species) are a family of antimicrobial molecules derived from nitric oxide and superoxide produced by the enzymatic activity of inducible nitric oxide synthase and NADPH oxidase respectively (Valko et al., 2006). Overproduction of free radicals can cause oxidative harm to

biomolecules, (lipids, proteins, DNA), ultimately leading to several chronic diseases like atherosclerosis, cancer, diabetics, rheumatoid arthritis joint pain, post-ischemic perfusion damage, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, aging and degenerative diseases in humans (Uttara et al., 2009). Herbs are traditionally defined as any part of a plant that is used in the diet for their aromatic properties with low nutritional value. But, more recently herbs are used as a source of various phytochemicals which possess powerful antioxidant activity. The demand for herbal medicines is superior to allopathic medicine due to their safety, fewer side effects, efficacy and good belief of the public in herbal medicines and their products. The secondary metabolites of plants are used as drugs and show many biological activities like antifungal, anticancer, antibacterial and antioxidants (Golla et al., 2014). *Cannabis sativa* Linn. is an annual herbaceous plant, (Synonym: Indian hemp, Ganja, Marihuana), the biological source is dried flowering tops of the cultivated female part of the plant *C. sativa*

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Family Cannabaceae (Kokate and Gokhl 2006). Plants growing in the tropical parts of India, Africa, and America produce narcotic substance whereas plant growing in temperate climates don't produce opiate substances and it is helpful in the generation of fiber and oil (Mohamed et al., 2009). More than 525 constituents have been identified from *C. sativa* L. The most particular class of *C. sativa* constituents are the C-21 terpenophenolic cannabinoids. Other phenolic constituents include flavonoids, spiro indans, dihydro stilbenes, phenanthrenes, dihydrophenanthrenes, Tetrahydro cannabinoids, sitosterol-3-*O*-D-glucopyranosyl-6'-acetate (Zuradi et al., 2006). It has been found that the cannabinoids in *C. sativa* particularly tetrahydrocannabinol (THC) and cannabidiol (CBD) showed potent antioxidant properties in various pre-clinical applications (Russo et al., 2006). The selected plant *C. sativa* possess various activities like antioxidant activity (Chen et al., 2012). The various parts of the plant are used in the treatment of several diseases, pain, whooping cough, asthma, sedative-hypnotic (Frankhauser et al., 2002) neurodegenerative diseases (Gerra et al., 2010) chemotherapy-induced nausea and vomiting, multiple sclerosis (Vara et al., 2011) anticoagulant (Iuvone et al., 2004) and glaucoma (McAllister et al., 2007) anticancer agents (Levndal et al., 2006; Costa 2004). This study was aimed to evaluate the antioxidant property and H₂O₂-induced oxidative stress protection activity of the aqueous extract of *C. sativa* and explore the underlying mechanism of action.

Material and methods

Plant material

The dried plant materials were obtained from authorized outlet (Rajkumar Sahu Contractor) Bhopal. The plant was authenticated by Dr. Zia-ul-Hasan. A voucher specimen of the plant has been deposited (418/BOT/ Safia/16). The plant material was ground to a coarse powder before extraction.

Preparation of the extract

Coarsely powdered plant parts (aerial parts) of *C. sativa* (35 gm) was defatted with petroleum ether (40-60°C) and successively extracted with methanol and distilled water for 36 hrs using Soxhlet apparatus. The extracts were evaporated to dryness under reduced pressure by rotary evaporator and the resulted dried residue was stored in airtight containers for further use (Uddin et al., 2014).

Chemicals and reagents

The chemicals used in the study were YPD (Difco Laboratories Detroit, Michigan), hydrogen peroxide (30%) (Merck, Mumbai), ascorbic acid (Sigma-Aldrich Mumbai), MitoSOX™ (Molecular probes, Invitrogen, USA), mounting solution (Vector Laboratories, Burlingame, CA), propidium iodide (Sigma-Aldrich, Mumbai). All other chemicals and reagents

used in this work were of analytical grade.

Yeast strains, media and growth conditions

The *Saccharomyces cerevisiae* wild-type strain BY 4743 and deletion strains ($\Delta trx2$, Δctt , Δcta) strains were purchased from Open Biosystems (Thermo Scientific) were grown up to OD₆₀₀ 0.6 to 1 in liquid YPD media (1% yeast extract, 2% glucose and 2% peptone) using incubator shaker 30°C and 180 rpm.

Preliminary phytochemical screening

The various phytoconstituents of the aqueous extract of *C. sativa* (ACS) (aerial parts) were analyzed to detect the carbohydrates, alkaloids, flavonoids, saponins, tannins, phenolic compounds, terpenoids, steroids, proteins and mucilage using standard tests (Tiwari et al., 2011).

Toxicological study

The aqueous extract (ACS) at different concentrations (200 µg to 1600 µg) were evaluated for toxicity in *S. cerevisiae*-BY 4743 (Garay et al., 2016).

Growth curve of yeast (BY 4743, $\Delta trx2$) under stress in presence of various extracts/ ascorbic acid

The cultures of BY 4743 and $\Delta trx2$ (OD₆₀₀=0.1), were transferred to different wells of a microplate. Further different concentrations of 10 µl ACS (400, 800, 1600 µg/ml) and 10 µl ascorbic acid (AA) (10 mM) were added in duplicate. Finally, 10 µl H₂O₂ (40 mM) was added to all the wells except the control and incubated at 30°C for 24 hrs in a microplate reader (BioTEK, USA) with continuous shaking at medium intensity. OD₆₀₀ was measured at every hour. Normal YPD was used as a blank (Wu et al., 2011).

Spot assay

The cultures of BY 4743 and $\Delta trx2$ (OD₆₀₀=0.1), was taken (1 ml) in a microtube in quadruplet and added ACS (400 µg/ml)/ AA (10 mM) all the tubes were incubated for 2 hrs, 23°C, at 180 rpm. Further, 40 mM H₂O₂ (100 µl) was added to all the tubes to get a final concentration of 4 mM, and incubated for 1 hr, 23°C at 180 rpm. The cells were harvested (12,000 rpm, 30 sec) and washed with sterile water. Finally, the cells were suspended in 1ml of sterile water. Further serial dilutions (10⁻¹, 10⁻² and 10⁻³) were made of a 96 well microtiter plate, and spotted (2.5 µl) onto YPD agar plate. Plates were incubated 23°C/48 hrs, then imaged by UVP Multidoc It (Bench Top Transilluminator) (Spencer et al., 2014).

Viability

Viability assay by plate method

A single colony of both wild strains BY 4743 and deletion strain $\Delta trx2$ was inoculated with YPD and were grown at

30°C at 180 rpm for overnight. Adjusted OD₆₀₀~0.6, both the cultures were divided into four groups each containing 1 ml. Except the control group, all the groups were treated with 4 mM H₂O₂. The test groups were treated with ACS (400µg/ml) and the standard group was treated with AA (10 mM). All the tubes were incubated for 3 hrs at 30°C in dark. After incubation, OD₆₀₀ was checked and an equal number of cells were taken from each tube. The cells were harvested, washed and finally suspended in 1000 µl of water. 10µl was taken from the cell suspension and diluted to 1000µl, from which 20 µl was spread on a YPD agar plate in triplicate. Incubated at 30°C for 48 hrs and the viable cell was expressed as the colony-forming unit. Colonies were counted after growth and then imaged after 48 hrs by UVP multi-doc It (Bench top Transilluminator) (Iracema et al., 2007).

Viability assay by propidium iodide staining

The cultures were prepared by inoculating single colony of both wild strains BY 4743 and deletion strain $\Delta trx2$ with YPD at 30°C at 180 rpm for overnight. Then, the culture of both the yeast strains was adjusted to an OD₆₀₀~0.6. The cultures were divided into four groups each having 1 ml. All the tubes were incubated for 3 hrs at 30°C, in dark. After incubation, the cells were pelleted and washed thrice with Milli-Q water. The cells were suspended in 200µl of PBS buffer and mixed with 0.2 µl of propidium iodide, to get a final concentration of 1 µg/ml from the stock (1mg/ml). After 5 min incubation in dark, the cells were harvested and washed with PBS. Finally, the stained cells were suspended in an appropriate amount of PBS. About 20µl cell suspension was mounted on a slide. The cells were visualized under a fluorescence microscope (Zeiss Apotome) at 100 X (Liesche et al., 2015).

Mitochondrial ROS Estimation by MitoSOX staining

The yeast BY 4743 strain and $\Delta trx2$ were stained by MitoSOX Red to determine the mitochondrial superoxide. The culture was prepared by inoculating single colony of both the strains in YPD at 30°C, 180 rpm for overnight. The overnight culture (OD₆₀₀~0.6) was divided into four groups, normal control, standard (AA 10mM), negative control (4 mM H₂O₂) and test (ACS 400µg/ml+H₂O₂). All the cultures were incubated for 3 hrs at 30°C, 180 rpm. The cells were harvested (12000 rpm, 30 sec), washed and suspended in 200 µl Milli-Q water. The cell suspension was mixed with 0.2 µl of MitoSOX Red from the stock (5 mM), to get a final concentration of 5µM. After 20 min incubation in dark, the cells were harvested and washed with PBS (3 times) to remove media and were finally suspended the stained cells in an appropriate amount of PBS. About 20 µl cell suspension was mounted on a slide. The cells were visualized under a fluorescence microscope (Zeiss Apotome) at 100 X (Liesche et al., 2015).

Results

Preparation of the extract

The calculated % yield of plant extract was found to be 4.58 % (1.60 g) of petroleum ether, 9.2 % (2.7 g) of methanolic extract and 1.28 % (0.35 g) of water extract.

Preliminary phytochemical screening

According to preliminary screening alkaloids, flavonoids, glycosides, tannins, saponins, terpenes were present in the aqueous extract of *C. sativa* (Table 1).

Toxicological study

In the toxicity study, the growth of *S. cerevisiae*- BY 4743 cells (untreated and treated) was monitored with ACS in different concentrations (200, 400, 800 and 1600µg/ml) as shown in the (Figure 1). All the treated groups exhibited normal growth pattern and there was no toxicity of extract observed at tested concentrations. So, these concentrations of plant extract were used for further studies.

Table 1. Phytochemical screening of *C. sativa*

Name of phytoconstituents	Aqueous extract of CS (<i>Cannabis sativa</i>)
Alkaloids	+
Flavonoids	+
Glycosides	+
Tannins	+
Saponins	+
Phytosterol	-
Resins	-
Terpenes	+

(+) Present, (-) Absent

Figure 1. In cytotoxicity study wild (BY 4743) type of yeast cell treated with different concentration of *C. sativa* (200, 400, 800 and 1600 µg/ml) on normal YPD plate and extract showed no toxicity.

Growth curve

We performed liquid growth assay of BY 4743 and $\Delta trx2$ (Figure 2) at concentration 400, 800 and 1600 $\mu\text{g/ml}$ of ACS. From the growth curve, it was observed that H_2O_2 -induced stress was overcome by ACS (400 $\mu\text{g/ml}$) in both the wild and $\Delta trx2$ strains of *S. cerevisiae*. After 12 hrs, the optical density of yeast cells treated with ACS (400 $\mu\text{g/ml}$) was even greater than the control group and found to be significant (** $P < 0.01$). AA was used as the standard antioxidant and it showed significantly (** $P < 0.01$)

improved growth of stressed cells comparable to the control.

Spot assay

We performed spot assay on wild strain as well as H_2O_2 sensitive deletion strains (Δctg , Δcta , $\Delta trx2$). All the strains of yeast treated with ACS (400 $\mu\text{g/ml}$) + H_2O_2 were able to overcome the stressing effect and showed a similar growth pattern as that of normal group (Figure 3).

Figure 2. (A) growth curve of wild (4743), (B) growth curve of $\Delta trx2$. Determination of arresting concentrations of oxidants on BY 4743 yeast cells and $\Delta trx2$ yeast cells ($\text{OD}_{600} \sim 0.01$) were challenged with H_2O_2 (4 mM) CTL- control group, H_2O_2 hydrogen peroxide (4 mM) containing group, AA –ascorbic acid (10mM) containing group, ACS different concentration 400 μg , 800 μg and 1600 $\mu\text{g/ml}$ cell density was monitored every hour for h at 30°C. Growth curve shows that treatment of ACS 400 $\mu\text{g/ml}$ was able to overcome the H_2O_2 stressed cell growth (** $P < 0.01$) (** $P < 0.01$).

Figure 3. (A) Normal: contain yeast culture, (B) Negative control: cells were stressed with H_2O_2 , these showed growth less than other groups (C) positive control: under H_2O_2 stressed and treated with AA these showed more cells than negative group (D) Test group contain H_2O_2 stressing agent and test drug ACS (400 $\mu\text{g/ml}$), here the deletion strains showed similar growth pattern as that of normal control group

Viability assay

A viability assay is an assay to determine the ability of cells or tissues to maintain or recover viability. Cell viability can be determined by various methods. We evaluated the cell viability by plate method and propidium iodide staining method.

Plate method

The viability assay was performed to study the protective activity of ACS against H_2O_2 induced oxidative stress in two yeast strains (BY 4743 and $\Delta trx2$). The percentage of viable cells was calculated using the number of colony-forming units (c.f.u.) where the control group is taken as the reference (100%). After 48 hours, 35% viability (wild BY 4743) and 33% cell viability ($\Delta trx2$) of yeast cells was observed in the H_2O_2 stressed group, while the cells treated ACS (400 $\mu g/ml$) + H_2O_2 showed an improved % viability i.e. 50% cell viability (BY 4743) and 57% cell viability ($\Delta trx2$) (** $P < 0.01$) (Figure 4).

Propidium iodide

Propidium iodide (PI) is an intercalating agent and a fluorescent molecule to evaluate cell viability. PI is commonly used for identifying dead cells in a population and as a counter stain fluorescent techniques. PI enters rupture cell membrane and impaired cell wall showed more fluorescence. After staining with PI in both strains BY 4743 (Figure 5) and $\Delta trx2$ (Figure 6) H_2O_2 stressed cell, a decreased viability was observed for both the strains (BY 4743, 57.15 ± 1.8 and $\Delta trx2$, 43.75 ± 9.72). At the same time, ACS (400 $\mu g/ml$) treated group

showed an increased viability (BY 4743, 92 ± 1.9 and $\Delta trx2$, 93 ± 1.5), similar to AA (10 mM) treated group (BY 4743, 95.35 ± 2.227 and $\Delta trx2$, 96 ± 1.4849), compared to negative control. The results indicate a significant (** $P < 0.01$) scavenging effect against the H_2O_2 -induced stress thereby protecting the cells from oxidative stress (Figure 7).

Figure 4 (A) CTL- control, (B) H_2O_2 -Negative control (Hydrogen peroxide), (C) AA-Ascorbic acid, (D) ACS-test group cell viability assay by plate method in wild and $\Delta trx2$ this graph showed % cell viability test group showed in wild 50% (** $P < 0.01$) and $\Delta trx2$ 57% (** $P < 0.01$) viable cell that will be comparable more than negative control. A&A1 - Control group (BY 4743) C&C1-positive control (H_2O_2 +AA); B&B1 -Negative control (H_2O_2) D&D1-Test group (H_2O_2 +ACS)

Figure 5. (A) Control group; (B) negative control stressed by (4 mM) H_2O_2 ; (C) positive control stressed by 4 mM H_2O_2 or group treated with 10 mM AA; (D) test group stressed by 4 mM H_2O_2 or treated with ACS 400 $\mu g/ml$. The negative control group showed more staining than the positive control or test group. ACS showed the same effect as AA. A&A1-Control group ($\Delta trx2$) C&C1-positive control (H_2O_2 +AA); B&B1-Negative control (H_2O_2 +yeast culture) D&D1-Test group (H_2O_2 +ACS)

Figure 6. (A) Control group; (B) negative control stressing by 4 mM H_2O_2 ; (C) positive control stressing by 4 mM H_2O_2 or group treated with 10 mM AA; (D) Test group stressing by 4 mM H_2O_2 or treated with ACS (400 μ g/ml). The negative control group showed more staining than the positive control or test group. ACS same effect comparable to as AA

Figure 7. This graph show % viability (A) Control group; (B) negative control stressing by (4 mM) H_2O_2 ; (C) positive control stressing by 4 mM H_2O_2 or group treated with 10 mM AA (D) test group stressing by 4 mM H_2O_2 or treated with ACS 400 μ g/ml. Showed significant (** $P < 0.01$) radical-scavenging H_2O_2 effect. **A&A1-Control group (BY 4743)** **C&C1-positive control (H_2O_2 +AA); B&B1-Negative control (H_2O_2)** **D&D1-Test group (H_2O_2 +ACS)**

MitoSOX staining

MitoSOX Red reagent is a fluorogenic dye particularly targeted to mitochondria in live cells. The oxidation of MitoSOX Red reagent by superoxides produces red fluorescence. The production of superoxides by mitochondria can be visualized in fluorescence microscopy by utilizing the MitoSOX Red reagent. MitoSOX Red reagent permeates live cells, where it selectively targets mitochondria and is quickly oxidized by superoxides (Girgih et al., 2011). In this study, the treated cells-both strains BY 4743 (Figure 8) and Δ trx2 (Figure 9) were stained with

MitoSOX to determine the amount of mitochondrial ROS. The negative control (H_2O_2 stressed) group showed more number of fluorescent cells which were evident by the high percentage of mitochondrial superoxides. While in the positive control, (AA + H_2O_2) showed less number of fluorescent cells (BY 4743, $6.4 \% \pm 0.070$ and Δ trx2, $8.7 \% \pm 0.353$). The test (ACS 400 μ g/ml+ H_2O_2) treated group showed the lesser number of fluorescent cells than the negative control group (BY 4743, $5.1 \% \pm 0.7071$ and Δ trx2, $3.59 \% \pm 0.417$), the percentage was found to be even less than that of AA. The results indicate that ACS was capable of scavenging the mitochondrial superoxide in a significant manner (** $P < 0.001$) and thereby showed the protective effect against H_2O_2 -induced oxidative stress (Figure 10).

Discussion

Hemp seed protein hydrolysate showed effective *in vitro* antioxidant properties. The ability of the peptides to scavenge hydroxyl and DPPH radicals, reduce ferric metal ions and chelate transition metal ions is indicative of their potential use for managing metabolic disorders that arise from excessive levels of ROS (Hong et al., 2015). It was demonstrated that the ethanolic extract of Hemp seeds, effectively inhibit H_2O_2 mediated oxidative stress and propose its beneficial effect as a therapeutic agent in preventing oxidative stress mediated diseases (Gavamukulya et al., 2014). The extract of Hemp seed hull, is proven to be a rich source of natural antioxidants, and its consumption as dietary supplements, help to prevent oxidative stress (Chen et al., 2012). Antioxidants are the substance that reduces damage due to free radical, *C. sativa* has long been known for its psychotropic effect. But

Figure 8. (A) control group; (B) negative control stressing by 4 mM H_2O_2 ; (C) positive control stressing by 4 mM H_2O_2 or group treated with 10 mM AA (D) test group stressing by 4 mM H_2O_2 or treated with ACS 400 $\mu\text{g/ml}$ MitoSOX stain was used for the detection of mitochondrial ROS level and the m-ROS was stained by red fluorescence. **A&A1-Control group ($\Delta trx 2$) C&C1-positive control (H_2O_2 +AA); B&B1-Negative control (H_2O_2 +yeast culture) D&D1-Test group (H_2O +ACS)**

Figure 9 (A) control group; (B) negative control stressed by 4 mM H_2O_2 (C) positive control stressed by 4 mM H_2O_2 or group treated with 10 mM AA; (D) test group stressing by 4 mM H_2O_2 or treated with ACS 400 $\mu\text{g/ml}$ MitoSOX staining was used for the detection of mitochondrial ROS level and the m-ROS was stained by red fluorescence.

recently research disclosed the wild range of pharmacological activity like anti cancer, anti diabetic etc (Girgih et al., 2011). The current study aimed to evaluate antioxidant activity of the *C. sativa* extract (aqueous) using eukaryotic model *S. cerevisiae* (both wild strain, $\Delta trx2$) against H_2O_2 induced oxidative stress. The ACS (400,800 $\mu\text{g/ml}$) was able to overcome the stressing effect of H_2O_2 and the treated cells showed a growth curve similar to that of normal control. We found that, surprisingly, ACS at low concentration (400 $\mu\text{g/ml}$) showed better protection compared to

that of higher concentration (800, 1600 $\mu\text{g/ml}$). So, we can infer that ACS is effective even at low concentration. In the viability assay, the % viability of the extract treated cells were increased approximately twice and twice times for BY 4743 and $\Delta trx2$ respectively. On staining with propidium iodide the measured viability of the live yeast cell treated group was improved viability levels were twice and twice the time for BY 4743 and $\Delta trx2$ respectively. The ACS also reduced mitochondrial ROS in yeast cells both BY 4743

Figure 10. This graph shows % stained cells by MitoSOX staining; A: control group; B: negative control stressing by 4 mM H₂O₂; C: positive control stressing by 4 mM H₂O₂ or group treated with 10 mM AA; D: test group stressing by 4 mM H₂O₂ or treated with ACS (400 µg/ml) significant result (*** P<0.001; ** P<0.01).

strain and $\Delta trx2$. In addition, the antioxidant properties of ACS are a promising strategy for therapeutic effects by avoiding disorders in the normal redox reactions in healthy cells. These activities of *C. sativa* extract may be attributed to the presence of various phytochemical constituents found to be such as alkaloids, flavonoids, glycoside, tannin, and saponins, terpenes which were reported earlier and confirmed in the present study. Typical phenolics that possess antioxidant activity have been characterized as phenolic acids and flavonoids. Phenolic compounds have been related to antioxidant activity, some studies have emphasized specific classes such as flavonoids and tannins. The higher phenol content in the aqueous extract would partly contribute to its higher antioxidant activity. This study provides experimental evidence and supports the medicinal use of this plant and lends pharmacological credence to the ethnomedical use in the traditional system of medicine. Also, this study demands further studies to elaborate its use, active constituents, and safety.

Conclusion

In this study, we have shown that ACS exhibited significant free radical scavenging activity and antioxidant activity against hydrogen peroxide radicals *in vivo*. This extract showed that regulation of redox state is critical for cell viability; activation, proliferation, and organ function and protect the oxidative stress and oxidative stress generated disease. The results show that the synergistic action of the phytoconstituents present in ACS has a lot of potential for the management of diseases like cancer, diabetes, neurodegenerative disorders etc.

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Conflict of Interest

Authors have no conflict of interest.

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