World Applied Sciences Journal 30 (3): 286-293, 2014
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2014.30.03.1750

Investigation of *Acacia modesta* Leaves for *In-vitro* Antioxidant Activity, Enzyme Inhibition and Cytotoxicity

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Abstract: In this study, the leaves of Acacia modestawas evaluated for total phenolic and flavonoid contentsas well as for its antioxidant activity, enzyme inhibition activity and cytoxicityactivity. The total phenolic and flavonoids contents of the methanol extract were determined by standard methods. The amount of total phenolic content was 2.5 gm per 100 gm and total flavonoids content was 0.8 gm per 100 gm of methanolic extract. The in-vitro antioxidant activity was assessed by using different methods such as; 2,2diphenylpicrylhydrazyl [DPPH]free radical scavenging activity,hydrogen peroxide scavenging activity, superoxide scavenging activity and ABTS [2, 2azobis-[3-ethylbenzothiazoline-6-sulphonic acid] free radical scavenging. In all these methods, antioxidant activities of the sample extract of *acacia modesta* leaves showed greater antioxidant activities than the ascorbicacid, used as a standard. The methanolic extract was observed to have significant inhibition activity for acetylcholinesterase. Additionally cytotoxicity was also evaluated by using brine shrimps assay and showed high cytoxicity with lower concentration of sample extract. In short our result showed evidence for the safe use of these medicinal plants as treatment for aliment in which free radicals are implicated.

Key words: *Acacia modesta* leaves · Scavenging activity · DPPH · ABTS · NBT · H_2O_2 · Inhibition activity

INTRODUCTION

The reactive oxygen species (ROS) which are produced in the bodyare potentially reactive derivative of oxygen and reactive nitrogen species (RNS) [1]. These reactive oxygen species (ROS) arenormally detoxified by various antioxidant species present in the body. Over production of these free radicals (ROS & RNS) or insufficient supply of antioxidant expose the body for free radical attack which causes oxidative damage to the different bio-molecules including lipid, proteins, lipoproteins and DNA [2, 3]. The oxidative damage is critical etiological factor involved in different chronic human diseases such as, diabetic mellitus, cancer, arthritis, neurodegenerative diseases, atherosclerosis and in the aging process [4]. Phenolic compounds andflavonoids are also widely distributed in plants which have been reported to exert multiple biological effects, including antioxidant free radicals scavenging abilities, anti-inflammatory and anti-carcinogenicetc [3]. Crude extract of medicinal plants are rich sources of phenolic which retard oxidative degradation of lipids while flavonoids are group of polyphenolic compounds has the property of free radical scavenging activity, inhibition of hydrolytic and oxidative enzyme and anti-inflammatory action [5]. Although several novel antioxidants have been investigated in many other species [6, 7], but still there is a demand to find more information concerning the antioxidant potential of plant species, as they are safe and also bioactive. Therefore recently considerable attention has been given to the identification of plants with antioxidant abilities. Although many medicinal plants have long been utilized by the local community, as health promoting agents but still the health benefits of many plants have not been scientifically studied [3]. *Acacia modesta* wall is traditionally using for medicinal, fuel and timber purposes in our country. The review of literatureshowed no study on the anti-oxidant activity of

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this species. The aim of this study wasto evaluateacacia modesta leaves for total phenolic and flavonoid contentsas well as for its in-vitro antioxidant activity, enzyme inhibition activity and cytoxicity activity. *Acacia modesta* plant leaves extract was tested for its free radical scavenging activities using in-vitro assays. Their corresponding level of total phenolic and total flavonoids were also quantified. Medicinal plant cytotoxicity was assessed by brine shrimps assay.

METRIALSAND METHODS

Chemicals: All reagents and solvents are of analytical grade. 2,2, diphenylpicrylhydrazyl [DPPH] and Folinciocalteus were from Fluka chemical and purchased in Pakistan from local market.Aluminum chloride, Sodium carbonate, Sodium phosphate, Rutin, Ascorbic acid, Gallic acid and all other solventswere of Sigma Aldrich chemicals. The ferric chloride, [$FeCl_3$] methanol, hydrogen peroxide, concentrated hydrochloric acid, was of Merck.

Plant Identification and Collection: The identified plant part was collected from the vicinity of FR Bannu District (Domel) Khyber Pakhtoonkhwa (April, 2012). Plant was further searched through internet, no work was found about this plant. On this base plant of *acacia modesta* was selected and then collected. After collection the leaves were separated and washed with tape water and dried in shed for two weeks at room temperature and change the dried leaves to powder form mechanically with electric grinder.

Prepration of Sample Extrect: Leaves powder of *acacia modesta* were soaked in 80% methanol. The solutions were shacking every day randomly. After 10 days the sample were filtered by using Whatmanns filter paper. The extracts of leaves were concentrated on rotary evaporator at 40°C. The concentrated plant extract was converted intolyophilizationbottle. The plant extract were freezed in bottle by using liquid nitrogen. The lyophilizer was settled at -40°C, pressure 50 torrs and after operation for several hours, obtained plant extract in solid form. This solid extract was used for further assays.

Preliminery Phytochemical Screening and Determination of Total Flavonoids and Phenolic Content
Preliminery Phytochemical Screening: The dry extract of acacia modest leaves was used for the determination of qualitative analysis of different phytochemical present in leaves extract. This showed presence or absence of different phytochemical constituents by using different standard methods.

Saponnins: For determination ofsaponnins in the methanolic fraction of acacia modest leaves the standard method of [8] was used. The 20mg methanolic extract was dissolved in 10ml distill water. The solution was boiled for 3 minutes and then filtered. To the cooled filtrate added 1ml olive oil which gave emulsions, showed the presence of saponnine in plant extract of leaves.

Cardiac Glycosides: For the determination of glycoside in methanolic extract the method of [9] was used. 1ml plant extract (1mg/ml) was mixed with 1ml of glacial acetic acid then added 1 drop of 5% ethanolic ferric chloride solution then poured 1ml of concentrated sulfuric acids along the side of test tube. Brownish ring appeared between the two layers indicated the presence of cardiac glycosides in the methanolic extract of plant leaves.

Terpenoids: The presence of terpeniods in the methanolic plant extract was determined according to the method of [8]. 5mg plant extract of leaves was dissolved in 5ml distilled water and then added few drops of chloroform and to this added carefully 3ml of concentrated sulfuric acid. Reddish brown layer was formed between the two layers showed the presences of terpeniods in the plant extract of*acacia modesta* leaves.

Coumarims: For presence of cumarims in leaves extract of acacia modesta the protocol of [8] was used. 0.3gm of leaves extract was dissolved in 10ml distilled water in a small beaker and covered with filter paper which was wetted with 1N of sodium hydrooxide solution. The beaker was placed in a boiling water bath for 5 minutes. Then removed the filter paper and examined under the UV Lamp. Yellow florescence was present indicated the presence of coumarims.

Flavonoids: The flavonoids were determined according to the method of [10]. Small quantity of methanolic extract of *acacia modesta* leaves was mixed with 10 ml of ethyl acetate and boiled for 3 minutes in a test tube and then filtered the solution. The 3 ml filtrate was takenand added 1ml of ammonium hydrooxide. Yellow color appeared show the presence of flavonoids in the extract.

Determination of Total Flavonoids: A total flavonoid was determined by using a method of [11]. Briefly, 0.25ml of each fraction of sample extract [1mg-5mg]dissolved in respective solvent and Rutin standard solution of each fraction [15-250 ug /ml]was mixed with 1.25 ml of distilled water in a test tube. Then added 75ul of 5% of sodium nitrite solution. After 6 minutes incubation added 150ul of 10% $AlCl_3$, again incubated for 5 minutes and added 0.5 ml of 1M, NaOH solution to each fraction made the volume up to 2.5 ml with distilled water and immediatelynoted the absorbance of each fraction at 510 nm. The results of sample were expressed as mg of rutin equivalents of total extractable compound.

Determination of Total Phenolic Content: Total phenolic content was determined by the Folin-Ciocalteu reagent method [12] with slight modification. Briefly the sample extract 0.5 mg was mixed with 0.5 ml of Folin reagent [previously diluted 1:1 with distilled water] and incubated for 5 minutes at room temperature. Then added drop wise 1ml of 2% Na_2CO_3solution. After 10 minutes incubation at room temperature, the absorbance was measured at 730 nm. Gallic acid monohydrate was used as a standard. The total phenolic content was measured as gram of galic acid equivalent [GAE] per 100 gram extract.

Antioxidant Evaluations

DPPH Scavenging Activity Assay: The DPPH [2, 2, diphenylpicrylhydrazyl] free radical scavenging capacity of the extract was estimated according to the method of [13]. Prepared the DPPH (2, 2, diphenylpicrylhydrazyl) solution by dissolving the 0.003gmof DPPH in 50ml methanol and also. prepared the stock solution of sample extract by dissolving 5mg per 5ml methanol. This solution was further diluted in methanol and prepared the desired concentration of 50µg/ml up to 500µg/ml of plant extract. Prepared the ascorbic acid stock solution and from this then prepared the desired concentration [50µg/ml - 500 µg/ml]. The absorbance of DPPH was recorded at 517nm wavelength. The solution of the required concentration was then converted into a test tube by taking 100µl sample and 900µl of DPPH solution and then incubated for 30 minutes at 37°C in water bath. The concentration of ascorbic acid solution in DPPH was used as standard. The absorbance of each concentration was taken at 517nm by using double beam spectrophotometer. The percentage of DPPH scavenger was calculated by the following formula,

DPPH % scavenging activity = A_{DPPH}-A_{Sample} x 100/A_{DPPH}

A_{DPPH} = absorbance of DPPH

A_{Sample} = absorbance of sample

Then calculated the % scavenging activity of sample and compared it with standard ascorbic acid scavenging activity.

Hydrogen Peroxide Scavenging Activity Assay: The ability of plant extract to scavenge hydrogen peroxide can be estimated according to the method of [14] with some modifications. A solution of hydrogen peroxide 40mM was prepared in phosphate buffer [50 mM, pH-7.4]. The concentration of hydrogen peroxide is determined by absorption at 320 nm using spectrophotometer. Plant extract [50 ug- 250ug/ml] in distilled water is added to hydrogen peroxide and absorbance at320 nm is determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The ascorbic acid was used as positive control. The scavenging activity of hydrogen peroxide is calculated as follow;

% Scavenged [H_2O_2] = [Ac – As] x 100/ Ac

where Ac is the absorbance of the control and As is the absorbance of the sample.

Determination of Superoxide Radical Scavenging Activity: Superoxide scavenging was determined by the nitro blue tetrazolium (NBT) reduction method (Nishi kini M. Rao *et al.,*). The reaction mixture consisted of 0.5ml of nitro blue tetrazolium (NBT) solution (1M NBT in 100mMl phosphate buffer, pH 7.4). 0.3ml NADH solution 1M NADH in 100mMl phosphate buffer, pH 7.4 and 0.1 ml of different fractions and ascorbic acid (50mMl phosphate buffer pH 7.4) was mixed. The reaction was started by adding 100 µl of PMS (phasphometha zone sulphate) solution (60 µM PMS in 100mM phosphate buffer pH7.4) in the mixture. The tubes were uniformly illuminated with an in candescent visible light for 15 minutes and the optical density was measured at 530nm before and after the illumination. The %age inhibition of superoxide generation values of the control and experimental tubes. The ability to scavenge the superoxide radical was calculated by using the following formula.

% scavenging of superoxide = (Ac-As)/Ac x 100

where Ac is the absorbance without sample and As is the absorbance with sample

Abts Radical Scavenging Activity: ABTS radical cat-ion scavenging activity of the extract was determined as described in [15] with modification.Stock solution was prepared by mixing equal volume of 7mM ABTS solution and 2.45mM potassium persulphate solution and incubated for 12hrs in the dark at room temperature to get the dark color solution containing free radicals of ABTS. The stock ABTS solution was diluted with 50% methanol and brings the absorbance up to0.668 nm at 745nmat room temperature. Free radical scavenging activity was evaluated by mixing 300µl of different concentration of sample extract of plant leaves and flowers (50µg/ml to 250µg/ml) with 300µl working standard. After mixing the solution the decrease in absorbance was noted exactly at 1minute and final absorbance up to 6minutes and data was recorded. The absorbance of ascorbic acid was used as positive control. The scavenging activity was calculated by %age activity of ABTS free radicals scavenged by the sample extract by the following formula.

% scavenging activity of ABTS= [(Ac-As)/Ac] x100

where Ac is the absorbance of the control and As is the absorbance of the sample extract.

Enzyme Inhibition Activity

Acetylcholine Esterase Activity Assay: The enzymatic activity was measured using an adaptation of the method described in [16]. Prepared the substrate solution by dissolving the substrate 0.9gm in 50ml distilled water and dissolved 0.003gm DNTB (5,5,dithiol,bis, (nitro benzoic acid)) in 100 ml phosphate buffer having pH 7.6. The sample stock solution was prepared by dissolving acacia modesta leaves methanolic extract 1mg in 1ml distilled water. Then used this stock solution by preparing serial concentration of 50µg to 150µg/ml. Initially mixed the solution in the following concentration 940µl DNTB to this added 50 ul of 50µg/ml of sample extract from stock solution, added 50µl distilled water, 10µl acetyl choline esterase enzyme and 80µl substrate. The absorbance of blank solution of 940µl DNTB and 60 µl distilled water was taken at 421nm.

Cytotoxicity Activity

Brine Shrimps Cytotoxicity Assay: To assess the cytoxicity of sample extract, the assay of the brine shrimps was performed as previously describe by some modification (Singlet VL, Rossi JA 1965) [17]. Eggs of brine shrimps were purchased and mixed with artificial sea salt water [prepared by dissolving 20 gm sea salt per liter of water. The eggs were allowed to hatch at room temperature for 24 hrs. To determine the cytoxicity of the plant extract, different concentration of the extract was tested. Ten brine shrimps larvae were then added to each test tube, containing the extract. After incubating for 24 hours at room temperature, the numbers of dead and surviving brine shrimps were countedfor evaluation of plant extract activity.

RESULTS AND DISCUSSION

Total Phenolic and Total Flavonoid Contents: The result of antioxidant activity of methanolic extract was due to the presence of phenolic compound as present in this species and there was a positive co-relation between the antioxidant activity and phenolic and flavonoids [18].

Plant phenolics acting as a primary antioxidant free radical scavengers, the presence of plant phenolic may be due to tannins and flavonoids [19]. This methanolic extract contains both constituents of tannins and flavonoids and act as an antioxidant. The amounts of total phenolics are 2.5 gm. per 100 gm. and total flavonoids is 0.8 gm per 100 gm of methanolic extract.

The result of the anti-oxidant activities and reducing power activity of the methanolic extract are due to presence of phenolic compounds as present in this species and there is the positive correlation between the anti-oxidant activity and phenolics, flavonides [30].20

Antioxidant Evaluations: Recently there has been upsurge of interest in therapeutic potential of plants, antioxidant in reducing free radical in inducing tissue injury. Although several synthetic antioxidant, such as butylatedhydroxyanisole (BHA) and butylatedhydroxytoluene (BHT) are commercially available, but are quit unsafe and their toxicity is a problem of concern. Hence strong restrictions have been placed on their applications and there is a trend to substitute them with naturally occurring antioxidant [21].

DPPH Scavenging Activity: In this study the antioxidant activity of the methanolic extract of *acacia modesta* leaves was determined using the DPPH scavenging assay, reducing power of the leaves extract and by investigating the total antioxidant capacity of the leaves extract. All these have shown the effectiveness of the leaves extract

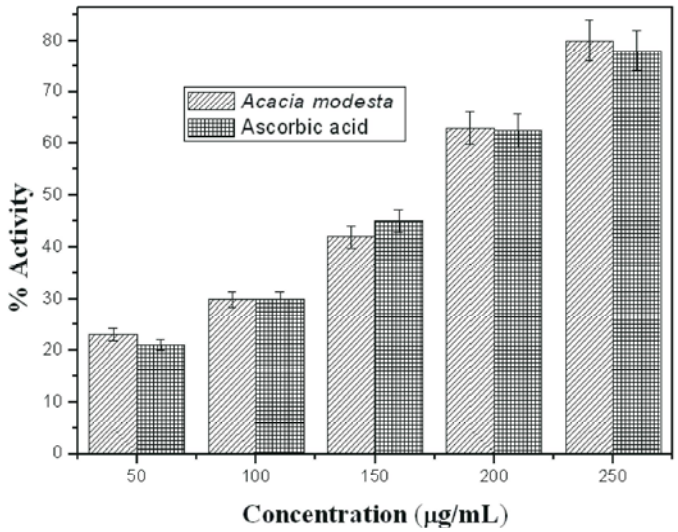

Fig. 1: DPPH scavenging activity of the methanolic extract of *Acacia modesta* leaves in comparison with a standard Ascorbic acid

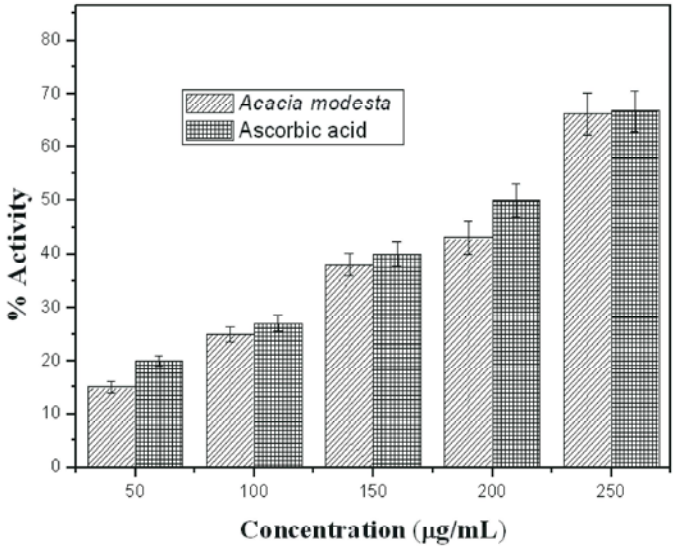

Fig. 2: Hydrogen peroxide scavenging activity of methanolic extract of *Acacia modesta* leaves in comparison with a standard ascorbic acid

as compared with the reference standard antioxidant ascorbic acid. The DPPH antioxidant assay is based on the ability of DPPH, stable free radical to change the color of the present antioxidant [22]. When DPPH accepts an electron donated by an antioxidant compound, the DPPH color is changed. This can be quantitatively measured from the change in absorbance. Comparison of the antioxidant activity of leaves extract and ascorbic acid is shown in Fig. 1. The methanolic extract of leaves showed high inhibition of DPPH activity, with 81% inhibition at concentration of 250 µg/ml as compared to ascorbic acid (250 µg / ml).

Hydrogen per Oxide Scavenging Activity: Hydrogen peroxide itself is nonreactive but it can cause damages to cell by producing free radicals in the body which react with biomolecules and lead to tissue damages which result various diseases [23]. A sample extract showed high scavenging activity of hydrogen per oxide compared with the scavenging activity of ascorbic acid used as standard as shown in Fig. 2. The scavenging ability of sample is 66.20% at 250 µg/mL which is equivalent to standard ascorbic acid i.e. 66.72% at 250ug/ml. This showed the best scavenging ability of the sample extract.

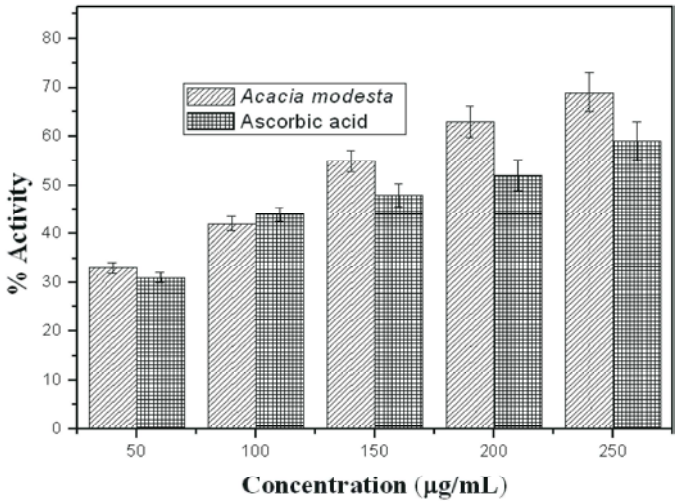

Fig. 3: Superoxide radical scavenging activity of methanolic extract of *Acacia modesta* leaves in comparison with a standard Ascorbic acid

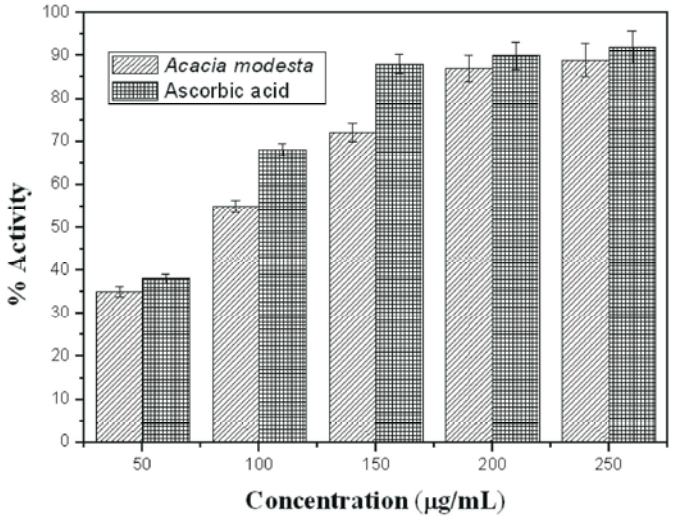

Fig. 4: ABTS scavenging activity of the methanolic extract of *Acacia modesta* leaves in comparison with a standard Ascorbic acid

Super Oxide Radical Scavenging Activity: Super oxide is harmful and damage to the tissue by producing various diseases it is precursor of the more reactive species which cause oxidative stress [24]. The scavenging activity of sample extract was correlated with standard ascorbic acid as shown in Fig. 3. Sample was found more reactive then the standard ascorbic acid. The percentage scavenging capacity of the extract is 69% while that of ascorbic acid is 59.73% at 250ug/ml.

Abts Scavenging Activity: The ABTS (2,2, azo-bis-(3-ethyl benzothiazoline-6-sulphonic acid) free radical scavenging capacity of the sample extract along with the standard ascorbic acid was recorded. It was found that the scavenging ability for the sample extract was equivalent to the standard ascorbic acid. The scavenging activity of the sample is 88.37% while that of ascorbic acid is 92% at 250ug/ml as shown in Fig. 4.

Enzyme Inhibition Activity

Actylcholinesterase Activity: Acetylcholine is involved in the signal transfer in the synapses. After being delivered in the synapses, acetylcholine is hydrolyzed giving choline and acetyl group in a reaction catalyzed by the enzyme acetylcholinesterase [25]. The molecular basis of the Alzheimer drugs used so for, take advantage of their action as acetylcholinesterase inhibitors, [26]. The sample extract showed some inhibitory activity against

acetyl choline esterase enzyme i.e.0.04mg, 0.05mg, 0.1mg concentration of extract shows 2.6%, 5.4% and 23.4% inhibition respectively.

Cytotoxicity Activity

Brine Shrimps Cytotoxicity Assay: The cytotoxicity of methanolic extract of *acacia modesta* was determined by its effect on brine shrimps [11]. At 20µg/ml concentration of extract all shrimps are alive, at 40µg/ml concentration of extract 8 were lived and 2 were dead, at 80µg/ml concentration 50%were alive, at 100µg/ml concentration of sample extract one was alive and 9 were dead and at 500µg/ml extract concentration all were dead. The effect of sample extract on shrimp was 50%at 80µg/ml concentration but at 100µg/ml the shrimps were 90% dead. Result showed that its small concentration show high cytotoxicity.

CONCLUSION

The methanolic extract of *Acacia modesta* leaves contained reasonable amount of total phenol and flavonoid and others phytochemicals which play important role in controlled oxidation process. The methanolicextractshowed strong antioxidantactivity by inhibitingscavenging activity ofDPPH,Hydrogen peroxide, Superoxide radicals, ABTS when compared with the standard Ascorbic acid. The results of this study show that the *Acacia modesta*leaves can be used as an easily accessible source of natural antioxidant. Acetyl choline esterase enzyme inhibition was measured 0.294 and cytotoxicity was 90% for brine shrimps cells when 100µg/mLof methanolic extract was used.

ACKNOWLEDGMENT

The authors appreciate department of Biotechnology, University of Science and Technology Bannu for providing support in carrying out this research work

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