Investigating the effect of borage (*Echium amoenum* L.) on chemical antimicrobial properties of rainbow trout (*Oncorhynchous mykiss*) fillet under refrigerator condition

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**ABSTRACT**

In the present study, antimicrobial and anti-oxidative effects of various concentrations of borage hydro alcoholic extract (500 and 1000 ppm) on shelf life of rainbow trout during storage in the refrigerator were investigated. Microbial assays include Total viable count (TVC) and Psychrophilic bacterial count (PTC), while chemical assays included Total volatile basic nitrogen (TVBN), acidity (pH) and Thiobarbituric acid (TBA) on 0, 4, 8, 12, 16 and 20 day intervals at 4 ± 1°C. Results indicated that it was significantly increased in all treatment during the time (p<0.05) and this increase was more severe at 500 ppm concentration of borage extract. Finally, the best condition for storage of rainbow trout was determined as 500 ppm of borage extract for 8 days at the refrigerator.

**Key words:** *Echium amoenum*, fillet, *Oncorhynchous mykiss*, storage process.

**INTRODUCTION**

Despite its high food value, fish is highly sensitive to oxidative deterioration and its quality decreases during storage as a result of oxidative and bacterial degradation (Mexia et al., 2009). Oxidative deterioration causes bad odor, unfavorable changes in taste, changes of nutritious structures and reduction of food value, while microbial decay causes serious harm for user’s health. Therefore, it is necessary and useful and the use of appropriate materials with anti-bacterial and anti-oxidation to improve quality, increase shelf life of meat and at the same time, prevent economic losses (Yin, 2003). Adverse effects of synthetic antioxidants have caused tissue including mutagenicity, toxicity and carcinogenicity, as well as being effective as natural antioxidants on the oxidation inhibition such that the use of natural antioxidants with plant source is recommended as an alternative to synthetic antioxidants (Sakanaka at al., 2005).

It is well documented that the anti-oxidative activity of some fruits and vegetables depends on their total phenolic contents (Mour et al., 2001). The anti-oxidative activity of the herbal phenolic compounds is mainly due to their chemical composition and redox properties that may play critical roles in the neutralization of free radicals, chelating of transitional metals and quenching mono and triple oxygen molecules. These properties are related to useful anti-oxidative effects of phenolics on the health which result from their inhibitory impact on the progress of many diseases related to oxidative stress, such as cardiovascular diseases, inflammatory bowel syndrome and Alzheimer (Ahmadi et al., 2007).

Borage (*Echium amoenum* L.) is a plant with anti-oxidative and anti-microbial properties. Borage is an herbaceous, biennial and perennial plant covered with soft and thin hairs and has a bluish-purple tubular flower. Its flowers are tender. It is effective for diseases such as meningitis, pleurisy, madness, obsession, repression, melancholy and cough (Sayah, 2005). In between different species of rainbow trout-breeding in terms of annual production, availability to the consumer and distribution, they have great importance between growers and consumers. In view of the economic and food value, the high percentage of production and methods of temporary
storage, supply of this fish, quality study and shelf life determined in the refrigerator, the impact of packaging and various additives to it, is one of the aspects of important qualitative studies on health and human nutrition (Ahmadi et al., 2007).

Fish plays a crucial role in the diet of many developing and developed countries. Owning to its high digestibility and suitable composition of the necessary amino acids such as lysine and methionine, fish meat is considered as a very useful food. The health benefits associated with polyunsaturated fatty acids and omega-3 fatty acids, stimulated the interest to increase seafood consumption (Jeon et al., 2002). Omega-3 fatty acid reduces blood cholesterol levels by preventing human exposure to cardiovascular disease and slowing the progression of cancer; it also helps to improve auto-immune disorders, enhance memory and vision (Shirazi et al., 2001).

In addition, fish meat is rich in vitamins and minerals. On the other hand, fish meat is aquatic and composed of relatively high protein, nitrogen compounds and abundant polyunsaturated fats in the muscles which are components of most perishable food (Razavi, 2006). Corruption fish muscle is caused by changes that occur during chemical reactions, such as fat oxidation reactions caused by fish natural enzymes (autolysis), and metabolic activity of micro-organisms which thus disappears sensory quality, organoleptic and its nutritional value (Ojagh et al., 2004).

Oxidative corruption causes unpleasant odor, adverse changes in taste and finally, changes in nutrients building, thereby reducing the nutritional value of the product, while corruption and microbial contamination leads to serious risks in taking food safety supplies. As such, using appropriate materials with antibacterial and antioxidant activity to improve quality and increase shelf life of meat and at the same time to prevent economic losses is necessary and useful (Yin, 2003). High capability of fish corruption (Liston, 1980) has led to maintaining the quality of fresh fish as one of the major issues of interest to the fish industry and consumers in this regard, considering the shelf-life product (the period when a food product under certain maintenance item is appropriate and safe for consumption) is important. For this purpose, the use of different techniques such as cooling product immediately after harvesting and storing it in ice, freezing (Aubourg and Gallardo, 2005), vacuum packing and modified atmosphere (Ozogul et al., 2004), gamma irradiation and UV, the use of anti-microbial substances such as organic acids (Al-Dagal and Bazarr, 1999) and salts of organic acids (Sallam, 2007), the use of natural and synthetic antioxidants (Banergee, 2006), the use of essential oils (Frangos et al., 2010) and the shell out (Fan et al., 2008) also covers the combined effect of food and oil (Shirazi, 2001) to increase shelf life of marine products and maintain the quality of the fish. In Iran, there is not a possibility of freezing immediately after fishing or on deck, so it is logical to choose the best way possible in the ice cover for the perfect fish, because on fillet fish, there is no possibility of ice cover, but through proper packaging in packages without air or proper cover and the use of antioxidants that can increase the shelf life (Rezaei et al., 2006).

Medicinal properties of borage were first discovered by the Romans in the third centuries BC and introduced to Europe. The Greek poet and historian, Homer introduced borage as a nepenthe herb. Pharmaceutical properties of borage were also remarked in primitive Islamic-Persian medicine, for example, in Tohfeh E Hakim (physician’s gift): The plant has warm fuzzies. Its flowers are tender and very fun. It is effective for diseases such as meningitis, pleurisy, madness, obsession, reppression, melancholy and cough. Its sweat is exhilarating and fun, and will strengthen the power of instinct." Renowned Iranian physician, Khorasani, in his book, ‘Makhzan Aldoyeh’ introduced borage as a remedy for cough, sore throat, pneumonia and shortness of breath and melancholy and treating a range of children's rash fever (Sayah et al., 2005). Therefore, the present study was conducted to evaluate the anti-oxidative and anti-microbial effect of borage extract on quality and shelf life of stored rainbow trout. The effect of borage extract on microbial and chemical properties of rainbow trout fillet stored in refrigerator was also investigated.

MATERIALS AND METHODS

Preparation of fish, borage extract and samples

Twenty rainbow trout (Oncorhynchus mykiss) with average weight of 700 g were purchased from Ilayi fish market in Sari as live fishes. The fishes were killed, washed with water, their heads and tails removed and the stomach content removed; after washing for the second time the fishes were transferred to Vastrivash laboratory located in Sari. Fillets prepared from the fishes were examined in terms of protein percentage, lipid, moisture and ash. Samples were taken randomly as control and test groups. Two kilo grams borage were picked up from Neka, milled and powdered and sent to Vastrivash laboratory of Sari for extraction. Samples were soaked in hydro alcoholic extract of borage (500 and 100 ppm per 100 g of fillet) for 30 s, packed in nylon bags and stored in the refrigerator at 4°C. On days 0, 4, 8, 12, 16 and 20 h, three fillets were randomly selected from each section and examined for determination of qualitative (chemical and microbial) parameters. All experiments were carried out with three replications.

Proximate analysis

Moisture percentage measurement

About 5 to 10 g of minced fish sample was placed in the
oven (105°C) for four hours and then transferred to desiccators; after cooling, the samples were weighed again and drying process continued until no significant change was observed in the sample and moisture content was calculated (AOAC, 2005) as follows:

Moisture percentage = \( \frac{\text{Final weight} - \text{Crucible weight}}{\text{Initial weight}} \times 100 \)

**Ash percentage measurement**

Electrical furnace was used to determine the ash percentage of the samples. Empty crucibles were placed at oven with temperature of 190°C for 60 min, cooled in desiccators and then weighed. 0.5 g of the sample previously put in the oven (65°C) for 48 h were poured in the crucible and the total weight of samples and crucibles measured; the samples were burnt for 5 h in the furnace with temperature of 550°C. The burnt samples were cooled at desiccators for 30 min and then weighed using digital balance with accuracy of 0.001 g; ash percentage was then calculated using this formula (AOAC, 2005):

Ash percentage = \( \frac{\text{Plant weight} - \text{Final sample weight}}{\text{Crucible weight}} \times 100 \)

**Protein percentage measurement**

To determine protein percentage of the samples, 0.5 g of the sample was poured in the test tube assigned for digestion and 10 ml of sulfuric acid (0.1N), a digestion pill containing copper sulfate and some drops of normal octane as anti-foam agent were added. In each set, operating the device of two tubes was considered as a witness. Digestion bath device is turned on previously and after putting the tubes in the target machine, the furnace temperature was gradually brought to a temperature of 420°C to be digested. During digestion, the samples turned brown at first, then transparent yellow color and in the end, almost all were transparent blue, which was a sign of complete digestion. This stage lasted about 4 h after digestion of samples and cooling them, thereafter, some distilled water was added to each tube and placed in the titration Kjeldahl device fully automated. Three containers containing hydrochloric acid 0.1 normal was connected to soda of 40% and distilled water, which according to the amount of nitrogen samples, the device automatically makes use of every bottle to the extent desired. After a few minutes titration of samples was carried out and the nitrogen content of the samples recorded on the display device by multiplying this number with 6.25 as the obtained the amount of protein samples (AOAC, 2005).

**Lipid percentage measurement**

15 g of the sample was weighed and poured in decanter with 60 ml of methanol and well homogenized. 30 ml of chloroform was added and decanter agitated. After 5 min, 30 ml chloroform was again added and the mixture left for 24 h for lipid extraction. After 24 h, 36 ml of distilled water was added for phase separation. After 2 h, the lower phase was collected in sanded balloon head and in Rotary to evaporate the solvent and stay just oil. Oil extracted and the amount of fat in percentage terms was obtained by the following equation (AOAC, 2005):

Lipid percentage = \( \frac{\text{Container and Lipid weight} - \text{Container weight}}{\text{Sample weight}} \times 100 \)

**Chemical tests**

**Measurement of thiobarbituric acid (TBA)**

TBA is widely used to measure lipid oxidation level in fish and it shows the amount of secondary oxidation products, especially aldehydes. TBA is an index of lipid oxidation based on Malondialdehyde (MDA) (Kostaki et al., 2009). TBA was measured using colorimetric method. 200 g of the mixed fish sample was poured in a 25 ml volumetric flask and reached to the volume by -1 butanol. 5 ml of the aforementioned mixture was added to dry lidded tube and then 5 ml of TBA reagent also added (TBA is produced by solving 200 mg of TBA in 100 ml of -1 butanol solvent after filtration). The lidded tubes were placed at bath (95°C) for 2 h and then cooled at room temperature. Then absorption rate (As) was then read at 532 nm as against distilled water (Ab). The amount of (TBA mg malondialdehyde in kg in fish tissue) was calculated using the following equation (Egan et al., 1997):

\[
\text{TBA} = \frac{(\text{As} - \text{Ab}) \times 200}{50}
\]

**Measurement of total volatile basic nitrogen (TVB-N)**

Total volatile basic nitrogen (TVB-N) is mainly composed of trim ethylamine, dim ethylamine, ammonia and other volatile compounds related to deterioration of sea foods produced by spoilage bacteria, autolytic enzymes, deamination of amino acids and nucleotide acids; respectively. TVB-N is a major indicator of meat spoilage and decay. 10 g of minced fish meat together with 2 g of magnesium oxide and 300 ml of distilled water was poured in Kjeldahl flask and some glass pearl and normal octanes (anti-foam) added. The flask was attached to the device and heated from beneath. A 250 ml Erlenmeyer flask containing 25 ml of 2% boric acid (2 g of boric acid in 100 ml of distilled water) and some drops of methyl red reagent (0.1 g methyl red in 100 ml of distilled water) was placed at the end of the device. Red methyl reagent is red in acidic medium and yellow in basic medium.
Distillation continues with over 30 min of boiling substances in balloons or gathering of about 125 cc of liquid in the Erlenmeyer flask. Boric acid solution soon after alkalinization and distilled by volatile nitrogen bases turned yellow in color. The Boric acid was titrated using 0.1 normal sulfuric acid until it turns red. The amount of TVB-N obtained in milligrams in one hundred grams of fish meat was then calculated according to the formula (Fan et al., 2008):

TVB-N = Amount of Consumption of Sulfuric Acid × 1.4 × 100/Sample weight
pH measurement
5 g of each sample was added to 45 ml of distilled water and mixed for 30 s in a mixer; the pH was determined using a digital pH meter calibrated with standards at pHs 4 and 7 (Sallam, 2007).

Microbial analysis of the samples
For counting the bacteria, 10 g of fish fillet meat was mixed and homogenized with 90 ml of 85% sodium chloride under aseptic condition and corresponding dilutions prepared. One milliliter of each dilution was used in pour plate assay. Total viable count (TVC) and psychrophilic bacterial count (PTC) were determined by counting the colonies in plate count agar medium at 37°C for two days and 7°C for ten days, respectively. All numbers were reported as log CFU/g (Frangos et al., 2010).

RESULTS
Table 1 shows the results of approximate analysis of fresh rainbow trout including moisture, ash, total protein and total lipid in terms of percentage and mean. Results of statistical analysis showed that there is significant interaction between time and treatments and hence, mean comparison was separately performed for different treatments during the storage period. In general, thiobarbituric variation showed that by passing of time, this index was significantly increased in all treatments (p<0.05) and this increase was more severe in 500 ppm concentration of borage extract. Thiobarbituric comparison in various treatment and in different storage periods showed that thiobarbituric content was not significantly different between 500 and 1000 ppm, respectively of borage extract (p>0.05) and these two treatments were not significantly different from the control (p>0.05).

In general, changes in volatile basic nitrogen showed that over time, it significantly increased in all treatments (p<0.05), and this increase was more intense in the extract of borage sample with a concentration of 500 ppm. Comparing the amount of total volatile basic nitrogen in treatment during different storage periods, it was revealed that the amounts of volatile basic nitrogen in extracts of borage treatment with concentrations of 500 and 1000 ppm, respectively significantly differed from the control treatment (p<0.05), as well as this amount at all times in the control sample was less than the rest of the treatments (p<0.05).

Table 2 showed that there is a negative and significant interaction between time and treatments. pH decreased in all treatments (p<0.05) and this decrease was more severe in borage extract with concentration of 500 ppm. Comparing the pH amount in treatments during different periods of maintenance was to suggest that the values of pH in the borage extracts treatment with a concentration of 500 ppm were significantly different from the control (p<0.05) and borage extracts treated with a concentration of 1000 ppm was not significantly different from the control treatments (p<0.05); in addition, this amount at all times, except for the fifth and sixth days in the control sample was greater than the rest of the treatments (p<0.05). On the other hand, the results of statistical analysis showed that there is significant interaction between time and treatment. For this reason, means comparison in different treatments were separately conducted during the storage period. In general, the total bacteria count showed significant increase over time in all treatments (P<0.05).

This increase in borage extracts sample intensified with a concentration of 500 ppm. Comparing the number of total bacteria treatment during different storage periods, it was indicated that the number of total bacteria in borage extracts treatment with concentrations of 500 and 1000 ppm was not significantly different from the control treatment (P>0.05). Finally, the results of statistical analysis showed that there is significant interaction between time and treatment. For this reason, means comparison in different treatments were separately conducted during the storage period.

In general, psychrophilic bacteria count showed significant increase in all treatments over time (P<0.05). This increase in borage extracts sample intensified with a concentration of 500 ppm. Comparing the number of total bacteria treatment during different storage periods, it was
Table 2: Variation of chemical variables (thiobarbituric acid, TVB-N, pH, TVC and psychrophilic bacteria) in different treatments during storage period.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatments</th>
<th>Storage period (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Thiobarbituric acid changes</td>
<td>Control</td>
<td>0.47 ± 0.01&lt;sup&gt;AD&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>500 ppm</td>
<td>0.48 ± 0.02&lt;sup&gt;AD&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1000 ppm</td>
<td>0.48 ± 0.02&lt;sup&gt;AD&lt;/sup&gt;</td>
</tr>
<tr>
<td>Changes in volatile basic nitrogen</td>
<td>Control</td>
<td>2.70 ± 0.41&lt;sup&gt;AD&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>500 ppm</td>
<td>2.72 ± 0.45&lt;sup&gt;AD&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1000 ppm</td>
<td>2.93 ± 0.35&lt;sup&gt;BE&lt;/sup&gt;</td>
</tr>
<tr>
<td>PH changes during maintenance process</td>
<td>Control</td>
<td>5.86 ± 0.05&lt;sup&gt;AC&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>500 ppm</td>
<td>5.83 ± 0.05&lt;sup&gt;AC&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1000 ppm</td>
<td>5.83 ± 0.05&lt;sup&gt;AC&lt;/sup&gt;</td>
</tr>
<tr>
<td>Changes in the number of total bacteria</td>
<td>Control</td>
<td>2.22 ± 0.03&lt;sup&gt;AD&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>500 ppm</td>
<td>2.23 ± 0.04&lt;sup&gt;AD&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1000 ppm</td>
<td>2.22 ± 0.04&lt;sup&gt;AD&lt;/sup&gt;</td>
</tr>
<tr>
<td>Changes in psychrophilic bacteria</td>
<td>Control</td>
<td>1.0 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>500 ppm</td>
<td>1.01 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>1000 ppm</td>
<td>1.00 ± 0.15</td>
</tr>
</tbody>
</table>

The numbers in a column with different letters (A, B and C) are significantly different, while the numbers in a row with different letters are significantly different (a, b and c, etc).

indicated that the number of total bacteria in borage extracts treatment with concentrations of 500 and 1000 ppm was not significantly different from the control treatment (P>0.05).

**DISCUSSION**

Although previous reports on composition of rainbow trout body suggest difference of these factors especially lipid content and the content of these compounds in the present study (Table 1) lie within the range reported in previous studies (Gonzalez-Fandos et al., 2005). Variation of chemical composition may be due to difference in feeding, fishing season, spawning cycle, genus differences, fish size, life region and other environmental factors (Sallam, 2007). For the reasons earlier mentioned, the variation in chemical composition led to changes in sensory properties such as taste, smell, texture, color and surface of fish which controlled microbial growth, oxidation rate and popular support for fish consumption. Lipid oxidation in fish due to high levels of unsaturated fatty acid after death is of great importance and is one of the main factors of undesirable flavor and taste in them (Guillén and Ruiz, 2004a, b). TBA test indicator was used to assess the degree of oxidation of lipid in fish. The trend of increasing this index during storage period may be due to the increase in free iron and other peroxide in the muscles, as well as aldehydes as a by-product of oxidation resulted in the defeat of hydro peroxide. It is important to note that, according to Aubourg and Gallardo (2005) that the amount of TBA may not be indicated in the actual degree of oxidation of lipids, especially when, Malone aldehydes do react with other compounds in fish. Such compounds can include amino acid, nucleotides and nucleic acids, proteins,
phospholipids and other aldehydes produced at the end of lipid oxidation. In such an approach, there is a lot of fish (Hosseini et al., 2008).

High content of TBA during storage period can be attributed to lipid oxidation and production of volatile metabolites in the presence of oxygen. Low TBA content in samples containing nettle may be due to anti-oxidative effect of phenolics (catechin and epicatechin) that decreases peroxide, because based on one molecule and two molecule mechanisms, when hydro peroxides content of fish muscle is low, formation rate of these compounds is higher than their degradation. In this case, based on the mechanism of a molecule, the amount of hydro peroxides start increasing in the fish muscle. With the passage of time and increase in the concentration of hydro peroxides based on two molecular mechanisms, these compounds break down quickly and looking for such a mechanism are reduced amounts of hydro peroxides. It should be noted that in this case, their decomposition rate is faster than the rate of formation. The results of this study are consistent with results of Hosseini et al. (2002) and Özoğul et al. (2004) on the European eel.

TVB-N is composed of ammonia and volatile amines considered as a main index of meat degradation and decomposition and its increase is related to activity of spoilage bacteria with indigenous enzymes (Fan et al., 2008). The amount of this index is different in different species and is affected by species, fishing area, age and gender (Ersoy et al., 2008). In general, the amount of total volatile nitrogen bases of fresh fish caught could be between 5 to 21 mg per 111 g of meat. However, some researchers noted in their study that this index cannot be used as a good criterion to judge the freshness of the fish. The total volatile basic nitrogen depends on the amount of bacteria that results in the destruction of bacteria. Degraded fish is a progressive proteolytic process, which is often carried out by microbial activity and to a lesser extent by autolysic enzymes (Hamzeh and Rezaei, 2012). pH of live fish muscle varies between 6.7 and 7 (Tokur et al., 2006). During and after rigor mortis, pH varies between 6 and 7.1 due to the production of lactic acid and according to species, age, genus and feeding conditions (Hamzeh and Masoud, 2012). pH affects connective tissue, peroxidants (such as increased iron solubility as a result of pH reduction) and anti-oxidants activities.

After death, pH is the most effective factor influencing meat tissue and decomposing connective tissue. One reason is that low changes of pH has great influence on connective tissue properties such that mechanical length of the tissue in pH=7.1 is four times longer than that at pH=6.2 (Huss, 1995). Fish has a small amount of carbohydrate (less than 0.5%) in your muscle tissue such that after the death of the fish lactic acid is produced following a low rate of glycolysis reaction and pH of fish meat after rigor mortis stage becomes higher than 6. This phenomenon is an important characteristics associated with fish meat. Analysis of nitrogen compounds in the fish maintenance period leads to an increase in meat pH in which part of this increase may be associated with alkaline compounds. Such an increase in pH could be an indicator of bacterial growth, reduced quality and finally fish spoilage (Gram and Huss, 1996). Analysis of nitrogen compounds in the fish maintenance period, which leads to an increase in meat pH and which is part of this increase, may be associated with alkaline compounds, such as ammonia and trim ethylamine due to protein degradation indicative of bacterial growth, reduced quality and finally, fish spoilage.

Corruption in fresh fish is part of the activity and growth of particular spoilage organisms that by production of metabolites can lead to undesirable flavors and the smell of fish and eventually, they become unusable. Gram-negative psychrophilic bacteria are the main groups of micro-organisms responsible for spoilage of fresh fish stored. Authorized bacterial load was reported for aerobic and Trophy cycle (psychrotroph) log CFU/g (Gram and Huss, 1996; Özyurt, 2009).

Based on the aforementioned issues, it can be concluded that fish meat, due to higher content of fatty acids, decays faster than red meat and chicken. Antioxidants can be used to avoid or postpone spoilage of fish meat and its products. Moreover, considering the increased demand for natural compounds, natural antioxidants can be suitable alternatives for synthetic preservatives in the food industry; thus, the present study was carried out to investigate anti-oxidative and anti-bacterial properties of hydroalcoholic extracts of borage on shelf life of rainbow trout fillet to deliver products with higher quality and safety to consumers.

Additionally, microbial analysis is indicative of the fact that in all treatments, there is an increase in the microbial load along with the passage of time, but this increase was less in the extract treatments at a concentration of 500 ppm. According to the results, it can be concluded that the borage hydroalcoholic extract at a concentration of 500 ppm is to maintain the quality of fish in terms of qualitative indicators of chemical, microbiological and sensory qualities and increase in the survival trout in the refrigerator as compared to without extract samples such that the plant extract could increase in 8 days the shelf life samples compared to the control samples.

REFERENCES


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