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Antioxidant and antihaemolytic activities of *Ferula foetida regel* (*Umbelliferae*)

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Abstract. – *Objectives:* **The Ferula genus (Umbelliferae) is a rich source of gum-resin and is much utilized in folklore medicine. This study is designed to examine antioxidant and antihaemolytic activities of *Ferula foetida regel* flower, stem and leaf extracts.**

Materials and Methods: **1,1-Diphenyl-2-picryl hydrazyl radical (DPPH), nitric oxide and H_2O_2 scavenging activities, Fe^{2+} chelating ability, reducing power and hemoglobin-induced linoleic acid peroxidation were used to evaluate antioxidant activities. Antihaemolytic activity was evaluated by H_2O_2 induced hemolysis in rat erythrocyte. Total phenolic compounds were determined as gallic acid equivalents and total flavonoid contents were calculated as quercetin equivalents from a calibration curve.**

Results: **The leaf aqueous-ethanol extract showed the highest activity in DPPH radical scavenging activity. All extracts showed weak nitric oxide scavenging activity. The stem extract had better activity in nitric oxide scavenging model than the other extracts (IC_{50} = 896.9 ± 21.9 µg ml^{-1}), but it was not comparable to quercetin (p<0.001). The leaf extract exhibited better H_2O_2 scavenging and Fe^{2+} chelating activity than the other parts. The extracts exhibited good antioxidant activity in linoleic acid peroxidation test but were not comparable to vitamin C (p<0.001). Extracts showed weak reducing power activity. The stem extract showed better antihaemolytic activity than the flower and leaf. The flower extract had higher phenolic contents. The extracts exhibited different levels of antioxidant and antihaemolytic activities in all tested models.**

Conclusions: **This study showed remarkable antioxidant and antihemolytic activities in *Ferula foetida*. Biological effects may be attributed to the presence of phenols and flavonoids in the extract. It is very promising for further biochemical experiments.**

Key Words:

Antioxidant activity, Chelating activity, DPPH, *Ferula foetida*, Flavonoid, Nitric oxide scavenging.

Introduction

It is commonly accepted that under situations of oxidative stress, reactive oxygen species (ROS) such as superoxide, hydroxyl and peroxyl radicals are generated. The ROS play an important role related to degenerative or pathological processes such as aging, cancer, coronary heart disease, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, cataracts, and inflammation[1,2]. Minimizing oxidative damage may well be one of the most important approaches to the primary prevention of these aging-associated diseases ad health problems, since antioxidants terminate direct ROS attacks and radical-mediated oxidative reactions, and appear to be of primary importance in the prevention of these diseases and health problems[3]. The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced *in situ* or externally supplied through foods and/or supplements. Endogenous and exogenous antioxidants act as free radical scavengers by preventing and repairing damage caused by ROS and, therefore, can enhance the immune defense and lower the risk of cancer and degenerative diseases[2,4]. The use of traditional medicine is widespread and plants still present a large source of natural antioxidants that might serve as leads for the development of novel drugs[1]. Consequently, the need to identify alternative natural and safe sources of food antioxidants arose and the search for natural antioxidants, especially of plant origin, has notably increased in recent years[5,6]. The *Ferula* genus (*Umbelliferae*) has been found to be a rich source of gum-resin[7]. This resin enjoys a reputation as a folklore medicine[8]. Sedative, carminative, antispasmodic digestive, expectorant, laxative, analgesic, anthelminitic, antiseptic and diuretic properties have been reported from the *Ferula* genus[9].

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It is also believed to have aphrodisiac and increasing sexual appetite[10]. This genus presents interesting phytochemical features, such as the occurrence of sesquiterpenes and sesquiterpene coumarins[8,11]. *Ferula foetida regel* is a perennial plant, which blooms once in its several years of life[12,13]. It is native to central Asia, Afghanistan and Iran[12]. Previously polysulfide derivatives[14] and sesquiterpene coumarins[15] were reported from *Ferula foetida*. *In vitro* fungi toxicity of the resin extract of *Ferula foetida* has been studied[16]. We have recently reported good antioxidant activity from *Ferula assafoetida*[9] and *Ferula gummosa*[17,18]. The aim of this study was to determine the antioxidant and antihemolytic activities of the hydroalcoholic extract of *Ferula foetida regel* leaf, flower or stems in order to understand the usefulness of this plant as a medicinal plant.

Materials and Methods

Chemicals

Trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydroxyl (DPPH), potassium ferricyanide and hydrogen peroxide (H_2O_2) were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Butylated hydroxyanisole (BHA), ascorbic acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, ethylenediaminetetraacetic acid (EDTA) and ferric chloride were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade or purer.

Plant Materials and Preparation of Freeze-Dried Extract

Ferula foetida regel was collected from Gadouk area, central Elburz, Iran, in 2009. The sample was identified by Dr. Bahman Eslami (Assistant Professor of plant systematic, Islamic Azad University, Ghaemshahr Branch, Iran). Voucher specimens were deposited in the Herbarium of Sari Faculty of Pharmacy (No GRF 32-34). A known amount of each sample (250 g) was extracted at room temperature for 24 h by percolation with ethanol/water (600 ml, 70/30 v/v). The extract was then separated from the sample residue by filtration through Whatman No. 1 filter paper. This procedure was repeated thrice. The resulting extract was concentrated over a rotary vacuum until a crude extract was obtained, which was then freeze-dried for complete solvent removal.

Determination of Total Phenolic Compounds and Flavonoid Content

Total phenolic compound contents were determined by the Folin-Ciocalteau method[19]. The extract samples (0.5 ml) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagents for 5 min and 2.0 ml of 75 g l^{-1} sodium carbonate was then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents. Total flavonoids were estimated as previously described[20]. Briefly, 0.5 ml solution of each extracts in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (UV–Visible EZ201, Perkin Elmer, USA). Total flavonoid contents were calculated as quercetin from a calibration curve.

Antioxidant activity

DPPH Radical-Scavenging Activity

The stable 1, 1-diphenyl-2-picryl hydroxyl radical (DPPH) was used for determination of free radical scavenging activity of the extracts[21] Different concentrations of extracts were added, at an equal volume, to the methanol solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm (UV–Visible EZ201, Perkin Elmer, USA). The experiment was repeated three times. Vitamin C, BHA and quercetin were used as standard controls. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Determination of Metal Chelating Activity

The ability of the *Ferula foetida regel* extracts to chelate ferrous ions was estimated in our recently published paper[22]. Briefly, different concentrations of each extract were added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixture was then shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solutions was measured spectrophotometrically at 562 nm (UV–Visible EZ201, Perkin Elmer, USA). The percentage inhibition of ferrozine-Fe^{2+} complex formation was

calculated as $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control, and A_1 of the mixture containing the extract or the absorbance of a standard solution. EDTA was used as a standard.

Assay of Nitric Oxide-Scavenging Activity

Sodium nitroprusside (10 mM), in phosphate-buffered saline (PBS), was mixed with different concentrations of extracts dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the extracts but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control[19,23].

Scavenging of Hydrogen Peroxide

The ability of the extracts to scavenge hydrogen peroxide was determined according to our recently published paper[9]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. All the extracts (0.1-3.2 mg ml^{-1}) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds was calculated as follows: % scavenged $[H_2O_2] = [(A_o - A_1)/A_o] \times 100$, where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract and standard.

Reducing Power Determination

The reducing power of extract was determined according to our recently published papers[24]. 2.5 ml of extract (25-800 mg ml^{-1}) in water were mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm (UV–Visible EZ201, Perkin Elmer, USA). Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Antioxidant Activity in A Hemoglobin-Induced Linoleic Acid Peroxidation Test

The antioxidant activity of extracts was determined by a modified photometry assay[25]. Reaction mixtures (200 ml) containing 10 ml of each extract (10-400 mg), 1 mmol/L of linoleic acid emulsion, 40 mmol/L of phosphate buffer (pH 6.5), and 0.0016% hemoglobin, were incubated at 37ºC for 45 min. After the incubation, 2.5 ml of 0.6% HCl in ethanol was added to stop the lipid peroxidation. The amount of peroxide value was measured in triplicate, using the thiocyanate method by reading the absorbance at 480 nm after coloring with 100 ml of 0.02 mol/l of $FeCl_2$ and 50 ml of ammonium thiocyanate (30 g/100 ml). Vitamin C was used as positive control.

Antihemolytic Activity of Extracts Against H_2O_2 Induced Hemolysis

The inhibition of rat erythrocyte hemolysis by the extracts was evaluated according to the procedure described by Ebrahimzadeh et al[26]. The rat erythrocyte hemolysis was performed with H_2O_2 as free radical initiator. To 100 µl of 5% (v/v) suspension of erythrocytes in phosphate buffered saline (PBS), 50 µl of each extracts with different concentrations (5-25 µg in PBS pH 7.4), which corresponds to 100-3200 µg of extract, was added. Then, 100 µl of 100 mM H_2O_2 (in PBS pH7.4) was added to it. The reaction mixtures were shaken gently while being incubated at 37°C for 3 h. The reaction mixtures were diluted with 8 ml of PBS and centrifuged at 2000×*g* for 10 min. The absorbance of the resulting supernatants was measured at 540 nm by spectrophotometer to determine the hemolysis. Likewise, the erythrocytes were treated with 100 µM H_2O_2 and without inhibitors (plant extracts) to obtain a complete hemolysis. The absorbance of the supernatants was measured at the same condition. The inhibitory effect of the extracts was compared with standard antioxidant vitamin C. To evaluate the hemolysis induced by extracts, erythrocytes were preincubated with 50 µl of extracts corresponding to 25 µg extracts for 1 h and the hemolysis was determined. Percentage of hemolysis was calculated by taking hemolysis caused by 100 µM H_2O_2 as 100%. The IC_{50} values were calculated from the plots as the antioxidant concentration required for the inhibition of 50% hemolysis.

Statistical Analysis

Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($p<0.05$) and the means separated by Duncan's multiple range tests. The EC_{50} values were calculated from linear regression analysis.

Results

Total phenol and flavonoids contents of extracts obtained from *Ferula foetida* leaf, flower, and stem are shown in Table I. The maximum of extractable polyphenol content was recorded in flower with 51.7 ± 2.3 mg gallic acid equivalent/g of extract, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.987$). In addition, maximum flavonoid contents were recorded in leaf extract with 20.9 ± 1.4 mg quercetin equivalent/g of extract, by reference to standard curve ($y = 0.0067x + 0.0132$, $r^2 = 0.999$). IC_{50} for DPPH radical-scavenging activity exists in Table 1. The IC_{50} values for vitamin C, quercetin and BHA were 5.05 ± 0.1, 5.28 ± 0.2 and 53.96 ± 3.1mg ml^{-1}, respectively. Results of iron chelating capacity were presented in Table I. Among the extracts, leaf extract showed better activity than others with IC_{50} = 302.2 ± 13.6 μgml^{-1}. EDTA showed very powerful activity (IC_{50} = 18 ± 1.5 μgml^{-1}). In scavengers of nitric oxide, percentage of inhibition was increased with increasing concentration of the extracts but the activity was very weak (IC_{50} for stem extract was 896.9 ± 21.9 μgml^{-1} vs. quercetin which was 20 ± 0.1 μgml^{-1}). The extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner. Results exist in Table 1. Leaf extract showed better activity than others (IC_{50} was 105.7 ± 4.7 mg ml^{-1}). The IC_{50} values for vitamin C and quercetin were 21.4 ± 1.1 and 52 ± 2.6 mg ml^{-1}, respectively. Figure 1 shows the dose response curves for the reducing powers of the extracts. It was found that the reducing powers of all extracts increased with the increase of their concentrations. There were no significant differences ($p>0.05$) among the extracts in reducing power. Their activities were not comparable with vitamin C ($p<0.001$). Tested extracts showed good activity in hemoglobin-induced linoleic acid system but there were significant differences between extracts and vitamin C ($p<0.01$) (Figure 2). The effects of extracts were tested and it was found that they did not show any harmful effects on erythrocytes. Results are shown in Table I. Stem extract showed better antihemolytic activity than other extracs (IC_{50} was 248.7± 16.2 $\mu g\ ml^{-1}$ vs. vitamin C which was 235 ± 9 $\mu g\ ml^{-1}$).

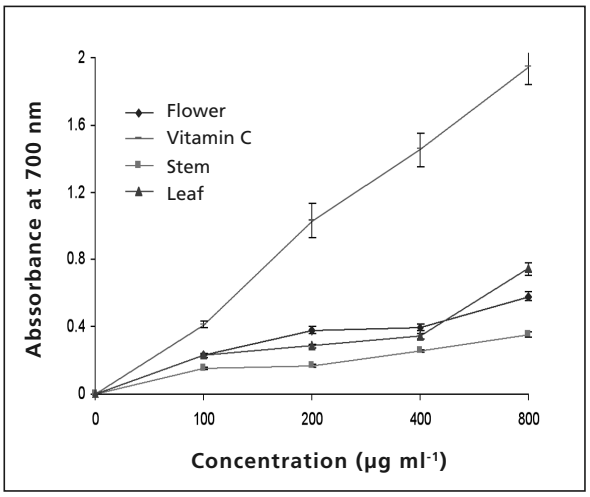

Figure 1. Reducing power of flower, stem and leaf of *Ferula foetida regel*. Vitamin C has been used as control.

Discussion

Extracts showed high level of total phenol and flavonoid contents. Phenols and polyphenolic compounds, such as flavonoids, are widely found

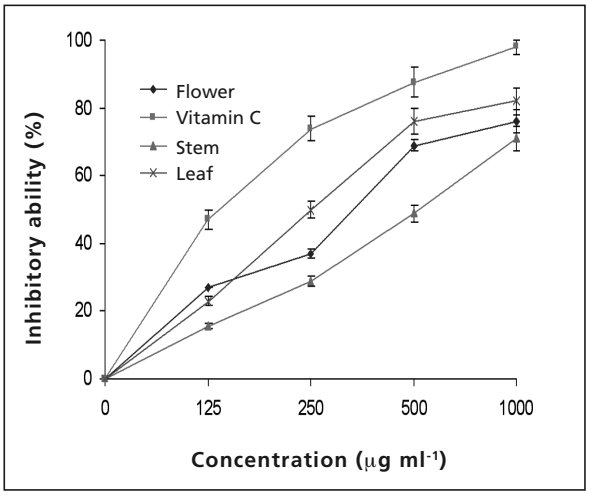

Figure 2. Effect of flower, stem and leaf extracts of *Ferula foetida regel* on hemoglobin-catalyzed linoleic acid peroxidation test. Vitamin C has been used as control.

Table I. Phenol and flavonoid contents, antioxidant and antihemolytic activities of flowers, stems and leaves of *Ferula foetida regel.*

Antihemolytic activity ($\mu g\ ml^{-1}$)	Fe^{2+} chelating ability, IC_{50} ($\mu g\ ml^{-1}$)[d]	H_2O_2 scavenging activity, IC_{50} ($\mu g\ ml^{-1}$)[c]	Nitric oxide scavenging, IC_{50} ($mg\ ml^{-1}$)[b]	DPPH free radical scavenging, IC_{50} ($\mu g\ ml^{-1}$)[a]	Total flavonoid contents ($mg\ g^{-1}$)	Total phenol contents ($mg\ g^{-1}$)	Sample
260.4 ± 11.5	775.5 ± 21.4	193.8 ± 8.6	1.2 ± 0.04	471.6 ± 19.4	20.7 ± 0.9	51.7 ± 2.3	Flower
253 ± 9.4	302.2 ± 13.6	105.7 ± 4.7	1.4 ± 0.03	192.5 ± 8.6	20.9 ± 1.4	49.4 ± 2.1	Leaf
248.7 ± 16.2	619.6 ± 18.9	150.2 ± 5.2	0.89 ± 0.01	775.9 ± 26.3	9.9 ± 0.3	36.7 ± 1.3	Stem

[a]IC_{50} of BHA was 53.96 ± 3.1, vitamin C, 5.05 ± 0.1 and quercetin 5.28 ± 0.2, respectively. [b]IC_{50} of quercetin was 20 ± 0.1. [c]IC_{50} for vitamin C and quercetin were 21.4 ± 1.1 and 52 ± 2.6, respectively. [d]EDTA used as control (IC_{50} = 18 ± 1.5 $\mu g\ ml^{-1}$). [f]IC_{50} of vitamin C was 235 ± 9.

in food products, derived from plant sources, and they have been shown to possess significant antioxidant activities[9]. DPPH is a stable nitrogen-centered free radical, the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers[27,28]. Phenol and flavonoid contents of this plant may lead to its agreable DPPH-scavenging activity[26]. Iron chelators reduce iron-related complications in human and thereby improves quality of life and overall survival in some diseases such as thalassemia major or Alzheimer's disease[29]. Bivalent transition metal ions play an important role as catalysts of oxidative processes[30]. These processes can be delayed by iron chelation and deactivation. The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular diseases[31]. Thus, minimizing Fe^{2+} concentration affords protection against oxidative damage. Many researches focused on some natural products, especially flavonoids that possess direct influence on iron (III) ions level within tissues[29]. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases. In this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The nitric oxide assay is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of NO compete with oxygen, leading to reduced production of nitrite ions. Extracts did not show any in scavenging NO.

Scavenging of H_2O_2 by extract may be attributed to its phenolics, and other active components which can donate electrons to H_2O_2, thus neutralizing it to water[32,33]. Although H_2O_2 itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important throughout food systems[9]. Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation[34]. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue[9] at 700 nm. Increasing absorbance indicates an increase in reductive ability. Reducing powers of extracts increased with the increase of their concentrations, but their activities were not comparable with vitamin C ($p<0.001$). Polyphenolic contents of all the sample extracts appear to function as good electron and hydrogen atom donors and, therefore, should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products. Similar observation between the polyphenolic constituents in terms of dose dependent and reducing power activity have been reported for several plant extracts[9].

Erythrocytes are considered as prime targets for free radical attack, owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the O_2 transport associated with redox active hemoglobin molecules, which are potent promoters of reactive O_2 species. Specially linoleic acid and arachidonic acid are targets of lipid peroxidation[35]. The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical[36,37]. Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. Tested extracts show good activity in hemoglobin-induced linoleic acid peroxidation test. Effect of extracts were tested and found that they did not show any harmful effects on erythrocytes.

Flavonoids interactions with cell membranes which generally serve as targets for lipid peroxidation (LP), constitute an important area of research[38]. Various model membrane systems like LDL and red blood cells (RBC) membrane comprising physiologically important membrane protein components offer a physiologically relevant and a relatively simple system for studying LP[39]. RBC has been chosen as an *in vitro* model to study the oxidant/antioxidant interaction since its membrane is rich in polyunsaturated fatty acids, which are extremely susceptible to peroxidation[40]. During recent years, a few interesting studies have been reported, indicating the protective effects of some plants extracts against oxidative damage in intact RBC membranes[26,37,40,41]. Hemolysis has a long history of use in measuring

free radical damage and its inhibition by antioxidants but only few studies have been performed with erythrocytes in whole blood. In this study, we used a biological test based on free radical-induced erythrocytes lysis in rat blood. Lipid oxidation of rat blood erythrocyte membrane mediated by hydrogen peroxide induces membrane damage and subsequently hemolysis. Stem extract showed better antihemolytic activity than other extracts (IC_{50} was 248.7± 16.2 μg ml^{-1} vs. vitamin C which was 235 ± 9 μg ml^{-1}). The antihemolytic activity of flavonoids has been previously reported and activity of the extract maybe results in high flavonoid content[9,38].

Conclusions

Our study improved remarkable antioxidant and antihemolytic activities in hydroalcoholic extract of *Ferula foetida regel* Boiss flower, stem and leaf. These effects maybe result of their high phenol and flavonoids contents. It is therefore very promising for further biochemical experiments, which will be focused on evaluating the mechanism of this activity.

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