The effect of heat treatment of java plum seed extract on its polyphenolics content and antioxidant activities

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Abstract. Butylated hydroxy anisole (BHA) is widely added in lipid and food lipid as a preservative and it is effective for inhibiting lipid peroxidation. However, BHA raised doubt would be the health impact for consumers. The methanolic extract of Java Plum seed (MEJS) contains a diverse group of phenolic compounds, it has potential as a natural antioxidant. The objective of the research was to determine the effect of heating treatment on total phenolics content and antioxidant activity of MEJS. Heating treatment applied on MEJS at 110, 120, 130, 140 and 150°C/10 minutes and heating time (10, 15, 20, 25 and 30 minutes/110°C). The results showed that heating of the extract at the temperature rose to 130°C should decrease the total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC) slightly, coincided with a decrease of the reducing power, but it could increase the free radical scavenging activity. Heating treatment at 110°C for about first ten minutes had a positive impact on increasing of its phenolics content and antioxidant activities. However, heating further at a higher temperature (>130°C) gave a bad influence on the free radicals scavenging activity. The extract heated at the 130°C/10 minutes gave the best EC50 value as 140 ppm.

1. Introduction
An artificial antioxidant such as butylated hydroxy anisole (BHA) and butylated hydroxytoluene (BHT) is widely used in lipid and food lipid as a preservative and they are effective for inhibiting the lipid oxidation [1,2]. However, artificial antioxidants have negative impacts on health. Therefore, that is required to replace it with an alternative antioxidant that is safe for human health. The Java Plum seeds (JS) extract contained a diverse group of phenolic compounds as such as (+)-catechin, gallic acid, ellagic acid, quercetin, (+)-epicatechin, rutin, kaempferol, and galloysl glucose group [3,4], meanwhile the methanolic extract of Java Plum seed (MEJS) containing rutin and (+)-catechin in abundant [5]. Many Java plum phenolic compounds mentioned above have been reported to possess potent antioxidant activity [4,6,7,8,9,10,11].

The antioxidant effect of plant phenolic compounds has been studied in relation to the retardation of lipid oxidation in the last decades [2,12]. Previous research showed that the MEJS was strong to act as reducing agents, radical scavenging, and hydrogen donors [4,5]. As a food additive, an antioxidant must be stable against food processing in particular heat treatment such as heating [13,14]. The heat treatment is
commonly applied to food processing for various purposes including inactivation of enzyme, and improving the color and texture. Its effectiveness is strongly influenced by the intensity of heating, methods, temperature, and long periods [14].

The heat treatment at the high temperature (> 180°C) could decrease the total phenolic content (TPC), flavonoid content (TFC), and free radical scavenging both 1,1-diphenyl 2-picrylhydrazyl (DPPH) and 2,2’-azino-bis (ABTS) radicals at the lower temperature 100-120 °C [15]. Heat treatment (autoclave) on grape seed extract (100 °C/0-60 min.) caused hydrolysis of gallic acid (70%), polyphenolic (61%), epicatechin (65%), procyanidin B1 (75%), and procyanidin B2 (73%). However, autoclave heat treatment could increase the gallic acid content (71%), gallic acid (100 %), and epicatechin-gallate (ECG) content to 129% in grape pomace. Autoclave heat treatment affects the antioxidant activity of both grape seed extract and grape pomace [16].

An antioxidant was sensitive toward heat treatment. Heating at the high temperature could decrease the antioxidant activities, even destructive to the structure of the chemical compounds [15,16,17]. Previous research showed that the antioxidant activity of white tea extract is still stable on heating up to 110°C, but its antioxidant activity declined by heating further [18]. Before the extract is applied to the food process, its antioxidant stability on heat treatment must be characterized. This study aimed to determine the effect of heat treatment on the total polyphenolics and antioxidant activity of MEJS.

2. Materials and methods

2.1. Materials
Java Plum (Syzygium cumini Linn.) seeds powder (60 mesh, 10% water content). Chemicals: methanol > 99.5% (Merck), gallic acid and tannic acid hydrate (Sigma-Aldrich, Belgium), (+)-catechin (Sigma Chemical Co. St. Louis USA), hydrochloric acid (HCl), ferro chloride (FeCl₂), ferryl chloride (FeCl₃), ammonium thiocyanate, potassium ferricyanide (III), trichloroacetic acid (TCA), phosphotungstic-acid, 1,1-diphenyl 2-picrylhydrazyl radical (DPPH) (Sigma-Aldrich Chemical Co.), Whatman filter paper (Whatman International, Ltd. England), Folin-Ciocalteu reagent and buffer phosphate. All reagents and chemicals were an analytical grade. The equipment used was analytic scales of Shimadzu AUW 120 (Shimadzu, Kyoto Japan), a rotary vacuum evaporator (IKARV10 Basic), freeze dryer (Virtis SP Scientific Sentry 2.0), oven Memmert UN 110 (Memmert, Germany), vortex (Velp Scientifica Europe), water-bath shaker (Julabo SW 22), UV-Visible spectrophotometer (UV1601 Shimadzu, Japan).

2.2. Sample preparation
The Java Plum fruits (Genthong var.) harvested on October 2018 (on the season), at the optimum maturity stage from a local farmer, at Semarang regency Central Jawa, Indonesia. After picking up the Java Plum fruits were stored at the cold storage (-18°C) until the use on May 2019. The Java Plum seeds were generated from Java Plum fruits that had been separated from the pulp. The seeds were cut into pieces with a sharp knife then dried using a cabinet dryer (55±5°C). That dried seeds were taken kernel and separated from husk and seed coats. The kernel was milled with a cutting mill then it was sieved so it was obtained Java Plum seeds powder (60 mesh, <10% water content. Java Plum seeds powder (JSP) was packed with airtight packaging and stored in the dark and dry room until it was used for the next step.

2.3. Proximate assay
Briefly, the JSP were analyzed proximately which including the analysis of water content assay referred to gravimetric methods of analysis AOAC [19], protein content assay by Kjeldahl extraction methods of analysis AOAC [19], lipid content by Soxhlet extraction methods of analysis AOAC [19], the crude fiber content was estimated as standard procedure in methods of analysis AOAC [19], ash content was analyzed
on dry matter basis in methods analysis of AOAC [19], and carbohydrate total was analyzed by the difference of analysis AOAC [19].

2.4. JSP extraction
JSP extraction was done using modified methods as described by Rohadi et al. [10]. Briefly, approximately 30 g of JSP was extracted using aqueous methanol 50% (v/v), at ratio materials: solvent (1:10), macerated on water–bath shaker (40 ±1°C/6 hrs. 100 rpm), thereafter the mixture filtered using Whatman filter paper. Thereafter the filtrate obtained was collected in the Erlenmeyer 1000 milliliters volume. The residue was extracted twice by using the same methods. The extracts were collected then concentrated using a rotary vacuum evaporator (IKA-RV 10 Basic Germany) to obtain the methanol free extract. The methanol free extract was freeze-dried (Virtis SP Scientific Sentry 2.0) for solvent residue removal and then the methanolic extracts of Java Plum seeds (MEJS) were obtained. Thereafter it kept in the refrigerator for further analysis. This extraction procedure was repeated three times.

2.5. Heat treatment of MEJS
Approximately 0.5 g of each sample was heated with an oven furnace (Memmert, Germany) at the temperature of 110, 120, 130, 140, and 150°C as long as for 10 minutes. Thereafter it was put out of the oven then it put into a desiccator until reached equilibrium temperature before using the next step. In the other part, with the same methods, as many 0.5 g of each sample was heated on an oven furnace (Memmert, Germany) at temperature 110°C in varied heating time (10, 15, 20, 25, and 30 minutes).

2.6. Total phenolic assay
The total phenolic compounds were determined using the Folin–Ciocalteu modified methods [20]. Briefly, 0.5 mg MEJS was diluted in 1 ml of methanol, and 0.5 ml of Folin -Ciocalteu reagent (1:1) was added and the mixture was homogenized. After 8 minutes, 4.5 ml of 2% of sodium carbonate (Na₂CO₃) was added and the mixture was mixed. Thereafter the mixture was stored in the dark room at the room temperature. As a result of the reaction, color was developed and absorbance at λ =765 nm was measured after 60 minutes (UV-1601 Shimadzu, Japan) against the blank. The same procedure was repeated using a standard solution of gallic acid. The result was expressed as a gram of gallic acid equivalent (GAE) per 100 grams of MEJS.

2.7. The total flavonoid assay
The total flavonoid compounds were determined using a method referred Ebrahimzadeh et al. [20]. Briefly amount 0.5 mg MEJS was diluted in 1.5 ml methanol, 0.1 ml aluminum chloride 10%, 0.1 ml 1 M potassium acetate, and 2.8 ml distilled water and then homogenized. After that, mixture incubated at the room temperature for 30 min. The absorbance was measured at 415 nm by spectrophotometer (UV-1601 Shimadzu, Japan). The total flavonoid content was calculated as (+)-catechin equivalent (CE) from a calibration curve as described previously.

2.8. The total tannin assay
The total tannins compound was determined using a method referred Palici et al. [21]. Briefly amount 2 mL of MEJS (0.01 – 0.001 %) added 1 mL of phosphotungstic-acid and 17 mL 50 % sodium bicarbonate (Na₂CO₃). Thereafter the mixture incubates as long 2 min. and then measured its absorbance. The absorbance was measured at λ= 750 nm by spectrophotometer (UV-1601 Shimadzu, Japan). The total flavonoid content was calculated as a tannic acid equivalent (TAE) from a calibration curve as describes previously.
2.9. DPPH radical scavenging activity assay

The DPPH radical scavenging activity was assayed using methods referred Vasi and Austin [7]. Briefly, amount 0.5 ml MEJS solution on various concentrations (25, 50, 100, 200, and 400 ppm) was added 0.5 ml of DPPH solution (100 µM) and then incubated at room temperature (37±2°C) for 15 min. The absorbance was recorded at λ = 517 nm. The reaction was then subjected to absorbance measuring at λ = 517 nm. Radical scavenging activity (RSA) was expressed as the percentage of inhibition of •DPPH free radical and calculated as equation 1.

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\text{Inhibition RSA-DPPH } \% = 1 - \left( \frac{\text{Abs. sample}}{\text{Abs. control}} \right) \times 100 \%
\] (1)

2.10. Reducing power assay

The reducing power activity was assayed using methods referred Vasi and Austin [7]. Briefly, amount 2.5 ml MEJS solution in various concentration (25, 50, 100, 200 and 400 ppm) was mixed with 2.5 ml buffer phosphate (0.2 M/pH= 6.6) and 2.5 ml 1% potassium ferricyanide solution, incubated at 50°C (in a water bath) for 20 min. and then received to terminate the reaction, 1 ml of TCA (10%) was added to each reaction. After that the solution then centrifuges (3,000 rpm/10 min). The upper layer of the mixture (2.5 ml) was mixed with 2.5 ml distilled water and 0.5 ml iron (III) chloride solution (0.1%) and subjected to absorbance measuring at λ = 700 nm. A higher absorbance indicates a higher total reducing power.

2.11. Statistical analysis

All the experiments in this study were done in triplicate and the results were expressed as mean ± SD. The data were analyzed through one-way of variance (ANOVA) using the statistical package for social sciences (SPSS) for Windows version (22.0) was used to analyzed data (SPSS Inc., Chicago, IL). Statistical significance was declared at \( p < 0.05 \).

3. Results and discussions

As the first of characteristic parameter of JSP revealed chemicals content were water content 9.1±0.1%, lipid 0.25 ± 0.04%, protein 3.89 ± 0.02%, crude fiber 2.14 ±0.07%, ash 1.07 ± 0.01% and carbohydrate (by difference) was 85.73 ± 0.1%. Previous research showed that Java plum seed was a solid waste (20.65%) of fresh fruits. And then JSP was rich in dietary fiber, sucrose (0.17%), fructose (2.78%), glucose (2.24%), and source of minerals as such as potassium 8,813 ppm, magnesium 2,162, ferrum 137, sodium 115 ppm, and some trace minerals as like calcium, phosphor and cuprum [9]. Yield extraction of JSP was 12.84 ± 0.8%, less than 13.89 ± 0.5% [9]. Its differences were caused due to the difference of freshness of sample and particles size. In addition, the use of (+)- catechin was as a standard on the total flavonoid assay of the sample exactly. Therefore (+)- catechin content on the MEJS was abundant than quercetin [5]. Many publications reported using (+)- catechin as a

3.1. Phenolic compounds

The phenolics compound of MEJS before heat treatment was expressed in three ways, these were; total phenolic, flavonoid and total tannins. The total phenolics was 34.73 ± 0.3 (g-GAE/100 g) and referred from a calibration curve \( y_1 = 7.145x - 0.034, R^2=0.986 \). The total flavonoid was 7.12±0.17 (g-CE/100 g) and referred from a calibration curve \( y_2 = 0.0012x - 0.0038, R^2 = 0.998 \) and the total tannins was 29.81 ±0.15 (g-TAE/100 g) and referred from a calibration curve \( y_3 = 0.84x + 0.031, R^2 = 0.996 \). Yield extraction of JSP was 12.84 ± 0.8%, less than 13.89 ± 0.5% [9]. Its differences were caused due to the difference of freshness of sample and particles size. The smaller size of JSP particles (increasing the mesh size) obtained a higher yield [9]. Vasi and Austin, reported that yield extraction of Java plum seed with 50% ethanol was 12.96% [7].
standard on the total flavonoid assay [22,23]. Vayuparp and Laksanalamal [1] reported that the grape seeds extraction using 50% ethanol, in maceration (50°C/6 hrs.1:10) obtained a yield as 14.86±0.03% and total phenolic as 32.86±0.04% (g-GAE/100 g dry extract).

3.2. Phenolic compounds and antioxidant activity assay

Table 1 showed the effect of the difference of heat treatment on total phenolics content (TPC), total flavonoids content (TFC), total tannins content (TTC), and in vitro antioxidant activity immediately (0 days) after the heat treatment.

Table 1. The effect of heat treatment on phenolic compounds and in vitro antioxidant activity of MEJS.

| Temperature (°C)/10 min. | Phenolic compounds | Antioxidant Activity* |
|--------------------------|--------------------|-----------------------|
|                          | TPC (g-GAE/100 g)  | TFC (g-CE/100 g)     | TTC (g-TAE/100 g) | RSA-DPPH (%)                          | FRAP (OD)            |
| 30/C                     | 31.76 ± 0.17b      | 7.12 ± 0.20a         | 29.81 ± 0.15a     | 81.16 ± 0.1d                         | 2.26 ± 0.002a        |
| 110                      | 33.93 ± 0.10a      | 6.95 ± 0.17b         | 26.26 ± 0.20b     | 83.15 ± 0.1c                         | 2.13 ± 0.001b        |
| 120                      | 31.15 ± 0.20b      | 6.41 ± 0.10c         | 21.59 ± 0.10c     | 84.73 ± 0.1b                         | 2.09 ± 0.005c        |
| 130                      | 27.34 ± 0.10c      | 5.47 ± 0.10d         | 18.07 ± 0.10d     | 84.94 ± 0.1a                         | 1.75 ± 0.002d        |
| 140                      | 19.54 ± 0.20d      | 5.17 ± 0.22e         | 12.71 ± 0.26e     | 79.41 ± 0.1e                         | 1.34 ± 0.003e        |
| 150                      | 17.84 ± 0.30e      | 3.84 ± 0.20f         | 10.07 ± 0.14f     | 76.74 ± 0.1f                         | 1.24 ± 0.004f        |

Mean in the same column with different alphabetical letters are significantly different (p<0.01)

*Antioxidant activity was measured at 400 ppm extract. c = control

The effect of heat treatment (oven) on phenolic compounds and in vitro antioxidant activity of the samples revealed interesting phenomena. In general, the increase of heating temperature (110-150°C) caused an extensive decrease in the TPC, TFC, and TTC of the samples as 48.7%, 46.06 %, and 66.2% respectively compared to the control (table 1). Similarly, the data mentioned above, heating time (10 minutes the second and so on) of MEJS caused a decrease in the TPC, TFC, TTC, and free radical scavenging activity (table 2). However, heating for about ten minutes first had a positive impact on increasing in its phenolic content and antioxidant activities (table 2).

Meanwhile, oven treatment means raising the temperature to 130°C/10 minutes could increase the free radical scavenging by 4.7 % (significantly). It might suggest that the heat treatment of MEJS (30-130°C) liberated phenolics compounds and thus increased the amounts of activity at these range temperatures, but raising the temperature to 150°C/10 minutes let the free radical scavenging declined. The decreasing of TPC, TFC, and TTC samples accordance with the decrease of the total reduction power. As the changes in the phenolic compound were not related to the changes in antioxidant activity. This was in line with what was stated by Chamorro et al. [16] that modifying the phenolic profile content due to different heat treatment methods was not related to changes in antioxidant activity. Similarly, with the statement mentioned above, Pop et al. [24] reported that heat treatment (70-100°C/15 min.) on the grape seed extraction was not significantly affected the radical scavenging activity on its extracts.

Anthocyanin degradation increased in temperature and time of pasteurization of Jamun (Eugenia jambolana) fruit juice [25]. Heating treatment (60-125°C/8-72 hr.) decreased procyanidin concentration significantly (p<0.05) in both blueberry and grape pomace [26]. It was suggested that a decrease in the TPC, TFC, and the TTC assay (Table 2) could be attributed to the degradation of proanthocyanins present in MEJS powder which in having higher molecular weight into the release of simple phenolic compounds.
like quercetin, rutin, and (+)-catechin. That’s why the radical scavenging activity increasing while the total phenolic compounds assay declined.

Table 2. The effect of heating time on phenolics changes and antioxidant activity of MEJS.

| Time-heating (min./110°C) | Phenolic compounds | Antioxidant Activity* |
|--------------------------|--------------------|-----------------------|
|                          | TPC (g-GAE/100g)   | TFC (g-CE/100 g)      | TTC (g-TAE/100 g) | RSA-DPPH (%) | FRAP (OD) |
| C                        | 31.76 ± 0.17b      | 7.12 ± 0.20c          | 29.81 ± 0.15c     | 89.04±0.16b  | 2.66± 0.01f |
| 10                       | 33.27 ± 0.14a      | 8.40 ± 0.20a          | 32.65 ± 0.10a     | 89.11±0.10ab | 2.68 ± 0.01e |
| 15                       | 31.67 ± 0.17b      | 7.70 ± 0.10b          | 31.51 ± 0.06b     | 89.32±0.10a  | 2.83 ± 0.01d |
| 20                       | 31.05 ± 0.10c      | 7.20 ± 0.10c          | 29.64 ± 0.12c     | 88.39±0.16b  | 2.86 ± 0.01c |
| 25                       | 30.60 ± 0.10d      | 6.71 ± 0.10d          | 28.15 ± 0.20d     | 87.24±0.01d  | 2.87 ± 0.01b |
| 30                       | 29.82 ± 0.16e      | 6.20 ± 0.20e          | 25.43 ± 0.10e     | 87.03±0.16d  | 2.88 ± 0.01a |

Mean in the same column with difference alphabetical letters are significantly different ($p<0.01$)

*Antioxidant activity was measured at 400 ppm extract. c = control

3.3. DPPH radical scavenging activity assay

DPPH free radical scavenging activity expressed as (% inhibition) was used for the assay in vitro antioxidant activity of MEJS samples (both heated and control). The effect of heating treatment against in vitro antioxidant activity of the samples served in figure 1. In general, the results showed that the increase of heating temperatures (110-130°C/10 min.) affected the increase of free radical scavenging activity from the samples significantly ($p<0.01$). While the increasing of the sample’s concentration (25-400 ppm) in each heating treatment gave a positive correlation ($r=0.89-0.99$) with the increase of antioxidant activity. However, the further increase in heating temperature (140-150°C) led to a decrease in the free radical scavenging activity significantly ($p<0.01$). It suggested that heating temperature had a considerable influence on the changes of phenolic compounds. Heating treatment at the lower temperature (< 130°C/10 min) induced breakdown the proanthocyanins are converted to the oligomeric and monomeric aglycone. On the other hand, the heating at the higher temperature (> 130°C/10 min.) induced the degradation of the monomeric aglycone, therefore the effect led to the decrease of the antioxidant activity [16]. On behalf of increasing the antioxidant activity on the samples heated at high temperature, Miranda et al. [27] told that it might be due to the generation and accumulation of Maillard derived melanoidins.

According to Chamorro [16], the type of the samples (an extract or not extract) influenced the type of the ongoing reaction. Pasteurization, sterilization, or thermosonication are less able to maintain anthocyanin retention than sonication process in Jamun fruit juice [25]. In addition, the anthocyanin degradation will increase together with the increase of temperature and time of pasteurization. However, we could not establish a relationship between the changes of phenolic compound and the antioxidant activity [11,15,25,28,29,30].
**Figure 1.** Free radicals scavenging activity of MEJS (25-400 ppm) in an air oven at various temperatures heating (110-150°C/10 min.). There is a significant effect of temperature heating against antioxidant activity ($p<0.05$). Each value is expressed as mean ± SD, n=3.

**Figure 2.** The reducing power activity of MEJS (25-400 ppm) in an air oven at various temperatures heating (110-150°C/10 min.). There is a significant effect of temperature heating against antioxidant activity ($p<0.05$). Each value is expressed as mean ± SD, n=3.
3.4. Reducing power assay
The total reduction ion Ferric (Fe$^{3+}$) expressed as (optical density-OD) was used for the assay in vitro antioxidant of MEJS samples (both heated and control). Effect of heating treatment against in vitro antioxidant activity of the samples served in figure 2. In general, the results showed that the increase of heating temperatures (110-150$^\circ$C/10 min.) affect the decrease of the reducing power activity from the samples significantly ($p<0.01$). Dry heating at a higher temperature ($>110^\circ$C) gave a negative correlation ($r=0.8-0.96$) with the reducing power. This finding is in line with previous research done by Reda [17]. While increasing of the sample’s concentration (25-400 ppm) in each heating treatment gave a positive correlation ($r=0.98-0.99$) with the increase of radical scavenging activity (figure 1), it was in the contrary with what had mentioned above, Nikousaleh & Prakash [31] reported that the heat treatment on clove exhibited significantly higher reducing power activity. The decrease of the optical density (OD) value of the samples means were less able to the reduction of ferric ion (Fe$^{3+}$).

3.5. Half maximal effective concentration (EC$_{50}$ value) in antioxidant properties
The antioxidant properties were normally expressed as EC$_{50}$ value (mg-extract per Liter) for comparison. Efficacy in antioxidant properties inversely correlated with EC$_{50}$ value. EC$_{50}$ value of the heated of MEJS were 200 ppm, 178.2; 156.1; 149; 22, and 250.1 ppm with the heating temperature 30, 110, 120, 130, 140 and 150$^\circ$C respectively. On behalf to antioxidant efficacy, heated the samples with 110, 120, and 130$^\circ$C/10 minutes were more effective as evidence by the lower EC$_{50}$ value compared to the control. Nevertheless, heating further at a higher temperature ($>130^\circ$C) gave a bad influence on the antioxidant activity. The enhanced antioxidant efficacy after heated might be attributed to the degradation of proanthocyanins present on MEJS into release of a free simple bioactive compounds like rutin, quercetin and (+)- catechin as aglycone. The effect of the heating applied on foods perhaps could decrease some of the identified phenolic compounds, but in the other hand could increase others depend on the methods, temperature, and time. But, in general, heating treatment demonstrated a slight decrease in some of the identified phenolic compounds [28]. In this study, there was a negative correlation between the TPC, TFC, and TTC with the free radical antioxidant activity. An antioxidant efficacy (EC$_{50}$) of the samples are presented in table 3.

| Sample | EC$_{50}$ (ppm) |
|--------|-----------------|
| MEJS   | 200             |
|        | 178.2           |
|        | 156.1           |
|        | 149             |
|        | 221             |
|        | 250.1           |

4. Conclusion
The heating treatment that was applied to MEJS at the temperature risen to 130$^\circ$C should decrease the TPC, TFC, and TTC slightly coincided with the decrease of the reducing power. The contrary heating treatment risen to 130$^\circ$C should increase the free radical scavenging activity of the extract. However, heating further at a higher temperature ($>130^\circ$C) gave a bad influence on the free radicals scavenging. Antioxidant activity of Java Plum (S. cumini Linn.) seed extract was unstable by heating at high temperature more than 130$^\circ$C/10 minutes. The extract heated at the 130$^\circ$C/10 minutes gave the best EC$_{50}$ value as 140 ppm. Concerning of the effect thermal treatment on the changes of the phenolics compound,
finally the present study revealed that there was no established relationship between the changes of phenolic compound and the antioxidant activity.

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