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Antioxidant and Free Radical Scavenging Activity of Different Fractions from Hawthorn Fruit

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Abstract

Hawthorn fruit is a conventional medicine used in treating cardiovascular diseases. Its therapeutic effects may relate to its antioxidant compounds. In this study, we evaluated the antioxidant activity of CH_2Cl_2, EtOAc, *n*-butanol and water fractions from 70% methanolic hawthorn fruit extract by total phenolic and flavonoid contents, total antioxidant activity, DPPH free radical scavenging activity, superoxide radical scavenging activity, reducing power assay, lipid peroxidation inhibitory activity and protective effect against hydroxyl-radical-induced DNA damage. Results showed that the EtOAc fraction contained significantly greater antioxidant activities than other fractions, which suggests that the potent EtOAc fraction should be used for further studies to identify the antioxidant compounds.

Key words: antioxidant activity, DNA damage, free radical, hawthorn fruit

INTRODUCTION

Oxidative damage appears to be related to the etiology of cardiovascular disease, diabetes mellitus, gastric ulcers, cancer, arthritis, Alzheimer's disease, Parkinson's disease and inflammation (1-3). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) including free radicals such as superoxide radical anion ($•O_2^-$), hydroxyl radicals (•OH), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) are constantly produced by severe oxidative stress, such as ultraviolet light, chemical pollution and normal metabolic processes, resulting in oxidative stress (4,5). ROS can react with cell membrane lipids, leading to lipid peroxidation and RNA, DNA and protein damage (6). Antioxidant compounds are thought to play an important role in the maintenance of good health. Scientific evidence demonstrated that antioxidants can protect the human body against free radical damage and can retard progression of many chronic diseases, such as cancer and heart disease (7-9). Natural antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens are mostly derived from grains, fruits and vegetables, and have been identified to have the potential in reducing disease risk (10,11).

Hawthorn (*Crataegus oxyacantha*) is a medicinal and spice plant. In pharmacological and toxicological studies, the fruit was considered to be generally safe and well-tolerated in the treatment of cardiovascular diseases, and angina pectoris of coronary heart disease (12,13). The extracts from hawthorn fruit are clinically effective in reducing blood pressure and total plasma cholesterol (14), and treating indigestion and retention of food due to improper diet or excessive intake of greasy food or meat. Some studies have established that hawthorn fruits are rich in proanthocyanidins and flavonoids (15-17). Such polyphenolic compounds can improve human health by reducing oxidative stress and providing activity associated with anti-cancer, anti-allergic, and anti-inflammatory effects (18,19). The phenolic profiles and antioxidative effects of cell suspensions, fresh fruits and medicinal dried parts from hawthorn have been investigated (20). However, the literature regarding antioxidant activities and pharmacological studies of dried hawthorn fruit are limited. Hence, the present work investigates the possible antioxidative effects of different fractions of dried hawthorn fruit. Antioxidant properties including total phenolic and flavonoid contents, total antioxidant activity, DPPH scavenging activity, superoxide radical scavenging activity, reducing power assay, lipid peroxidation inhibitory activity and protective effect against hydroxyl-radical-induced DNA damage were measured.

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MATERIALS AND METHODS

Sample preparation

Hawthorn fruit is the bright red berries of the *Crataegus phaenopyrum*. The fruits were collected from native trees in Chuncheon, South Korea, in autumn 2008. Freshly-harvested whole hawthorn fruits were shade dried and then finely powdered. A 50 g powder was extracted twice with 1 liter 70% methanol. The extract was filtered through filtration by vacuum (100-mm, Whatman, Maidstone, UK). The solvent was evaporated under reduced pressure using a vacuum rotary evaporator (CCA-1110, Eyela, Tokyo, Japan). The coarse extract was successively extracted with dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) and H_2O in a continuous Soxhlet extractor.

Determination of total phenolic and flavonoid contents

0.1 mL of each fraction (1 mg/mL) was mixed with 0.9 mL reagent solution (0.1 N Folin-Ciocalteu reagent, and 7.5% sodium carbonate). The reaction mixture was incubated at room temperature for 30 min against a blank. The total phenolic content was done with respect to the standard curve of tannic acid, and was expressed as tannic acid equivalent (21).

Total flavonoid content in hawthorn fruit fractions was evaluated by a colorimetric method described by Liu et al. (22), with slight modification. 0.25 mL of each fraction, 1.25 mL distilled water, and 75 μL of 5% sodium nitrite solutions were mixed together and incubated at room temperature for 6 min. 150 μL of 10% aluminum chloride hexahydrate solution was added and incubated for a further 6 min. The reaction was terminated by adding 0.5 mL of 1 M sodium hydroxide. 0.275 mL distilled water was added to bring the total mixture volume to 5 mL. The absorbance was measured at 510 nm against the blank. The total flavonoid content was determined with respect to the standard curve of catechin, and was expressed as catechin equivalent.

Determination of total antioxidant activity

The capacity of hawthorn fruit fractions on total antioxidant activity was investigated according to the method of Prieto et al. (23) with several modifications. Briefly, 0.2 mL of each fraction with concentrations at 12.5～200 μg/mL was mixed with 0.6 mL of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate solutions). The reaction mixture incubated at 95°C for 90 min. The absorbance of the cooled mixture was measured at 695 nm against a blank. The total antioxidant activity was expressed as the absorbance of the sample. The higher absorbance value indicated higher antioxidant activity. L-ascorbic acid was used as a standard.

Determination of DPPH free radical scavenging activity

The effect of hawthorn fruit on 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging was evaluated by the method described by Zhang et al. (24) with several modifications. Briefly, a 50 μL of 0.1 mM DPPH solution was added to varying concentrations of each fraction, vortex-mixed and incubated at room temperature for 30 min. The absorbance was measured at 515 nm against a blank. The scavenging ratio by each sample was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)}=[A_0-(A_1-A_s)]/A_0\times100$$

where A_0, A_1, A_s was the absorbance of the control, fractions of sample and blank.

The scavenging ratio by pure compound was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)}=(A_0-A_1)/A_0\times100$$

where A_0, A_1 was the absorbance of the control and antioxidant compound (in this experiment, we used α- tocopherol as standard).

Inhibition (IC_{50}) was calculated from the graph of DPPH scavenging activity against fraction concentration.

Determination of superoxide anion radical ($\bullet O_2^-$)-scavenging activity

The $\bullet O_2^-$ scavenging activity of different hawthorn fruit fractions was determined by the method described by Ewing and Janero (25) with some modifications. 50 μL aliquot of each sample solution was mixed with 1 mL of 0.1 M phosphate buffer solvent (pH 7.4), containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 50 μM nitro blue tetrazolium (NBT), 78 μM b-nicotinamide adenine dinucleotide (NADH) and 3.3 μM phenazine methosulfate (PMS). After incubation at 25°C for 8 min, the absorbance was measured at 560 nm. Gallic acid was used as the positive control.

Determination of reducing power activity

The reducing power of hawthorn fruit fractions was described by Rumbaoa et al. (26). Briefly, 1 mL of fraction or control solutions with concentrations 0～1000 μg/mL were mixed with 2.5 mL sodium phosphate buffer (0.2 mM, pH 6.6) and 1 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After terminating the reaction by adding 1 mL of 10% trichloroacetic acid (TCA) solution, the mixture was centrifuged at 3000 rpm for 10 min. One mL upper layer solution was mixed with 1 mL of distilled water and 0.2 mL of 0.1% ferric chloride and the absorbance was measured at 700 nm. The increased absorbance indicated increased reducing power activity.

Determination of lipid peroxidation inhibitory activity

The lipid peroxidation inhibitory effect of hawthorn fruit fractions was estimated by measuring thiobarbituric acid reacting substances (TBARS) (27,28) with modification. A 2 g chook liver was homogenized (1%, w/v) in 50 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 3000 rpm at 4°C for 15 min and the supernatant used for assay. Liver peroxidation was induced by Fe^{2+}-H_2O_2. Briefly, 1% prepared liver homogenate was incubated with 30 mM potassium chloride, 0.16 mM ferrous ammonium sulphate, 0.6 mM ascorbic acid, and different concentrations of fractions (1~10 mg/mL). After incubation at 37°C for 1 hr, 0.4 mL of the incubation mixture was mixed with 0.2 mL of 8.1% sodium dodecyl sulphate (SDS), 1.5 mL of 0.8% 2-thiobarbituric acid (TBA) and 1.5 mL of 20% acetic acid (pH 3.5). The total volume of the mixture was brought up to 4 mL with distilled water. The mixture was incubated for another 1 hr in a boiling water bath. The cold mixture was vortex-mixed with 5 mL *n*-butanol. The absorbance of the upper layer was measured at 532 nm after centrifugation. L-ascorbic acid was used as a standard.

Genomic DNA isolation

Genomic DNA was extracted from HEK 293 cells (embryonic kidney cell line). The cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Before treatment, HEK 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented (10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin) and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for three days. After being washed twice with PBS, the collected cells were dissolved in the extraction buffer (1 M Tris-HCl, 0.5 M EDTA, 5 M sodium chloride and 20% SDS) and incubated at 64°C for 10 min. The DNA was extracted three times with phenol : chloroform : isoamylalcohol (25:24:1) solution on ice for 5~10 min and centrifuged at 13,000 rpm for 15 min at 4°C. Supernatant was mixed with isopropanol and centrifuged at 13,000 rpm for 10 min at 4°C. The DNA was washed by 70% ethanol, then, dissolved in distilled water and kept in a refrigerator until further analysis.

Determination of protective effect against hydroxyl-radical-induced DNA damage

To measure the protective effect of hawthorn fruit fractions on DNA damage induced by hydroxyl radical, 0.5 μg genomic DNA from HEK 293 cells in 5 μL of 50 mM phosphate buffer (pH 7.2) was mixed with 2 μL of 1 mg/mL sample of the hawthorn fruit fraction, 4 μL of 30% H_2O_2 and 3 μL of 5 mM ferrous sulphate. The mixture was incubated at 37°C for 30 min. The DNA was analyzed on 1% agarose gels and DNA bands were visualized by ethidium bromide staining and a Mini BIS image analysis system (DNR Bio-Imaging Systems Ltd., Kiryat Anavim, Israel). Densitometric analysis was done with image analysis software (Quantity one; Bio-Rad, Hercules, CA, USA). The protect percentage of DNA damage was calculated by comparing the proportion of control genomic DNA.

Statistical analysis

All experiments were conducted in independent triplicate (n=3) and data were expressed as mean±SD.

RESULTS AND DISCUSSION

Total phenolic and flavonoid contents

Phenolic compounds and flavonoids are widely distributed in plants, vegetables, and fruits, and have been reported to have free radical scavenging and antioxidant activities (29). Diverse biological actions, such as anti-inflammatory, anticarcinogenic and anti-atherosclerotic functions are considered part of these activities (30). Hence, phenolic and flavonoid compounds are the usual components analyzed when measuring antioxidant activity. Some of phenolic compounds present in hawthorn fruit include protocatechuic acid, chlorogenic acid, epicatechin, quercetin, rutin, isoquercitrin and hyperoside (31). In this study, the total phenolic and flavonoid contents of hawthorn fruit fractions were expressed as tannic acid and catechin equivalent, respectively (Table 1). Among the fractions, the EtOAc fraction showed significant phenolic and flavonoid contents (140.18±2.16 tannic mg/g, 56.52±1.33 catechin mg/g). Since the phenolic compounds are considered to be major contributors to the antioxidant capacity of plants, the relatively large phenolic content in the EtOAc fraction was suggestive for further research on isolating antioxidant compounds.

Table 1. Total phenolic and flavonoid contents of different fractions from hawthorn fruit

Fractions	Total phenolic (tannic mg/g)[1)]	Total flavonoid (catechin mg/g)[2)]
CH_2Cl_2	32.00±1.90	4.46±0.17
EtOAc	140.18±2.16	56.52±1.33
n-BuOH	43.50±1.05	14.07±1.62
H_2O	21.16±0.19	5.83±0.74

[1)]Tannic acid was used as a standard for measuring of the total phenolic content.

[2)]Catechin was used as a standard for measuring of the total flavonoid content.

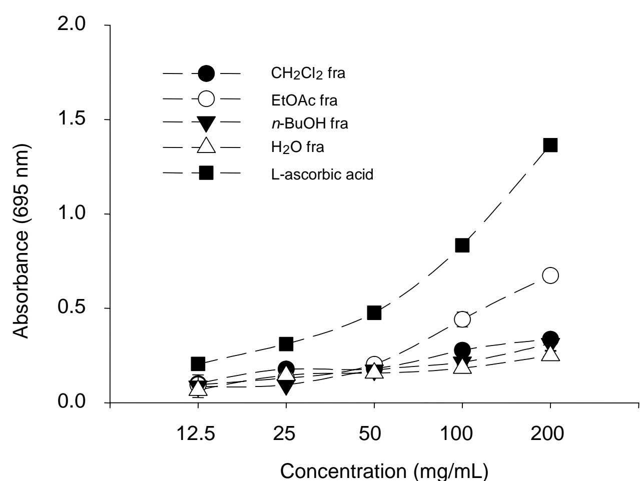

Fig. 1. Total antioxidant activity of different fractions from hawthorn fruit. L-Ascorbic acid was used as a positive control.

Table 2. Free radical scavenging activity of hawthorn fruit fractions

Fractions	DPPH radical scavenging activity (IC_{50}: μg/mL)[1)]	Superoxide radical scavenging activity (IC_{50}: mg/mL)[1)]
CH_2Cl_2	138.10±4.05	11.50±0.023
EtOAc	30.95±0.44	1.32±0.011
n-BuOH	87.35±1.10	5.95±0.019
H_2O	165.52±3.63	–
Standards		
α-Tocopherol	5.71±0.20	–
Galic acid	–	300.727±0.008

[1)]IC_{50}: the effective concentration at which DPPH radicals were scavenged by 50%.

Total antioxidant activity

The total antioxidant activity of hawthorn fruit fractions was measured according to the molybdenum blue assay. The formation of "isopoly-molybdenum blues" shows intense blue color and usually been used as a sensitive test for reducing reagents. The high absorbance values indicated that hawthorn fruit fractions possessed antioxidant activity. The total antioxidant activity of hawthorn fruit fractions and ascorbic acid was showed in Fig. 1. The natural antioxidant compound ascorbic acid was used as the positive controls, as described in other reports (32). The results showed that the EtOAc fraction had higher total antioxidant activity than any other fractions compared to positive control. The strong total antioxidant activity was probably due to the presence of flavonoids, carotenoids, ascorbic acid and other bioactive compounds (33). The content of phenolic compounds could be used as an important indicator of antioxidant activity (34). Hence, the EtOAc fraction exhibited significantly total antioxidant activity that may be attributed to its chemical composition and high phenolic and flavonoid contents.

Free radical scavenging activities

We investigated the free radical-scavenging activities of hawthorn fruit fractions by DPPH free radical and superoxide radical scavenging assays (Table 2). DPPH is an unstable free radical compound commonly used to determine the free radical-scavenging ability of various samples (35-38). α-Tocopherol is an important chain-breaking antioxidant which used as a positive control in DPPH radical scavenging assay (39). In this study, we expressed the scavenging activity of hawthorn fruit in IC_{50} values. All hawthorn fruit fractions showed some DPPH free radical scavenging activity, with the EtOAc fraction appearing to be the most effective, with IC_{50} values reached 30.95±0.44 μg/mL. Superoxide radical anion ($•O_2^-$) is an important part of ROS species. Its contribution to the redox imbalance in cells has harmful physiological consequences (40). Ljubuncic et al. (41) have measured the $•O_2^-$ scavenging activity of *Crataegus aronia* extracts by xanthine-xanthine oxidase (X-XO) system. In this study, we valued the activity by triad NADH/reduced PMS/dioxygen as a source of $•O_2^-$ radical. The EtOAc fraction showed highest $•O_2^-$ scavenging activity among the fractions, while the H_2O faction had scant effect. The strong free radical scavenging activity of the EtOAc fraction might be due to its high phenolic and flavonoid contents, which are related to the scavenging activity of phenolic and flavonoid compounds.

Reducing power activity

The antioxidant potential of a compound may be indicated by its reducing power activity (42), as there is a positive correlation between antioxidant activity and reducing power (43,44). The reducing activity associated with the presence of reductones, which act as primary and secondary antioxidants, occurs by breakage of the free radical chain through the donation of a hydrogen atom (45-47). The reducing power of hawthorn fruit fractions showed a dose-dependent response (Fig. 2). Compared to the positive control (α-tocopherol), the EtOAc fraction exerts the highest activity than other fractions. However, the α-tocopherol and EtOAc fractions showed different responses at different concentrations. Lower than 400 μg/mL, the α-tocopherol exhibited higher activity than EtOAc fraction, whereas EtOAc fraction showed stronger activity at 400～1000 μg/mL concentration. The CH_2Cl_2 and H_2O fractions showed similar activity to each other, but lower than *n*-BuOH fraction. The reducing capability of a com-

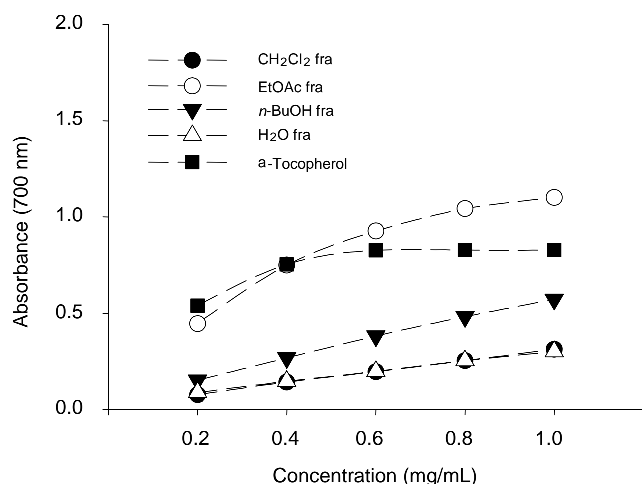

Fig. 2. Reducing power activity of the different fractions from hawthorn fruit. α-Tocopherol was used as a positive control.

pound may serve as a significant indicator of its potential antioxidant activity (48), thus the significant antioxidant activity of the EtOAc fraction appears to be at least partially related to its reducing power activity.

Lipid peroxidation inhibitory activity

Lipid peroxidation (LPO) is a process induced by free radicals that mediate chain reactions. Some products of the LPO process can damage DNA, RNA and enzymes. Hence, LPO has been used as an indicator of oxidative stress in cells and tissues (49). The results were measured at 532 nm at thiobarbituric acid reacting substances (TBARS) form. L-ascorbic acid as the positive control showed that LPO of chook liver homogenate was induced by Fe^{2+}-ascorbate. As shown in Fig. 3, the extracts displayed significant inhibitory activity on lipid peroxidation, even at 1 mg/mL concentration. The EtOAc fraction showed the highest protective effect in a dose-dependent manner. However, there was scarcely any activity of CH_2Cl_2 or H_2O fractions even at 10 mg/mL concentration. Since free radicals are the source of lipid peroxidation (50), the significantly protective effect of the EtOAc fraction may relate to the stronger free radical scavenging activities.

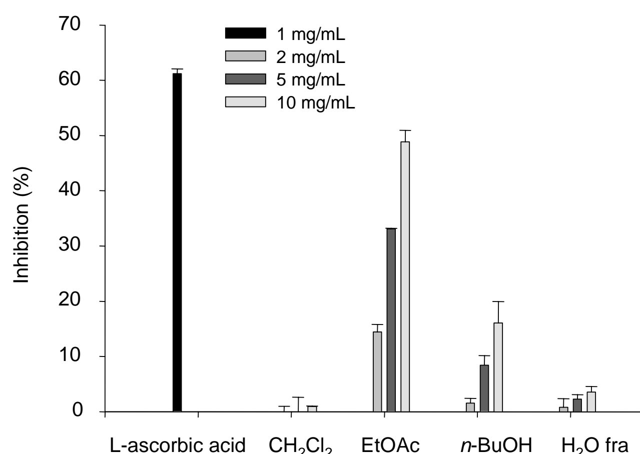

Fig. 3. Inhibitory activity of different fractions from hawthorn fruit on lipid peroxidation. L-Ascorbic acid was used as a positive control.

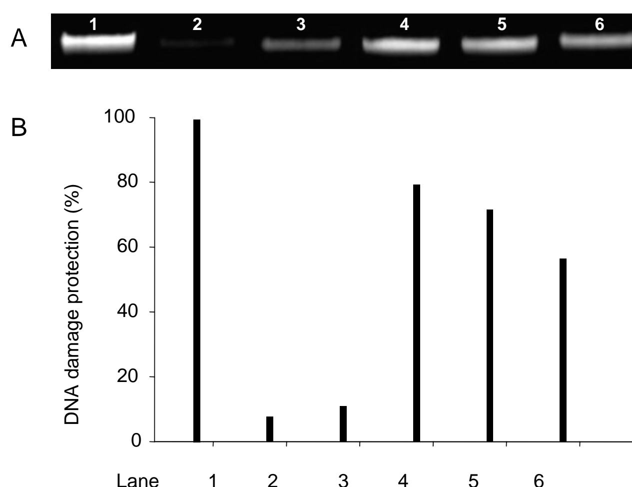

Fig. 4. (A) Agarose gel electrophoretic patterns of the DNA (genomic DNA from HEK 293 cells) damage induced by hydroxyl radicals in the presence of hawthorn fruit fractions. (B) Histogram showing the protective effect (%) on DNA damage of the fractions on densitometric measurements. Line 1, DNA incubated without Fenton's reagent; Line 2, DNA incubated with Fenton's reagent; Line 3~6, DNA incubated with Fenton's reagent in the presence of CH_2Cl_2 fraction, EtOAc fraction, *n*-BuOH fraction and H_2O fraction, respectively.

Protective effect against hydroxyl-radical-induced DNA damage

DNA is another sensitive biotarget for free radical mediated oxidative damage (51). Free radicals can induce chemical modifications in DNA. The hydroxyl radical (•OH) is recognized as a DNA-damaging agent of physiological significance. The results of the DNA-protection assay are shown in Fig. 4. Sample 1 was the genomic DNA control. Sample 2 was completely destroyed by hydroxyl radical. The protective effect of 1 mg/mL fractions is distinctly exhibited in Sample 3 (CH_2Cl_2 fraction treated). The following treatments were assayed in Sample 4, Sample 5, and Sample 6: EtOAc fraction treated, *n*-BuOH fraction treated, and H_2O fraction treated, respectively. According to the densitometric analysis, the EtOAc, *n*-BuOH and H_2O fractions exhibited obviously protective effects, as shown in the histogram as 79.7%, 71.9% and 57%, respectively. However, the protective effect of the CH_2Cl_2 fraction was very low. DNA damage has been implicated in all stages of carcinogenesis and other degenerative diseases (52); therefore, the protective effect of some hawthorn

fruit fractions on DNA damage may be valuable in preventing cancer and other degenerative diseases.

CONCLUSION

In conclusion, the EtOAc fraction of hawthorn fruit exhibited potent antioxidant properties, expressed by the total phenolic and flavonoid contents, reducing power and activities on total antioxidant condition, the ability to scavenge DPPH radical, and inhibit lipid peroxidation and oxidative DNA damage. The *in vitro* antioxidant activity of the hawthorn fruit did not provide much insight into the exact pharmaceutical mechanisms of its activity; hence, the *in vivo* efficacy of EtOAc fraction against oxidative damage needs to be evaluated further studies.

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