

Postprandial glycemic effects, acute oral tolerability, and exploratory DNA-damage assessment of an aqueous *Anethum graveolens* L. seed extract

Mohamed Bouhrim^{1*} , Ayoub Farihi² , Abdellah Baraich², Salah Laaraj^{3*} ,
Fatiha Chigr¹, Bruno Eto⁴, Ennouamane Saalaoui², Mohamed Bnouham²

¹ Biological Engineering Laboratory, Faculty of Sciences and Techniques, Sultan Moulay Slimane University, P.O. Box 523, Mghila, Beni Mellal 23000, Morocco

² Laboratory of Bioresources, Biotechnology, Ethnopharmacology, Faculty of Sciences, Mohammed First University, BP 717, Oujda 60000, Morocco

³ Environmental, Ecological, and Agro-Industrial Engineering Laboratory LGEEAI, Moulay Slimane University (USMS) Faculty of Science and Technology (FST) Beni Mellal, Morocco

⁴ Laboratories TBC, Laboratory of Pharmacology, Pharmacokinetics and Clinical Pharmacy, UFR3S, Department of Pharmacy, University of Lille, 3, rue du Professeur Laguesse, B.P. 83, F-59000 Lille, France

* Corresponding author's e-mail: m.bouhrim@usms.ma; salah.laaraj@usms.ma

ABSTRACT

Anethum graveolens L. (dill) has a long history of medicinal use and has been investigated for potential glucose-modulating properties; however, evidence regarding its acute effects on postprandial glycemia and its toxicological profile remains limited. This study evaluated the acute oral tolerability, DNA-damaging potential, and postprandial glycemia-modulating effects of an aqueous seed extract of *A. graveolens* (AGAE). The extract was prepared by decoction from seeds purchased in the Béni Mellal-Khénifra region of Morocco. Acute oral toxicity was assessed in Swiss albino mice after single oral doses of 0.5, 1, and 2 g/kg followed by a 14-day observation period. DNA-damage responses were explored *ex vivo* in leukocytes obtained from a single male Wistar rat using the alkaline comet assay after exposure to AGAE concentrations ranging from 1 to 100 µg/mL. The effects of AGAE (400 mg/kg, p.o.) on postprandial glycemia were investigated in normoglycemic mice using oral glucose, sucrose, and starch tolerance tests, with glibenclamide and acarbose as reference drugs. In the glucose tolerance test, AGAE significantly reduced blood glucose at 60 min ($p < 0.001$), although the overall area under the curve (AUC) was not significantly affected. Following sucrose loading, AGAE did not significantly alter blood glucose concentrations or the AUC. In contrast, after starch loading, AGAE significantly reduced blood glucose at 60, 90, and 150 min ($p < 0.001$) and significantly decreased the AUC ($p < 0.001$). No mortality or overt clinical or behavioral signs of acute toxicity were observed at doses up to 2 g/kg. In the comet assay, no clear concentration-dependent pattern was observed across the evaluated DNA-damage descriptors, although higher tail-length and % tail-DNA values were observed at 100 µg/mL. Overall, AGAE exhibited acute glycemia-modulating activity in normoglycemic mice, with the most pronounced effect observed following starch loading, while showing no overt acute oral toxicity at the tested doses. Further studies are required to clarify the mechanisms underlying its effects on starch-derived glycemia and to characterize its genotoxic and repeated-dose safety profiles.

Keywords: *Anethum graveolens* L., aqueous seed extract, postprandial glycemia, oral carbohydrate tolerance tests, acute oral toxicity, alkaline comet assay.

INTRODUCTION

The global burden of diabetes mellitus (DM) continues to increase, driven in part by population ageing, urbanization, sedentary lifestyles, and dietary patterns characterized by high intakes of refined carbohydrates and energy-dense foods (1,2). DM is a heterogeneous metabolic disorder characterized by chronic hyperglycemia resulting from impaired insulin secretion, reduced insulin action, or both (3). Persistent hyperglycemia contributes to oxidative and metabolic disturbances, including the formation of advanced glycation end-products (AGEs), which are implicated in the development and progression of microvascular and macrovascular diabetic complications (4). According to the International Diabetes Federation, approximately 589 million adults aged 20–79 years were living with diabetes worldwide in 2024, corresponding to a global prevalence of 11.1%; this number is projected to reach approximately 853 million by 2050 (5). These estimates underscore the need for effective strategies to prevent and control hyperglycemia and its associated complications.

Current pharmacological management of diabetes includes insulin and several classes of glucose-lowering agents. Although these treatments are effective, their long-term use may be limited by adverse effects and variability in therapeutic response. For example, insulin and sulfonylureas may increase the risk of hypoglycemia and weight gain, whereas metformin is commonly associated with gastrointestinal adverse effects (6). These limitations, together with the continued use of traditional medicines in many populations, have stimulated interest in medicinal plants as potential sources of bioactive compounds for the management of metabolic disorders (7). Numerous plant species have been traditionally used for glycemic control, and several contain secondary metabolites capable of modulating pathways involved in carbohydrate digestion, glucose absorption, insulin signaling, and oxidative stress (8).

Morocco possesses a rich tradition of medicinal-plant use, including the use of several species for the management of diabetes and related metabolic disorders (9). Among these plants, *Anethum graveolens* L. (Apiaceae), commonly known as dill, is an annual aromatic herb widely cultivated and used as both a culinary spice and a medicinal plant (10). Different parts of *A. graveolens*, particularly the fruits commonly referred to as seeds, have been traditionally used for gastrointestinal

complaints, including flatulence, colic, and intestinal spasms, and have also been associated with galactagogue and other traditional medicinal applications (10). In addition, *A. graveolens* has been reported in traditional and experimental contexts related to glycemic control (11).

Pharmacological investigations have attributed a broad range of biological activities to *A. graveolens*, including antioxidant, antimicrobial, analgesic, anti-inflammatory, glucose-lowering, and lipid-lowering effects (11–13). These activities have been associated with its chemically diverse secondary metabolites. The volatile fraction of dill is characterized by compounds such as carvone, limonene, dillapiole, myristicin, and α -phellandrene, although the relative abundance of these constituents varies according to plant part, geographical origin, developmental stage, and extraction procedure (10). Non-volatile constituents reported in *A. graveolens* include flavonoids, phenolic acids, coumarins, tannins, and other phenolic compounds (13,14). Such chemical diversity provides a plausible basis for the multiple pharmacological effects attributed to the species.

Control of postprandial glycemia is an important component of diabetes management. Digestion of dietary carbohydrates involves pancreatic α -amylase, which hydrolyzes starch into smaller oligosaccharides, followed by intestinal brush-border α -glucosidases that generate absorbable monosaccharides. Consequently, modulation of carbohydrate digestion represents an established pharmacological strategy for attenuating postprandial increases in blood glucose (15). Oral sucrose and starch tolerance tests may therefore provide functional evidence of whether a botanical preparation modifies the glycemic response to complex carbohydrates, although these models do not by themselves demonstrate direct inhibition of α -glucosidase or α -amylase *in vivo*.

Previous studies have reported glucose-lowering and metabolic effects of *A. graveolens*, including reductions in blood glucose following repeated administration of aqueous preparations in diabetic rodents (16,17). An oral glucose tolerance study has also demonstrated an acute glucose-modulating effect of an ethanolic *A. graveolens* seed extract in rabbits (18). However, whether an aqueous seed preparation acutely modulates postprandial glycemia differently according to the carbohydrate substrate remains poorly characterized. To the best of our knowledge, the effects of an aqueous *A. graveolens* seed extract on oral

sucrose and starch tolerance have not previously been evaluated *in vivo* within the same experimental framework as an oral glucose challenge. Therefore, the present study comparatively investigated the acute glycemic responses to glucose, sucrose, and starch following administration of an aqueous *A. graveolens* seed extract in normoglycemic mice. Acute oral tolerability was evaluated as a preliminary *in vivo* safety endpoint, while an exploratory *ex vivo* alkaline comet assay was performed to characterize DNA-damage responses in rat peripheral-blood leukocytes. The study was designed primarily to characterize the carbohydrate-dependent pharmacological profile of the extract and to generate hypotheses for subsequent mechanistic and toxicological investigations.

MATERIALS AND METHODS

Materials and reagents

All chemicals and reagents used in this study were of analytical grade. D-glucose, sucrose, starch, acarbose, and glibenclamide were obtained from Merck. Reagents used for the alkaline comet assay included calcium- and magnesium-free phosphate-buffered saline (PBS), low-melting-point (LMP) agarose, normal-melting-point (NMP) agarose, Tris-based neutralization buffer, ethidium bromide, and hydrogen peroxide (H_2O_2). Lysis and alkaline electrophoresis solutions were prepared as described in the corresponding comet assay procedure. Unless otherwise specified, all aqueous solutions were prepared using distilled water.

Plant material and preparation of the aqueous extract

Commercial samples of *A. graveolens* L. seeds were purchased from a local herbalist in the Béni Mellal-Khénifra region, central Morocco. The botanical identity of the plant material was authenticated by a botanist at the Department of Biology, Faculty of Sciences and Techniques, Sultan Moulay Slimane University, Morocco. A voucher specimen was deposited in the Herbarium of the Faculty of Sciences and Techniques, Sultan Moulay Slimane University, Béni Mellal, Morocco, under the reference number AG-2024. The seeds were washed, air-dried at room temperature, and ground into a fine powder. Three parallel extraction preparations were performed under identical

experimental conditions. For each extraction, 100 g of powdered seed material was decocted in 1 L of distilled water for 30 min. The resulting decoctions were filtered through Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator until a dry powdered extract was obtained. Extraction yield was calculated from the mass of recovered dry extract relative to the initial mass of dry seed material. The mean extraction yield was approximately $7.63 \pm 0.34\%$, corresponding to approximately 7.63 ± 0.34 g of dry extract per 100 g of starting plant material. Following yield determination, the dry powders obtained from the three parallel extraction preparations were pooled to constitute a single experimental batch of AGAE used throughout the subsequent experiments. The pooled powdered extract was stored at 4 °C in a glass container protected from light with aluminum foil and was used within a few days of preparation. Immediately before each experimental administration, the required amount of AGAE powder was freshly dissolved in distilled water. The administered doses were expressed as mass of recovered dry extract per kilogram of body weight.

Experimental animals and ethical approval

Adult Swiss albino mice (25–30 g) used for the oral glucose, sucrose, and starch tolerance tests and the acute oral toxicity study were obtained from and maintained at the animal facility of the Faculty of Sciences and Techniques, Sultan Moulay Slimane University, Béni Mellal, Morocco. These experiments were conducted at Sultan Moulay Slimane University. The male Wistar rat used as the biological donor for the comet assay was obtained from the animal facility of the Faculty of Sciences, Mohammed First University, Oujda, Morocco. Blood collection and the subsequent *ex vivo* comet assay were performed at Mohammed First University. Animals were housed under standard laboratory conditions (22 ± 2 °C, 50–60% relative humidity, and a 12 h light/12 h dark cycle), with free access to standard laboratory diet and tap water except during the fasting periods required for specific experimental procedures. The experimental protocol was reviewed and approved by the Ethics Committee for Laboratory Animal Research of the University of Lille (approval No. D5935010) within the framework of the scientific collaboration involving the authors and the University of Lille laboratory. In the

absence of a local institutional animal ethics committee, the experimental protocol was submitted for ethical review before initiation of the animal experiments. The approved protocol covered the animal procedures reported in the present study. Animal handling and experimental procedures were conducted in accordance with the principles outlined in the Guide for the Care and Use of Laboratory Animals (19).

Animals meeting the study eligibility criteria were allocated to experimental groups by random assignment while maintaining comparable distributions of sex and body weight across groups. For the carbohydrate-tolerance experiments, baseline blood glucose was also considered to avoid major pre-treatment imbalance between groups. No formal blinding procedure was applied during animal treatment or blood-glucose measurements, as investigators were aware of group allocation. For the comet assay, slides were assigned coded identifiers before microscopic analysis. Animals or samples could be excluded in the event of impaired health status, clinical abnormalities, failure to comply with the experimental conditions, technical problems compromising measurement reliability, or inability to obtain the predefined experimental endpoint. No animals or samples were excluded from the analyses.

Exploratory *ex vivo* assessment of DNA damage using the alkaline comet assay

Blood collection and ex vivo treatment

Fresh peripheral blood was collected from a single male *Wistar* rat ($n = 1$ biological donor) under pentobarbital anesthesia via the retro-orbital route. The blood sample was diluted in 2 mL of calcium- and magnesium-free phosphate-buffered saline (PBS, pH 7.4). Aliquots of this single blood sample were incubated with AGAE at concentrations ranging from 1 to 100 $\mu\text{g/mL}$ for 1 h at 37 °C. PBS-treated cells served as the negative control, whereas hydrogen peroxide (H_2O_2 ; 250 $\mu\text{mol/L}$) was used as the positive genotoxic control (20). After incubation, 10 μL of the treated blood-cell suspension was used for the comet assay.

Alkaline comet assay

The alkaline comet assay was performed according to the method of Singh et al. (21), with modifications based on Ouahhoud et al. (22). Following treatment, the samples were centrifuged

at 4500 rpm for 10 min. The supernatant was discarded, and the resulting leukocyte pellet was re-suspended in 1 mL of PBS and washed three times.

The leukocyte suspension was mixed with 0.5% (w/v) low-melting-point agarose prepared in PBS and layered onto microscope slides previously coated with 1.5% (w/v) normal-melting-point agarose. The slides were then immersed in lysis solution and incubated in the dark at 4 °C for 1 h.

Following lysis, the slides were transferred to an alkaline electrophoresis buffer (pH > 13) and subjected to electrophoresis for 20 min at 25 V and 300 mA while the temperature was maintained at approximately 4 °C. After electrophoresis, the slides were neutralized three times with Tris buffer (pH 7.5). DNA was stained with 25 μL of ethidium bromide, and the slides were covered with coverslips before fluorescence imaging.

Comet imaging and DNA-damage analysis

DNA comets were visualized using a ZOE™ Fluorescent Cell Imager (Bio-Rad, USA) using the red fluorescence channel. Slides were assigned coded identifiers before microscopic analysis. Images were analyzed using Comet Assay IV software (Perceptive Instruments, UK). DNA damage was characterized using tail length, percentage of DNA in the tail (% tail DNA), and tail moment. For each treatment condition, we prepared two technical replicate slides from aliquots of the same peripheral-blood sample obtained from a single biological donor. Fifty nuclei were randomly selected and scored per slide, corresponding to 100 nuclei per treatment condition. Individual nuclei and replicate slides were considered technical subsamples and not independent biological replicates. Accordingly, the comet assay was regarded as an exploratory experiment intended to describe DNA-damage response patterns rather than to provide population-level statistical inference regarding the genotoxic potential of AGAE. No separate cytotoxicity or cell-viability assay was performed.

Oral carbohydrate tolerance tests in normoglycemic mice

The effects of AGAE on postprandial glycaemic responses were evaluated in normoglycemic mice using oral glucose, sucrose, and starch tolerance tests according to previously described procedures (23,24). Before each experiment, the

mice were fasted for 14 h with free access to water. For each test, mice were randomly allocated into three groups ($n = 6$ per group; male-to-female ratio, 1:1): a control group receiving distilled water (10 mL/kg, p.o.), an AGAE-treated group receiving the extract (400 mg/kg, p.o.), and a positive-control group. AGAE was freshly dissolved in distilled water immediately before administration. The extract was administered at a volume of 10 mL/kg body weight; therefore, the 400 mg/kg dose corresponded to an AGAE concentration of 40 mg/mL. The administered dose was calculated on the basis of the mass of recovered dry extract. Glibenclamide (2 mg/kg, p.o.) was used as the reference drug for the glucose tolerance test, whereas acarbose (10 mg/kg, p.o.) was used for the sucrose and starch tolerance tests. Baseline blood glucose was measured immediately before treatment administration (t_0) and again 30 min after treatment. The animals then received the corresponding oral carbohydrate load: D-glucose (2 g/kg) for the glucose tolerance test, sucrose (2 g/kg) for the sucrose tolerance test, or starch (3 g/kg) for the starch tolerance test. Blood glucose levels were subsequently monitored at 60, 90, and 150 min according to the experimental timeline to evaluate the postprandial glycemic response.

Acute oral toxicity study in mice

The acute oral toxicity of AGAE was evaluated in Swiss albino mice following a previously described experimental approach (24). Before administration, the animals were fasted and randomly allocated into four groups ($n = 6$ per group; male-to-female ratio, 1:1). The control group received distilled water (10 mL/kg, p.o.), whereas the treatment groups received a single oral dose of AGAE at 0.5, 1, or 2 g/kg by gavage. AGAE dosing preparations were freshly prepared in distilled water immediately before administration and were administered at a constant volume of 10 mL/kg body weight. The doses of 0.5, 1, and 2 g/kg therefore corresponded to extract concentrations of 50, 100, and 200 mg/mL, respectively. All doses were calculated on the basis of the mass of recovered dry extract. Body weight was recorded before treatment and again at the end of the 14-day observation period to assess overall body-weight evolution. Following administration, the animals were closely observed during the first 10 h for mortality and clinical or behavioral signs of toxicity and were subsequently monitored daily for 14 days for delayed signs of toxicity.

Statistical analysis

Data from the oral glucose, sucrose, and starch tolerance tests are expressed as mean $\pm$ standard error of the mean (SEM). Blood glucose concentrations measured over time were analyzed using two-way repeated-measures analysis of variance (ANOVA), with treatment as the between-subject factor and time as the within-subject factor, followed by Bonferroni-adjusted multiple-comparison tests when appropriate. Areas under the blood glucose concentration-time curves (AUCs) were calculated for each animal using the trapezoidal rule and compared among groups using one-way ANOVA followed by Dunnett's multiple-comparison test. For the comet assay, all measurements originated from a single biological donor. Individual nuclei and replicate slides therefore represented technical subsamples rather than independent biological replicates. Consequently, comet-assay data were summarized descriptively, and no inferential statistical comparisons among AGAE concentrations and controls were performed. The comet-assay findings were therefore considered exploratory. Acute toxicity outcomes, including mortality and clinical signs, were analyzed descriptively. Statistical analyses of the carbohydrate-tolerance experiments were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). For inferential analyses of the carbohydrate-tolerance experiments, differences were considered statistically significant at $p < 0.05$.

RESULTS

Exploratory assessment of DNA-damage responses in rat leukocytes

Exposure to H_2O_2 (250 μ mol/L), used as the positive assay control, produced markedly greater DNA migration than that observed in PBS-treated cells across the evaluated comet descriptors, supporting the responsiveness of the assay under the experimental conditions. Across the AGAE-treated conditions, tail length, % tail DNA, and tail moment remained broadly comparable with the negative-control values at the lower and intermediate concentrations. At the highest tested concentration (100 μ g/mL), higher tail-length and % tail-DNA values were observed relative to the negative control, whereas a corresponding increase in tail

moment was not apparent. No clear concentration-dependent pattern was observed across the three evaluated DNA-damage descriptors (Figure 1).

Effect of AGAE on oral glucose tolerance in normoglycemic mice

Oral administration of AGAE (400 mg/kg) significantly modified the glycemic response to an oral glucose challenge in normoglycemic mice. Two-way repeated-measures ANOVA revealed a significant treatment $\times$ time interaction [F (8,60) = 10.71, $p < 0.0001$], together with significant effects of time [F (4,60) = 58.04, $p < 0.0001$] and treatment [F (2,15) = 10.08, $p = 0.0017$]. Bonferroni-adjusted post hoc comparisons showed that blood glucose at 60 min was significantly lower in AGAE-treated mice than in vehicle-treated controls (1.762 vs. 2.423 g/L, $p < 0.001$), whereas no significant differences were observed at 0, 30, 90, or 150 min ($p > 0.05$). Glibenclamide significantly reduced blood glucose at 60 and 90 min ($p < 0.001$) and at 150 min ($p <$

0.05) compared with the vehicle-treated group (Figure 2A). Analysis of the area under the blood glucose concentration-time curve revealed a significant overall difference among groups [one-way ANOVA, F (2,15) = 10.17, $p = 0.0016$]. The AUC was lower in AGAE-treated mice (181.8 ± 6.0 g.min/L) than in vehicle-treated mice (217.2 ± 18.6 g.min/L); however, this difference was not statistically significant according to Dunnett's multiple-comparison test ($p > 0.05$). In contrast, glibenclamide significantly reduced the AUC to 133.2 ± 12.0 g.min/L ($p < 0.001$ vs. vehicle). These findings indicate that AGAE transiently attenuated the post-glucose glycemic peak without significantly reducing overall glycemic exposure during the observation period (Figure 2B).

Effect of AGAE on the glycemic response to oral sucrose in normoglycemic mice

Oral administration of AGAE (400 mg/kg) did not significantly alter the glycemic response to oral sucrose loading compared with the vehicle-treated

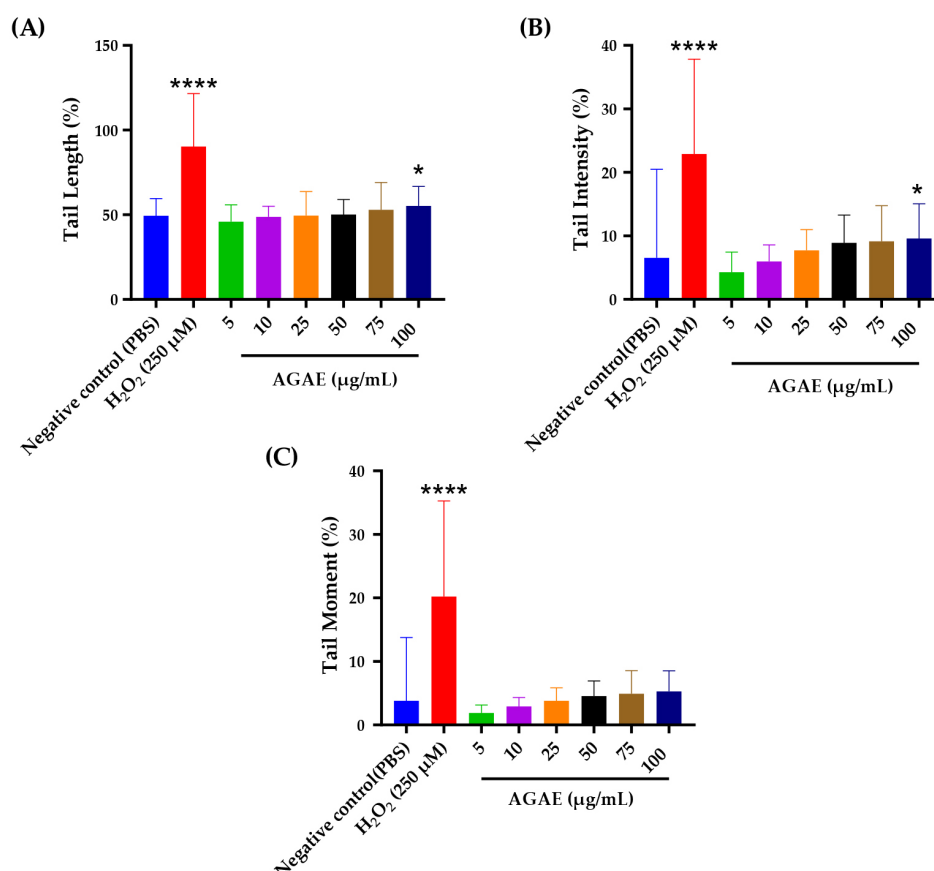


Figure 1. Effect of AGAE on DNA damage parameters in rat leukocytes assessed using the alkaline comet assay. (A) Tail length, (B) tail DNA intensity (% tail DNA), and (C) tail moment. PBS-treated cells served as the negative control, and H_2O_2 (250 $\mu\text{mol/L}$) was used as the positive control. Data are expressed as mean $\pm$ SEM

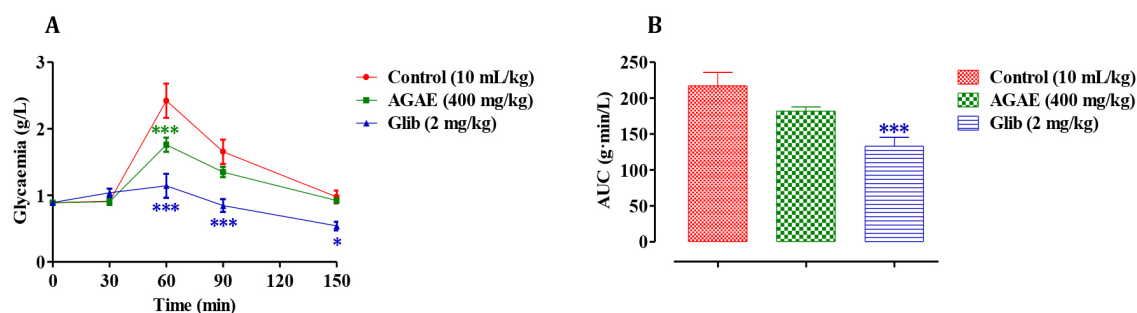


Figure 2. Effect of AGAE and glibenclamide on blood glucose responses during the oral glucose tolerance test in normoglycemic mice. (A) Blood glucose concentrations over time. (B) Corresponding area under the curve (AUC). Data are expressed as mean $\pm$ SEM ($n = 6$). Time-course data were analyzed using two-way repeated-measures ANOVA followed by Bonferroni-adjusted post hoc comparisons, whereas AUC values were analyzed using one-way ANOVA followed by Dunnett's multiple-comparison test. * $p < 0.05$ and *** $p < 0.001$ versus the vehicle-treated group.

group. Two-way repeated-measures ANOVA revealed a significant treatment $\times$ time interaction [$F(8,60) = 4.075$, $p = 0.0006$], as well as significant effects of time [$F(4,60) = 36.47$, $p < 0.0001$] and treatment [$F(2,15) = 5.271$, $p = 0.0185$]. Bonferroni-adjusted post hoc comparisons showed no significant differences between AGAE- and vehicle-treated mice at 0, 30, 60, 90, or 150 min ($p > 0.05$). In contrast, acarbose (10 mg/kg) significantly reduced blood glucose compared with the vehicle-treated group at 60 min ($p < 0.001$) and at 90 and 150 min ($p < 0.05$) (Figure 3A).

Analysis of the post-sucrose area under the blood glucose concentration-time curve revealed a significant overall difference among groups [one-way ANOVA, $F(2,15) = 5.32$, $p = 0.018$]. AGAE did not significantly reduce the AUC compared with vehicle-treated mice (144.0 ± 7.8 vs. 151.8 ± 16.2 g·min/L; Dunnett's test, $p > 0.05$). In contrast,

acarbose significantly reduced the AUC to 105.0 ± 6.0 g·min/L ($p < 0.05$ vs. vehicle) (Figure 3B).

Effect of AGAE on the glycemic response to oral starch in normoglycemic mice

Oral administration of AGAE (400 mg/kg) markedly attenuated the glycemic response to oral starch loading in normoglycemic mice. Two-way repeated-measures ANOVA revealed a significant treatment $\times$ time interaction [$F(8,60) = 6.973$, $p < 0.0001$], together with significant effects of time [$F(4,60) = 68.46$, $p < 0.0001$] and treatment [$F(2,15) = 23.61$, $p < 0.0001$]. Bonferroni-adjusted post hoc comparisons showed no significant differences between AGAE- and vehicle-treated mice before starch administration (0 and 30 min; $p > 0.05$). Following starch loading, however, blood glucose concentrations were significantly

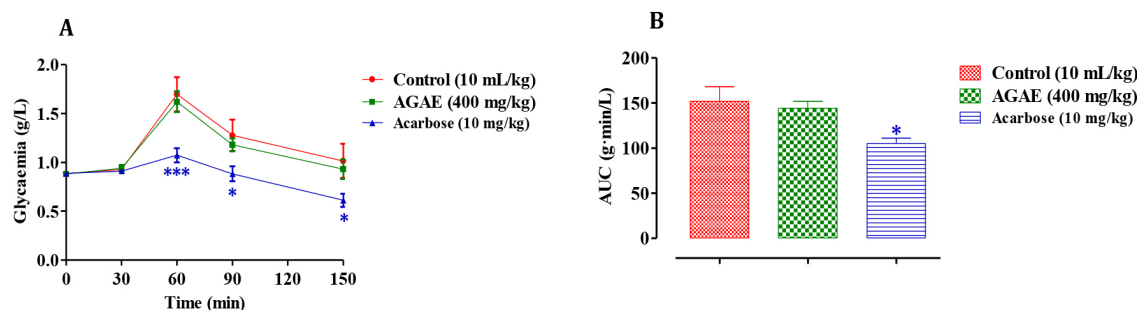


Figure 3. Effect of AGAE and acarbose on blood glucose responses during the oral sucrose tolerance test in normoglycemic mice. (A) Blood glucose concentrations over time. (B) Corresponding area under the curve (AUC). Data are expressed as mean $\pm$ SEM ($n = 6$). Time-course data were analyzed using two-way repeated-measures ANOVA followed by Bonferroni-adjusted post hoc comparisons, whereas AUC values were analyzed using one-way ANOVA followed by Dunnett's multiple-comparison test. * $p < 0.05$ and *** $p < 0.001$ versus the vehicle-treated group.

lower in AGAE-treated mice at 60 min (0.975 vs. 1.507 g/L, $p < 0.001$), 90 min (0.700 vs. 1.158 g/L, $p < 0.001$), and 150 min (0.489 vs. 0.779 g/L, $p < 0.001$). Acarbose (10 mg/kg) significantly reduced blood glucose compared with the vehicle-treated group at 90 min (0.780 vs. 1.158 g/L, $p < 0.001$), whereas the differences observed at 60 and 150 min were not statistically significant ($p > 0.05$) (Figure 4A).

Analysis of the area under the blood glucose concentration–time curve confirmed a significant overall treatment effect [one-way ANOVA, $F(2,15) = 22.39$, $p < 0.0001$]. AGAE significantly reduced the AUC from 135.38 ± 4.60 g.min/L in vehicle-treated mice to 89.75 ± 5.47 g.min/L (Dunnett's test, $p < 0.001$). Acarbose also significantly reduced the AUC to 108.30 ± 4.41 g.min/L ($p < 0.01$ versus vehicle) (Figure 4B).

Acute oral tolerability of AGAE in mice

Oral administration of AGAE at single doses of 0.5, 1, and 2 g/kg resulted in no mortality or overt clinical or behavioral signs of toxicity during the 14-day observation period. Locomotion and mobility, food and water intake, and general behavior remained apparently normal, and no piloerection, hair loss, diarrhea, abnormal agitation, anorexia, or isolation behavior was observed (Table 1). No apparent abnormality in body weight was observed between baseline and the end of the 14-day observation period. Therefore, no observable acute toxicity was detected following a single oral administration of AGAE at doses up to 2 g/kg under the present experimental conditions.

DISCUSSION

An important feature of the present study is the comparative evaluation of three distinct carbohydrate challenges using the same aqueous seed extract and a common acute experimental framework. To the best of our knowledge, previous studies have not characterized the effects of an aqueous *A. graveolens* seed extract on glucose-, sucrose-, and starch-derived glycemic responses within the same *in vivo* experimental design. The resulting pattern was not indicative of a uniform glucose-lowering action: the effect was transient following glucose administration, absent following sucrose loading, and substantially more pronounced and sustained following starch loading. This carbohydrate-dependent profile, particularly the predominant effect on starch-derived glycemia, represents the principal pharmacological observation of the present study and provides a rationale for subsequent mechanistic investigation.

Following oral glucose administration, AGAE significantly reduced blood glucose at the early post-load peak. Similar glucose-lowering effects have previously been reported for *A. graveolens* preparations. Mishra demonstrated that repeated oral administration of aqueous and ethanolic seed extracts reduced blood glucose concentrations in alloxan-induced diabetic mice (17). Abbasi Oshaghi et al. also reported a reduction in fasting blood glucose following prolonged administration of an aqueous *A. graveolens* extract in streptozotocin-induced diabetic rats, together with improvements in oxidative and glycation-related parameters (16). Although

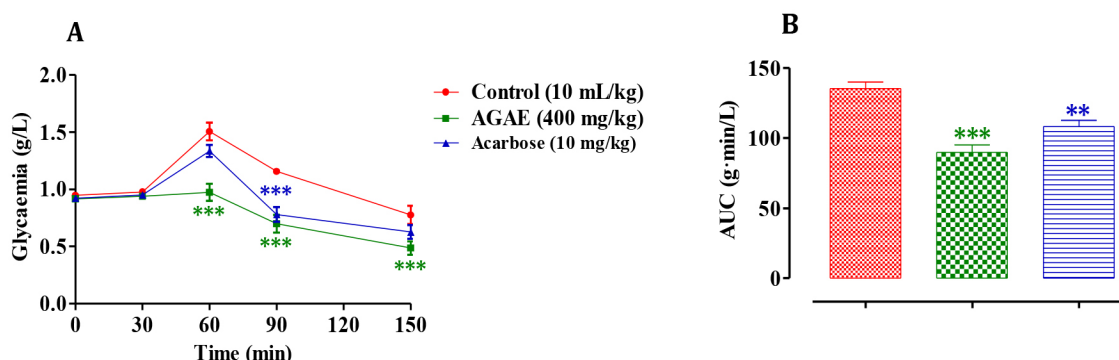


Figure 4. Effect of AGAE and acarbose on blood glucose responses during the oral starch tolerance test in normoglycemic mice. (A) Blood glucose concentrations over time. (B) Corresponding area under the curve (AUC). Data are expressed as mean $\pm$ SEM ($n = 6$). Time-course data were analyzed using two-way repeated-measures ANOVA followed by Bonferroni-adjusted post hoc comparisons, whereas AUC values were analyzed using one-way ANOVA followed by Dunnett's multiple-comparison test. ** $p < 0.01$ and *** $p < 0.001$ versus the vehicle-treated group.

Table 1. Clinical observations during the 14-day acute oral tolerability assessment of AGAE in mice.

Clinical observation	Distilled water	AGAE		
	10 mL/kg	0.5 g/kg	1 g/kg	2 g/kg
Locomotion and mobility	Normal	Normal	Normal	Normal
Piloerection or hair loss	Absent	Absent	Absent	Absent
Diarrhea	Absent	Absent	Absent	Absent
Abnormal agitation	Absent	Absent	Absent	Absent
Anorexia	Absent	Absent	Absent	Absent
Corner isolation behavior	Absent	Absent	Absent	Absent
Mortality	Absent	Absent	Absent	Absent
Food intake	Normal	Normal	Normal	Normal
Water intake	Normal	Normal	Normal	Normal
Body-weight evolution	Normal	Normal	Normal	Normal

Note: “Absent” indicates that the specified clinical sign was not observed. Body-weight gain was qualitatively recorded as normal; numerical body-weight data are not presented in this table.

these studies were conducted in diabetic animals using repeated treatment, they support the ability of aqueous preparations of *A. graveolens* to influence glucose homeostasis.

Clinical investigations have also reported metabolic effects of dill supplementation. Mobasser et al. observed changes in insulin sensitivity and lipid-related parameters following supplementation with *A. graveolens* in patients with type 2 diabetes, although fasting blood glucose was not significantly reduced (25). Haidari et al. reported improvements in insulin-related indices and several metabolic parameters after dill supplementation in patients with type 2 diabetes (26). Differences among experimental and clinical studies may reflect variations in plant material, dose, extraction procedure, treatment duration, and metabolic status.

The response observed during the oral glucose tolerance test cannot be attributed to inhibition of carbohydrate-hydrolyzing enzymes because glucose was administered directly. Processes affecting intestinal glucose uptake, gastric emptying, insulin secretion, insulin sensitivity, or peripheral glucose utilization may therefore contribute to the early reduction in glycemia (27). These mechanisms were not directly measured, and the glucose tolerance test alone does not allow discrimination among them. Nevertheless, the response to glucose indicates that the activity of AGAE is not exclusively dependent on the digestion of complex carbohydrates.

A different response was observed following sucrose administration. AGAE did not significantly reduce blood glucose at any measured

time point and did not significantly alter the post-sucrose AUC. In contrast, acarbose significantly attenuated the glycemic response, confirming the sensitivity of the experimental model to pharmacological modulation of carbohydrate digestion. Sucrose is predominantly hydrolyzed at the intestinal brush border by sucrase-isomaltase before monosaccharide absorption (28). The absence of a significant effect of AGAE in this model therefore indicates that the extract did not markedly alter sucrose-derived glycemia under the experimental conditions used.

The response to starch was substantially different. AGAE significantly reduced blood glucose concentrations at 60, 90, and 150 min and markedly decreased the post-starch AUC. The persistence of the effect at several post-load time points contrasted with the transient response observed after direct glucose administration and with the absence of a significant response after sucrose loading. Acarbose also significantly decreased the starch AUC, confirming that the experimental model was responsive to interference with carbohydrate digestion.

Starch digestion involves sequential hydrolysis by pancreatic α -amylase and intestinal brush-border enzymes before the resulting monosaccharides become available for absorption (15). However, the preferential attenuation of glycemia observed following starch loading should not be interpreted as direct evidence of α -amylase inhibition. Rather, the differential responses observed following starch, sucrose, and glucose challenges indicate a carbohydrate-dependent glycemic response and suggest that one or more processes

influencing starch-derived glycemia may be involved. These processes could occur at the level of starch digestion, subsequent oligosaccharide hydrolysis, intestinal absorption, gastric transit, or downstream metabolic regulation. The present tolerance tests do not discriminate among these possibilities. Direct enzymatic assays and complementary intestinal and metabolic measurements are therefore required before a specific mechanism can be established.

Previous phytochemical investigations provide a basis for considering interactions between dill constituents and carbohydrate digestion. Hadi et al. characterized extracts and decoctions prepared from *A. graveolens* seeds from Morocco and identified numerous secondary metabolites, including phenolic and flavonoid constituents (29). Compounds reported included umbelliferone, 3-hydroxyflavone, coumarin-related constituents, and other phenolic molecules. The use of a seed decoction in that investigation is relevant to the aqueous preparation evaluated here.

Jang et al. characterized a water extract of *A. graveolens* seeds by UHPLC-MS/MS and identified several phenolic acids and flavonoids, including caffeic acid, caffeoylquinic acids, rutin, and derivatives of quercetin, kaempferol, and isorhamnetin (30). Belabas et al. also reported the presence of phenolic compounds, including chlorogenic acid, rutin, and kaempferol, in an ethanolic *A. graveolens* seed extract (13). Although extraction procedures strongly influence qualitative and quantitative phytochemical profiles, these studies demonstrate that dill seeds contain several classes of compounds capable of interacting with metabolic and digestive processes.

Phenolic compounds and flavonoids have been extensively investigated for their effects on starch digestion. Sun et al. described the ability of numerous plant polyphenols to inhibit α -amylase activity, with the magnitude of inhibition depending on molecular structure and interactions with the enzyme (31). Martínez-González et al. further demonstrated structure-dependent inhibition of α -amylase by several flavonoids (32). Such interactions may involve hydrogen bonding and hydrophobic interactions with regions involved in enzyme activity (32). Chlorogenic acid, previously identified in *A. graveolens* seed preparations, has also been shown to interact with pancreatic α -amylase. Zheng et al. reported that chlorogenic acid

inhibited porcine pancreatic α -amylase through a mixed-type mechanism and induced changes in enzyme conformation (33). Caffeoylquinic acids and related phenolic compounds have likewise been identified in aqueous dill seed extracts (30). These findings provide possible mechanistic hypotheses for future investigation; however, they cannot be directly extrapolated to the specific AGAE preparation evaluated in the present study. However, the phytochemical composition of the specific extract evaluated in the present experiments was not characterized, and the observed effect cannot be assigned to an individual compound or chemical class.

Importantly, the phytochemical profiles reported in previous studies should not be considered representative of the specific AGAE batch evaluated in the present study. The qualitative and quantitative composition of botanical extracts may vary according to plant origin, plant material, processing conditions, storage, and extraction procedure (34). Although the extraction procedure, dry-extract yield, storage conditions, and administered dry-extract doses were defined in the present study, the specific experimental batch was not subjected to chromatographic fingerprinting, quantitative phytochemical characterization, or determination of individual marker compounds. Furthermore, no formal stability study was performed. Consequently, the observed pharmacological effects cannot presently be attributed to any specific constituent or chemical class, and chemical equivalence with other *A. graveolens* preparations cannot be assumed. Future studies should therefore incorporate chromatographic fingerprinting, quantitative marker-based standardization, batch-to-batch comparison, and formal stability assessment.

The contrasting responses to sucrose and starch further support a carbohydrate-dependent glycemic response to AGAE. However, the present tolerance tests do not identify the biological process responsible for this differential pattern. The more pronounced response following starch loading could involve digestive, absorptive, gastrointestinal, hormonal, or metabolic processes, either individually or in combination. Therefore, the present findings should not be interpreted as evidence of a specific action on starch digestion or on a particular carbohydrate-hydrolyzing enzyme.

The comet assay provided useful preliminary information regarding the DNA-damage

response of rat peripheral-blood leukocytes exposed *ex vivo* to AGAE. Across the tested concentrations, AGAE did not produce a clear concentration-dependent increase in the evaluated DNA-damage parameters. Tail length, % tail DNA, and tail moment remained broadly comparable with the negative-control values at the lower and intermediate concentrations. At 100 µg/mL, higher values of tail length and % tail DNA were observed, whereas tail moment remained largely unchanged. Taken together, these findings indicate that AGAE did not produce a consistent pattern of DNA damage under the experimental conditions used. The response of the H₂O₂ positive control further confirmed the ability of the assay to detect DNA-damaging effects. Previous investigations of aqueous and hydroalcoholic dill preparations have reported considerable antioxidant activity associated with their phenolic constituents (13,29,30). Such properties may contribute to the biological behavior of these preparations, although antioxidant activity cannot by itself be considered evidence of absence of genotoxicity. However, because the experiment was based on blood obtained from a single biological donor, the technical replicate slides and individual nuclei do not provide independent biological replication. Therefore, the comet assay findings should be regarded as preliminary, and confirmation using independent biological donors is required before conclusions regarding the genotoxic potential of AGAE can be established. In addition, no separate cytotoxicity or cell-viability assay was performed; therefore, potential cytotoxic effects at the tested concentrations cannot be excluded and should be assessed in future confirmatory experiments.

The toxicological profile of aqueous preparations should also be distinguished from that of chemically different preparations such as essential oils. Lazutka et al. reported genotoxic and cytotoxic effects of dill essential oil in experimental systems (35). Essential oils are enriched in volatile lipophilic constituents and differ substantially in composition from aqueous seed extracts (10). Consequently, toxicological findings obtained with essential oils cannot be directly extrapolated to AGAE.

The acute oral toxicity experiment showed no mortality or overt clinical or behavioral signs after single administration of AGAE at 0.5, 1, or 2 g/kg during the 14-day observation period.

The highest tested dose was fivefold greater than the 400 mg/kg dose used in the carbohydrate tolerance experiments. Belabas et al. similarly reported an absence of overt acute toxicity following administration of an ethanolic *A. graveolens* seed extract at a high oral dose (13). Hosseinzadeh et al. investigated aqueous and ethanolic seed extracts and reported relatively low acute toxicity, although the LD₅₀ values in that study were determined following intraperitoneal administration and are therefore not directly comparable with oral administration (36). The available findings nevertheless indicate relatively good acute tolerability of several dill seed preparations while emphasizing the influence of extraction procedure and route of exposure.

Overall, AGAE produced a carbohydrate-dependent glycemic response characterized by a transient effect following glucose administration, no significant effect following sucrose loading, and a pronounced reduction in starch-derived glycemia. Previous phytochemical investigations of *A. graveolens* seeds have demonstrated the presence of phenolic acids, flavonoids, coumarins, and related constituents, several of which belong to chemical classes known to modulate carbohydrate digestion. The pronounced response observed following starch administration provides a basis for future mechanistic investigation, but the present experiments do not establish whether this effect arises from digestive, absorptive, gastrointestinal, hormonal, or downstream metabolic processes.

Further studies should include chromatographic characterization and standardization of AGAE, followed by direct assays of pancreatic α -amylase, intestinal α -glucosidases, and intestinal glucose transport. Bioassay-guided fractionation could help identify the constituents associated with the effect on starch-derived glycemia. Evaluation of different doses and confirmation in diabetic animal models would clarify the pharmacological relevance of the acute responses observed in normoglycemic mice. Repeated-dose toxicity studies incorporating biochemical, hematological, histopathological, and reproductive endpoints should be complemented by confirmatory genotoxicity experiments using several independent biological donors, predefined experimental units, appropriate cytotoxicity or cell-viability measurements, and statistical analyses based on biologically independent replicates.

CONCLUSIONS

The aqueous seed extract of *Anethum graveolens* L. produced a distinct carbohydrate-dependent pattern of acute glycemic responses in normoglycemic mice. The effect was transient following glucose administration, was not significant following sucrose loading, and was most pronounced following starch administration, where both blood glucose concentrations and overall glycemic exposure were significantly reduced. These comparative findings provide preliminary in vivo evidence that the pharmacological response to AGAE depends on the carbohydrate challenge, but they do not establish the underlying molecular mechanism. Single oral doses up to 2 g/kg produced no mortality or overt clinical signs under the tested conditions. In the exploratory *ex vivo* comet assay, AGAE did not produce a clear concentration-dependent pattern of DNA damage across the evaluated parameters under the experimental conditions used. Further studies should include chromatographic fingerprinting, quantitative determination of appropriate marker compounds, batch-to-batch comparison, and formal stability assessment to achieve rigorous chemical standardization of AGAE, followed by direct assays of pancreatic α -amylase, intestinal α -glucosidases, and intestinal glucose transport and confirmation of its pharmacological effects in appropriate diabetic models.

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