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Effect of Sustained Systemic Administration of Ginger (*Z officinale*) Rhizome Extracts on Salivary Flow in Mice

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ABSTRACT

Objective: This study aimed to evaluate the effect of methanol (70% v/v), ethanol (80% v/v), dimethyl sulfoxide (DMSO; 100% v/v) extracts of ginger rhizome (GR), and 6-shogaol on the pilocarpine-stimulated salivary flow rate in C57BL/6 mice.

Methods: Three extracts of ginger (*Zingiber officinale*) rhizome prepared by maceration using the respective solvents and 6-shogoal were reconstituted in normal saline with 0.2% DMSO. Thirty C57BL/6 15-week-old mice were divided into 5 groups: Group 1, saline; Group 2, 70% methanol extract; Group 3, 80% ethanol extract; Group 4, 100% DMSO extract; and Group 5, 6-shogaol. The baseline pilocarpine-stimulated salivary flow rate was measured at the age of 15 weeks (15th week), and treatment solutions were administered by intraperitoneal injection from the 16th to 18th week. The stimulated salivary flow rate during treatment weeks was recorded for each group, and its difference with baseline was analysed using paired-sample *t* test. The change in salivary flow rate between the treatment groups and the control group was analysed using one-way analysis of variance.

Results: Groups 2, 3, 4, and 5 showed a significant increase in salivary flow rate when compared to baseline ($P < .05$). The increase in salivary flow rate in all 4 treatment groups was significant when compared to the control group ($P < .05$). Group 4 produced the highest increase in salivary flow rate; however, the differences amongst the treatment groups did not reach statistical significance ($P > .05$).

Conclusions: All GR extracts (70% methanol, 80% ethanol, 100% DMSO) and 6-shogaol were equally effective in increasing the pilocarpine-stimulated salivary flow rate in C57BL/6 mice when administered systemically as a sustained dose for 3 weeks.

Introduction

Saliva in the oral cavity plays an important role in mastication, swallowing, and speech.[1] Hyposalivation, an objective reduction in the quantity of saliva, may lead to complications such as dysphagia, dysgeusia, halitosis, lingual papillae atrophy, burning sensation on the tongue, increased risk of oral infections, and dental caries.[2]

Hyposalivation is seen commonly in menopausal women and geriatric patients. It arises from various causes such as head and neck radiotherapy, medications, Sjogren's syndrome, insulin-dependent diabetes, and depression.[3] Apart from pathologic causes, hyposalivation has also been associated with aging but the association remains controversial today.[4-8] Few animal studies have identified certain mechanisms involved in hyposalivation including salivary cytokine dysregulation, oxidative stress associated with aging, and

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down-regulation of aquaporin 5 (AQP5), which is a water channel protein that is essential for the normal volume of salivation.[4,9-12]

The complications of hyposalivation such as oral discomfort, infection, and dental caries can be devastating to patients who experience it.[13, 14] Current treatment modalities for this condition include local stimulation, systemic stimulation provided by pharmacologic agents such as pilocarpine and cevimeline, as well as saliva substitutes in the forms of liquid, spray, and gel.[15,16] Unfortunately, these agents need to be used continuously and often produce adverse effects like dizziness, excessive sweating, and dyspepsia.[17,18]

Some have investigated traditional remedies using plant extracts as alternative hyposalivation management.[19-22] Ginger rhizome (GR) is one of the promising candidates which has been traditionally used to treat a wide range of diseases such as motion sickness, arthritis, and gastrointestinal disorders.[23,24] Recent studies have also shown that it has antioxidant, anticancer, anti-inflammatory, antiapoptotic, antihyperglycemic, antihyperlipidemic, and antiemetic actions.[25-27] In the context of hyposalivation, an animal experiment demonstrated that 80% ethanol GR extract was uniquely effective in increasing the salivary flow in rats compared to the other 6 types of herbal extracts tested in that study.[28] Other clinical trials have also shown that the administration of ginger infusion and ginger extracts improved the salivary flow in patients who experienced xerostomia due to pathologic causes such as diabetes mellitus, radiation, and smoking.[29-31] Whilst a majority of studies have demonstrated the an enhancing effect of ginger on salivary flow, one animal experiment that used 70% methanol GR extract failed to show a positive effect, and it was suggested to be caused by the variation in the extraction process.[32] Different extraction methods can affect the composition of the resultant extract, which can in turn produce varying effects on the salivary flow rate.[33-37] One of the objectives of the present study was to validate the conflicting results on methanol and ethanol extracts of GR on salivary flow rate by evaluating the effect of 70% methanol GR extract and 80% ethanol GR extract made by a standardised extraction process on the salivary flow rate of C57BL/6 mice.

On the other hand, being a dipolar aprotic solvent, dimethyl sulfoxide (DMSO) has been proved as an effective solubilising agent in cell culture assays when investigating natural substances. It has been added to plant extracts before applying to the cell culture medium to facilitate solubilisation of nonpolar molecules.[38,39] Although DMSO is not commonly used to make plant extracts, it has been found to provide an efficient means of extracting chlorophyll from intact plant tissue.[40] Therefore, DMSO may have the potential of effectively extracting the bioactive compounds from GR which could also affect the outcome. Thus, the present study also aimed to evaluate the effect of 100% DMSO extract of GR on salivary flow in C57BL/6 mice and compare it with the salivary flow rates of the mice treated with 70% methanol and 80% ethanol extracts of GR.

Whilst natural plant extracts have gained much attention in becoming a major source for new drug development, isolation and purification of active ingredients could maximise the therapeutic benefit. In ginger, phenolic compounds such as gingerols and shogaols have been shown to have a therapeutic role in disease control via modulation of various biological activities such as anticancer, anti-inflammatory, antimicrobial, and antioxidant activities.[41-44] One of its bioactive compounds, 6-shogaol, has been of interest in the present study because of the increased proportion of shogaols in dried ginger due to the conversion of gingerols to shogaols during the drying process,[34,45] and 6-shogaol has been found to be a more potent bioactive compound than gingerols in terms of the antioxidant and free radical–scavenging potentials.[46,47] The latter correlates well with the oxidative stress mechanism of hyposalivation.[9] Therefore, the third objective of this study was to evaluate the effect of 6-shogaol on the salivary flow rate in C57BL/6 mice.

Material and methods

GR extract preparation

Ginger (*Z officinale*) rhizomes of were collected from Bentong, Pahang, Malaysia (3°21′17.8″N 101°48′51.3″E) in October 2019. They were authenticated taxonomically at the herbarium of University Kebangsaan Malaysia, and a specimen was deposited in the herbarium with the voucher number ID023/2020. The plants were carefully washed with distilled water to remove dirt and soil. Then, they were sliced into a standard thickness of 2 mm and shade-dried for 3 days. Maceration was done by immersing 20 mg of dried ginger slices in 100 mL of respective solvents (70% methanol, 80% ethanol, and 100% DMSO) for 48 hours at room temperature. It was noted that 70% methanol and 80% ethanol were chosen as the solvents in this experiment to validate the discrepancy between their effects on salivary flow as demonstrated in previous studies.[28,32] The potent solubilising agent 100% DMSO was included in the experiment as a solvent to explore its effectiveness in extracting bioactive compounds from GR.[38-40] After maceration, the extracts were filtered using 110 mm qualitative filter papers (Advantec) and evaporated at 50 °C with a rotary evaporator (Buchi).[28] The concentrated extracts solutions were subjected to the freeze-drying process using a freeze dryer (LabConCo) and the resultant dried powder extracts were stored in the freezer at less than −81 °C until they were used for analysis. To prepare the injection solution, the extracts were weighed and dissolved in the base solution of saline with 0.2% DMSO to obtain a final concentration of 0.3 mg/mL. The potent solubilising vehicle DMSO at a concentration of 0.2% v/v was specified with normal saline as a background medium for all testing in the present study.[48-50]

Analysis of major bioactive compounds in GR extracts

Analysis of all 3 GR extracts (70% methanol, 80% ethanol, and 100% DMSO) for the major bioactive compounds (6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol) was done using a high-performance liquid chromatography (HPLC) 1260 system (Agilent Technologies, Inc.) equipped with an auto-injector and diode array detector (DAD). All the HPLC solvents and reagents were purchased from ThermoFisher. Ultra-pure

water was obtained using the UV Water Purification system (Sartorius).

Then, 70% methanol, 80% ethanol, and 100% DMSO dried extracts of GR were individually weighed and dissolved in HPLC-grade methanol by sonicating for 30 min to prepare a 1-mg/mL stock solution for each extract. The solution was filtered through a 0.22-μm filter. The stock solution was further diluted with HPLC-grade acetonitrile to produce the required concentrations before injecting into the HPLC system. For each extract, 20 μL of the solution were injected into HPLC.

All the standards were purchased from ChromaDex. The standards were dissolved in HPLC-grade acetonitrile to obtain the required concentrations before they were injected into the HPLC system. The separation was carried out on ODS GENESIS C18 (250 $\times$ 4.6 mm, 5 μm, Hichrom Ltd) using a solvent system composed of 0.1% formic acid aqueous solution (A) and acetonitrile (B) at a flow rate of 1.0 mL/min. The gradient elution was carried out as follows: 0 to 5 minutes, 50% to 60% B; 5 to 18 minutes, 60% to 78% B; 18 to 29.5 minutes, 78% to 78% B; 29.5 to 30.5 minutes, 78% to 100% B; 30.5 to 38 minutes, 100% to 100% B, 22 to 32 minutes, 100% to 100% B. The quantification of bioactive compounds was carried out by measuring the absorbance at 282 nm.

6-Shogaol preparation

To prepare the injection solution, 6-shogaol (Merck) was weighed and dissolved in the base solution of saline with 0.2% DMSO to obtain a final concentration of 0.12 mg/mL. All the injection solutions (70% methanol extract, 80% ethanol extract, 100% DMSO extract, and 6-shogaol) were kept in dark containers at room temperature.

Mice husbandry

This animal study was approved by the University Joint Committee of the Research and Ethics Committee (grant number: BDS I-01–2019(18)) on March 28, 2019. The experiment was performed in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.[51,52] A priori sample size was determined by performing Student *t* test on the pilot study. With a 5% significance threshold, 80% statistical power, expected effect size of 2.13, and standard deviation of 1.31, the sample size calculated was 6 per arm.

Thirty well-characterised 10-week-old C57BL/6 mice (12 males and 18 females) were purchased from Brain Research Institute Monash Sunway (Monash University, Selangor, Malaysia). Their weight ranged from 17.5 g to 24.3 g with an average of 20.4 g at that age. They were housed in a well cross-ventilated room with a controlled temperature of 25 °C and humidity of 40% under a light-dark cycle of 12 hours at the animal house facility, International Medical University (Kuala Lumpur, Malaysia). The mice were kept in ventilated cages and provided with standard mice pellets and purified reverse-osmosis water. They were distributed randomly in such a way that each cage contained 5 mice from 5 different groups.

Saliva collection

In this study, the pilocarpine-stimulated salivary flow was measured. When the mice reached the age of 15 weeks, saliva was collected following an intraperitoneal injection of pilocarpine (Sigma-Aldrich) at 5 mg/kg body weight and recorded as baseline saliva.[10,53-55] The saliva collection method was modified from a previous study.[55] The mouse was placed into a 5-mL conical tube with the end carefully removed to just allow head protrusion. The saliva was collected into a pre-weighed microcentrifuge tube (Eppendorf) using a suction device for 5 min. After the saliva collection, the tube was weighed and recorded. The salivary flow rate (mg/min) was calculated by dividing the weight of saliva secreted by 5 min. The researcher who did the saliva collection and data recording was blinded to the mice labelling and the administration of solutions.

Animal treatment

There were 6 mice in each group (n = 6). After baseline saliva collection was done when the mice were at 15 weeks old (15th week), the treatment began at the 16th week and continued for 3 weeks, including the 18th week. The 3 extracts and 6-shogaol were administered to the respective treatment groups 3 times a week on alternate days (Monday, Wednesday, and Friday) by intraperitoneal injection using a 26-gauge, 0.5-inch needle and 1-mL syringe (Terumo). The dose of administration of GR extract was 10 mg/kg body weight, whereas the dose of 6-shagaol was 4 mg/kg body weight. As for the control group, the sham procedure was performed by administrating saline with 0.2% DMSO in a similar fashion as the treatment groups. The pilocarpine-stimulated saliva was measured every Saturday morning from the 16th to the 18th week, as described in the previous section.

Data analysis

As the salivary flow rate was recorded specifically for each mouse, each mouse was acting as its negative control prior to the intervention. To evaluate the effect of GR extracts and 6-shogaol on salivary flow for each group, the salivary flow rate during the treatment weeks (mean of 16th to 18th week) was compared with the baseline (15th week) in each group. Paired-sample *t* test was used to analyse whether there was a significant difference between pretreatment and treatment weeks.

Second, the change in salivary flow rate of each mouse was calculated from the difference between the baseline salivary flow rate (15th week) and the mean salivary flow rate during the treatment weeks (16th to 18th week). The mean change in salivary flow was obtained for each group. One-way analysis of variance (ANOVA) with post hoc Tukey's honestly significant difference (HSD) test was used to analyse the differences in mean change in salivary flow rate amongst all 5 groups.

Statistical analysis was carried out using IBM SPSS Statistics software for Windows, version 25.0 (IBM Corp. *P* values $<.05$ were considered to indicate statistical significance.

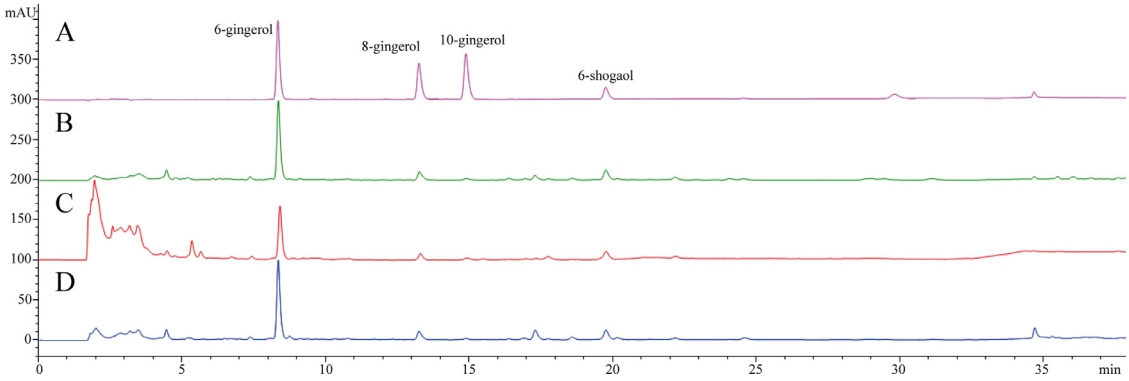

Fig. 1 – A, High-performance liquid chromatography (HPLC) chromatogram of reference standards. B, HPLC chromatogram of 100% dimethyl sulfoxide ginger rhizome (GR) extract. C, HPLC chromatogram of 70% methanol GR extract. D, HPLC chromatogram of 80% ethanol GR extract.

Results

Identification of 6-, 8-, and 10-gingerols and 6-shogaol in GR extracts

The HPLC analysis of the 3 GR extracts (70% methanol, 80% ethanol, and 100% DMSO extracts) was carried out with standard compounds of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol (Figure 1). As shown in the chromatograms of all extracts, the 4 peaks coinciding with the standards indicate the presence of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol in them. Among them, 6-gingerol was the most abundant compound in all 3 GR extract while the other 2 gingerols and 6-shogaol were present in small quantities.

Effect of GR extracts on salivary flow in mice

In the control group (Group 1), 4 (67%) mice showed a decrease in salivary flow rate and 2 mice showed an increase in salivary flow rate as they aged from 16 weeks old to 18 weeks old. In contrast, the majority of the mice in the treatment groups (Group 2 to 5) showed an increase in salivary flow rate during the same period: 5 out of 6 (83%) mice in Group 2, 5 out of 6 (83%) mice Group 3, all 6 mice (100%) in Group 4, and 5 out of 6 (83%) mice in Group 5. The mean salivary flow rates at baseline (15th week) and treatment weeks (16th to 18th week) are shown in the Table, along with the mean change in salivary flow rate for each group. All the treatment groups showed increase in mean change in salivary flow rates, whereas the opposite is true for the control group, which showed the smallest mean change in salivary flow rate (−1.95 mg/min). Group 4 (100% DMSO GR extract) showed the highest increase in salivary flow rate amongst all groups.

When the mean salivary flow rate during treatment weeks of Groups 2 to 5 were compared with their baseline salivary flow rate using paired sample t-test, there was a statistically significant differnce (Group 2: P = .019, Group 3: P = .021, Group 4: P = .007, and Group 5: P = .011) The control group (Group 1) did not show a statistically significant difference between its baseline and treatment weeks' salivary flow rate (Figure 2).

The difference in the mean change in salivation between the groups was analysed with one-way ANOVA followed by post hoc Tukey's HSD test. Statistical analysis showed that the increase in salivary flow in Group 2, 3, 4 and 5 was statistically significant when compared with the control group (Group 2: P = .048, Group 3: P = .035, Group 4: P = .007, and Group 5: P = .037). Although Group 5 showed the highest increase in salivary flow rate, there was no statistically significant difference amongst the 4 intervention groups ($P > .05$; Figure 3).

Table – Stimulated saliva secretion of mice.

Experimental treatment	No. of mice	Mean of salivary flow rate (mg/min) ± SD		
		Week 15	Average of weeks 16 to 18	Change in salivary flow rate[a]
Control	6	23.95 ± 4.39	22.00 ± 2.24	−1.95 ± 5.61
70% methanol GR extract	6	21.42 ± 2.90	27.22 ± 5.97	5.80 ± 4.14
80% ethanol GR extract	6	21.39 ± 7.10	27.56 ± 5.21	6.17 ± 4.56
100% DMSO GR extract	6	20.86 ± 7.32	28.80 ± 5.34	7.94 ± 4.42
6-shogaol	6	20.23 ± 3.49	26.35 ± 2.77	6.12 ± 3.79

Saliva secretion of mice in 5 min after injection of pilocarpine at week 15 (baseline) and the average of 16 to 18 treatment weeks for the control and 4 treatment groups. Mean data for each group were shown.

[a] The change in salivary flow rate was calculated from the difference between week 15 and the average of week 16 to week 18.DMSO, dimethyl sulfoxide; GR, ginger rhizome.

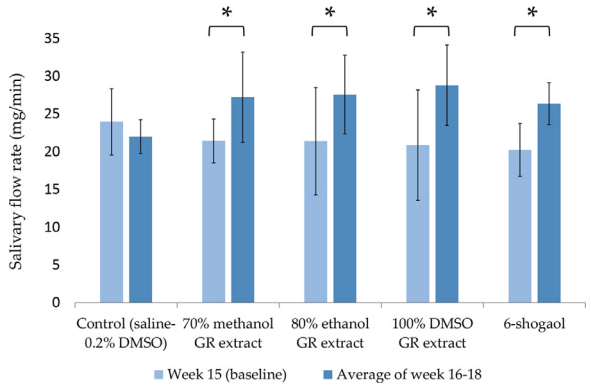

Fig. 2 – Effect of ginger rhizome (GR) extracts and 6-shogaol on salivary flow in mice. The salivary flow rate at the 15th week (baseline) and the mean of 16th to 18th week in the control and the intervention groups (n = 6). All data are mean ± SD. *P < .05, paired-sample t test.

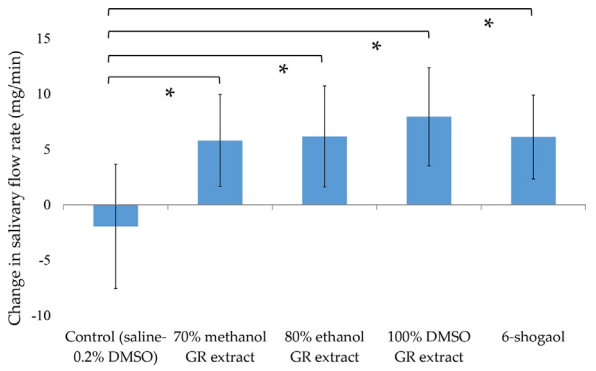

Fig. 3 – Effect of ginger rhizome (GR) extracts and 6-shogaol on salivary flow in mice. The changes in salivary flow rate between the 15th week (baseline) and the mean of 16th to 18th week in the control and intervention groups (n = 6). All data are mean ± SD. *P < .05, one-way analysis of variance with post hoc Tukey's honestly significant difference test.

Discussion

Reduced salivary flow may have repercussions in the oral cavity and could impact the quality of life. Over the past few decades, herbal medicines have gained considerable attention when exploring alternative treatment modalities for hyposalivation.[20-22] The present study aimed to explore the possibility of different extracts of GR in producing sustained improvement in the salivary flow of mice under standardised settings.

Based on the results, the 3 extracts of GR and 6-shogaol increased the mice's salivary flow significantly, whereas the control group mice experienced a decrease in salivary flow on average. According to the result found in the control group, the reduction in salivary flow is consistent with previous findings that a gradual reduction in salivary flow was seen in young mice at the age of 6 weeks old to 24 weeks old. The researchers hypothesised that age-related hypermethylation of the *AQP5* gene would lead to downregulation of the *APQ5* protein that is essential for the normal secretion of saliva.[10] However, the increase in salivary flow rate in the treatment groups suggests that the active compound in the treatment groups could compensate for the age-related hyposalivation and produce additional salivary flow compared to baseline values.

All the treatment groups showed a sustained significant increase in salivary flow in mice. The previous experiment on animals demonstrated the stimulatory effect of ginger on saliva secretion by measuring the salivary flow rate immediately following administration of the ginger extract.[28] This saliva stimulation was hypothesised to be attributed to the cholinergic effect of ginger , as it was previously shown that ginger has a parasympathomimetic effect on the postsynaptic M3 muscarinic receptors and also a possible repressive effect on presynaptic muscarinic autoreceptors, which are found in salivary glands.[56,57] Interestingly, the current study showed that systemic administration of ginger extract could cause a sustained positive effect on the salivary flow in mice. This was demonstrated by the study design in which saliva was collected 1 day after the administration of the extracts. The interval of 1 day should have allowed for cessation of the transient cholinergic effect of ginger constituents as described in the previous study.[28] Therefore, the sustained increase in salivation may result from mechanisms other than the transient cholinergic effect of ginger's constituents. One of the possible actions of ginger's constituents is the scavenging of free radicals.[58,59] The antioxidant property may be able to reduce oxidative stress in the salivary gland, which was shown to be one of the factors that causes hyposalivation.[9,60,61] Similar outcomes were also found in human studies, which demonstrated improved salivary flow after long-term intake of ginger extract.[29-31]

In the present study, 70% methanol extract of GR was able to increase salivary flow significantly, which was in conflict with the animal study done by Chamani et al.[32] It is possible that the disparity in the extraction process could have contributed to the ineffectiveness of 70% methanol ginger extract in the previous study. The concentration of methanol ginger extract used in the previous study was 100 mg/kg body weight, which was 10-fold the current study's extract concentration (10 mg/kg body weight). This difference in ginger extract concentration may have caused an alteration in its properties as studies have proved that ginger exhibits both cholinergic and anticholinergic properties.[56,62] Hence, it is plausible that at higher concentrations, ginger extract's anticholinergic properties become more pronounced, causing a negative result.

We hypothesised that 6-shogaol in GR extracts has a major contributory role in enhancing salivary flow. First, studies have shown that the proportion of 6-shogaol in ginger increased after the conversion of gingerols to shogaols in the drying process.[34,45] Second, 6-shogaol is a more potent bioactive compound than gingerols in terms of the antioxidant activity and oxygen-scavenging potential.[9,46,47,60,61] In accordance with those facts, the present study also demonstrated that 6-shogaol was similarly effective in improving salivary

flow amongst the experimental groups. However, the analysis of GR extract showed that 6-shogaol was present in a rather small amount, whilst the highest proportion was 6-gingerol. This can be explained by the poor conversion of gingerols to shogaols due to the present study's drying process at room temperature, which can be rectified by using higher temperature, as shown in previous studies.[34,45,63] Despite the HPLC analysis showing the highest abundance of 6-gingerol in the GR extracts, it cannot be conclusively proved that it was the main contributory agent to salivary flow enhancement due to the likelihood of synergistic effect between different molecules in the extracts.

The current study has 2 potential strengths. First, it is unique as it investigated healthy participants who may have experienced age-related hyposalivation, whereas previous trials were conducted amongst participants associated with pathologic conditions such as smoking, diabetes mellitus, and postradiation complications.[29-31] Thus, it may be possible that GR can benefit elderly individuals who experience hyposalivation unrelated to pathologic conditions. Second, ginger extraction using 100% DMSO as a solvent has not been carried out before. The current study showed that the process was feasible, and 100% DMSO can be used as an effective solvent due to its excellent solubilising ability when it comes to extracting complex macromolecules from plants. The weakness of this study was the absence of a negative control group that might capture any effect 0.2% DMSO vehicle had on the salivary flow of the mice. Besides, the mechanism contributing to the increase in salivary flow is unknown. To explore the mechanism at play, future studies would be valuable to evaluate the effect of GR extracts on the expression of specific genes such as *AQP5* in salivary glands of mice, the oxidative stress, and salivary cytokine profile. In addition, future animal experiments may be carried out by administering GR extracts via the oral route. If it is proved to be effective, it will be a more feasible method of administration when exploring the alternative treatment for hyposalivation in human trials.

Conclusions

All GR extracts (70% methanol, 80% ethanol, 100% DMSO) and 6-shogaol were equally effective in increasing the pilocarpine-stimulated salivary flow rate in C57BL/6 mice when administered systemically with sustained doses for 3 weeks.

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