



## Original Article

# Novel effects of rooibos extract on tear and saliva secretion mediated by the muscarinic acetylcholine receptor 3 in mice

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## ABSTRACT

**Objectives:** Sicca syndrome is characterized by dry mouth and eyes and results in a reduction of the patient's quality of life. Various natural plants, including certain herbs, have long been employed to alleviate such symptoms. Rooibos grown in South Africa is one of the potent herbal plants used for the treating dry mouth. However, the precise mechanism of action by which rooibos alleviates symptoms of dryness remains unclear.

**Methods:** The *in vivo* effects of rooibos extract (RE), which comprises eriodictyol-6-C-glucoside, on the secretory function of saliva and tears were analyzed after intraoral RE administration using wild-type C57BL/6 (B6) mice. In addition, the mechanisms of RE were investigated after administration of a muscarinic acetylcholine receptor 3 (M3R) antagonist.

**Results:** Tear and saliva volumes in mice increased significantly and in a dose-dependent manner following intraoral RE administration compared to those in mice in the control group administered H<sub>2</sub>O. An experiment performed using darifenacin administration revealed that the effects of RE on secretory function were exerted via M3R.

**Conclusion:** These results suggest that RE administration is an effective treatment for symptoms of dryness and may be used in clinical settings against sicca syndrome.

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## 1. Introduction

*Aspalathus linearis* (rooibos), a herbal plant grown in South Africa, has become popular as a health tea and cosmetic ingredient [1]. Rooibos tea is caffeine-free, low in tannins, and contains a variety of minerals and polyphenols. There is increasing evidence suggesting that rooibos has preventive effects against certain metabolic syndromes, including diabetes mellitus, obesity, and hypertension [2–8]. Any of the components of rooibos may play a potent role in antioxidative reactions in the body [9]. However, the effects of rooibos on homeostatic functions remain unclear.

Xerostomia, defined as dry mouth and eyes, is induced by various disorders and factors, including Sjögren's syndrome, mucocle, infections, salivary gland tumors, drug and radiotherapy side effects, aging, and mental stress [10]. Herbal tea, including rooibos tea, is generally known to remedy thirst, and may stimulate saliva and tear secretion [11,12].

Recently, eriodictyol-6-C-glucoside (E6CG) was identified as a component of rooibos extract (RE) that activates muscarinic acetylcholine receptor 3 (M3R) *in vitro* [13,14]. In addition, a clinical study indicated that E6CG significantly improved mouth, eye, and skin dryness in humans [12]. However, the mechanisms via which E6CG enhances saliva and tear secretions *in vivo* remain unclear. Experimental confirmation of secretion mechanisms in humans is complex and studies using mice should contribute to understanding these effect. Currently, food containing RE is considered as a “food with functional claims”.

In this study, the *in vivo* effects of ER, including the effects of E6CG on saliva and tear secretion, were analyzed in wild-type mice.

Abbreviations: E6CG, eriodictyol-6-C-glucoside; M3R, muscarinic acetylcholine receptor 3; ANOVA, one-way analysis of variance; PBS, phosphate buffered saline; RE, rooibos extract; SEM, standard error of mean.

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In addition, the secretory mechanisms of E6CG-simulated exocrine glands were evaluated *in vivo* to investigate the potential effects of this novel functional component extracted from rooibos on mouth and eye dryness.

## 2. Materials and methods

### 2.1. Animals

Male C57BL/6 mice aged 8 weeks were purchased from the Japan SLC Laboratory (Shizuoka, Japan) and maintained in a specific pathogen-free colony with food and water provided *ad libitum*. The mice were fasted 15–18 h prior to experiments. All animal experiments were approved by the animal research committee of Tokushima University (Permit Number T29-115) and performed in accordance with the guidelines by the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of the Environment, the Science Council of Japan. All experiments were performed after administration of anesthesia, and all efforts were made to minimize suffering.

### 2.2. Rooibos extract

Rooibos extract (Pure Rooibos Extract L), containing 0.34% E6CG, was purchased from Maruzen Pharmaceuticals Co., Ltd. (Hiroshima, Japan). To prepare RE, fermented rooibos tea leaves were subjected to extraction with hot water, filtered using activated carbon to remove bitter ingredients such as quercetin, supplemented with dextrin, and spray-dried.

### 2.3. Measurement of tear and saliva secretion

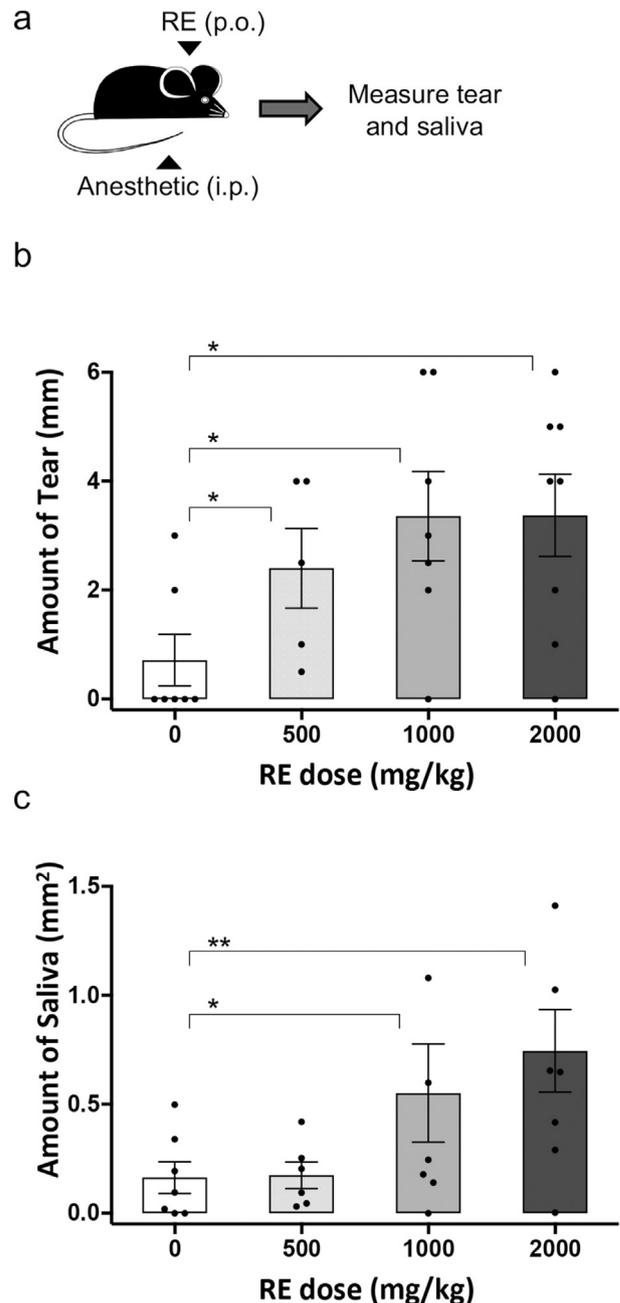
All mice were weighed, anesthetized with ketamine (60 mg/kg body weight (bw); Daiichi Sankyo Co., LTD., Tokyo Japan) and xylazine (6 mg/kg bw; ICN Biomedicals, Inc. Irvine, CA, USA), and administered RE orally. To examine the effects of RE on saliva secretion mechanisms, darifenacin was intraperitoneally administered 15 min prior to RE administration. Small volumes of saliva were measured as previously described [15]. Briefly, mice were laid on sloping hot plates (37 °C) with head tilted slightly downwards and, 5 min after RE administration, oral pH test paper (4 mm in diameter; Sun Dental Corp., Tokyo, Japan) was placed under the tongue for 10 min. Next, the test paper was removed and photographed, and the stained areas were quantified using the National Institutes of Health (NIH) ImageJ analysis software. Tear secretion volume was determined by measuring the length of the phenol red thread (Showa Yakuhin KaKo Co., Ltd., Tokyo, Japan) in contact with the eye for 10 min [16]. Darifenacin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffered saline (PBS) to concentrations of 250 µg/mL and 50 µg/mL, and both dilutions were adjusted to contain 0.5% DMSO. All reagents, including the control (H<sub>2</sub>O and 0.5% DMSO in PBS), were administered at a dose of 10 µL/g bw.

### 2.4. Statistical analysis

Differences between individual groups were determined using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test, and  $p < 0.05$  was considered to be statistically significant. Data are presented as means  $\pm$  standard error of mean (SEM). Outliers as determined by Mahalanobis distance were excluded before statistical analysis.

## 3. Results

The protocol used in the first experiment is shown Fig. 1a. RE was orally administered simultaneously with an intraperitoneal injection of anesthetic. Tear volume was measured using phenol red cotton thread. To detect saliva volumes, oral pH test paper was placed on the mice's sublingual portion touching the aperture of a Wharton duct, 5 min following RE administration and for 10 min. The tear volumes of RE-administered mice (500, 1000, and 2000 mg/kg) were significantly higher than those of control group mice and this increase was dose-dependent (Fig. 1b). In addition,



**Fig. 1.** Effects of rooibos extract (RE) on tear and saliva secretion. (a) Male C57BL/6 (B6) mice aged 8 weeks were intraorally administered RE (0, 500, 1000, and 2000 mg/kg) following anesthesia. Tear and saliva volumes were measured for 10 min, 5 min following RE administration. (b) Tear secretion. Data are shown as means  $\pm$  standard error of the mean (SEM,  $n = 5-8$ ); \* $p < 0.05$ . (c) Saliva secretion. Data are shown as means  $\pm$  SEM ( $n = 5-7$ ); \* $p < 0.01$ .

the saliva volumes of RE-administered mice (1000 and 2000 mg/kg) were significantly higher than those of the control mice, and this increase was also RE dose-dependent. However, there were no differences in saliva volume between the mice that were administered 500 mg/kg RE and controls (Fig. 1c).

To understand the *in vivo* mechanisms underlying the effects of RE on tear and saliva secretions in wild-type mice, an M3R antagonist, darifenacin, was intraperitoneally administered to mice prior to RE administration and anesthetic injection (Fig. 2a) [17]. Administration of darifenacin had no effect on tear secretion (Fig. 2b), although RE-induced tear secretion was significantly reduced by darifenacin administration (2.5 mg/kg; Fig. 2b). Moreover, RE-induced saliva secretion was significantly suppressed by darifenacin pre-treatment (0.5 and 2.5 mg/kg; Fig. 2c). These results indicate that RE elicits a potent effect on the enhancement of tear and saliva secretion via M3R.

#### 4. Discussion

In the present study, RE was used as an activator of M3R to enhance tear and saliva secretion. Following oral administration of RE, secretion of both tears and saliva was significantly increased in

RE-treated mice compared with that in control mice. Moreover, *in vivo* experiments using an M3R antagonist demonstrated that RE-induced saliva and tear secretion may be regulated via M3R within salivary and lacrimal gland cells. RE appears to be an important functional component for control of dryness, including that of the mouth and eyes.

E6CG has been identified as an active component of rooibos and is considered to be an antioxidant marker [9]. Among the numerous components of RE, E6CG may play a role in the activation of M3R in exocrine gland cells. In addition, a clinical study of healthy humans using RE containing E6CG demonstrated that RE intake improved mouth, eye, and skin dryness [12]. In the present study, a similar effect of RE on tear and saliva secretion was observed in wild-type mice in a dose-dependent manner. Moreover, no side effects on systemic organs were noted in treated mice.

M3Rs are widely expressed in various organs and tissues, such as smooth muscles, endocrine glands, lungs, pancreas, brain, and exocrine glands [18]. M3Rs are coupled with G proteins of the Gq class that activate phospholipase C following inositol trisphosphate and intracellular calcium increases and are potent signals for secretion from exocrine glands, including lacrimal and salivary glands [19]. In this study, experiments using darifenacin, an M3R antagonist, demonstrated that the effects of RE on lacrimal and salivary gland cells were mediated by an M3R response. In addition, the effects of low-dose RE (500 mg/kg) on tear and saliva secretion differed. At a dose of 500 mg/kg RE, tear secretion was significantly increased compared with that in control mice, whereas no difference was noted in saliva secretion between controls and RE-treated mice. This suggests that there may be a different M3R expression pattern between lacrimal and salivary gland cells. Furthermore, there may be a different signaling pathway for secretory function in these cells.

In conclusion, oral RE administration significantly increased the secretion of both tears and saliva in wild-type mice via M3R. Rooibos should be further studied as a food or supplement with functional claims to improve xerostomia as well as eye and skin dryness. Additional research on functional extracts from natural plants, such as E6CG, may be useful for clinical application to manage dryness.

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#### Conflict of interest

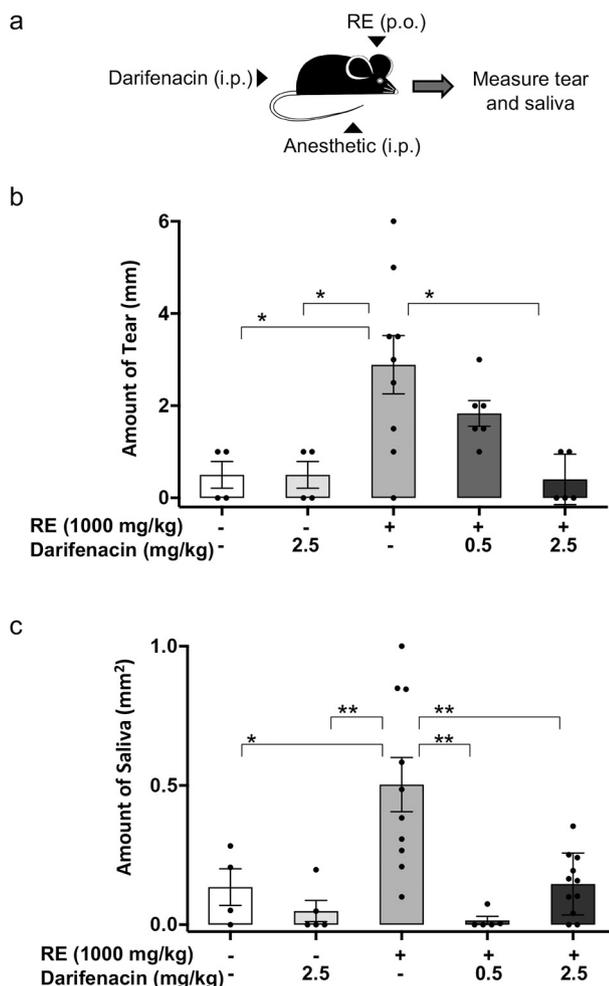
The authors declare that they have no potential conflicts of interest.

#### Ethical approval

All animal experiments were approved by the Committee on Animal-Experiments of Tokushima University (Permit Number T29-115).

#### CRediT authorship contribution statement

**Rieko Arakaki:** Data curation, Formal analysis, Methodology, Investigation, Writing - original draft. **Aya Ushio:** Data curation, Formal analysis. **Satoru Kisoda:** Data curation, Formal analysis. **Mami Sato:** Data curation, Formal analysis. **Yoshitaka Nakamura:** Data curation, Formal analysis, Methodology, Resources. **Kanako Yuyama:** Data curation, Formal analysis, Methodology, Resources.



**Fig. 2.** Effects of RE on tear and saliva secretions mediated by muscarinic acetylcholine receptor 3 receptor (M3R). Male B6 mice were pretreated via intraperitoneal injection of darifenacin (0, 0.5, and 2.5 mg/kg), followed by 1000 mg/kg RE oral administration. Tear and saliva volumes were measured for a 10-min period, 5 min after RE administration. (b) Tear secretion. Data are shown as means  $\pm$  SEM ( $n = 4-7$ ); \* $p < 0.05$ . (c) Saliva secretion. Data are shown as means  $\pm$  SEM ( $n = 4-7$ ); \* $p < 0.05$ , \*\* $p < 0.001$ .

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