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***Russula cutefracta* inhibits antigen-induced degranulation and Syk and MAPK phosphorylation in rat basophilic leukaemia cells**

To the Editor,

Russula cutefracta (synonymous with *Russula cyanoxanthus*) is a mushroom of the genus *Russula* commonly found in parts of Europe and Asia. Although species of *Russula* have rarely been used for medicinal preparations, extracts of other mushrooms species are used as traditional remedies for various diseases in Asian countries. Recent evidence indicates that the mushroom lectins, polysaccharides, and other molecules have general anti-tumour, anti-inflammatory, and immunodulatory properties. Here, we investigated the effects of *R. cutefracta* on mast cell degranulation, an important step in allergic reactions.

Mast cells are important for immediate hypersensitivity and the inflammatory response and the rat basophilic leukaemia cell line, RBL-2H3, has proven useful for studying the biology of mast cells.¹ Allergy, or immediate (Type I) hypersensitivity, is one of four types of hypersensitivity.² The binding of antigen to the high affinity IgE receptor (FcεRI) on the surface of mast cells and basophils induces the release of histamine, prostaglandin, arachidonic acid metabolites, proteases, heparin sulphate, serotonin, leukotrienes, and proinflammatory cytokines from secretory vesicles called granules.^{3,4}

Basophils and mast cells express high levels of FcεRI consisting of an α chain, a β chain, and two disulfide-linked γ chains.^{3,4} The α chain of FcεRI contributes to high affinity IgE binding whereas the β and γ chains, which contain immunoreceptor tyrosine-based activation motifs (ITAMs), initiate the downstream signalling cascade leading to release of preformed and newly synthesised mediators.^{3,4} The spleen tyrosine kinase (Syk) plays an essential role in the IgE-dependent activation of mast cells. For example, cross-linking of FcεRI results in phosphorylation of the ITAMs on the β and γ chains by Lyn, a Src family kinase.⁵ The phosphorylated γ chains then bind and activate Syk, which has an essential role for FcεRI-proximal signalling pathways.^{6,7} However, many more kinases including Fyn, PI3-kinase,

PLC-γ, and MAPKs are important for mast cell activation and degranulation.^{3,4} Therefore, the inhibition of these kinases by natural compounds found in *R. cutefracta* might suppress the release of hypersensitivity-inducing mediators in mast cells, thereby leading to inhibition of the allergic response.

As a first step toward using *R. cutefracta* as a treatment for allergy, dried *R. cutefracta* (80 g) was used to prepare *R. cutefracta* extract (RCE) by three extractions with ethanol. After filtration, excess water was removed with a rotary evaporator under vacuum at 50 °C. Next, we determined whether RCE is toxic by assessing the viability of mast cells. RBL-2H3 cells were treated with various concentrations of RCE for 12 h and cell viability was determined by MTT assay. Untreated cells were used as a negative control (Fig. 1A). We did not observe any increase in cell death due to RCE, although we tested a range of concentrations from 10 μg/ml to 100 μg/ml (Fig. 1A). Therefore, in the range of concentrations used in our study, RCE was not toxic to mast cells.

Mast cell degranulation is a cellular process whereby secretory vesicles release hypersensitivity-inducing molecules including histamine, proteoglycans and proteases. β-Hexosaminidase is another molecule present within mast cell granules and this marker has been used widely to monitor degranulation.⁸ Mast cells were incubated with DNP-specific IgE (20 ng/ml) overnight and various concentrations of RCE (10 μg/ml, 20 μg/ml, and 100 μg/ml) for 30 min, resulting in a dose-dependent decrease in degranulation, as measured by β-hexosaminidase release (Fig. 1B). In comparison, our control experiments with untreated cells or cells receiving antigen stimulation only resulted in no degranulation and complete degranulation, respectively (Fig. 1B). Another control experiment using PP2 (10 μM), a general Src-family kinase inhibitor, also inhibited degranulation and RCE at 30 μg/ml and 100 μg/ml were as potent as PP2 in inhibiting degranulation (Fig. 1B). We believe that this is the first demonstration of the anti-allergy properties of *R. cutefracta*.

An increase in cytosolic Ca²⁺ ionophore concentration ([Ca²⁺]_i) is thought to be important for mast cell degranulation.⁹ Also, it is known that thapsigargin, a potent inhibitor of Ca²⁺-ATPase in the endoplasmic reticulum membrane and ionomycin, a Ca²⁺ ionophore, cause mast cell degranulation without FcεRI activation.¹⁰ To determine whether degranulation resulting from an increase in

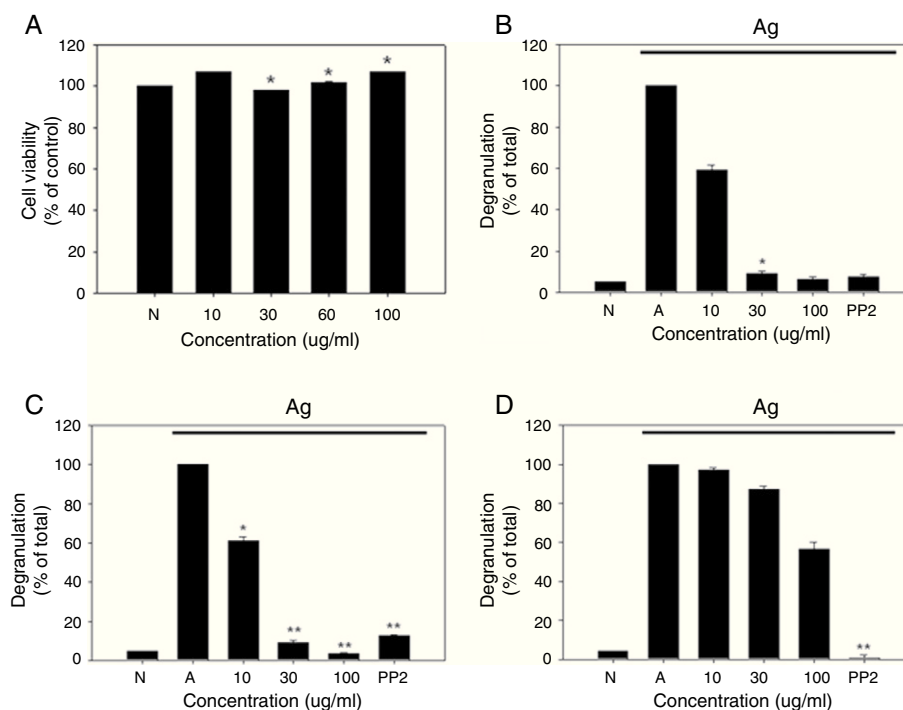


Figure 1 The effect of *R. cutefracta* on RBL-2H3 degranulation. (A) RBL-2H3 cells were treated by the indicated concentrations of RCE for 12 h and cell viability was determined by MTT assay. (B) Degranulation of RBL-2H3 cells was measured by the release of β -hexosaminidase. (C and D) Thapsigargin and ionomycin-stimulated degranulation. Thapsigargin (300 nM) and ionomycin (1 μ M) were used to stimulate degranulation of RBL-2H3 cells. PP2 (10 μ M) is a general Src-family kinase inhibitor; N, no treatment; A, antigen stimulation only. The values are expressed as the mean \pm SEM from three independent experiments (* $P < 0.05$ and ** $P < 0.01$).

cytosolic $[Ca^{2+}]$ can be inhibited by RCE, RBL-2H3 cells were incubated with DNP-specific IgE (20 ng/ml) overnight, treated with RCE at various concentrations (10 μ g/ml, 20 μ g/ml, and 100 μ g/ml) for 30 min, and treated with thapsigargin (300 nM) or ionomycin (1 μ M) for 10 min. RCE treatment after thapsigargin pre-treatment resulted in a dose-dependent inhibition of mast cell degranulation, similar to that seen with DNP-IgE-induced degranulation (compare Fig. 1B and C). Ionomycin could also cause degranulation, but the inhibition of degranulation after subsequent treatment with RCE was weaker than that observed after thapsigargin treatment (compare Fig. 1C and D). Together, these data provide evidence that non-Fc ϵ RI-mediated mast cell degranulation could also be inhibited by *R. cutefracta*.

To understand how *R. cutefracta* suppresses mast cell activation and degranulation, we examined its effects on Fc ϵ RI-mediated signalling events by Western blot analyses, including phosphorylation of Syk and the MAP kinases (ERK1/2, JNK1/2 and p38). Syk is a key mediator of immunoreceptor signalling in mast cells; it is activated upon engagement of the Fc receptor and it is a critical link to many downstream events including calcium mobilisation.⁴ Treating the mast cells with increasing concentrations of RCE resulted in a gradual decrease in Syk phosphorylation (as detected by α -P-Syk Tyr525/526 antibody), thus verifying the dose-dependency of RCE and mast cell degranulation (Fig. 2A). Some secondary signalling events following KIT activation in mast cells, including phosphorylation of the mitogen-activated protein kinases (MAPKs), are important events in the transcriptional regulation of activated mast cells.⁴ When mast cells were treated with increasing con-

centrations of RCE, we observed significant decreases in JNK phosphorylation (as detected by α -P-JNK Thr183/Tyr185 antibody) and p38 phosphorylation (as detected by α -P-p38 Thr180/Tyr182 antibody) and a slight decrease in ERK1/2 phosphorylation (as detected by α -P-Erk1/2 Thr202/Tyr204 antibody) (Fig. 2B). Together, these results support our observation that RCE could inhibit mast cell degranulation by inhibiting cell signalling intermediaries. Furthermore, it is likely that RCE inhibits mast cell degranulation by preventing the phosphorylation of regulatory molecules downstream of Fc ϵ RI, such as Syk and the MAP kinases.

In conclusion, ours is the first study demonstrating that *R. cutefracta* inhibits degranulation in mast cells. Furthermore, only a few other mushroom species including *Flammulina velutipes*, *Hypsizigus marmoreus*, and *Agaricus blazei* are known to protect against hypersensitivity. Therefore, *R. cutefracta* merits further evaluation as a natural treatment for allergy.

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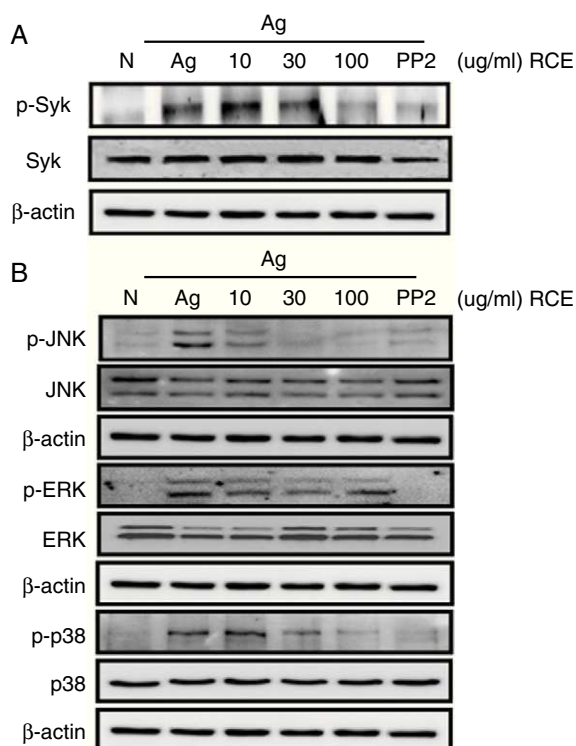


Figure 2 The effect of *R. cutefracta* on Syk and MAPK phosphorylation. RBL-2H3 cells were treated as described to initiate degranulation, with or without RCE treatment. The cell lysates were subjected to Western blot analysis to detect phosphorylated forms of (A) Syk and (B) MAP kinase. PP2 (10 μM) is a general Src-family kinase inhibitor; N, no treatment; A, antigen stimulation only.

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