to fish physiology, predominate over inorganic processes in determining trace element concentrations in otolith carbonate. Presumably the reasons for the changes in discrimination by the fish for and against the various ions will be clarified when more is known about seasonal variations in fish physiology, and their precise relationship to environmental conditions.

Inasmuch as otolith microchemistry provides an environmental record, long-lived species and fossil otoliths might provide evidence of past environments, despite the fact that the underlying biological processes are not yet defined. In addition, information on the life histories of individual fish, particularly those of migratory habit, should be contained within their otoliths, and it should be possible to reveal this information by microchemical analysis. The work described here has shown the value of FAB-SIMS as a tool for the elemental analysis of intact calcareous samples, giving high spatial resolution.

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Inhibitory effects of flavonoids on several venom hyaluronidases

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Abstract. In vitro studies showed that the flavonoid aglycones apigenin, luteolin and kaempferol inhibited the hyaluronidase activity of five different venoms dose-dependently. They were also able to delay the venom action when injected into mice. Naringenin, catechin and flavonoid glycosides had no effect. The flavonoids with unsubstituted hydroxyl groups at C-positions 5, 7 and 4′, a double bond between carbons 2 and 3, as well as a ketone group at position 4, exhibited potent inhibitory actions on the venom hyaluronidases.
Key words. Flavonoids-hyaluronidase inhibition-venom action.

Flavonoids are benzo-γ-pyrone derivatives which are ubiquitous in plants[1] and they exhibit a great variety of pharmacological effects in biological systems[2,3]. They are also present as important constituents in several preparations used in folk medicine[4]. Various plant extracts have been reported to be able to neutralize the lethal activity of snake venoms[5,6]. Nevertheless, it must be borne in mind that such effects on the lethal activity do not necessarily correlate with the neutralization of specific pharmacological or enzymatic activities[7].

Hyaluronidase (EC 3.2.1.35), an enzyme widely distributed in mammalian testes, leech heads, invasive bacteria, and venoms of snakes, bees, scorpions and poisonous fishes[8,9] depolymerizes hyaluronic acid, a major constituent of animal connective tissues, which is essential for maintaining the integrity of the extracellular matrix[10]. Venoms of snake, bee and scorpion contain hyaluronidases with molecular weights ranging from 33,000 to 110,000[11,12], but little is known about the molecular structures of these enzymes. The activity of venom hyaluronidases shows a broad pH range in vitro, with an optimum pH at 4.0–5.0[9,10,12]. The end products of the action of these enzymes on hyaluronic acid are mainly tetrasaccharide units[9].

Tu and Hendon[11] supplied experimental evidence that this enzyme plays a role as a 'spreading factor' in the toxic action of venom. Despite the fact that hyaluronidase in itself is not a toxin[13], it may contribute to local or systemic envenomation by accelerating venom absorption and diffusion into the tissues of the victim[14]. Therefore, inhibition of this enzyme could play an important role in the therapy of venom poisoning.

Earlier work reported by Rodney et al.[15], and investigations carried out in our laboratory[16], showed that several structurally related flavonoids exhibited potent inhibition of bovine testis hyaluronidase. The present report is an investigation on the effects of a series of 13 flavonoids (table 1) on the hyaluronidase activity present in several commercially available snake, scorpion and bee venoms. In addition, venoms of two different species of snakes, namely the rattlesnake (*Crotalus atrox*) and the Malayan Cobra (*Naja naja sputatrix*), were used as models for an

Table 1. Structure of flavonoids used in this study

Compounds	Class	Hydroxylation pattern	Substitution
Apigenin	Flavone	5,7,4′	
Apiin	Flavone	5,4′	R_7 = Apiose-glucose
Luteolin	Flavone	5,7,3′,4′	
Luteolin-7-glucoside	Flavone	5,3′,4′	R_7 = Glucose
Kaempferol	Flavonol	3,5,7,4′	
Myricetin	Flavonol	3,5,7,3′,4′,5′	
Myricitrin	Flavonol	5,7,3′,4′,5′	R_3 = Rhamnose
Quercetin	Flavonol	3,5,7,3′,4′	
Quercitrin	Flavonol	5,7,3′,4′	R_3 = Rhamnose
*Naringenin	Flavanone	5,7,4′	
Phloretin	Chalcone	4,2′,4′,6′	
**Catechin	Flavan-3-ol	3,5,7,3′,4′	

* Flavonoids without double bond between $C_{2,3}$.
** Flavonoids without carbonyl group at C_4 and double bond between $C_{2,3}$.

in vivo study to investigate the possible application of flavonoids as antagonists of venom action.

Materials and methods

Hyaluronic acid (human umbilical cord); hexadecyltrimethyl ammonium bromide (Cetrimide) and the venoms of: honey bee (*Apis mellifera*), scorpion (*Scorpio maurus palmatus*), eastern diamondback rattle snake (*Crotalus adamenteus*), western diamondback rattle snake (*Crotalus atrox*) and Malayan Cobra (*Naja naja sputatrix*) were obtained from Sigma Chemicals Co. (St. Louis, USA).

Flavonoids were purchased from Extrasynthese, Genay, France. Other chemicals used were of the best analytical grade available.

Male Swiss mice (20 ± 2 g b.wt) were obtained from the Animal Laboratory Centre, National University of Singapore.

Hyaluronidase activity was determined according to the modified method of Xu et al.[13]. Incubations contained 100 µg hyaluronic acid and 100 µl of venom in 1 ml of 0.2 M acetate buffer, pH 5.0, containing 0.15 M NaCl, and were carried out for 45 min at 37 °C. Turbidity-reducing activity was expressed as a percentage of the remaining hyaluronic acid, taking the absorbance of a tube to which no venom was added as 100%. The venom concentrations that gave a turbidity-reduction of approximately 50% were used for subsequent experiments (table 2).

Table 2. Hyaluronidase activity of venoms

Venom species	Amount of venom (µg, dry weighting) required to hydrolyze 50% of hyaluronic acid
Apis mellifera	3 ± 0.5
Scorpio maurus palmatus	15 ± 0.9
Crotalus adamenteus	30 ± 1.0
Crotalus atrox	20 ± 0.9
Naja naja sputatrix	35 ± 1.1

Results are shown as mean ± SEM (n = 3).

The flavonoids were added in absolute ethanol, except for apigenin and apiin which were dissolved in dimethyl sulfoxide (100% DMSO). Aliquots of the flavonoid solutions at the final concentration range of 5–250 µM were preincubated with various venom solutions for 15 min at 37 °C. A set of control assays which contained the venom and the solvent without flavonoid were carried out simultaneously. The final concentration of the solvents used was 1% (v/v). After the preincubation, the reaction was started by adding hyaluronic acid (100 µl) and the mixture was incubated for a further 45 min. Blanks containing only the buffer and flavonoid solutions or solvents were run in parallel. All incubations were carried out in triplicate. The reaction was terminated by adding 2 ml of cetrimide (2.5% w/v) in 2% (w/v) NaOH solution, and optical density was read against appropriate blanks at 400 nm after 30 min. The percentage inhibition was calculated as previously described[16], i.e.

$$\text{Inhibition (\%)} = \frac{\text{Activity of control} - \text{activity in presence of flavonoids}}{\text{Activity of control}} \times 100$$

The approximate median lethal doses (LD_{50}s) of the venoms of *Crotalus atrox* (CA) and *Naja naja sputatrix* (NNS) were determined after subcutaneous (s.c.) injection of 0.1 ml of each venom into male mice (n = 4; b.wt = 20 ± 2 g). The venom dose that caused 2 out of 4 mice to die (after 24 h) was taken as LD_{50}. To study the effects of flavonoids on the lethality of the venoms, dosages amounting to 5 × LD_{50} of the venoms were used. Five mice were used for each set of treatments. The $5LD_{50}$ for CA was 22.5 µg/g b.wt and for NNS was 10 µg/g b.wt.

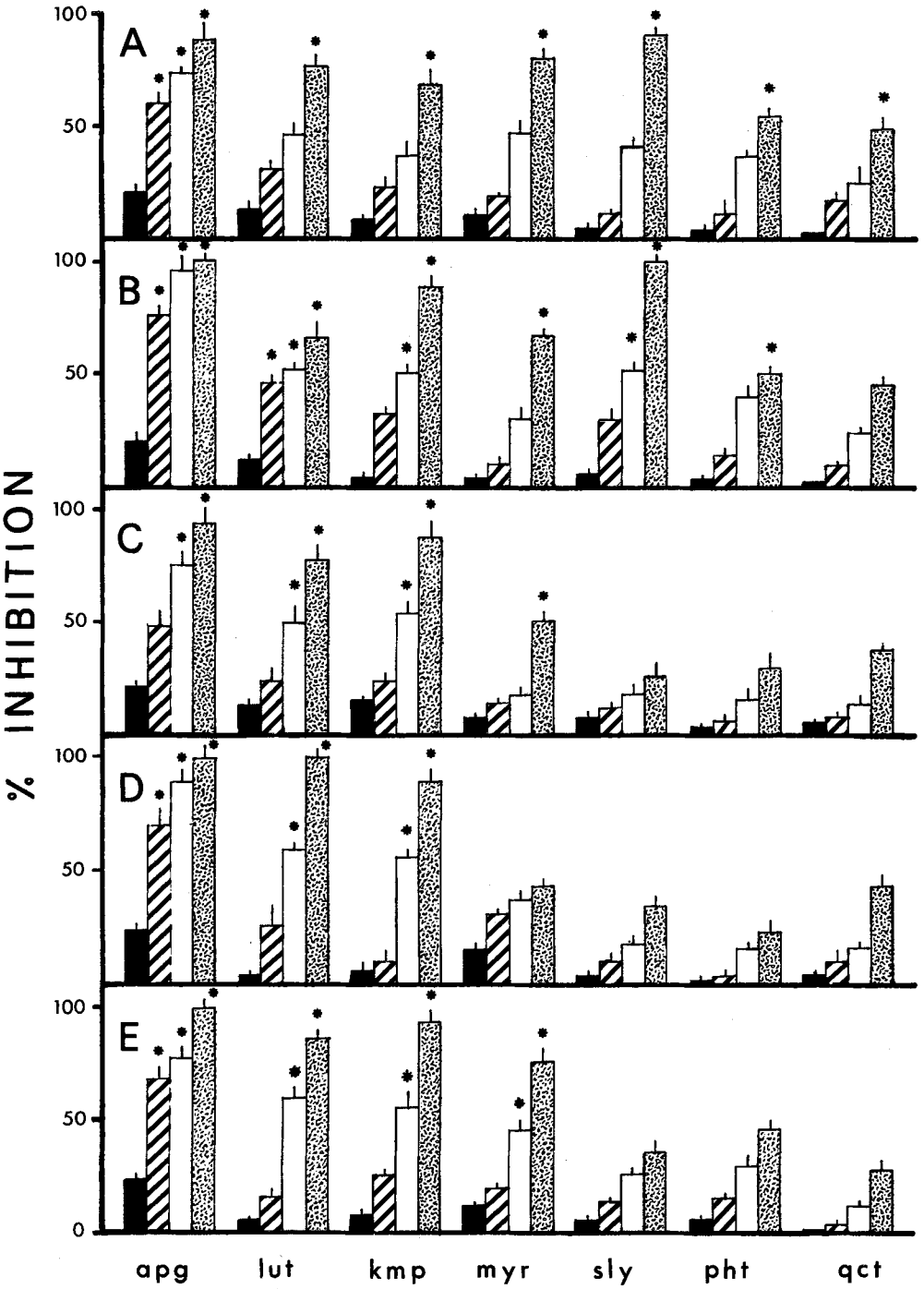
The flavonoids luteolin and kaempferol (dissolved in ethanol) or apigenin (dissolved in DMSO) were further diluted with 0.9% NaCl to give appropriate concentrations. Each of the flavonoid solutions was mixed with the venom solution ($5LD_{50}$) at room temperature 5 minutes before s.c. injection. The survival time (time between s.c. injection and death) was then monitored. The final concentration of the solvents (ethanol or DMSO) present in the venoms was 2.5% (v/v). The mean survival times of mice after the injection of venom ($5LD_{50}$) plus ethanol (2.5% v/v) or venom ($5LD_{50}$) plus DMSO (2.5% v/v) were also measured. Since the difference in survival times between these two treatments was insignificant, the mean value was used as control.

Results

All of the venoms tested showed hyaluronidase activity. The venoms of *Apis mellifera* and *Naja naja sputatrix* exhibited the highest and the lowest activities, respectively (table 2). The venom of *Apis mellifera* (bee venom) has previously been reported to have a high concentration of enzyme (2–3% of dry venom) as well as a high rate of hyaluronidase activity[17].

The percentage inhibition due to each of the flavonoids was determined with respect to control assays (without flavonoids) carried out in the presence of solvent. The solvents did not cause any significant inhibition of the venom hyaluronidases (result not shown). The effect of flavonoids at concentrations above 250 μM could not be determined, as their solubilities were poor at higher concentrations.

Naringenin, catechin and the 4 flavonoid glycosides myricitrin, apiin, luteolin-7-glucoside and quercitrin (table 1) were generally inactive as inhibitors of all five venom hyaluronidases tested (results not shown). The



Inhibition of venom hyaluronidase by flavonoids. Venoms were preincubated with different concentrations of flavonoids (■ = 25, ▨ = 50, □ = 100 and ▩ = 250 μM) for 15 min prior to the addition of hyaluronic acid. Flavonoids are abbreviated: apg = apigenin, lut = luteolin, kmp = kaempferol, myr = myricetin, sly = silybin, pht = phlotretin and qct = quercetin. Venoms used were A = *Apis mellifera*, B = *Scorpio maurus palmatus*, C = *Crotalus adamenteus*, D = *Crotalus atrox* and E = *Naja naja sputatrix*. Results are expressed as mean ±SEM (n = 3). *Significantly different from the control, $p < 0.01$ (Student's t-test).

Table 3. Effect of flavonoids on the mean survival time of mice injected with venoms

Venom or venom + flavonoid	Survival time (min)	% Increase in survival time
$5LD_{50}$ CA (control)	93 ± 9	
$5LD_{50}$ CA + 0.04 µmol lut	215 ± 16*	131
$5LD_{50}$ CA + 0.1 µmol lut	356 ± 17*	283
$5LD_{50}$ CA + 0.04 µmol kmp	170 ± 9*	83
$5LD_{50}$ CA + 0.1 µmol kmp	256 ± 13*	175
$5LD_{50}$ CA + 0.04 µmol apg	185 ± 13*	99
$5LD_{50}$ CA + 0.1 µmol apg	245 ± 15*	163
$5LD_{50}$ NNS (control)	35 ± 5	
$5LD_{50}$ NNS + 0.04 µmol lut	52 ± 6*	49
$5LD_{50}$ NNS + 0.1 µmol lut	67 ± 11*	91
$5LD_{50}$ NNS + 0.04 µmol kmp	45 ± 4*	29
$5LD_{50}$ NNS + 0.1 µmol kmp	66 ± 9*	89
$5LD_{50}$ NNS + 0.04 µmol apg	50 ± 10*	43
$5LD_{50}$ NNS + 0.1 µmol apg	56 ± 7*	60

Abbreviations: CA = *Crotalus atrox*, NNS = *Naja naja sputatrix*, lut = luteolin, kmp = kaempferol and apg = apigenin. The LD_{50}s were determined by s.c. injection of the venom into mice. The $5LD_{50}$s of CA and NNS correspond to 22.5 µg/g and 10 µg/g respectively. Mean survival times were determined by injecting (s.c.) $5LD_{50}$ venom or $5LD_{50}$ venom plus flavonoids into mice (n = 5, b.wt = 20 ± 2 g). Results are expressed as mean ± SE.

*Significantly different from the control, $p < 0.05$ (Student's t-test).

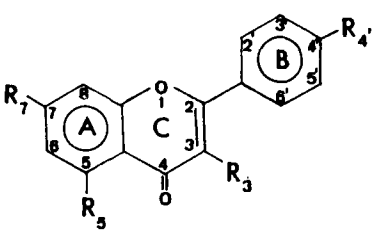
aglycones apigenin, luteolin, kaempferol, silybin, myricetin, phloretin and quercetin exerted dose-dependent inhibition in the concentration range of 25–250 µM (fig.). Silybin was a very potent inhibitor (100% inhibition at 250 µM concentration) for the bee and scorpion venom hyaluronidases (fig. A and B), but exhibited less than 40% inhibition on the snake venom hyaluronidases (fig. C, D and E). It is possible that this difference between silybin and the other flavonoids is due to its different structure (table 1). Apigenin, luteolin and kaempferol could efficiently inhibit all of the 5 venom hyaluronidases tested (>80%). Myricetin, phloretin and quercetin showed variable inhibitions ranging from 50–80%, 30–50% and 30–50% respectively (fig.).

The mean survival times of mice after the injection of the venom plus a flavonoid were significantly prolonged (table 3), and this effect was also dose-dependent for both the venoms studied. However, the prolongation of survival time was more pronounced with the rattlesnake venom than with the Malayan Cobra venom. The mean survival time of mice injected with rattlesnake venom plus one of the following flavonoids (0.1 µmol) luteolin, kaempferol and apigenin was increased by 283%, 175% and 163% respectively, but for the cobra venom with similar treatment, the increase was only in the range of 60%–100%.

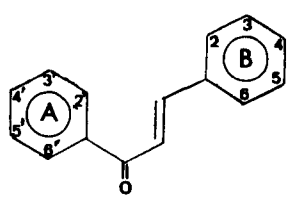
Discussion

We have previously established that flavonoids which have a $C_{2,3}$ double bond, unsubstituted hydroxyl groups at 5,7,4′ and a ketone group at position 4 had a potent inhibitory effect on bovine testis hyaluronidase [16]. In the present investigation, the effects of flavonoids (with unsubstituted hydroxyls at positions 5, 7 and 4′) on hyaluronidase activity in several snake, bee and scorpion venoms have been studied.

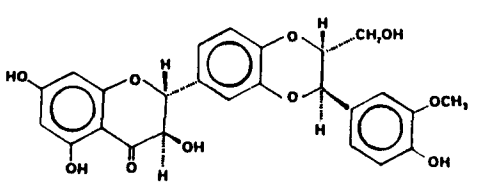
The results indicate that flavones, flavonols and chalcones possess the general ability to inhibit venom hyaluronidases. However, there are some variations in activity related to structural features in the compounds. The presence of a glycoside substituent completely destroys the ability of the flavonoids to inhibit the enzyme activity (result not shown). Such effects have also been reported for a few other enzymes [18, 19]. The structure-activity comparison of the flavonols differing in the number and position of the B ring hydroxyl groups showed that kaempferol, which contains a mono hydroxyl group, is the most potent inhibitor, followed by myricetin, containing a pyrogallol configuration, then quercetin, containing a catechol configuration (fig. and table 1). The effects of these hydroxyl configurations on potency are different from those reported for a series of model phenolic compounds tested on several other enzyme activities [20].



flavone



chalcone



silybin

The importance of the $C_{2,3}$ double bond was indicated by the high potency of apigenin (fig.) whilst the corresponding flavanone, naringenin, which lacks the $C_{2,3}$ double bond, was inactive (result not shown). Ferrell et al.[21] suggested that the saturation of the $C_{2,3}$ double bond could disrupt the planarity and the conjugation of the C ring and therefore contribute to the inactivity. In addition, the presence of a keto group at C_4 is important for inhibitory activity because catechin (table 1), which lacks such substitution, was inactive (result not shown).

The conversion of flavone (apigenin and luteolin) to chalcone (phloretin) by the elimination of the oxygen and the opening of the C ring reduced the potency in all of the venoms tested, as can be seen by the results presented in the figure.

We have reported that several flavonoids, namely apigenin, luteolin, kaempferol and silybin inhibited bovine testis hyaluronidase in a competitive manner[16]. We did not attempt kinetic studies in the present investigation. Nevertheless, the structure-activity related effect of flavonoids on venom hyaluronidases is consistent with that observed for bovine testis hyaluronidase[16]. Thus it is reasonable to suggest that in general their mode of action could be similar.

In view of the important role played by hyaluronidase in the spread of venom in an envenomated victim[11], the possible application of flavonoids as antagonists of venom action was investigated. Snake venoms were selected for our study, since death from 'snake-bite' is common. As the flavonoids luteolin, kaempferol and apigenin were potent hyaluronidase inhibitors (in vitro), they were tested to see whether they could also significantly delay the venom action when injected into mice. The effect of flavonoids was determined by monitoring the mean survival time and not by the determination of LD_{50}, because various research groups have demonstrated that the former method is more sensitive and useful[22, 23]. Although our in vivo studies showed that the three flavonoids were effective in delaying the venom action when injected into mice (table 3), one cannot rule out the possibility that the flavonoids, besides inhibiting the hyaluronidase activity, might also affect the activities of the various other components present in venoms, which may act synergistically[22, 23] to induce toxicity. Therefore, more detailed studies to find out the effects of flavonoids on the various venom components need to be carried out, to ascertain whether flavonoids might be of therapeutic use in venom poisoning.

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