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Structure–activity studies of flavonoids as inhibitors of hyaluronidase

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The flavonoids are a group of naturally occurring low molecular weight benzo-γ-pyrone derivatives, ubiquitous in plants [1]. Their pharmacological and pharmaceutical functions have been reviewed by Havsteen [1]. The effects exerted by flavonoids on animal systems include anti-inflammatory and anti-allergic activity, lipid peroxidation, RNA, DNA and protein synthesis, antiviral activity, capillary fragility and inhibition of tumour promotion [2, 3].

Hyaluronidases (EC 3.2.1.35) are enzymes which depolymerize or hydrolyse hyaluronic acid presumably by splitting glucosaminidic bonds to yield oligosaccharides [4]. This enzyme was earlier claimed to be involved in allergic effects [5], migration of cancer cells [6], inflammation, petechial haemorrhages following its injection in mesentery preparations and also the increase in permeability of the vascular system [5–7].

Tu and Hendon [8] reported that hyaluronidase plays a definite role in the penetration of venoms through tissues of a victim and can be considered as a spreading factor. The mechanism of malignant invasiveness was reported to be dependent upon the continuous release of lysosomal glycosidases (hyaluronidases) [6]. Further postulations were made that if a method of inhibiting tumour cell glycosidases (hyaluronidases) were found, it would be possible not only to restrain malignant invasiveness but also to retard malignant cell proliferation [6]. This information initiated our interest to investigate the effects of flavonoids on hyaluronidase. Bovine testis hyaluronidase, an easily available enzyme was employed as the model for our investigation. There have been a limited number of earlier reports describing the effects of a few flavonoids on the action of hyaluronidase *in vivo* and *in vitro* [9–11]. However, the present study was designed to investigate the structure–activity relationship and effects of a series of 31 flavonoids *in vitro* on bovine testis hyaluronidase. The kinetic studies of several potent flavonoids were further examined.

In view of the important role played by hyaluronidase in the aetiology and/or development of diseases [5–8] it would be very useful to find potent inhibitors of this enzyme for they could be used to develop new therapeutic agents.

Materials and Methods

Bovine testis hyaluronidase (sp. act. 290 National Formulary Unit/mg protein); human umbilical cord hyaluronic acid and hexadecyltrimethylammonium bromide (Cetrimide) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Flavonoids were obtained from Extrasynthese (Genay, France) and Sigma. Condensed tannin was supplied by Dr L. Butler, Purdue University, U.S.A. Other chemicals used were of the best analytical grade available.

Flavonoids were dissolved either in absolute ethanol or dimethylsulfoxide (100% DMSO). Stock solution (freshly prepared for each assay) had 25 μmol/mL for each flavonoid. Aliquots (10 μL) of the flavonoid solution were used in the assay mixture to give a final concentration of 250 μM.

Hyaluronidase activity was determined by the modified method of Xu *et al.* [12]. The assay system contained hyaluronic acid (100 μg), the enzyme (15 μg) and test compound (10 μL) in 1.0 mL of 0.2 M acetate buffer, pH 5.0 containing 0.15 M NaCl. Hyaluronidase (15 μg/mL) was preincubated with flavonoids (250 μM) for 15 min at 37° and the volume was made up to 900 μL with acetate buffer. Control tubes contained the enzyme plus 10 μL of either absolute ethanol or 100% DMSO and buffer in a final volume of 900 μL. After preincubation, the assay was commenced by adding hyaluronic acid (100 μL) to each tube and incubated for 45 min. All incubations were carried out in triplicate. Reactions were terminated by the addition of 2 mL of cetrimide (2.5% w/v) in 2% (w/v) NaOH solution ('stop reaction' solution). The percentage of inhibition was calculated as follows:

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

Table 1. Inhibition of bovine testis hyaluronidase by flavonoids

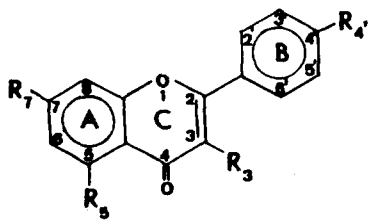
Polyphenolic compounds	Class	Hydroxylation pattern	Substitution	Activity % hyaluronic acid hydrolysed	% Inhibition
Control (nil) containing ethanol (solvent used)				47.8 ± 1.1	
Condensed tannin	Proanthocyanidin	Flavan-3-ol polymer		0.9 ± 0.9*	100
Luteolin	Flavone	5,7,3′,4′		11.5 ± 0.8*	76
Kaempferol	Flavonol	3,5,7,4′		21.0 ± 0.4*	56
Silybin	Silymarin			24.8 ± 0.6*	48
Myricetin	Flavonol	3,5,7,3′,4′,5′		31.5 ± 1.9*	34
Morin	Flavonol	3,5,7,2′,4′		33.0 ± 0.8*	31
Quercetin	Flavonol	3,5,7,3′,4′		33.9 ± 0.7*	29
Butein	Chalcone	3,4,4′,6′		37.8 ± 0.7	21
Hesperetin†	Flavanone	5,7,3′	R_4=OCH_3	40.6 ± 0.7	15
Taxifolin†	Flavanonol	3,5,7,3′,4′		40.5 ± 0.8	15
Phloretin	Chalcone	4,2′,4′,6′		41.1 ± 0.9	14
Flavanone†	Flavanone			41.6 ± 1.0	13
Fisetin	Flavonol	3,7,3′,4′		42.0 ± 1.0	12
Catechin‡	Flavan-3-ol	3,5,7,3′,4′		42.5 ± 0.8	11
Epicatechin‡	Flavan-3-ol	3,5,7,3′,4′		42.6 ± 0.6	11
Naringenin†	Flavanone	5,7,4′		43.0 ± 0.7	10
Flavone	Flavone			46.9 ± 0.7	0
Chrysin	Flavone	5,7		48.0 ± 0.8	0
Dihydrofisetin†	Flavanonol	3,7,3′,4′		47.6 ± 0.8	0
Rutin	Flavonol	5,7,3′,4′	R_3=Rutinose	40.5 ± 1.0	15
Isoquercitrin	Flavonol	5,7,3′,4′	R_3=Glucose	42.1 ± 0.9	12
Hesperidin†	Flavanone	5,3′	R_7=Rhamno-glucose R_4=OCH_3	43.5 ± 1.1	9
Quercitrin	Flavonol	5,7,3′,4′	R_3=Rhamnose	43.9 ± 0.9	8
Naringin†	Flavanone	7,4′	R_5=Rhamno-glucose	46.7 ± 1.0	2
Phloridzin	Chalcone	4,2′,4′	R_4= β-D-glucose	47.4 ± 0.7	0
Control (nil) containing DMSO (solvent used)				45.6 ± 1.2	
Apigenin	Flavone	5,7,4′		15.0 ± 1.1*	67
Diosmetin	Flavone	5,7,3′	R_4=OCH_3	41.0 ± 2.9	10
Diadzein	Isoflavone	7,4′	Ring B attached to C_3	42.4 ± 2.1	7
Apiin	Flavone	5,4′	R_7=Apiose-glucose	40.1 ± 3.0	12
Luteolin-7-glucoside	Flavone	5,3′,4′	R_7=-Glucose	39.2 ± 1.8	14
Diosmin	Flavone	5,3′	R_7=Rutinose R_4=OCH_3	44.8 ± 1.3	0

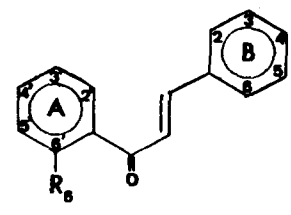
The assays were carried out as described in Materials and Methods. Condensed tannin was present at approximately 50 μM whilst all the other flavonoids were present at 250 μM concentrations. Results are means ± SE of three experiments.

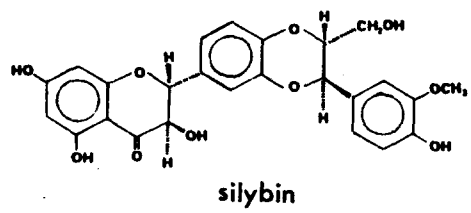
* Statistically different from control, $P < 0.01$ (Student's *t*-test).

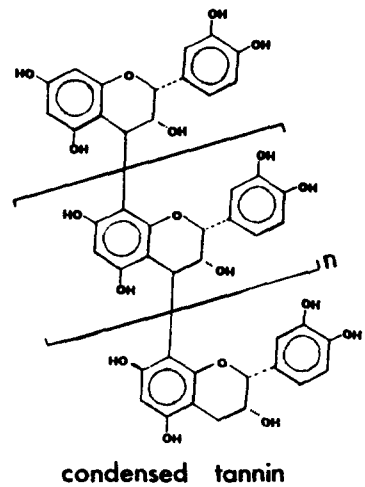
† Flavonoids without double bond between $C_{2,3}$.

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









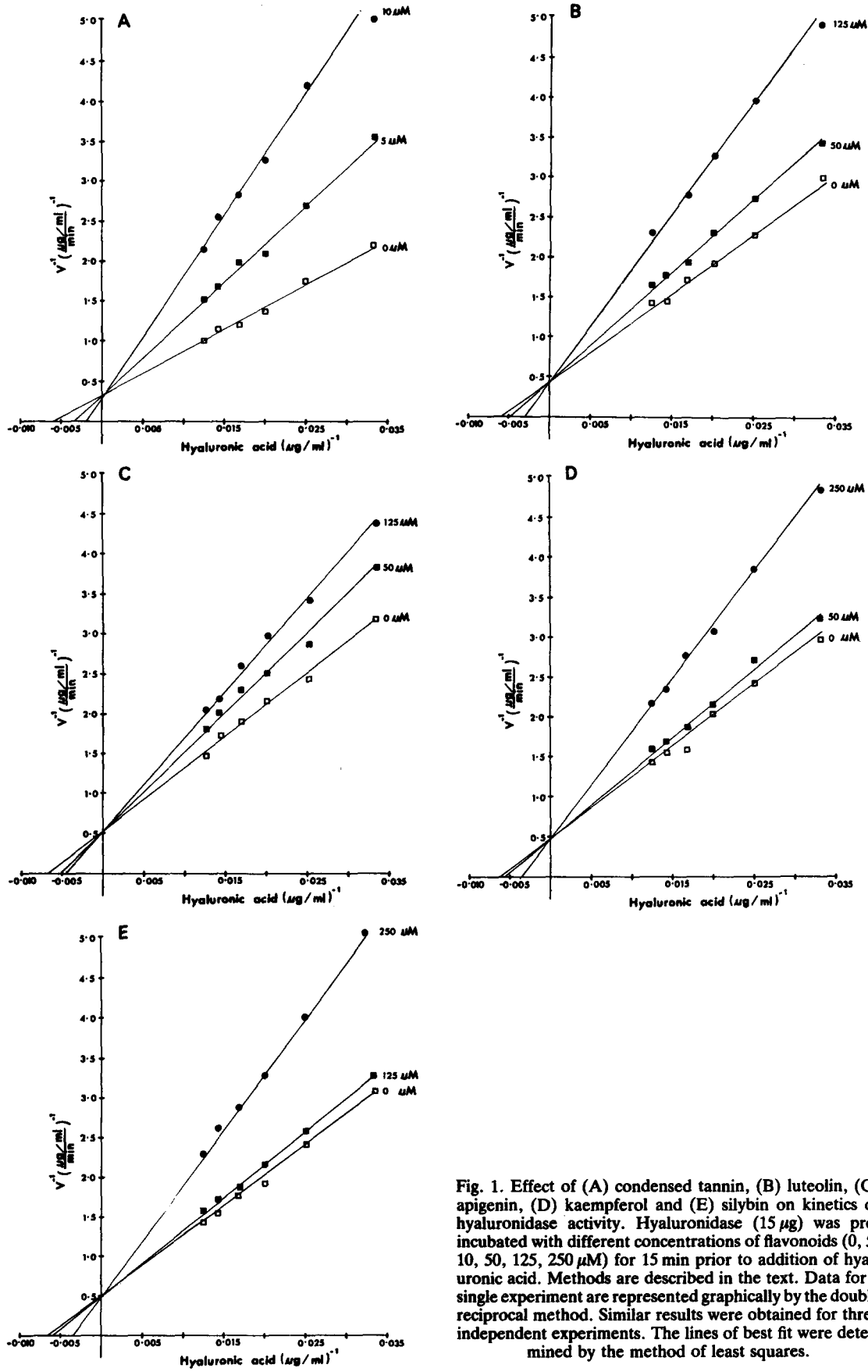


Fig. 1. Effect of (A) condensed tannin, (B) luteolin, (C) apigenin, (D) kaempferol and (E) silybin on kinetics of hyaluronidase activity. Hyaluronidase (15 μg) was pre-incubated with different concentrations of flavonoids (0, 5, 10, 50, 125, 250 μM) for 15 min prior to addition of hyaluronic acid. Methods are described in the text. Data for a single experiment are represented graphically by the double reciprocal method. Similar results were obtained for three independent experiments. The lines of best fit were determined by the method of least squares.

In the kinetic studies, activity (v) was expressed as μg/mL/min hyaluronic acid hydrolysed. The O.D. of the various 'standard' incubations i.e. assay tubes which did not contain the enzyme was plotted against the corresponding initial amount of substrate added. Amount of substrate hydrolsed was determined from this standard curve.

Results and Discussion

Thirty-one flavonoids (Table 1) were examined for their effects on bovine testis hyaluronidase. The percentage inhibition of each of the flavonoids was determined with respect to control assays run simultaneously. The amount of ethanol or DMSO used in the assay system did not cause any significant inhibition of the enzyme (result not shown). The ID_{50} for most of the flavonoids tested could not be determined as their activity at 250 μM was very low, and their solubilities were poor at higher concentrations.

Condensed tannin (a polymer of flavan-3-ols), luteolin, apigenin, kaempferol and silybin inhibited hyaluronidase in a concentration-dependent manner with varying potency over the range of 0 to 250 μM (results not shown). Condensed tannin showed 100% inhibition at a concentration of approximately 50 μM. This was followed by luteolin, apigenin, kaempferol and silybin with inhibitions of 76.1, 66.5, 56.1 and 47.4%, respectively, at a concentration of 250 μM. Myricetin, quercetin, morin and butein, showed inhibitions within the range of 20 to 30% whilst the other flavonoids studied exhibited less than 15% inhibition (Table 1).

Luteolin, apigenin and quercetin were at least two-fold more potent in their inhibitory effects on the bovine testis hyaluronidase than their corresponding glycosides, luteolin-7-glucoside, apiin, quercitrin and rutin, respectively (Table 1). Similar effects were also reported for a few other enzymes [13, 14].

The $C_{2,3}$ double bond flavonoids (flavones, flavonols) exhibited higher potency than those flavonoids lacking the double bond (flavanones, flavanonols and flavan-3-ols) (see Table 1). This laboratory has reported that such structure also confer upon the flavonoid potent antiperoxidative properties [15].

Hydroxyl substituents at positions 5, 7 and 4′ contributed to a more potent inhibitory effect for the flavonoids luteolin, apigenin, kaempferol, myricetin, quercetin and morin. Our earlier report has shown that flavonoids having 5,7-dihydroxyl substituents can bind to the active site of the cytochrome P450 and that the inhibition potency may arise from their different binding affinity to this enzyme [16]. Luteolin, hesperetin and diosmetin are very similar in structure except that the latter two compounds have methoxy substitution in position 4′. As a result, diosmetin and hesperetin exhibited reduced inhibitory activity (Table 1). The 4′-methoxy substitution was reported by Ferriola *et al.* [17] to cause steric hindrance in the essential structural feature of the flavonoid molecule that is required for inhibitory activity on rat brain protein kinase C.

Rodney *et al.* [10] reported that 3′,4′-dihydroxyl substitutions confer greater inhibitory properties than those singly hydroxylated type of flavonoids. Our present findings however, showed that the 3′-hydroxylation was not of significant importance because kaempferol and apigenin, which lacked such hydroxylation pattern could exert 56.1 and 66.5% of inhibition, respectively (see Table 1). It must be pointed out that Rodney *et al.* [10] had based their observation on eriodictyol and narigenin which are flavanone group of compounds. The 3-hydroxyl substitution in ring C was shown to be unimportant because luteolin and apigenin were potent inhibitors whilst the 3-hydroxylated flavones (flavonols) exerted lower inhibitions (Table 1).

Five most potent inhibitors, namely condensed tannin, luteolin, apigenin, kaempferol and silybin, were selected for further enzyme kinetic studies. Lineweaver–Burk plots (Fig. 1A–E) were used to determine the mode of inhibition, with least squares lines fitted to the data. The results were consistent with Michaelis–Menten kinetics for these flavonoids. Within each compound there were no statistically significant differences in the maximal enzyme velocities (V_{max}) determined at each inhibitor concentration. This showed the inhbition was for all cases a competitive one.

Low molecular weight compounds have been shown to effectively inhibit hyaluronidase, primarily because of their capability to associate in solution [4]. An acidic function (hydroxyl, carboxyl, phosphate or sulphate) is essential for the formation of the effective unit (aggregate or micelle) with multiple negative charge [4]. Further investigation is required to ascertain the possibility of aggregate formation by low molecular weight flavonoids to function as effective inhibitory units.

Heparin is well known to be a competitive inhibitor of hyaluronidase [18]. In our present study, heparin was also used (results not shown) and it exhibited only 30% inhibition within the same concentration range as the other compounds studied. Heparin has been suggested to act as a competitive inhibitor by virtue of its chemical structural similarity to the natural substrate hyaluronic acid [4]. Similar explanation was given for the inhibitions observed for sulfated hyaluronic acid, acetylated and nitrated hyaluronic acid and various other hyaluronic acid which like heparin were strongly bound to but not depolymerized by hyaluronidase [4].

However, in contrast, it is interesting to note that condensed tannin and the monomeric flavonoids used in the present study having no structural similarities to the substrate could also exert competitive inhibition on hyaluronidase. Asquith and Butler [19] reported that proteins with high contents of proline and other hydrophobic amino acids have high affinity for tannin. It has been shown that biological effects of tannin are due to its propensity for binding proteins, involving hydrogen bonding and hydrophobic interactions [20]. Considering the numerous benzene rings and hydroxyl groups present in the condensed tannin structure (Table 1), it is pertinent to suggest that the inhibitory effect may have resulted through conformational change or denaturation of the enzyme, caused by hydrogen bonding and/or hydrophobic interactions between the enzyme and the condensed tannin.

In summary, the order of decreasing potency for five most potent flavonoids as inhibitors of hyaluronidase was found to be: condensed tannin < luteolin < apigenin < kaempferol < silybin. Kinetic studies of these inhibitors showed that their mode of inhibition was competitive. Aglycones were stronger inhibitors than their corresponding glycosides. The following flavonoid structure conferred potent inhibitory effect: a double bond between carbons 2 and 3; unsubstituted hydroxyl groups at positions 5, 7 and 4′ and a ketone group at position 4.

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