Hyaluronidase and collagenase inhibitory activities of the herbal formulation *Triphala guggulu*

Venil N Sumantran[1,]*, Asavari A Kulkarni[1], Abhay Harsulkar[1], Asmita Wele[2], Soumya J Koppikar[1], Rucha Chandwaskar[1], Vishakha Gaire[2], Madhuri Dalvi[2] and Ulhas V Wagh[1]

[1]*Interactive Research School for Health Affairs (IRSHA),* [2]*Department of Ayurvedic Studies, Bhartiya Vidyapeeth Deemed University Medical College Campus, Dhankawadi, Pune 411 043, India*

**Corresponding author (Fax, 91-44-2447 0442; Email, venils@hotmail.com)*

Myrrh (*guggulu*) oleoresin from the *Commiphora mukul* tree is an important component of antiarthritic drugs in Ayurvedic medicine. Clinical data suggest that elevated levels of hyaluronidase and collagenase type 2 enzymes contribute significantly to cartilage degradation. *Triphala guggulu* (TG) is a *guggulu*-based formulation used for the treatment of arthritis. We assessed the chondroprotective potential of TG by examining its effects on the activities of pure hyaluronidase and collagenase type 2 enzymes. *Triphala shodith guggulu* (TSG), an intermediate in the production of TG, was also examined. A spectrophotometric method was used to assay Hyaluronidase activity, and to detect potential Hyaluronidase inhibitors. Aqueous and hydro-alcoholic extracts of TSG showed weak but dose-dependent inhibition of hyaluronidase activity. In contrast, the TG formulation was 50 times more potent than the TSG extract with respect to hyaluronidase inhibitory activity. A validated X-ray film-based assay was used to measure the gelatinase activity of pure collagenase type 2. Hydro-alcoholic extracts of the TG formulation were 4 times more potent than TSG with respect to collagenase inhibitory activity. Components of *Triphala* were also evaluated for their inhibitory activities on hyaluronidase and collagenase. This is the first report to show that the T2 component of *Triphala* (*T. chebula*) is a highly potent hyaluronidase and collagenase inhibitor. Thus, the TG formulation inhibits two major enzymes that can degrade cartilage matrix. Our study provides the first *in vitro* preclinical evidence of the chondroprotective properties of TG.

[Sumantran V N, Kulkarni A A, Harsulkar A, Wele A, Koppikar S J, Chandwaskar R, Gaire V, Dalvi M and Wagh U V 2007 Hyaluronidase and collagenase inhibitory activities of the herbal formulation *Triphala guggulu*; *J. Biosci*. **32** 755–761]

1. Introduction

Historically, the medicinal oleoresins produced by *Commiphora mukul* were known as myrrh. In Ayurvedic literature, myrrh oleoresins are referred to as *guggulu*. *Guggulu* destroys *ama* (bodily wastes and toxins), boosts metabolism and strength, and rejuvenates the body. Excessive *ama* plays a major role in the aetiopathology of rheumatoid arthritis. A study reported the anti-inflammatory activity of crystalline steroids from *C. mukul* resin (Arora *et al* 1971). In a clinical trial, *C. mukul* capsules administered to patients with osteoarthritis (OA) of the knee led to significant improvement without side-effects (Singh *et al* 2003). However, there are no *in vitro* studies examining the chondroprotective mechanisms underlying the anti-arthritic activity of *C. mukul*.

This study focused on *Triphala guggulu* (TG), a major *guggulu*-based formulation for the treatment of acute arthritis

Keywords. Collagenase; hyaluronidase; *Triphala guggulu* (TG); *Triphala shodith guggulu* (TSG)

Abbreviations used: ADAMTS, disintegrin containing metalloproteinases with thrombospondin motifs; HA, hyaluronic acid; HPTLC, high-performance thin layer chromatography; MMP, matrix metalloproteinase; OA, osteoarthritis; TG, *Triphala guggulu*; TSG, *Triphala shodith guggulu*

and rheumatism (Svoboda 1992). In Ayurvedic materia medica, 'triphala' is a combination of three powdered fruits, namely, *amalaki* or T1 (*Phyllanthus emblica*), *haritaki* or T2 (*Terminalia chebula*), and *bibhitaki* or T3 (*Terminalia belerica*). Historically, *triphala* was used for wound healing and ear–nose–throat (ENT) diseases (Svoboda 1992). *Triphala* is also part of several oral formulations because it acts as a *rasayana* (rejuvenating agent). Since *triphala* and *guggulu* (*C. mukul*) have wound healing and rejuvenating activities, we hypothesized that a TG formulation may inhibit enzymes that destroy connective tissue. *Triphala shodith guggulu* (TSG), an intermediate in the production of TG, was also tested.

Two assays were developed to test this hypothesis. First, we tested the effects of the TSG and TG formulations on the activity of hyaluronidase, an enzyme that destroys the hyaluronic acid (HA) backbone of cartilage matrix. Indeed, the serum level of HA is a reliable biomarker of arthritis progression (Pavelka *et al* 2004). Second, we tested the effects of both formulations on the gelatinase activity of collagenase type 2, since matrix metalloproteinases (MMPs)-1, -3 and -8 express collagenase activity which degrades cartilage matrix (Murphy and Lee 2005). Inhibition of hyaluronidase and/or collagenase by the TG formulation would in part account for its reported chondroprotective activity.

In this report we conclusively show that TG has potent hyaluronidase inhibitory activity and moderate collagenase type 2 inhibitory activity. These data provide the first *in vitro* preclinical evidence of the chondroprotective properties of TG. Interestingly, the TSG intermediate (in the production of TG) is a weak inhibitor of both enzymes.

2. Materials and methods

2.1 *Materials*

Bovine testicular hyaluronidase and the potassium salt of human umbilical cord HA were obtained from Calbiochem Corporation, USA. Hyaluronidase assays were performed on sterile 96 well plates (Tarson Corporation, India). Collagenase type 2 was obtained from Life Technologies, Gibco, USA. Guggulsterone Z was purchased from Natural Remedies, Bangalore, India. The *guggulu* resin, herbs for the preparation of *Triphala*, and *P. longum* were procured from the Ayurvedic Pharmacy of Bharatiya Vidyapeeth Deemed University Medical College Campus, Pune.

2.2 *Preparation of* Triphala shodhit guggulu *(TSG) and* Triphala guggulu *(TG)*

Three successive steps are involved as per Ayurvedic protocol (Chunekar *et al* 1995).

2.2.1 *Preparation of* Triphala *decoction: Triphala* powder (20 g) is boiled in 160 ml of water until 40 ml water is left. Therefore, the final concentration of *Triphala* powder in the decoction is 0.50 mg/ml (20 g *guggulu* in 40 ml).

2.2.2 *Purification of* guggulu *with* Triphala *to produce TSG: Guggulu* resin from *C. mukul* (10 g) is dissolved in *Triphala* decoction (40 ml). The resulting solution is filtered through muslin cloth, and termed *Triphala shodith guggulu* (TSG). TSG contains 6 g *guggulu* resin and has a final concentration of 0.15 mg/ml (6 g resin in 40 ml decoction). Filtered TSG solution is dried and stored as TSG powder.

2.2.3 *Preparation of TG from TSG:* Filtered TSG solution is heated and *Triphala* (6 g) and *P. longum* powders (2 g) of 60 mesh size are added to formulate TG (Shaha 1985). Thus, the TG formulation is a mixture of *Triphala*, TSG and *P. longum* in a proportion of 3:3:1. After preparation, the TSG and TG powders were stored in inert containers at room temperature with a dessicant.

2.3 *HPTLC analysis*

The physicochemical properties of the *guggulu* formulations matched recommended specifications (Indian Herbal Pharmacopoeia 2002). The identities of methanolic extracts of the TSG and TG formulations were confirmed by high-performance thin layer chromatography (HPTLC) fingerprint analysis using standard guggulsterone Z (Banerjee 2002) detected at 254 nm.

2.4 *Preparation of extracts of TSG and TG for enzyme inhibition studies*

All extracts of *guggulu* powders were freshly prepared before each experiment.

2.4.1 *Aqueous extracts:* TSG or TG powders were solubilized in distilled water (20–40 mg/ml) by limited autoclaving (5 lb pressure, 7 min) to yield extracts with reproducible activity.

2.4.2 *Hydro-alcoholic extracts:* An appropriate volume of absolute alcohol was added to pre-weighed TSG or TG powder and the mixture vortexed. After 12–18 h, distilled water was added to each tube for 1–3 h to obtain a final concentration of 20–40 mg/ml TSG or TG in 50% ethanol.

2.5 *Hyaluronidase assay*

Hyaluronidase was assayed by a highly sensitive spectrophotometric method, based on precipitation of

HA with cetylpyridinium chloride, which is used for high throughput screening for hyaluronidase inhibitors (Tung *et al* 1994). Enzyme (800 U/ml) and HA substrate (0.40 mg/ml) were incubated at 37°C for 1 h. Enzyme activity was measured by monitoring the percentage of undigested HA substrate in the cetylpyridinium chloride precipitate at absorbance $_{415}$nm (A_{415} nm) after the enzyme reaction.

The following formulae were used for calculations: (i) A_{415} nm value of intact undigested HA substrate was set at 100%. (ii) % Enzyme activity = (100%) – {A_{415} nm of HA + enzyme/A_{415} nm of HA x 100} (iii) Per cent digestion of the substrate by enzyme in the presence of herbal extract was calculated after correcting for absorbance of the herbal sample alone (*guggulu*) at A_{415} nm.

2.5.1 *Assay validation:* Hyaluronidase assays were standardized to get strong, reproducible activity. This assay was validated by demonstrating that pure tannic acid (0.07 mg/ml), a known hyaluronidase inhibitor (Girish and Kemparaju 2005), gave 75–80% enzyme inhibition.

2.6 *Assay of gelatinase activity of collagenase type 2*

A modified, validated, gelatin zymography method was used to assay gelatinase activity of type 2 collagenase (Sumantran *et al* 2007). We then attempted to use this method to test the effects of the TSG and TG formulations on gelatinase activity. However, this method did not work because the hydro-alcoholic extracts of TG and TSG did not evenly permeate the gel, and resulted in smeared bands of gelatin hydrolysis. The modified zymography method using water extracts of TSG formulation did not give reliable results, because TSG is sparingly soluble in water. Since it was important to compare the effects of TSG versus TG on collagenase activity, we continued with our efforts to obtain hydro-alcoholic extracts of TG and TSG formulations. In addition, we developed a different method to assay for gelatinase activity of type 2 collagenase. The method is termed as the gelatinase spot assay. It was standardized and validated before use (*see* below).

2.6.1 *Gelatinase spot assay for collagenase type 2:* Pure type 2 collagenase (0.125% w/v in 50 mM Tris–HCl buffer, pH 6.8) was assayed after preincubation with varying concentrations of the TG and TSG extracts, or solvent control (50% hydro-alcohol) for 20 min. Then, 5 μl spots of each reaction mixture were pipetted (in duplicate) onto pre-washed, unexposed X-ray film. After exposure for 15 min, films were washed to visualize enzyme activity, as indicated by clear spots of hydrolysed gelatin (Harsulkar *et al* 1998). Densitometry was done with a gel documentation system (Alpha Imager using Alpha Ease FC software, Alpha Innotech).

2.6.2 *Validation of gelatinase spot assay:* O-phenanthroline (a known zinc chelator), is an inhibitor of metalloproteinases (Murphy and Lee 2005). This chemical reproducibly inhibited the gelatinase activity of type 2 collagenase with a minimum inhibitory concentration (MIC) value of 2 mM (data not shown), and \served as a positive control.

2.6.3 *Rationale for gelatinase spot assay:* We considered the possibility of using a MMP kit to measure effects of the TSG and TG formulations on the gelatinase activity of type 2 collagenase. These kits use biotinylated or fluorescent-labelled gelatin as substrate and a specific MMP inhibitor. When we compared our gelatinase spot assay and the MMP kits, we found that both methods were semi-quantitative. The gelatinase spot assay detects collagenase protein in a range of 2.5–0.50 μg, whereas the MMP kit measures nanogram levels of MMP protein. Thus, our spot assay was less sensitive than the MMP kit. However, the kit requires this sensitivity because it is used for detecting MMP activity in body fluids or cell culture samples. In contrast, the sensitivity of the gelatinase spot assay was adequate to measure the activity of pure collagenase enzyme and its response to inhibitors.

In summary, the gelatinase spot assay for type 2 collagenase activity was validated, reproducible, reliable and sensitive enough for our stated purpose.

3. Results

3.1 *Hyaluronidase inhibitory activity of* guggulu *formulations*

3.1.1 *Hydro-alcoholic extracts of TSG and TG:* The TSG and TG formulations inhibited hyaluronidase activity in a dose-dependent manner. Table 1 shows that TSG and TG caused complete enzyme inhibition at concentrations of 4 mg/ml, and 2 mg/ml, respectively. Thus, the hydro-alcoholic extract of TG was twice as potent as TSG with respect to hyaluronidase inhibition.

Table 1. Per cent inhibition of hyaluronidase by TSG and TG formulations

Concentration of TSG or TG in enzyme reaction (mg/ml)	Enzyme inhibition by TSG (%)	Enzyme inhibition by TG (%)
4 mg/ml	100 ± 29.19	84.60 ± 8.71
2 mg/ml	46.86 ± 8.10	88.60 ± 9.55
1 mg/ml	26.16 ± 2.22	58.27 ± 6.12

Under standard conditions, 85–95% of hyaluronic acid (HA) is digested by the enzyme, i.e. the enzyme is 85–95% active. Columns 1 and 2 show the per cent enzyme inhibition with decreasing concentrations of hydro-alcoholic extracts of *Triphala shodith guggulu* (TSG) and *Triphala guggulu* (TG), expressed as mean ± SD of 5 experiments.

3.1.2 *Aqueous extracts of TSG and TG:* Although TSG was sparingly water soluble, it caused dose-dependent inhibition of hyaluronidase activity. At a concentration of 5 mg/ml, TSG showed complete enzyme inhibition. At 4 mg/ml, TSG caused a 72.82 ± 1.32% inhibition (n = 3); whereas 2 mg/ml of TSG caused 30.80 ± 3.85% enzyme inhibition (n = 3) (data not shown).

To summarize, high concentrations of aqueous and hydro-alcoholic extracts (2–5 mg/ml) of TSG were required to inhibit the enzyme. Hence, the TSG formulation contained weak inhibitors of hyaluronidase.

Next, we compared the hyaluronidase inhibitory activities of aqueous versus hydro-alcoholic extracts of the TG formulation. A high concentration of hydro-alcoholic extract of TG (2 mg/ml) was required to give 90% enzyme inhibition (first 2 columns of table 2). In contrast, much lower concentrations of aqueous extracts of TG (0.10 mg/ml, last 2 columns of table 2) were sufficient to cause 85% enzyme inhibition. Therefore, the aqueous extract of the TG formulation is 20 times more potent than the hydro-alcoholic extract of TG in inhibiting hyaluronidase (ratio of TG concentrations required to give complete enzyme inhibition: 2.0/0.10 mg/ml = 20).

3.2 *Potency of hyaluronidase inhibitory activity of TG versus TSG formulations*

Tables 1 and 2 showed that the TG formulation (but not TSG) was a potent hyaluronidase inhibitor. Since aqueous extracts of TG (not TSG), are prescribed in ayurvedic therapy, we compared the potencies of aqueous extracts of TSG versus TG, with respect to hyaluronidase inhibitory activity. Thus, 5 mg/ml of TSG versus 0.10 mg/ml of TG formulations (table 2) were required to demonstrate complete enzyme inhibition, suggesting that hyaluronidase inhibitors in the TG formulation are 50 times more potent than those present in TSG extracts (ratio of 5 mg/ml/0.10 mg/ml).

3.3 *Collagenase inhibitory activity of* guggulu *formulations*

Using the gelatinase spot assay for collagenase type 2, we tested the effects of the TSG and TG formulations on enzyme activity. Such data allowed the estimation of relative potencies of collagenase type 2 inhibitors present in TSG versus TG formulations.

3.3.1 *Triphala guggulu:* Figure 1A shows data for the TG formulation (hydro-alcoholic extracts). The enzyme alone (0.125%) showed strong activity (lane 1). Incubation of the enzyme with the solvent for TSG (50% alcohol) did not significantly alter its activity (lane 2). A concentration of 2.5 mg/ml TG showed maximal enzyme inhibition (lane 5). Densitometric analysis showed that TG (2.5 mg/ml) caused 80% enzyme inhibition, whereas lower concentrations of 1.25 and 0.625 mg/ml of TG extract (figure 1A, lanes 6, 7) caused 39% and 27% enzyme inhibition, respectively.

3.3.2. *Triphala shodith guggulu:* Figure 1B shows data on the TSG formulation (hydro-alcoholic extracts). Lanes 1 and 2 show that the enzyme (0.125%) incubated with/without the solvent for TSG (50% alcohol) retained strong activity. A concentration of 10 mg/ml TSG showed maximal enzyme inhibition (lane 3). Densitometric analysis showed that TSG (lanes 3 and 4**:** 10.0 and 5.0 mg/ml TSG) caused 86% and 66% enzyme inhibition, respectively. Lower concentrations of TSG (lanes 5–7**:** 2.50, 1.25 and 0.625 mg/ml) caused 50% enzyme inhibition each.

In summary, figure 1 shows that the TG extract is four times more potent than TSG with respect to inhibition of collagenase activity (ratio of the concentrations of TSG to TG which gave maximal enzyme inhibition was 10/2.50). Densitometric aanlysis also showed that each formulation inhibited enzyme activity in a dose-dependent manner.

Table 2. Hyaluronidase inhibition by extracts of *Triphala guggulu* (TG)

	Hydro-alcoholic extract	Hydro-alcoholic extract	Aqueous extract	Aqueous extract
Test sample	Without TG	With TG (2.0 mg/ml)	Without TG	With TG (0.10 mg/ml)
Hyaluronic acid (HA)	0.579 ± 0.038	0.562 ± 0.052	0.489 ± 0.033	0.528 ± 0.040
HA + enzyme	0.043 ± 0.013	0.545 ± 0.051	0.057 ± 0.031	0.511 ± 0.039
Enzyme activity	**92.58%**	3.02%	88.34%	4.00%
Per cent enzyme inhibition		**89.56%** (92.58–3.02)		**84.34%** (88.34–4.00)

Each value represents absorbance (A_{415} nm) of hyaluronic acid expressed as mean ± SD of 4 experiments. Formulae used to calculate enzyme activity in the presence/absence of the TG formulation are explained in section 2.5. Hyaluronidase activity in absence of *Triphala guggulu* (TG) extract is 93%. Incubation of enzyme and substrate with hydro-alcoholic extracts of TG (2 mg/ml) caused 89% enzyme inhibition. In contrast, incubation of enzyme and substrate with 20-fold lower concentration (0.10 mg/ml) of aqueous extract of TG was sufficient to cause 84% enzyme inhibition.

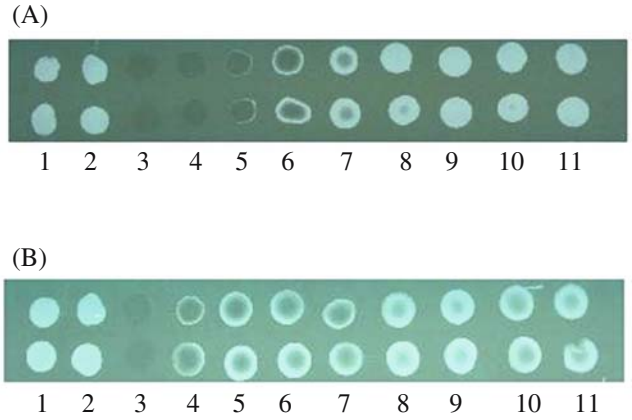

Figure 1. (A) Lanes 1, 2 show equal enzyme activity in the absence and presence of 50% alcohol (the solvent for TG). A 20 mg/ml hydro-alcoholic stock of TG was used. The concentration of TG required to give complete enzyme (0.125%) inhibition is 2.50 mg/ml (Lane 5). Lanes 6, 7 show partial enzyme inhibition with decreasing concentrations of TG (1.25, 0.625 mg/ml). Very low concentrations of TG (0.312, 0.156, 0.078 and 0.040 mg/ml) did not inhibit the enzyme (Lanes 8–11). **(B)** Lanes 1, 2 show equal enzyme activity in the absence and presence of 50% alcohol (the solvent for TSG). A 20 mg/ml hydro-alcoholic stock of TSG was used. The concentration of TSG required to give complete enzyme (0.125% inhibition) is 10 mg/ml (Lane 3). Lanes 4–11 show partial inhibition of enzyme activity with decreasing concentrations of TSG (5.0, 2.50, 1.25, 0.625, 0.312, 0.156, 0.078 and 0.04 mg/ml).

3.4 *Hyaluronidase and gelatinase inhibitory activities of components of* Triphala

The data in sections 3.2.2, 3.3, 3.4 and 3.5 indicated that components of *Triphala* synergized with the *guggulu* resin in TSG enhanced the hyaluronidase and gelatinase inhibitory activities of the TG formulation. Therefore, we tested the effects of aqueous extracts of each of the 3 component fruit powders of *Triphala* (T1, T2 and T3,) on the activities of both enzymes. These results are summarized in table 3.

3.4.1 *Hyaluronidase inhibitory activity of the components of* Triphala*:* Table 3 shows that the aqueous extracts of components of T1 (*P. emblica)* and T2 (*T. chebula*) caused complete hyaluronidase inhibition at 0.30 mg/ml and 0.15 mg/ml, respectively. However, T3 *(T. bellerica)* extracts did not cause significant enzyme inhibition. As expected, the aqueous extracts of T1, T2 and T3 (combined in a ratio of 1:1:1 to produce *Triphala*), caused complete hyaluronidase inhibition at a concentration of 0.30 mg/ml.

We draw three important conclusions from table 3. First, the T1 and T2 components of *Triphala* must synergize with TSG to cause a 50-fold potentiation of the hyaluronidase inhibitory activity of TG (0.10 mg/ml) with respect to TSG (5 mg/ml). Second, the T2 component of *Triphala* is the most potent hyaluronidase inhibitor. Indeed, the concentrations of T2 versus TG required for complete hyaluronidase inhibition were almost equal (0.15 mg/ml for T2 and 0.10 mg/ml for TG). Third, both T2 and TG were unpurified herbal drugs evaluated for hyaluronidase inhibition in a primary screen. In this context, their capability for hyaluronidase inhibition has pharmaceutical significance, i.e. crude herbal extracts with this degree of potency for inhibition of a target molecule would be considered as potential 'lead compounds' in a drug screening programme.

The potent hyaluronidase inhibitory activity of the TG formulation and its component *T. chebula* provide preclinical data with therapeutic potential.

Table 3. Potency of hyaluronidase and gelatinase inhibition by test formulations

Test drug	Hyaluronidase activity	Gelatinase activity of collagenase type 2
T1 *(P. emblica)*	0.30 mg/ml	0.625 mg/ml
T2 *(T. chebula)*	*0.15* mg/ml	*0.08* mg/ml
T3 *(T. bellerica)*	No significant inhibition	0.30 mg/ml
Triphala (T1+T2+T3 in equal proportion)	**0.30** mg/ml	**0.15** mg/ml
TG (*Triphala guggulu*)	***0.10*** mg/ml	***2.50*** mg/ml
TSG (*Triphala shodith guggulu*)	5 mg/ml	10 mg/ml

These values indicate the concentrations of fresh aqueous extracts of each formulation required to give complete enzyme inhibiton in 3 successive experiments. *Triphala* is a potent inhibitor of hyaluronidase and gelatinase activities (*see* numbers in bold). *Triphala* contains equal amounts of 3 fruit extracts (T, T2 and T3). The T2 component of *Triphala* is the most potent with respect to hyaluronidase and gelatinase inhibition (*see* italicized numbers). The T1 and T2 components of *Triphala* synergize with TSG to enhance the hyaluronidase inhibitory potency of the TG formulation (numbers italicized and in bold). All 3 components of *Triphala* can synergize with TSG to enhance the gelatinase inhibitory potency of the TG formulation (numbers italicized and in bold).

3.4.2 *Gelatinase inhibitory activity of the components of* Triphala*:* We tested the effects of the 3 component fruit powders of *Triphala* (T1, T2 and T3) on the gelatinase activity of collagenase type 2 (0.125%). Table 3 shows that each of the components of *Triphala* caused significant and reproducible inhibition of collagenase. Interestingly, as was observed with hyaluronidase, the T2 component of *Triphala* was the most potent collagenase type 2 inhibitor. As expected, water extracts of T1, T2 and T3 combined in a ratio of 1:1:1 to produce *Triphala* decoction (0.15 mg/ml) caused complete enzyme inhibition. Our data are consistent

with a report showing that *Triphala* extract (1.50 mg/ml) inhibited gelatinase activity of human periodontal tissue (Abraham *et al* 2005).

These data suggest that the 3 components of *Triphala* can individually synergize with the TSG formulation to cause a 4-fold potentiation of the collagenase inhibitory activity of the TG formulation as compared with TSG.

In addition to the 3 components of *Triphala* (T1, T2 and T3), it was important to test *P. longum.* As mentioned in section 2.2.3*, P. longum* is present in TG, but not in the TSG formulation, and could account for the greater hyaluronidase and gelatinase inhibitory activities of the TG formulation. However, this was not the case, since aqueous extracts of *P. longum* consistently failed to inhibit the activities of both enzymes (data not shown).

3.5 *Protease inhibitory activity of* Triphala guggulu

Since gelatin is a substrate for many proteases, we also tested the effect of the hydro-alcoholic extracts of TSG and TG formulations on the gelatinase activity of trypsin (0.125%) using the spot assay we had developed. Results showed that the TSG and TG formulations inhibited the gelatinase activity of trypsin with MIC values of 10 mg/ml, and 5 mg/ml, respectively; suggesting that these *guggulu* formulations may be non-specific inhibitors of a wide range of proteases. However, an earlier report showed that human trypsin is activated in certain forms of rheumatoid arthritis (Stenman *et al* 2005.). Therefore, the trypsin inhibitory activity of the TG formulation may contribute to its chondroprotective activity. This point is reinforced by reports stating that all four classes of proteases (Evans 1991), namely, the zinc MMPs, serine proteases, cysteine proteases and the disintegrin containing metalloproteinases with thrombospondin motifs (ADAMTS) proteases (Porter *et al* 2005) contribute to the degradation of cartilage matrix, bone resorption and inflammation in chronic arthritis and rheumatism.

4. Discussion and conclusions

The anti-arthritic activity of *guggulu* resin (*C. mukul)* has till date been ascribed to its anti-inflammatory actions (Arora and Kapoor 1971; Svoboda 1992). *Triphala* extracts were shown to scavenge nitrous oxide (NO) free radicals *in vitro*, which may in part account for their anti-inflammatory activity (Jagetia *et al* 2004). This is the first report showing that the TG formulation is a highly potent inhibitor of hyaluronidase (table 2). This finding becomes important when we consider that expensive injections of hyaluronic acid into the synovial cavity are administered to patients with chronic arthritis (Tanaka *et al* 2002). Clearly, a safe and potent hyaluronidase inhibitor is urgently required in the clinic, and there are none!

Our results also show that the TG formulation is a moderate inhibitor of the gelatinase activity of collagenase type 2 (figure 1) and trypsin. This finding may be physiologically significant, since both these enzymes can be activated in arthritis. This is also the first report to show that the T2 component of *Triphala* (*T. chebula*) is a highly potent inhibitor of hyaluronidase and collagenase (table 3). Such a level of potency of *T. chebula* (0.10 mg/ml inhibits both enzymes completely) has pharmaceutical significance for drug development programmes.

In summary, inhibition of hyaluronidase and collagenase activity by the TG formulation may be an important mechanism underlying its chondroprotective effects. Notably, TSG, an intermediate in the production of TG formulations, is a weak inhibitor of both hyaluronidase and collagenase. These data assume significance since it is TG (rather than TSG) which is used in ayurvedic therapy.

4.1 *Other inhibitors of hyaluronidase and collagenase type 2*

Plant-derived inhibitors of hyaluronidase and collagenase type 2 include curcumin, quercetin and aristolochic acid, which are potent inhibitors of snake venom hyaluronidase (Girish and Kemparaju 2005). Polyphenols of blackberry fruits also inhibit hyaluronidase (Marquina *et al* 2002). Green tea polyphenols (Demeule *et al* 2000) show gelatinase inhibitory activity.

Some of the prescribed anti-arthritic drugs have hyaluronidase or gelatinase inhibitory activity. Thus, the anti-inflammatory drugs indomethacin and dexamethasone (0.1–0.2 mM) are moderate inhibitors of hyaluronidase (Girish and Kemparaju 2005). The drugs nimesulide (Pelletier *et al* 1993) and doxycycline (Smith *et al* 1998) inhibited collagenase and gelatinase in cartilage derived from osteoarthritis patients *in vitro*.

In this context, the hyaluronidase and gelatinase inhibitory activities of the TG formulation containing *C. mukul* resin is promising and warrants further study. Future studies will address the active molecules of TG which cause hyaluronidase and gelatinase inhibition.

Acknowledgements

The authors acknowledge and sincerely thank Mr M Krishnan and the staff of Anchrom Corporation, Mumbai, for performing HPTLC analyses of the *guggulu* formulations. We thank Ms Trushna Chinchwade for assistance with the collagenase assays. Funding and support was provided by the Interactive Research School for Health Affairs (IRSHA), Bhartiya Vidyapeeth Deemed University, Pune.

References

Abraham S, Kumar M S, Sehgal P K, Nitish S and Jayakumar N D 2005 Evaluation of the inhibitory effect of triphala on PMN-type matrix metalloproteinase (MMP-9); *J. Periodontol.* **76** 497–502

Arora R B, Kapoor V, Gupta S K and Sharma R C 1971 Isolation of a crystalline steroidal compound from *Commiphora mukul* and its anti-inflammatory activity; *Indian J. Exp. Biol*. **9** 403–404

Banerjee P 2002 *Quality control of herbal drugs* (New Delhi: Business Horizons) pp 723–725

Chunekar K C and Pandeya G S 1995 *Bhavaprakashnighantu* 10th edition (Varanasi: Chaukhabha Bharati Academy) p. 206

Demeule M, Brossard M, Page M *et al* 2000 Matrix metalloproteinase inhibition by green tea catechins; *Biochem. Biophys. Acta* **1478** 51–60

Girish K S and Kemparaju K 2005 Inhibition of *Naja naja* venom hyaluronidase by plant derived bioactive components and polysaccharides; *Biochemistry (Mosc)* **70** 948–952

Evans C H 1991 The role of proteinases in cartilage destruction; *Agents Actions Suppl.* **32** 135–152

Harsulkar A M, Giri A P, Gupta V S, Sainani M N, Deshpande V V, Patankar A G and Ranjekar P K 1998 Characterization of *Helicoverpa armigera* gut proteinases and their interaction with proteinase inhibitors using gel X-ray film contact print technique; *Electrophoresis* **19** 1397–1402

Indian herbal Pharmacopeia Revised edn. 2002 (Mumbai: Indian Drug Manufacturers Association) pp 493–496

Jagetia G S, Rao S K, Baliga M S and Babu K 2004 The evaluation of nitric oxide scavenging activity of certain herbal formulations in vitro: a preliminary study; *Phytother. Res.* **18** 561–565

Marquina M A, Coroa G M, Araujo L, Buitrago D and Sosa M 2002 Hyaluronidase inhibitory activity from the polyphenols in the fruit of blackberry (*Rubus fruticosus* B); *Fitoterapia* **73** 727–729

Murphy G and Lee M H 2005 What are the roles of matrix-metalloproteinases in cartilage and bone repair?; *Ann. Rheum. Dis.* **64** 44–47

Pavelka K, Forejtova S and Olejarova M 2004 Hyaluronic acid levels may have predictive value for the progression of knee osteoarthritis; *Osteoarthritis Cartilage* **21** 277–278

Porter S, Clark I M, Kevorkian L and Edwards D R 2005 The ADAMTS metalloproteinases; *Biochem J*. **386** 15–27

Pelletier J P and Martel-Pelletier J 1993 Effects of nimesulide and naproxen on the degradation and metalloprotease synthesis of human osteoarthritic cartilage; *Drugs* (*Suppl. 1*) **46** 134–139

Shaha N C 1985 *The encyclopedia of ayurvedic formulations* (New Delhi: B Jain Publishers) Vol 2, pp 360–361

Singh B B, Mishra L C, Vinjamury S P, Aquilina N, Singh V J and Shepard N 2003 The effectiveness of *Commiphora mukul* for osteoarthritis of the knee: an outcomes study; *Altern. Ther. Health Med.* **9** 74–79

Smith G N Jr, Yu L P Jr, Brandt K D and Capello W N 1998 Oral administration of doxycycline reduces collagenase and gelatinase activities in extracts of human osteoarthritic cartilage; *J. Rheumatol.* **25** 532–535

Stenman M, Ainola M, Valmu L, Bjartell A, Guofeng M, Stenman U-H *et al* 2005 Trypsin-2 degrades type 2 collagen and is expressed and activated in mesenchymally transformed rheumatoid arthritis synovitis tissue; *Am. J. Pathol.* **167** 1119–1124.

Sumantran V N, Kulkarni A, Boddul S, Chinchwade T, Harsulkar A, Patwardhan B, Chopra A and Wagh U V 2007 Chondroprotective potential of root extracts of *Withania somnifera* in osteoarthritis; *J. Biosci.* **32** 299–307

Svoboda R E 1992 *Ayurvedic life health and longevity* 1st edition (New Delhi: Penguin Books) p 221, 258–25

Tanaka N, Sakahashi H, Sato F, Hirose K, Ishima T and Ishi S 2002 Intra-articular injection of high molecular weight hyaluronan after arthrocentesis as treatment for rheumatoid knees with joint effusion; *Rheumatol. Int*. **4** 151–154

Tung J S, Mark G E and Hollis G F 1994 A microplate assay for hyaluronidase and hyaluronidase inhibitors; *Anal. Biochem.* **223** 149–152

MS received 19 December 2006; accepted 7 April 2007

*e*Publication: 3 May 2007

Corresponding editor: Stuart A Newman