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Crataegus monogyna and *Centella asiatica* Extracts as Inhibitors of Cathepsin S

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Abstract Cathepsin cysteine proteases such as cathepsin S are responsible for the unfavorable and irreversible dysregulation of extracellular matrix (ECM) proteolysis found in cardiomyopathy, heart valve diseases and atherosclerosis. Cathepsin S plays a central role in ECM remodeling, which has been implicated in the development and progression of cardiovascular diseases. The current study aimed to evaluate the aqueous and ethanol extracts of the berries of *Crataegus monogyna* and the leaves of *Centella asiatica* for inhibition of cathepsin S activity using an *in vitro* fluorescent assay. Dried and ground berries of *Crataegus monogyna* and the dried and ground leaves of *Centella asiatica* were used to prepare the aqueous and organic extracts. A fluorometric enzyme assay was used to evaluate the inhibition of cathepsin S by the plant extracts. The aqueous extracts of *Crataegus monogyna and Centella asiatica* produced a maximum enzyme inhibition of 6% and 9.6% respectively, at a maximum of 200 µg/ml. The organic extract of *Centella asiatica* produced a maximum inhibition of 14%, occurring with the 100 µg/ml concentration. The ethanol extract of *Crataegus monogyna* produced a concentration-dependent inhibition of cathepsin S enzyme activity for all concentrations, with a maximum inhibition of 71.7% obtained with the highest concentration of 200 µg/ml. This demonstrates that the organic extract of *Crataegus monogyna* plays a crucial role in the inhibition of cathepsin S *in vitro and* could serve as a viable source for the isolation of cathepsin S inhibitors.

Keywords *Crataegus monogyna*, Cathepsin S, Cardiovascular Disease, *Centella asiatica*

1. Introduction

Cardiovascular diseases (CVDs) are the number one cause of death from non-communicable diseases globally and are estimated to account for 23 million deaths yearly, by the year 2030 [1-4]. Cathepsin cysteine proteases are proteolytic enzymes involved in the regulation of the remodeling of extracellular matrix (ECM) and have been implicated in the unfavorable and irreversible dysregulation of ECM proteolysis present in the development and progression of CVDs such as cardiomyopathy, heart valve diseases and atherosclerosis [5-7]. Cathepsin S has been reported to promote cardiovascular inflammatory diseases, with the enzyme activity elevated in the presence of atherosclerotic plaques [7-9]. Cathepsins also show differential expression in various stages of atherosclerosis, and the deficiency of cathepsin S has been demonstrated to reduce atherosclerosis development *in vivo* in gene knockout mice [10,11]. Thus, inhibition of the enzyme activity is considered to be an ideal target for the development of new agents for the prophylaxis of cardiovascular diseases due to its intricate involvement in the various pathological processes of cardiovascular disease [11,12].

Plant extracts have a history of use in the prevention and treatment of diseases and are a viable source of lead compounds for drug development [13]. *Centella asiatica* (L.) Urban is used as a culinary vegetable and a medicinal herb in the treatment of small wounds, burns, psoriasis, scleroderma, photo aging skin, cellulite, and striae [14]. Phytochemical compounds isolated from the plant have exhibited anti-inflammatory, antioxidant, antibacterial, antiulcer, cardioprotective, cytotoxic, and antitumor effects [14-17]. *Crataegus monogyna* Jacq. is traditionally used as a valuable medicinal herb and has been reported to increase blood flow to the heart muscles, decrease the contraction rate of cardiomyocytes, treat cardiac arrhythmias, angina, and cardiovascular diseases, as well as control hypertension [18-21]. The plant has also shown clinical efficacy in the treatment of congestive heart failure [22].

To help reduce the prevalence and death burden associated with CVD, the need for new effective treatments has led to continuing research into new therapies, with medicinal plant drug discovery playing an important role in providing leads for new drug compounds [23]. The aim of this study was to evaluate the aqueous and ethanol extracts of the berries of *C. monogyna* and leaves of *C. asiatica* for inhibition of cathepsin S activity using an *in vitro* fluorescent assay.

2. Materials and Methods

2.1. Preparation of Extracts

The dried and ground berries of *Crataegus monogyna* and the dried and ground leaves of *Centella asiatica* were purchased from Warren Chemical Specialists (Cape Town, South Africa) and Brenntag Phytochemicals (Cape Town, South Africa) respectively. To prepare aqueous and organic extracts of the plants, plant material (500 g) was macerated for 72 hours with occasional agitation with 1.8 L of ethanol or distilled water in a 5 L glass jar. Extracts were filtered and the excess solvent was removed via evaporation (for ethanol) or freeze-drying (for distilled water) to yield the organic and aqueous extracts respectively. Extracts were stored in opaque, air-tight jars at -80^{o}C until required for enzyme assay.

2.2. Fluorometric Enzyme Assay

A fluorometric enzyme assay as described by Tato *et al* [24] was used to evaluate the inhibition of cathepsin S activity by the plant extracts. The cathepsin S inhibitor screening kit (Sigma-Aldrich Chemical Corporation, Johannesburg, South Africa) for this assay contained the following: CTSS reaction buffer (15 ml); Cathepsin S, human; CTSS substrate, Z-VVR-AFC, 10 mM (0.2 ml) and CTSS Inhibitor Z-FF-FMK, 1 mM (20 ml). Four concentrations (10 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml) of the aqueous and ethanol extracts of the plants were prepared by dissolving the dried extracts in CCTS reaction buffer solution. For experiments, 10 µl of each extract concentration was pipetted into a black, flat, and clear-bottomed 96-well plate. Cathepsin S (1 µl) and CTSS reaction buffer (49 µl) were added to each well, mixed by horizontal shaking, and incubated at room temperature with protection from light for 15 minutes. Following incubation, 40 µl of enzymatic reaction mix (38 µl of CTSS reaction buffer and 2 µl of CTSS substrate, Z-VVR-AFC) was pipetted into each well, and the well plate was transferred to a Bio Tek Synermix® microplate reader (Agilent, Santa Clara, USA). Fluorescence was measured in kinetic mode, every minute, for 60 minutes at room temperature with the excitation and emission wavelengths set at 400 nm and 505 nm respectively. For a blank control, 50 µl of each extract concentration was added to 40 µl of CCTS reaction buffer without cathepsin S, while for a positive (inhibitor) control the plant extract was replaced by 10 µl of the cathepsin S inhibitor solution (1 µl of cathepsin S inhibitor (Z-FF-FMK) and 9 µl of CCTS reaction buffer) included in the kit (table 1).

2.3. Data Analysis

All extracts and controls were replicated six times and the mean fluorescence units for each dose were calculated, plotted on a graph of fluorescence units versus time, and the slope was calculated on GraphPad Prism 8 (San Diego, California). The slope obtained from the negative control was subtracted from that obtained for the corresponding concentration of test extract to obtain a corrected measurement, which was then used to determine % relative inhibition using the formula;

$$\%\ \text{Relative Inhibition} = \frac{\text{Slope}_{\text{EC}} - \text{Slope}_{\text{sample}} \times 100\%}{\text{Slope}_{\text{EC}}}$$

Where EC = enzyme control

Table 1. Enzyme activity assay protocol

	Plant extracts	CTSS inhibitor solution	Cathepsin S	CCTS reaction buffer	Enzymatic reaction mix (38 µl of CTSS reaction buffer and 2 µl of CTSS substrate, Z-VVR-AFC)
Wells for plant extracts	10 µl		1 µl	49 µl	40 µl
Wells for blank control				60 µl	40 µl
Wells for positive control		10 µl	1 µl	49 µl	40 µl

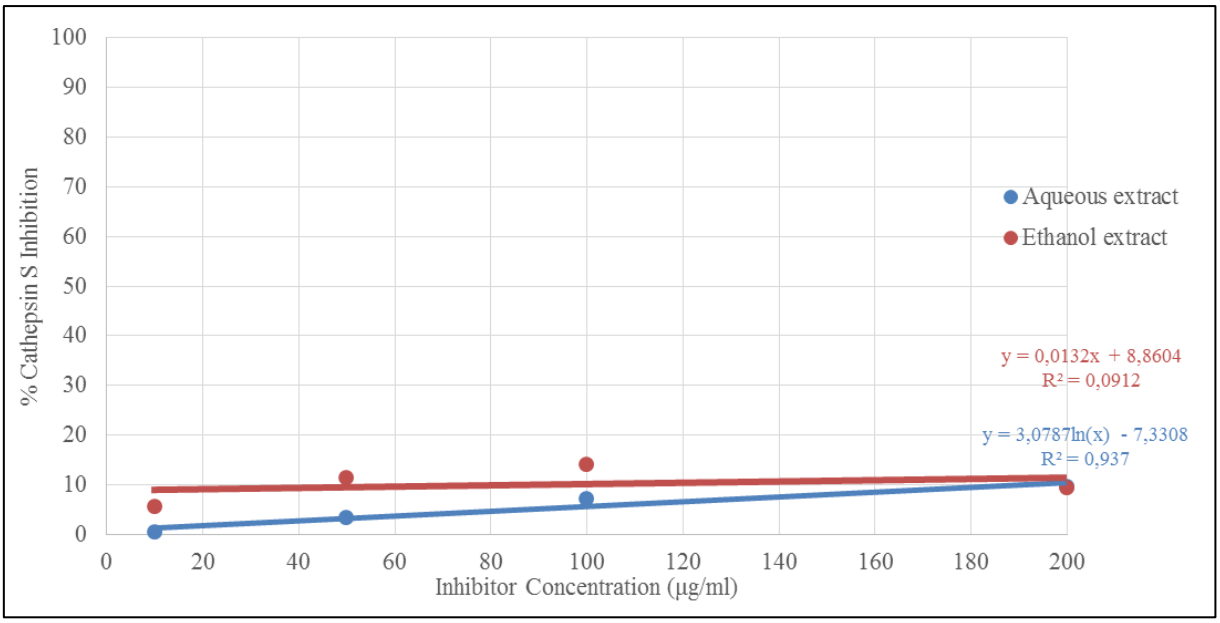

Figure 1. Percentage inhibition of Cathepsin S enzyme activity by the aqueous and ethanol extracts of *C. asiatica*

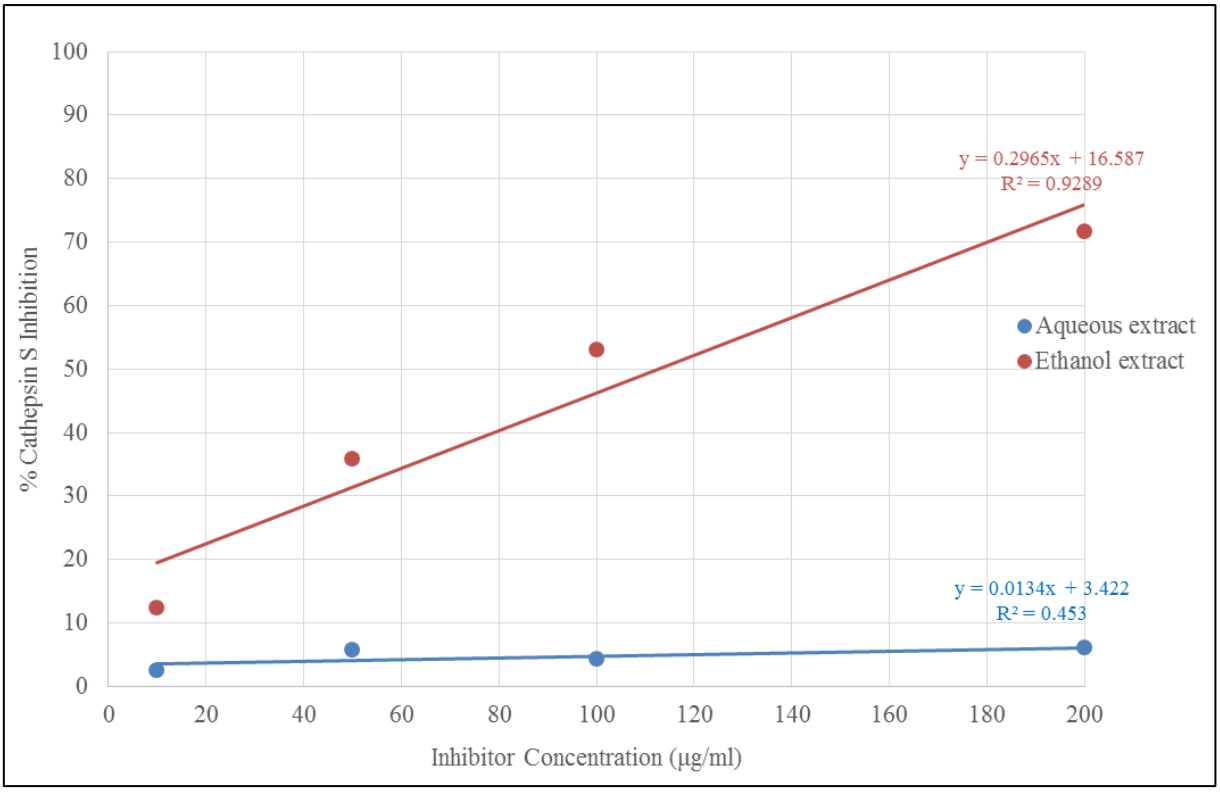

Figure 2. Percentage inhibition of Cathepsin S enzyme activity by the aqueous and ethanol extracts of *C. monogyna*

3. Results

Maceration of 1 kg of the dried and ground berries of *C. monogyna* and the dried and ground leaves of *C. asiatica* with 3.6 L of ethanol yielded 42.5 g and 28.4 g of dried extracts, with a percentage yield of 4.25% and 2.84% respectively. Maceration with distilled water on the other hand yielded 13.4 g and 7.9 g of freeze-dried extracts with a percentage yield of 1.34% and 0.79% respectively.

The aqueous and organic extracts of *C. asiatica* (10 – 200 µg/ml) produced slight inhibition of enzyme activity for all concentrations used. Maximum enzyme inhibition with the aqueous extract of *C. asiatica* (9.6%) occurred with the highest (200 µg/ml) concentration while the maximum enzyme inhibition with the ethanol extract of *C. asiatica* (14%) occurred with the 100 µg/ml concentration

(table 2, figure 1).

C. monogyna aqueous extract produced a maximum inhibition of 6% with the highest concentration tested, while the ethanol extract of *C. monogyna* produced dose-dependent inhibition of cathepsin S enzyme activity for all concentrations, with the maximum inhibition (71.7%) obtained with the highest (200 µg/ml) concentration tested (table 2 and figure 2). An IC50 of 83.5 µg/ml was calculated for the ethanol extract of *C. monogyna*.

4. Discussion

The ethanol extract of *C. monogyna* produced a dose-dependent inhibition of cathepsin S, with a 71.7% maximum inhibition of enzyme activity. Although cathepsin inhibitors, including inhibitors of cathepsin S, have been extensively studied for their anti-inflammatory and anti-cancer properties, very few researchers have investigated medicinal plants used in the treatment of cardiovascular disease for cathepsin S inhibition [25,26]. As one of the few studies on plant extracts or compounds as cathepsin S inhibitors, Hsin *et al.* [27] reported that hispolon isolated from *Phellinus igniarius* mediated the downregulation of cathepsin S expression and inhibited cell metastasis in cancer cell lines. The cathepsin S inhibitor RO5444101 was reported to attenuate the progression of atherosclerotic lesions in mice by reducing cathepsin S immunoreactivity, while LY3000328 has been reported to reduce cathepsin S activity in plasma [12,28]. LY3000328 was also reported to provide protection against atherosclerotic activity in apoe-deficient mice fed a high-fat diet [12]. It is possible that the ethanol extract of *C. monogyna* could likewise provide a protective cardiovascular effect either by preventing atherosclerotic activity or by attenuating the progression of existing lesions via the mechanisms reported with cathepsin S inhibitors in other studies [12,28].

The results are also suggestive of significant differences in the extraction efficiency of ethanol and distilled water. Both are polar solvents often used to extract bioactive compounds from plant material, however, the differences in extraction efficiency noted here may also explain the differences in enzyme inhibition observed with the two *C. monogyna* extracts [29]. *C. monogyna* extracts have been reported to exhibit neuroprotective, hepatoprotective, cardioprotective, and nephroprotective effects, and to reduce cardiovascular risk factors such as hypertension and hypercholesterolemia [30-33]. These effects have been attributed to the presence of polyphenolic compounds in the plant which are extracted by polar solvents [34,35]. Although both ethanol and distilled water are polar in nature, ethanol is known as an excellent solvent for polyphenol extraction [36,37]. As such, it is possible that the cathepsin S inhibitory effects of the plant may be due to polyphenolic compounds, but this needs to be further investigated. Although compounds isolated from *C. asiatica* have been reported to possess anti-inflammatory properties, the results of this study indicate that the plant does not contain compounds that inhibit cathepsin S enzyme activity [14].

5. Conclusion

The ethanol extract of *C. monogyna* inhibits cathepsin S enzyme activity and this holds significant potential for the development of either extracts or compounds for the prevention and treatment of cardiovascular disease of inflammatory origin.

Table 2. Percentage inhibition of cathepsin S enzyme activity by aqueous and ethanol extracts of *C. monogyna* and *C. asiatica*.

Concentration (µg/ml)	*C. monogyna*		*C. asiatica*	
	Aqueous extract	Ethanol extract	Aqueous extract	Ethanol extract
10	2.5	12.4	0.4	5.5
50	5.8	35.9	3.2	11.4
100	4.2	53.1	7.1	14
200	6	71.7	9.6	9.3

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