Coagulant Protein from plant materials:
Potential Water Treatment Agent

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“You must not lose faith in humanity. Humanity is an ocean; if a few drops of the ocean are dirty, the ocean does not become dirty.”

Mahatma Gandhi

Abstract

Access to fresh water is a human right, yet more than 780 million people, especially in rural areas, rely on unimproved sources and the need for finding ways of treating water is crucial. Although the use of natural coagulant protein in drinking water treatment has been discussed for a long time, the method is still not in practice, probably due to availability of material and limited knowledge. In this study, about hundred different crude extracts made from plant materials found in Southern India were screened for coagulation activity. Extracts of three Brassica species (Mustard, Cabbage and Cauliflower) were showing activity comparable to that of Moringa oleifera and were further investigated. Their protein content and profile were compared against each other and with coagulant protein from Moringa. Mustard (large) and Moringa seed proteins were also studied for their effect against clinically isolated bacterial strains. The protein profiles of Brassica extract showed predominant bands around 9kDa and 6.5kDa by SDS-PAGE. The peptide sequence analysis of Mustard large identified the 6.5kDa protein as Moringa coagulant protein (MO2.1) and the 9kDa protein band as seed storage protein napin3. Of thirteen clinical strains analysed, Moringa and Mustard large were proven effective in either aggregation activity or growth kinetic method or both in all thirteen and nine strains respectively. To my knowledge this is the first report on the presence of coagulant protein in Brassica seeds. Owing to the promising results Brassica species could possibly be used as a substitute to Moringa coagulating agent and chemicals in drinking water treatment.

Keywords: antimicrobial effect; Brassica; coagulant protein; MO2.1; Moringa oleifera; Mustard; napin; thermo-resistance; plant material; protein extraction; salt extract.
List of publications:

This thesis is based on the following publications, which are referred to by their roman numerals:


III. Chelliah R, **Bodlund I**, Sankaran K, Rajarao GK. Antibacterial activity of Mustard and Moringa seed extracts against pathogenic organisms (Manuscript)

Related publication:


Contribution to papers:

I. Principal author, performed minor part of the experimental work. Writing of the manuscript.

II. Principal author, took part in outlining the experiments, performed most of the experimental work and writing of the manuscript.

III. Participated in planning of the experiments, prepared seed extracts and isolated coagulant proteins and took part in the preparation of the manuscript.

Related publication I: Principal author, took part in outlining the experiments and performed all experimental work and writing of the manuscript.
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Cab</td>
<td>Cabbage</td>
</tr>
<tr>
<td>Cau</td>
<td>Cauliflower</td>
</tr>
<tr>
<td>CE</td>
<td>crude extract</td>
</tr>
<tr>
<td>DBP</td>
<td>disinfection by-product</td>
</tr>
<tr>
<td>HWT</td>
<td>household water treatment</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix-assisted laser desorption/ionization-time of flight</td>
</tr>
<tr>
<td>MDG</td>
<td>Millennium Development Goal</td>
</tr>
<tr>
<td>MO</td>
<td><em>Moringa oleifera</em></td>
</tr>
<tr>
<td>MS/MS</td>
<td>mass spectrometry/mass spectrometry</td>
</tr>
<tr>
<td>MusL</td>
<td>Mustard Large</td>
</tr>
<tr>
<td>MusS</td>
<td>Mustard Small</td>
</tr>
<tr>
<td>MusY</td>
<td>Mustard Yellow</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NOM</td>
<td>natural organic matter</td>
</tr>
<tr>
<td>NTU</td>
<td>Nephelometric turbidity units</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SODIS</td>
<td>solar water disinfection</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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</table>
1 Introduction

1.1 Background

The Millennium Development Goal (MDG) aims at halving the portion of the population without sustainable access to safe drinking water by 2015. The indicator for the MDG is “use of an improved drinking water source” as defined by the World Health Organisation (WHO). Although 2 billion people have gained access to improved water sources between 1990 and 2010, more than 780 million people are still relying on unimproved sources such as surface water and unprotected wells for drinking water (WHO/UNICEF 2011).

A recent report (WHO/UNICEF 2011) points out the inequities of the access to improved drinking-water sources in the world (Figure 1).

![Worldwide use of improved drinking water sources in 2008.](image)

**Figure 1** Worldwide use of improved drinking water sources in 2008.
The increase of accessibility of potable water has been lowest in the least developed countries and amongst the poorest people and there are great disparities between the regions of the world. The situation is worst in sub-Saharan Africa, where 40% still do not have improved water and in this area the population growth exceeds the number of people that have gained access to improved water sources.

There is also a great inequity between genders in the sustentation of water. In households without access to drinking water on the premises the responsibility for collecting water falls on women in 64% of the cases, the tribute of men, girls and boys are 24, 8 and 4% respectively. This is a significant burden and very time-consuming, in some cases girls have to stay home from school to help their mothers in the household.

Although a water source is considered as improved, this does not take into account if the water is of good quality. In Southern-Asia, the number of people that have access to water from boreholes has increased by 310 millions over the last 20 years; nevertheless, the quality of this water is of concern and might contain microorganisms or other contaminants (WHO/UNICEF 2011).

For instance, in India, rapid population growth and other factors such as industrial discharge, agricultural run-off and poor sanitation practices put the long-term availability and quality of the potable water at stake. Water from boreholes is often too hard and although most people in the urban area have access to water treated by the municipality through tap or protected pumps; this water is rarely fit for drinking unless treated first. Microbial contamination through faecal contamination in water is the major reason for the poor water quality, transmitting
a large number of diseases. The pathogens present in the drinking water include: *Shigella* species, *Salmonella* species, *Klebsiella* species, *Escherichia coli*, *Enterobacter* species, and parasites such as *Giardia lamblia* and *Entamaeba histolytica*.

Unsafe drinking water, along with poor sanitation and hygiene, accounts for around 10% of diseases worldwide including 4 billion cases of diarrhoea disease annually, causing 1.8 million deaths (WHO 2005). In particular, children, elderly and people suffering from chronic diseases are affected.

Microbial contamination can be removed by household water treatment (HWT) and the most commonly used is boiling of the water. HWT is more likely to be put in practice by people who do have access to improved water sources than those who do not. This is believed to be due to economical, social and educational reasons. HWT also reduce the risk of post-collection contamination during transport (Wright et al., 2004).

Water treatment processes exists in many parts of the world to provide safe drinking water and most commonly the municipalities carry out this service through physical and chemical processes.

### 1.2 Conventional water treatment processes

Drinking water treatment involves a number of combined processes based on the quality of the water source such as turbidity, amount of microbial load present in water and the others include cost and availability of chemicals in achieving desired level of treatment.
Generally drinking water treatment protocols consist of two major steps: coagulation/flocculation and disinfection (Figure 2). Commonly alum (aluminum sulfate) is used as a coagulation agent, as it is efficient and relatively cost-effective in developed countries; while, disinfection is achieved by the addition of chemical disinfectants like chlorine-based compounds.

![Figure 2 Schematics over conventional water treatment process.](image)

**Coagulation/flocculation and sedimentation**

Turbidity is caused by suspended particles and natural organic matter (NOM) present in the water. These particles are negatively charged and are therefore repelling each other, making them unable to aggregate and settle. The particles are carriers of unwanted contaminants and pathogenic organisms. In order to decrease the turbidity of the inlet water some positively charged chemical is added. This will destabilize the particles by neutralization of the negative charges. Flocculation is the agglomeration of these particles into large size particles known as flocs, which will settle by gravity, i.e. sedimentation (Figure 3). If the water is highly turbid, a flocculant aid is necessary in addition to the coagulant. Turbidity of water is most commonly measured by turbidimeter and expressed in Nephelometric Turbidity Units (NTU).
Prolonged usage of such chemical substances cause adverse health effects like autonomous and central nerve systemic disorders including Alzheimer's disease (Crapper et al., 1973; Martyn et al., 1989; Gupta et al., 2005; Domingo, 2006) and inhibits bone mineralisation (Mjöberg et al., 1997). Moreover, if proteolytic additives are required along with alum it becomes an expensive process.

In the sedimentation process, the settled particles (sludge) is removed and the water filtered before entering the disinfection step. The sludge is unstable and contains accumulated pathogenic organisms and should therefore be treated. Dewatering is one of the most important steps in reducing sludge volume and has a very big impact on total sludge treatment costs. It is desirable if the sludge volume is as low as possible, i.e. the flocs are really tight hence the sludge more compact as this will facilitate the sludge disposal.
Disinfection

The chemicals most commonly used for disinfection of the water are chlorine, ozone, chlorine dioxide, and chloramines, and these chemical disinfectants are all removing microorganisms present in the water. However, they are powerful oxidants and will react with NOM and contaminants in the water, and form disinfection by-products (DBPs). These DBPs have been associated with increased risk for cancer and other health related issues (Morris et al., 1992; Cantor et al., 1998; Villanueva et al., 2006; Richardson et al., 2007).

In addition to the health concerns, such complicated methods are difficult to adopt, especially in poor or developing countries, where cost-effective and simple drinking water treatment methods are needed. Therefore, usage of safe, traditional water treatment agents from natural sources becomes essential.

Although the technology and the efficiency of treating water are rapidly increasing it will take time before these techniques will be available for all. This is an urgent matter and it is very important to find alternative means of treating water in areas where the advanced treatment methods are not yet available. There is no universal solution for solving the problem with water supply in the world, every place and village is unique, and hence it is desirable to find alternative methods for water treatment to access potable water. Moreover, due to the emerging threat of climate changes WHO is now recommending small-scale water treatment rather than relying on large-scale treatment plants that constitute a more fragile system.
1.3 Water treatment with natural coagulants

Traditionally, natural coagulants of plant origin have been used at household level. Women in rural areas of Sudan (Jahn & Dirar, 1979), Tanzania (Marobhe et al., 2007a) and India (Saif et al., 2012) treat their water with Moringa seed powder prior to use as drinking water.

1.3.1 Moringa oleifera coagulant protein

The genus *Moringa* is a tropical plants belonging to the family of Moringaceae, 14 species have been identified so far and all possess coagulant properties in varying degrees (Jahn, 1988). The most extensively studied, as water treatment agent are seed extracts of *Moringa oleifera* (MO), commonly called Moringa. Various parts of this plant such as the leaves, roots, seed, bark and fruit, possess many beneficial qualities such as antitumor, anti-inflammatory, antibacterial and antifungal activities, and are being employed for the treatment of different ailments in the indigenous system of medicine, particularly in South Asia (Anwar et al., 2007).

Preparation of Moringa seed extracts

Extraction of MO coagulant agent prepared with sodium chloride (NaCl) solution is more efficient than extraction with distilled water due to the salting-in mechanism in proteins, wherein a salt increases protein-protein dissociations and protein solubility as the salt ionic strength increases (Okuda et al., 1999).

Coagulation activity

Crude extract (CE) of MO have been reported to show coagulation activity (Jahn, 1988; Muyibi & Evison, 1995; Ndabigengesere et al., 1995) similar to that of alum for samples with high turbidity (Ghebremichael et al., 2005). MO is biodegradable, and unlike alum, does not significantly affect the pH and conductivity of the water.
after the treatment (Ndabigengesere et al., 1995; Yarahmadi et al., 2009). Further it produces significantly less sludge volume than alum (Ndabigengesere et al., 1995). Moringa has also been shown efficient for conditioning of sewage sludge (Wai et al., 2009) and as a natural adsorbent for the treatment of dairy industry wastewater (Vieira et al., 2010). The concerns are that MO CE may not be an efficient coagulant for low turbid water (Muyibi & Evison, 1995) and that concentration of organic matter in the treated water increases considerably with the dosage of MO CE. This leads to changes in colour, odour and taste of the treated water if it is stored for a long time. Therefore it is suggested that the extracts should be purified before treating the water, in order to decrease the amount of organic matter added (Ndabigengesere, 1998). A simple methods for purification of the coagulating proteins has been developed by Ghebremichael et al (Ghebremichael et al., 2005).

**Mechanisms of action**

The suggested mechanism of coagulation property of MO protein is supposed to be that positively charged proteins bind to part of the surface of negatively charged particles through electrostatic interactions. This leads to the formation of negatively and positively charged areas of the particle surface. Due to particle collision and neutralization, formation of flocs with a net-like structure take place (Figure 4) (Ndabigengesere et al., 1995; Gassenschmidt et al., 1995). The poor performance of CEs in low turbid water could be explained by low rate of contacts between particles in such water.
Figure 4 Coagulation/flocculation mechanism of *Moringa oleifera* protein.

**Protein structure**

The coagulating agent in Moringa seed has been identified as a protein with a molecular mass of about 6.5 kDa and the isoelectric point is above pI 10. Amino acid analysis and sequencing showed a total of 60 residues and this peptide has been reported to the protein database and given the name MO2.1 (SwissProt ID:P24303) (Gassenschmidt et al., 1995). A 3D structure of the protein has been suggested by Okoli et al. (Okoli, 2012). The peptide has eight positively charged amino acids (7 arginines and 1 histidine) and 15 glutamine residues. It has been shown that glutamine-rich regions in proteins cause their aggregation (DePace et al., 1998).

There are significant homologies between MO2.1 and common seed storage proteins such as 2S albumin and a part of the large chain of both napin and
mabinlin (Broin et al., 2002; Suarez et al., 2005). Napins are initially synthesized as a precursor protein, which is proteolytically cleaved to generate a smaller A chain of 4,000 Da and a larger B chain of 9,000 Da held together by disulfide bonds (Ericson et al., 1986). Such homologies may be useful for explaining the flocculent activity of other seeds that have been traditionally used in water clarification.

**Thermo-resistance of MO coagulant protein**

The protein is found to be thermo-resistant and possesses coagulation activity after 5 hours of heat treatment at 95°C (Ghebremichael et al., 2005). Since this property makes them less susceptible to deterioration, it can be seen as a prerequisite for natural coagulants to be thermo-resistant. Due to the high average temperatures (35–40°C) in tropical countries, they would not be suitable if they were heat sensitive.

**Effect of MO against microorganisms**

MO extract has flocculating properties towards bacteria (Broin et al., 2002; Ghebremichael et al., 2005) and bacteriostatic effect has been observed against several human pathogens. However, the inhibition of *Escherichia coli* growth was found to be transitory, with resumption of growth after 3–6 hours (Suarez et al., 2003). In addition, MO were able to reduce the number of the parasite *Schistosoma mansoni* cercariae (Olsen, 1987) and helminth eggs (Sengupta et al., 2012). Moringa extract has been suggested as a complement to solar water disinfection (SODIS) due to its bacteriostatic effect and ability to clarify and decolorize water before SODIS treatment (Wilson & Andrews, 2011).
1.3.2 Other natural coagulants

Coagulation activity has also been reported from other plant materials such as: Cactus (*Opuntia spp.*) (Zhang et al., 2006; Miller et al., 2008), common bean (*Phaseolus vulgaris* (Šeiban & Antov, 2006), red bean (*Phaseolus vulgaris* sp.), sugar maize and red maize (*Zea mays* sp.) (Gunaratna et al., 2007), chestnut and acorn (Šeiban et al., 2009) *Cactus latifaria* and seeds of *Prosopis juliflora* (Diaz et al., 1999), *Cassia angustifolia* (Sanghi et al., 2002), Grape seeds (Jeon et al., 2009), Nirmali seeds (*Strychnos potatorum*) (Babu & Chaudhuri, 2005), *Jatropha curcas* and Guar gum (Pritchard et al., 2009), *Vigna unguiculata* and *Parkinsonia aculeate* (Marobhe et al., 2007b), Tannins (Ozacar & Sengil, 2000; Ozacar & Şengil, 2003; Beltrán-Heredia et al., 2010; Sánchez-Martín et al., 2010), *Enteromorpha* extract (green algae) (Zhao et al., 2012) and Algal alginate (Devrimci et al., 2012).

Thermo-resistance of coagulant protein was also confirmed in seeds from *V. unguiculata, P. aculeate* (Marobhe et al., 2007b), red bean, sugar maize and red maize (Gunaratna et al., 2007). Further, a coagulant protein with a molecular mass of around 6.0 kDa, similar to that of MO, was identified in both *V. unguiculata* and *P. aculeate* (Marobhe et al., 2007b).

Although many natural coagulants have been identified, most of them are not feasible due to cost and availability worldwide, thus difficult to implement for water treatment. Moreover, the availability throughout the year is an important factor to take into consideration. Some of them are competing with food-crops or do not show effect against microorganisms. This calls for a systematic study in order to identify other natural coagulants that might be more applicable for water treatment.
1.4 Objective

The creative idea of women in villages in Asia and Africa for household water treatment using natural plant materials deserves to be acknowledged. Therefore, the potential natural coagulants should be assessed for its application in HWT. These findings should be evaluated for potential use in advanced water treatment technology and knowledge for improved HWT should be transferred back to the villages.

Southern India was selected as a good location to perform these experiments, as it constituted a good representative for tropical climates and the results and findings can be applicable to other parts of the world. This area is having both water scarcity and limited water treatment hence, there is an urgent need for efficient processes.

The aim of the work presented in this thesis was to identify new plant materials with coagulating properties. These should have competitive characteristics such as being cost effective, easily available and having an effect against microorganisms.

This was done by an extensive screening study with many various plant materials from fruits, vegetables, nuts and pulses. The materials showing the best potential coagulating agent were further examined with respect to physico-chemical properties such as protein content, protein profile and thermo-resistance/activation and the active agent was identified in order to better understand the molecules responsible for coagulation property. This information could add knowledge to the field of natural coagulants and help in understanding the specific seed storage protein responsible for coagulating ability.
In addition to the coagulation activity of the seed extract, it is desirable to find out whether the extract possesses antibacterial activity and can be used as a water treatment agent. This was tested by aggregation of bacterial cells and growth inhibition of pathogenic microorganisms causing waterborne diseases. The active coagulant from the newly identified plant materials was further analysed in pond water for their potential application in water treatment.
2 Experimental technique

2.1 Preparation of coagulant protein

The seeds were removed from the dried pods or from fruits and ground to fine powder, other plant material such as leaves were dried and ground to a paste. Lipid extraction was thereafter conducted with ethanol as described by Ghebremichael (Ghebremichael et al., 2005). All the experiments were performed at least three times in replicates and the results are discussed.

Extraction of soluble proteins from all dried cake solids was prepared (5% w/v) using distilled water or 0.5 M sodium chloride (NaCl) solution. A flowchart of the coagulant extracting process is depicted in Figure 5.

Figure 5 Schematic of coagulant protein extraction from plant material.
The CEs were further purified by Sepharose ion exchange method as described by Ghebremichael et al. (2005). Sepharose ion exchanger was prepared by using 10mM Ammonium acetate solution. The aqueous crude extract was added to the equilibrated sepharose ion exchanger and mixed gently for 30 minutes. Finally the protein was eluted using NaCl. The obtained liquid is referred to as coagulant protein (CP).

2.2 Protein analysis

Protein content of the extracts was estimated using protein-dye binding method (Bradford, 1976) and a standard curve was plotted using known concentrations of Bovine Serum Albumin (BSA). Before the antimicrobial experiments, the protein content of the extracts was estimated by Lowry’s method (Lowry et al., 1951).

The protein profile and the molecular mass of the protein were determined by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970; Fling & Gregerson, 1986).

In order to analyse the stability of coagulant proteins, the CEs were heated at 95°C for 5 hours. Protein content and protein profiles, before and after the heat treatment, were compared and coagulation activity was tested.

Protein bands from SDS-PAGE were excised and processed according to Ghebremichael et al. (2005) with some modifications. The mass spectra were acquired (after calibration with standard peptides) using Mass Spectrometer (Waters, USA). The proteins were identified by GPS explorer software using the
Mascot software program; sequence information and other details were obtained from SwisProt and UniProt.

2.2.1 Coagulation activity

Water sample for coagulation activity tests was prepared as 1% kaolin solution using tap water. Coagulation activity test was carried out with kaolin synthetic clay solution as described by Ghebremichael (Ghebremichael et al., 2005). Crude extract (10µl) was added to the clay solution, mixed instantly and measured the initial absorbance at 500 nm (A₅₀₀) using a spectrophotometer. The solution was allowed to settle and thereafter A₅₀₀ was measured again. The percentage of coagulation activity was calculated using the following formula: \( \frac{(t₀-t)/t₀) \times 100} \), where \( t₀ \) is the A₅₀₀ measured instantly after the sample has been homogenized and \( t \) is the A₅₀₀ measured after 60 or 90 minutes.

2.2.2 Effect against microbial strains

The bacteria were grown in nutrient broth at 37°C in shaking incubator overnight and diluted to an initial absorbance of 0.1 at 600 nm. The two characteristics to be investigated were: i) the ability of the extracts to aggregate bacterial strains, and ii) the effect of the extracts on the growth of the bacterial strains. See paper III for detailed description.
3 Present investigation

3.1 Screening for coagulants (Paper I)

Around 100 plant materials from 75 different plant species were collected in and around Chennai region, India. From these plant materials, both water and salt (NaCl) crude extracts were prepared for coagulation activity test, i.e. 200 extracts in total. Among the samples tested, only seven extracts other than *Moringa oleifera* (MO), showed more than 50% activity as can be seen in Figure 6.

In all cases there was higher activity in salt extracts than in the water extracts, implying the salting-in phenomenon (Okuda et al., 1999). The coagulation activity of Mustard, Cabbage, and Cauliflower crude seed extracts showed more than 80% activity and the results are comparable to that of MO. Moreover, Mustard and Cabbage were the only water CEs showing more than 70% coagulation activity. Interestingly, these plant seeds belong to the same genera, *Brassica*.
Cabbage and Cauliflower are food-crops and the availability and costs of seeds are of concern. On the other hand, Mustard is a spice cultivated abundantly for oil production and different Mustard varieties are cultivated in various parts of the world. It would be of interest to test the coagulation activity from different Mustard varieties available in Southern India.

Although the activity of the salt extracts was slightly higher for these seeds, water extracts were used for further studies in order to develop a simple protocol for preparing coagulant protein as a potential candidate in water treatment.

### 3.2 Coagulants from *Brassica* species (Paper I & II)

The crude seed extracts were made from Cauliflower (Cau), Cabbage (Cab) and three types of Mustard seeds: Mustard large (MusL), Mustard Small (MusS) and Mustard Yellow (MusY), belonging to the family *Brassica*, except MusY. Mustard Yellow (*Sinapsis alba*) was previously in the *Brassica* family but is now considered as a member of the *Sinapsis* family.

#### 3.2.1 Protein content and profile

There was a reduction in protein content after heat treatment in all the crude seed extracts (Table 1).

<table>
<thead>
<tr>
<th>Crude Extract</th>
<th>Protein content (mg/ml)</th>
<th>Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before heating</td>
<td>After heating</td>
</tr>
<tr>
<td>Cauliflower (Cau)</td>
<td>2.4</td>
<td>2.2</td>
</tr>
<tr>
<td>Cabbage (Cab)</td>
<td>1.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Mustard Yellow (MusY)</td>
<td>4.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Mustard Large (MusL)</td>
<td>2.9</td>
<td>2.7</td>
</tr>
<tr>
<td>Mustard Small (MusS)</td>
<td>0.9</td>
<td>0.8</td>
</tr>
</tbody>
</table>
The protein profile of the CEs from different seeds before and after heat treatment revealed by SDS-PAGE is shown in Figure 7. There are several protein bands visualized on samples before heat treatment, while after heating, only the 9 kDa and 6.5 kDa protein was observed with similar intensities. This indicates that some of the proteins have been denatured during the heating process (Table 1). The heat-stable low-molecular weight protein could possess coagulation activity.

**Figure 7** Protein profile for *Brassica* CEs before and after heating (marked with Δ). The loaded amount of protein was around 10 µg/well. Marker used was low molecular weight marker from Sigma (6.5-66 kDa).

### 3.2.2 Coagulation activity before and after heating

MusS and MusL, which had the highest activity before heating showed only a slight increase in coagulation activity after heating whereas the other extracts clearly gained activity upon heating (Figure 8). This suggests some partial hindrance that was removed upon heating. MusL and MusS, whose coagulation activity did not increase dramatically, on the other hand did not lose much protein upon heating. The possible mechanism of thermo-activation of coagulant protein remains to be clarified. The thermo-resistance of coagulant protein are in accordance with previous findings from other natural coagulants (Ghebremichael et al., 2005; Marobhe et al., 2007b) and since only two protein bands of 9 and 6.5
kDa (marked with arrows in Figure 7) are remaining in heated extracts these are likely to be responsible for the coagulation property. Hence it is interesting to investigate the peptide sequences of the low molecular weight molecules.

**Figure 8** Coagulation activity for various crude extracts before and after heat treatment (90min). The kaolin solution as blank is marked with a line.

### 3.3 Mass Spectrometric analysis of protein (Paper II)

The purification protocol developed for MO (Ghebremichael et al., 2005) was not found equally efficient for MusL, after purification two protein bands with a molecular weight of 6.5 and 9 kDa were still visible on SDS-PAGE gel (Figure 9). Hence, after SDS-PAGE the protein bands of 6.5 and 9 kDa were excised and digested from peptide analysis by MALDI-TOF to disclose their identity.
The MS/MS analysis of these two trypsin digested protein bands from SDS-PAGE revealed totally six peptide fragments (Table 2). From the 6.5 kDa protein band, only one sequence was obtained whereas five peptide fragments were obtained from the 9 kDa protein band.

<table>
<thead>
<tr>
<th>No.</th>
<th>Mol. weight</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.5 kDa</td>
<td>QAVQLTHQQQGQVGPQQVR</td>
</tr>
<tr>
<td>2</td>
<td>9 kDa</td>
<td>GPQGPQQRPPLLQQGNCNELHQEEPLVCPTLK</td>
</tr>
<tr>
<td>3</td>
<td>9 kDa</td>
<td>GQQGQQLQQVISR</td>
</tr>
<tr>
<td>4</td>
<td>9 kDa</td>
<td>QQGQGQQGQQLQQVISR</td>
</tr>
<tr>
<td>5</td>
<td>9 kDa</td>
<td>QQVRQQQGQQLQQVISR</td>
</tr>
<tr>
<td>6</td>
<td>9 kDa</td>
<td>KVCNIPQVSVCPPQK</td>
</tr>
</tbody>
</table>

Figure 9 SDS-PAGE gel of Mustard and Moringa extracts. Lane 1: MusL CE, 2: MusL CP, 3: Marker Sigma-Aldrich 6.5-66 kDa, 4 MO CP, 5 MO CE. The CPs shown on the gel are crude extracts first eluted with 0.3 M NaCl followed by elution with 0.6 M NaCl. The box marks the two protein bands from MusL CP.
Peptide sequence 1 (Table 2) obtained from 6.5 kDa protein band showed a sequence coverage of 37% with flocculent protein of *Moringa oleifera* (MO2.1; SwissProt ID: P24303) (Figure 10).

The other five peptides from 9 kDa band correlated with napin3 protein (SwissProt ID: P80208) (Figure 10). Napin3 consists of two chains, small (4.2 kDa) and large (9.8 kDa) and the identified peptides and the size of the band are matching the large chain. Peptide sequences 3, 4 and 5 were found to overlap each other.

<table>
<thead>
<tr>
<th>P24303</th>
<th>MO2X_MOROL Flocculent-active proteins MO2.1 and MO2.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>QGPGRQPQDFQRGQQGQLRNISPPRCPSSLRQAVQLTHQQGQQVGPGQQVRQMYRVASNIPST</td>
<td>QAVQLTHQQGQQVGPGQQVR</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P80208</th>
<th>2SS3_BRANA Napin-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small chain: SAGPFRIPKCRKEIFQQAQHLRACQQWLHMKAMQSGSG</td>
<td></td>
</tr>
<tr>
<td>Large chain: PQGPQQRPPLLQQCCNELHQEEPLCVCPTLGAS41</td>
<td>PQGPQQRPPLLQQCCNELHQEEPLCVCPTLK</td>
</tr>
<tr>
<td>42RAVKQQVQOGQQGQQGQOLQQVQISR</td>
<td>43QVQVQRQOGQQGQOLQQVQISR</td>
</tr>
<tr>
<td>44VQCGQQGQQLQQVQISR</td>
<td>45VCNIPQSVCPFK</td>
</tr>
<tr>
<td>46QQGQQGQLQQVQISR</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 10** Sequence alignment of identified peptides in Table 2.

The homology of MO2.1 with other seed storage proteins like 2S albumin, napin and mabinlin has been reported earlier (Broin et al., 2002; Suarez et al., 2005). Sequence alignment between MO2.1 and napin3 heavy chain showed 23 identical and 22 similar residues out of 60 and 88 amino acids respectively (Figure 11).
Similar to MO2.1, napin3 heavy chain is also rich in positively charged and glutamine residues, which could explain the coagulating properties. Out of 88 amino acids, there are 10 positively charged, only 3 negatively charged and 20 glutamines residues. It could be speculated that these proteins might have synergistic coagulation activity in Mustard since both 6.5 and 9 kDa protein was found. The coagulant property of the 9 kDa protein from Mustard remains to be evaluated.

In the present investigation, MusL seed extract was found to posses 6.5 and 9kDa protein having coagulation activity and these peptide sequences are homologous with MO coagulant protein. Therefore, it is desirable to find out their antibacterial properties and evaluate the potential use as water treatment agent.

### 3.4 Effect against clinical isolated strains (Paper III)

The effect of coagulant protein from MO and MusL were analysed for bacterial aggregation and reduction in bacterial growth. Table 3 summarises the results of thirteen clinical strains against crude and isolated coagulant proteins. Different
concentrations were tested to find out the optimum dosage for cell aggregation and growth reduction of bacteria. The optimum concentration of Moringa and Mustard seed extracts and the effect of cell aggregation can be seen in Table 3.

Table 3: Comparative analysis of Moringa and Mustard soluble seed protein towards thirteen different clinical pathogens

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Species</th>
<th>Moringa</th>
<th>Mustard</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein concentration (mg/ml) CE/CP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><em>Escherichia coli</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td><em>Enterococcus faecalis</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td><em>Shigella flexneri</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td><em>Shigella dysenteriae</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td><em>Salmonella typhi</em></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td><em>Salmonella paratyphi A</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td><em>Salmonella paratyphi B</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td><em>Salmonella typhimurium</em></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td><em>Serratia marcescens</em></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td><em>Enterobacter species</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td><em>Proteus mirabilis</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td><em>Staphylococcus aureus</em></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ indicates effect  
- indicates no effect

In conclusion, based on the above findings, both Moringa and Mustard seeds appear to have potential to be used for the removal of microorganisms, which can be readily applied for water treatment. Both these seed extracts showed good aggregation property; Mustard seed extract needs higher dosage level when compared with seed extract of Moringa. The studies showed that Moringa and
Mustard seed protein have properties such as aggregation and reduced bacterial growth (Paper III). Similar results were observed with *P. aculeata* and *V. unguiculata* seed protein showing aggregation and inhibition of bacterial growth (Marobhe et al., 2007b).

Since Mustard coagulant proteins possess similar coagulation and heat resistant properties to that of Moringa, it is interesting to investigate the application of Mustard seed extract for water treatment.

### 3.5 Potential applications of natural coagulants

#### 3.5.1 Effect of coagulant protein on turbid water from pond (Paper II)

To further confirm the coagulation activity of Mustard seed extract, highly turbid water was collected from a pond in Maduravoil, Chennai, India and treated with MusL and MO CE. Both extracts showed good coagulation activities. In fact, MusL showed better coagulation activity than MO (Figure 12) indicating the possible variability in efficiencies of plant coagulant proteins according to the nature of clay and other suspended particles.
Figure 12 Coagulation activity of Moringa and Mustard Large CE in turbid pond water. Samples for measurement of turbidity is taken after 60, 120 and 180 minutes. Pond water alone was used as control.

3.5.2 Water treatment with plant seeds in Tanzania

In a similar experiment conducted in Tanzania (Related publication I), two plant seeds were investigated regarding their coagulation activity and reduction of faecal coliforms. i) *Azadirachta indica*, commonly known as Neem, a tropical, fast growing, evergreen tree (Girish & Shankara, 2008) with many biological and pharmacological activities attributable to different parts of the plant (Atawodi & Atawodi, 2009). ii) The plant called Groundnut, a wild nut that is called “karanga”, which means Groundnut in Swahili.
The preliminary results with highly turbid water from Ruvu River clearly indicate that Groundnut CE is very efficient. As an example it can be mentioned that 75 mg of Groundnut seed powder (3.3 mg protein) in one litre of river water managed to reduce the turbidity from 1000 to 2 NTU in 30 minutes, thus 99.8 % reduction. Moreover, it was observed that water treated with Groundnut gave visibly smaller and more compact flocks than the water treated with alum (Figure 13).

![Figure 13](image)

Figure 13 Water with initial turbidity 1000 NTU treated with Groundnut CE (to the left) and alum (to the right).

Jar test experiments showed that both Neem and Groundnut CEs have the ability to reduce the turbidity of river water with the initial turbidity of 200 and 450 NTU by 95% or more. The removal efficiencies of faecal coliforms by Neem and Groundnut CEs were 81 and 61 % respectively. It is difficult to state the antibacterial mechanism of CEs, although bacterial flocculation and settlement along with other coagulated colloidal particles could have been involved. However, the Neem CE seem to possess antimicrobial properties due to its remarkable reduction in bacterial count while the residual turbidity remained higher than that observed for Groundnut CE.
Among hundred different materials tested for coagulation activity, the present study revealed the presence of coagulant proteins in *Brassica* seed extracts. The coagulant proteins are remained active even after heat treatment at 95°C for 5 hours. Coagulant proteins with molecular mass of approximately 6.5 and 9 kDa were identified in all *Brassica* extracts and peptide sequence analysis of Mustard large confirms the presence of coagulant protein with approximately 6.5kDa protein similar to MO2.1 and a 9kDa protein similar to napin3 seed storage protein. The napin3 peptide sequence has homology with MO2.1 suggesting that both 6.5 and 9kDa protein could be involved in coagulation activity. This research has further established that Mustard large seeds have an effect against microorganisms. To extend the study, some experiments were conducted with water from pond and river, and the results were promising. To my knowledge this is the first report on coagulating agent present in *Brassica* species.

The low cost of the raw material and the availability of Mustard throughout the year are the added advantages and Mustard large coagulating agent might be a good complement to Moringa and chemicals in drinking water treatment. The technology of using natural coagulants for treatment of water is most appropriate in developing countries, especially in rural areas, where they cannot afford the high cost of conventional coagulants.
5 Further studies

The prospect of using Brassica species for water treatment is dependant on availability of the raw material and the costs of the extraction process. In order to really make it a viable option it would be most preferable if residues from other processes such as oil industry could be used. In a preliminary study, Mustard cake from oil industry and seed extracts from Brassica napus (Rapeseeds) provided by Lantmännen SW Seed AB showed promising coagulation property. This could constitute additional materials in large-scale production of coagulants for water treatment processes. Furthermore, it could reduce the cost of production and increase the availability of the material. Moreover, it is interesting to find out the differences in coagulation property of 6.5 and 9 kDa protein in Mustard seed extracts. Based on these findings large-scale production of coagulant protein will be studied for water treatment. It is of interest to test the potential coagulant in a pilot study with different water quality and contaminants from different localities. The results of these findings will lead to the development of household water treatment methods and a transfer of scientific knowledge to the rural people who are using natural coagulants.
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Tack! ֻ Gracias!  Thank you!  Asante!
7 References


