Isolation and Characterization of Cationic Peptides from Selected Plants

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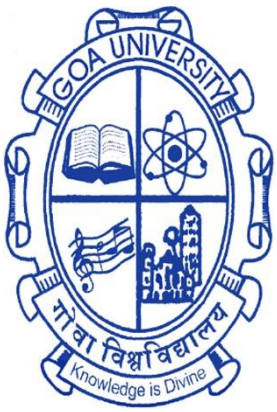

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DECLARATION

I, Sreekala S., hereby declare that this thesis represents work which has been carried out by me and that it has not been submitted, either in part or full, to any other University or Institution for the award of any research degree.

Place: Goa University

Date: Sreekala S.

CERTIFICATE

I hereby certify that the above Declaration of the candidate, Sreekala S., is true and that the work was carried out under my supervision.

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Sreekala S.

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Chapter 1

Introduction and Literature Review

1. Introduction

A diverse flora of medicinal plants has been used worldwide since ancient times as a part of traditional, folklore, Unani, Siddha as well as Ayurveda systems of healthcare to cure many complex maladies (Patwardhan et al. 2004; Vaidya and Devasagayam 2007; Ekor 2014). As per WHO estimates, more than 60% of the world population prefers natural products for primary health care, where the plant-based products outnumber any other natural products in respect of safety, cost-effectiveness and efficacy (Ekor 2014; Atanasov et al. 2015; WHO 2019). It is worth mentioning that the therapeutic use of most of traditional herbal formulations stemmed from empirical knowledge rather than precise interpretation of the active components present in these multi-compound mixtures (Ekor 2014; Anand et al. 2019). As a result, there is a remarkable interest in exploring traditionally known medicinal properties of various plant species and the potential phytochemical constituents using elaborate scientific approaches, thereby expanding the chemical-structural scaffold libraries for novel drug development (Sen and Chakraborty 2016; Anand et al. 2019; Lautie et al. 2020; Atanasov et al. 2021).

A heterogeneous array of phyto-biologics such as alkaloids, flavonoids, polyphenols, phenolic acids, glucosinolates, saponins, isothiocyanates, tannins, carotenoids, peptides, proteins, etc. with broad spectrum bioactivities and pharmacological properties have been identified from several plant species (Campos-Vega and Oomah 2013; Mohanraj et al. 2018). Among these bioactive molecules, plant-derived antimicrobial peptides which are produced as a part of plant defense mechanism encompass a distinct chemical space in natural product-based drug discovery due to their unique structural stability as well as functional characteristics.

1.1. Cationic Antimicrobial Peptides

Cationic Antimicrobial Peptides (CAPs) are ubiquitous natural antibiotics that exist in almost all organisms as an important constituent of the innate immune system. As an evolutionarily ancient molecular weapon, CAPs played an inevitable role in successful evolution of multicellular organisms (Hancock 1999; Zasloff 2002; Reddy et al. 2004; Jenssen et al. 2006; Rathinakumar and Wimley 2010; Lei et al. 2019). In general, they share many common characteristic features such as:

- Short chain length – usually of <100 amino acid residues and Mol. wt. <10kDa.
- Cationic or basic nature at physiological pH.
- Consisting of two or more basic amino acids (arginine or lysine) and at least 50% hydrophobic amino acid residues.
- Amphipathic nature, the alignment of hydrophilic and hydrophobic amino acid residues allowing electrostatic and hydrophobic interaction with lipid bilayers of microbial cell membranes.
- Broad spectrum bioactivities and immuno-modulatory effects.

The CAPs are expressed by specific sets of genes that are either constitutive or inducible by external stimuli (Rathinakumar and Wimley 2010; Lei et al. 2019). They exhibit diverse structural features which are primarily based for their defensive functions (Koehbach and Craik 2019). Majority of CAPs that have been isolated from insects, animals and plants are linear peptides that fold into their final conformation upon interacting with microbial membranes (Brogden et al. 2003; Nawrot et al. 2014; Wu et al. 2018). Nevertheless, the presence of cysteine-rich cyclic peptides was also reported from all major life forms (Nagy et al. 2015; Mikulass et al. 2016). Based on their secondary structures, antimicrobial peptides have been classified into four groups:

i. α-helical peptides – with hydrophobic and hydrophilic amino acid residues aligned along opposite sides and forming helical structures (Tossi et al. 2000). e.g. Magainin, Cecropin A, Cathelicidin, Melittin, Dermaseptin, etc.
ii. Peptides with β-sheet structure – with one or two disulfide bridges and forming hairpin or loop like structures (Jin et al. 2005). e.g. Lactoferricin, Tachyplesin
iii. Cysteine-rich peptides – with more than two intramolecular disulfide bonds (Nagy et al. 2015; Mikulass et al. 2016). e.g. Human defensins (HNP-1, 2 &3), plant defensins.
iv. Peptides rich in specific amino acids such as arginine, histidine, glycine and tryptophan or proline (Huan et al. 2020). e.g. Histatin (histidine), coleoptericin (glycine), tritripticin, holotricin (glycine & histidine), pyrrhocoricin (proline), indolicidin (tryptophan).

1.2. Modes of action of CAPs

Mode of action of CAPs is heavily reliant on their structural as well as molecular features (Bechinger and Gorr 2017; Huan et al. 2020). Their cationic or basic nature facilitates electrostatic interaction with negatively charged microbial membrane components, while the hydrophobic nature facilitates membrane penetration, which finally leads to the disruption of microbial cells (Li et al. 2017). Microbicidal effects exerted by CAPs may vary for different microbial species. While phospholipid bilayers of microbial cell membranes are mainly targeted for peptide activity, growing evidences suggest that some CAPs could cross the microbial membrane and interfere with intracellular activities such as DNA or protein synthesis, cell wall synthesis and protein folding (Cascales et al. 2011; Buccini et al. 2021).

Several hypothetical models have been proposed to explain the interactions of CAPs with phospholipid bilayers of microbial membrane and their mechanism of antimicrobial action (Wimley 2010; Kumar et al. 2018; Raheem and Straus 2019):

a) *The barrel stave model* – according to this model the hydrophilic and hydrophobic regions of α-helical peptide monomers contact with head groups and acyl chains of membrane phospholipids, respectively, assemble at the membrane surface and form transmembrane pores with the hydrophilic region of the peptide on the luminal side. This dissipates the transmembrane potential of microbial cells. Non-ribosomally synthesized Alamethicin (*Trichoderma viridae*) and gene-encoded Pardaxin from Red Sea Moses specifically act based on this model (Wimley 2010; Kumar et al. 2018; Raheem and Straus 2019).

b) *The carpet model or the detergent model* – this model suggests that the peptides electrostatically bind with microbial membrane components such as teichoic acids of Gram-positive bacteria and lipopolysaccharides of Gram-negative bacteria and traverse the phospholipid bilayer by forming a carpet-like structure. The accumulation of peptides up to a threshold concentration collapse the phospholipid bilayer and cause cell disruption. This model described the activity of human Cathelicidins, Dermaseptin from the frog genus *Phyllomedusa* and Cecropin from hemolymph of the giant silk moth *Hyalophora cecropi* (Wimley 2010; Kumar et al. 2018; Raheem and Straus 2019).

c) *The toroidal pore model* – which describes the activity of Magainins (*Xenopus laevis*), Protegrins (porcine leukocytes) and Melittin (honeybee venom). Here the peptides aggregate on the microbial membrane by interacting with phospholipid head groups of lipid monolayers and form toroidal shaped transmembrane pores with inner peptide lining and lipid flip-flop characteristics (Wimley 2010; Kumar et al. 2018; Raheem and Straus 2019).

d) *The aggregate or channel-forming model* – is similar to the toroidal model, with subtle variations. The peptides aggregate on the microbial membrane in a concentration or voltage dependent manner by electrostatically interacting with membrane cardiolipins, followed by peptide oligomerization, membrane permeabilization and pore formation. This model applies to the activity of Sapecin from *Sarcophaga peregrina* and Mastoparan from wasp venom (Wimley 2010; Kumar et al. 2018; Raheem and Straus 2019).

1.3. CAPs from Plant sources

Cationic peptides with broad spectrum antimicrobial activity against phytopathogens as well as human pathogens have been isolated from different plant parts including stem, root, leaves, flowers and seeds of various species (Nawrot et al. 2014; Li et al. 2021). Most of the plant antimicrobial peptides are cysteine-rich, with multiple disulfide bonds that form stable compact structures resistant to chemical, thermal and proteolytic degradation (Craik et al. 1999; Weidmann and Craik 2016). Plant peptides were classified into many subgroups mainly based on amino acid composition and structural characteristics (Tam et al. 2015; Li et al. 2021). The major families of plant antimicrobial peptides include thionins, defensins, hevein-like peptides, knottin-type peptides, cyclotides, snakins, lipid transfer proteins, α-hairpinin families and other unclassified Cys–rich peptides.

i. *Thionins:* They are prototypic cationic peptides with 45-48 amino acid residues and 3-4 disulfide bonds. They are ribosomally expressed and their expression can be induced upon microbial invasion (Florack and Stiekema 1994). Thionins elicit microbial toxicity by directly interacting with microbial membrane lipids. More than 100 distinct thionins have been identified from monocot and dicotyledonous plants. Based on

ii. structural folding, thionins are classified into three types *viz.*, α, β and γ thionins (Stec 2006). The α/β thionins are divided into five subclasses: Type I thionins (Purothionins), Type II (Hordothionins), Type III (Viscotoxins), Type IV (Crambins) and Type V (Hellothionin).

iii. *Defensins*: They are the most abundant plant AMPs, being basic, cysteine–rich with 4–5 disulfide bonds and generally consisting of 45–54 amino acids (Lacerda et al. 2014). They are involved in diverse biological functions including antimicrobial activity, biotic stress response, trypsin and α-amylase inhibitory effects, plant growth and development, etc. Majority of defensins were identified from seeds and roots. Plant defensins are reported to be highly stable at high temperature (>85°C), low pH, and in proteolytic and oxidative environments (Lacerda et al. 2014; Sher Khan et al. 2019). Two types of defensins have been identified based on the number of Cys–residues: 8C–defensins (NaD1, Fabatins) and 10C defensins (PhD1, PhD2).

iv. *Hevein–like peptides*: They are basic peptides of 29–45 amino acids, with 3–5 disulfide bonds, besides several Cys and Gly residues and conserved aromatic amino acid residues as present in hevein domains of lectins (Slavokhotova et al. 2017). Similar to the hevein lectins, hevein-like peptides inhibit the growth of fungal pathogens by targeting chitin in fungal cell walls (Porto et al. 2012). The WAMP, a 10C hevein like peptide from seeds of *Triticum kiharae* was reported to have high inhibitory activity against plant, fungal as well as bacterial pathogens (Odintsova et al. 2009).

v. *Knottin–type peptides:* They are small, basic, mostly linear peptides, composed of ~30 amino acid residues. The Cys–residues (6C) of these peptides with conserved disulfide bonds form a cysteine knot motif, hence being collectively known as Cysteine-knot peptides (Silverstein et al. 2007). They are considered as the largest groups of plant cationic peptides with distinct molecular structures and sequence diversity. Majority of plant knottin–type peptides are identified as enzyme (α-amylase or protease) inhibitors exhibit high chemical, thermal and enzymatic stability. They serve an important role in plant immune response by conferring antimicrobial, insecticidal and pesticidal activities (Molesini et al. 2017). PAFP-S isolated from *Phytolacca americana* seeds models the typical structure of knottin–type antifungal peptides, while Mj-AMP1 and

vi. Mj-AMP2 are knottin–type antimicrobial peptides identified from seeds of *Mirabilis jalapa* (De Bolle et al. 1996).

vii. *Cyclotides:* They are naturally occurring cyclic peptides considered as a subfamily of plant knottin–type peptides, and comprise of 28–37 amino acid residues and three or more intramolecular disulfide bonds (de Veer et al. 2019). Cyclotides generally exhibit high sequence similarities and have a cyclic backbone that makes them resilient to thermal and proteolytic degradations (Colgrave and Craik 2004). Cyclotides function as plant defense molecules and possess antimicrobial and insecticidal properties. Kalata B1 from *O. affinis*, Cycloviolacin from *V. hederaceae*, Vtri from *V. tricolor*, Cliotides, etc. are some previously characterized cyclotides (Jennings et al. 2001; Colgrave and Craik 2004; Svangard et al. 2004; Chen et al. 2006; Oguis et al. 2019).

viii. *Snakins*: They are Cys–rich (12C) peptides identified with broad spectrum antimicrobial activity, consist of two long α-helices linked by disulfide bonds, and demonstrate partial structural similarity to thionins (Su et al. 2020). Snakin-1 (63 amino acids) and snakin-2 (66 amino acids) from *Solanum tuberosum* were found active against fungal as well as Gram-negative and Gram-positive bacterial pathogens (Segura et al. 1999; Berrocal-Lobo et al. 2002). A homolog of snakin-2 with 64 amino acid residues isolated from French bean exhibited chitin binding ability and was involved in fungal growth inhibition.

ix. *Lipid transfer proteins:* LTPs are small, cationic molecules of 70–90 amino acid residues, with eight cysteine residues (8C). They possess non–specific lipid transfer activity and antimicrobial activities (Salminen et al. 2016).

x. *α-Hairpinin family:* Constitutes Lys/Arg rich plant peptides with helix-loop-helix secondary structures stabilized by disulfide bonds (Slavokhotova and Rogozhin 2020). Only a few numbers of α-hairpinin peptides have been reported so far, of which MBP-1, a 33 amino acid peptide identified from maize kernel showed antimicrobial activity against bacterial and fungal pathogens *in vitro* (Duvick et al. 1992). The MiAMP2 peptides (55 amino acid residues) from *Macadamia integrifolia* nut kernel exhibited inhibitory effect against several plant fungal pathogens (Marcus et al. 1999).

xi. *Unclassified cysteine-rich plant peptides:* This group consists of several plant antimicrobial peptides that lack structural and sequence homology to other peptide families. Ps-AFP1 (38 amino acids) from the root of *Pisum sativum* has eight Cys residues with a novel αβ-trumpet fold capable of binding to the fungal cell wall (Mandal et al. 2013). The Ib-AMPs 1-4 are four basic peptides (of 20 amino acids) isolated from *Impatiens balsamina* seeds and found to be active against various fungal and Gram-positive bacterial pathogens (Tailor et al. 1997).

1.4. CAPs as potential drug candidates

Prolonged use of conventional antibiotics can cause microbial mutation and multi-drug resistance which have already been linked to severe health consequences, as reported globally. Consequently, there is an urge to discover novel antibiotics that could effectively combat various microbial infections as well as antibiotic resistance. The CAPs have several unique characteristics that give them an edge over conventional antibiotics which were developed mainly based on the concept of having a single mode of action or single primary target (Yeung et al. 2011; Mwangi et al. 2019; Mahlapuu et al. 2020). Nature has chosen a very distinct evolutionary pattern for host-defense antimicrobial cationic peptides, favoring the design of efficient antibiotics that exhibit small molecular size, broad spectrum bioactivities, high efficiency at lower doses, low potential for microbial resistance development, ability to counteract the effect of pathogenic virulence factors, ability to synergize with host immune response, thermal stability, enzymatic and chemical stability, etc. (Yeung et al. 2011; Bahar and Ren 2013; Spohn et al. 2019). The unique mechanism of action of CAPs based on the fundamental differences between the design of cell membranes of microbes and multicellular organisms enables them to function in a target specific manner (Brogden 2005; Sani and Separovic 2016). An outstanding quality of CAPs is the 'peptide promiscuity' which arms them to exploit several weak spots of the target organisms and exert activity against a broad range of microbial pathogens (Sani and Separovic 2016). The probability of acquisition of microbial resistance against CAPs is rather low, unless the sensitive microbes redesign their membrane by altering the composition or arrangement of membrane lipids (Brogden 2005; Yeung et al. 2011; Bahar and Ren 2013; Sani and Separovic 2016; Spohn et al. 2019). As an

evolutionarily ancient defense molecule CAPs have been functioning effectively for billions of years.

1.5. Motivation behind the present study

With the above background information, it is now evident that a novel therapeutic approach using CAPs as potential drug candidates has emerged a proficient method to counter the comparatively short half-life of conventional antibiotics and the rapidly escalating antibiotic resistance issue. The search for such specific molecules from certain plants traditionally sourced for their medicinal (in particular antimicrobial) properties was hence considered a likely productive venture and prompted the present investigation, with the following objectives in mind:

- *Screening of plants for cationic peptides with antimicrobial activity*
- *Optimizing the extraction procedures of cationic peptides from selected plants based on antimicrobial activity*
- *Purification of one most potent cationic peptide*
- *Characterization of the selected cationic peptide*
- *Search for other potential bioactivities*

Chapter 2

Screening of plants for antimicrobial cationic peptides and optimization of extraction procedures

2.1. Plants selected for primary screening

Nineteen plant species have been short-listed for the present study (Table 2.1), primarily based on the reported use of their extracts as traditional medicines as well as on hitherto identified antimicrobial molecules from them, as gleaned through reviews of literature. Priority was given to those plant species from which antimicrobial activity due to cationic peptides remained unexplored.

Table 2.1: Plant samples used for the primary screening

1. ***Plectranthus amboinicus*** (Indian borage) – Lamiaceae family		
Traditional/Medicinal uses	**Bioactivities/Phytochemicals identified**	**Parts used in the present study**
• Essential oil extracts to treat asthma, cold, cough, fever, respiratory diseases, skin diseases, etc. (Lukhoba et al. 2006; Arumugam et al. 2016). • Raw leaves used for culinary purpose.	• Antifungal, antibacterial, larvicidal & antioxidant activities of Carvacrol, p-Cymene, α-Terpinolene, β-caryophyllene, thymol & phenolic compounds identified from essential oil/leaf extracts (Murthy et al. 2009; Da Costa et al. 2010; Arumugam et al. 2016). • Antitumor activities of Flavone (Luteolin) & flavanols isolated from hydro alcoholic leaf extracts (Gurgel et al. 2009; Arumugam et al. 2016). • Antiepileptic activity of alkaloids, flavonoids & saponins detected from aqueous/ ethanolic leaf, stem & root extracts (Lukhoba et al. 2006; Arumugam et al. 2016).	Leaves
2. ***Calotropis gigantea*** (Crown flower plant) – Apocynaceae family		
• Bark to treat spleen, liver and enteric diseases (Kumar et al. 2011; Jahan et al. 2016). • Root and leaves to treat respiratory diseases. • Milky juice effective against arthritis, cancer & snakebite. • Root, stem and leaf extracts as fungicide and mosquito repellent (Kumar et al. 2011)	• Antitumor effect of anhydrosophoradiol-3-acetate (A3A) isolated from alcoholic flower & root extracts (Jahan et al. 2016; Habib and Karim 2013). • Antimicrobial, insecticidal, anti-asthmatic, anti-inflammatory, anti-lipoxygenase, antioxidant, wound healing & hepatoprotective activities of alcoholic/organic solvent extracts, aqueous extracts of leaf, flower & latex (Jahan et al. 2016; Sharma et al. 2015; Alafnan et al. 2021).	Leaves

3. ***Aloe vera var. chinensis*** (Aloe vera)- Asphodelaceae family		
• Leaf gel as a moisturizer, anti-irritant to reduce chafing of nose and to treat skin ailments (Gupta and Malhotra 2012; Sanchez et al.2020). • Used as an ingredient in cleansing soaps shampoos or creams.	• Wound healing effect of a low molecular weight glycoprotein (Maenthaisong et al. 2007; Sanchez et al.2020). • Anti-tumour activity of glycoprotein and polysaccharide (acemannan) fractions (Manirakiza et al. 2021). • Anti-inflammatory action of acetylated mannan in aloe gel (Hamman et al. 2008). • Antimicrobial & anti-viral activities of lectins & anthraquinone derivatives found in aloe gel (Hamman et al. 2008). • Immunomodulatory activities of the polysaccharides (Hamman et al. 2008; Sanchez et al.2020). • Moisturizing & anti–aging properties of *A. vera* gel by enhancing collagen & elastin production (Manirakiza et al. 2021; Hamman et al. 2008). • Hypoglycaemic and hypolipidemic effects of aloe gel (Hamman et al. 2008; Sanchez et al.2020).	Leaves
4. ***Andrographis paniculata*** (Creat) - Acanthaceae family		
• Leaves, root or whole plant to treat bacterial infections, respiratory diseases, malaria, jaundice, intermittent fever, inflammation, stomach aches & pyrexia (Okhuarobo et al. 2014; Hossain et al. 2014). • Dietary supplement for cancer & diabetes prevention and cure. • Whole plant as an antidote for snake bite, insect bite (Okhuarobo et al. 2014). • An important constituent of at least 26 Ayurvedic formulas in Indian pharmacopoeia (Okhuarobo et al. 2014; Hossain et al. 2014).	• Antibacterial & antifungal activities of Andrographolides & arabinogalactan proteins isolated from aqueous extracts (Singha et al. 2003; Chao and Lin 2010). • Anti-inflammatory, antioxidant, antidiabetic, immunomodulatory, cytotoxic, anti-angiogenic & hepato-renal protective activities of andrographolide or its derivatives isolated from aqueous/organic solvent extracts of the whole plant/different plant parts (Chao and Lin 2010). • Antimalarial activities of four xanthones isolated from root extracts (Dua et al. 2004; Chao and Lin 2010).	Leaves

5. ***Allium sativum*** (Garlic) – Amaryllidaceae family		
• Garlic bulbs to treat respiratory diseases, arthritis, cancer, high cholesterol, cardiovascular diseases, diabetes, parasitic infestations, digestive diseases, toothache, gynaecologic diseases, snake & insect bites, etc. (Rana et al. 2011; Bayan et al. 2014).	• Antibacterial, antifungal, antiviral, anti-parasitic, antidiabetic, antiatherosclerosis, antithrombotic & immunomodulatory activities (Mikaili et al. 2013; El-Saber Batiha et al. 2020). • Anticancer activities of diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), S-allyl cysteine (SAC) and diallyl thiosulfinates (Lai et al. 2013; El-Saber Batiha et al. 2020). • Antihypertensive, hypolipemic & antioxidant properties of aqueous/aged garlic extracts (Dillon et al. 2003).	Bulbs
6. ***Allium cepa*** (Onion) – Amaryllidaceae family		
• *A. cepa* bulbs were used to treat bronchitis, cold, fever, skin burns, bruises, tuberculosis, diabetes, cataracts, jaundice, high blood pressure, high cholesterol, heart disease, varicose veins, insect stings, etc (Marrelli et al. 2018).	• Antidiabetic activity of allyl propyl disulphide & S-methyl cysteine sulfoxide purified from organic solvent extracts of *A. cepa* bulbs (Kumari et al. 1995). • Anticancer, antioxidant & anti-inflammatory potential of *A. cepa* flavonoids (Apigenin & quercetin) and sulphur containing compounds (Marefati et al. 2021). • Antibacterial effect of *A. cepa* aqueous extracts against Gram positive and Gram-negative bacteria (Elnima et al. 1983).	Bulbs
7. ***Moringa oleifera*** (Drumstick)– Moringaceae family		
• Root, bark, flowers & leaves are antifungal, antibacterial, antiviral, antianemic, analgesic, hypoglycaemic, anti-inflammatory, antilithic, antiepileptic, antiparalytic, antispasmodic and diuretic (Anwar et al. 2007). • Crushed seeds function as flocculating agent and used for water purification process (Beltran-Heredia et al. 2009). • Fresh & cooked leaves are given in influenza and catarrhal infections (Anwar et al. 2007).	• Antiproliferative and apoptotic properties of the *M. oleifera* leaf extract, rich in quercetin, kaempferol and phenolics compounds (Khor et al. 2018). • Hypoglycaemic, antidyslipidemic and anti-inflammatory activities of isothiocyanates & benzyl isothiocyanate from aqueous leaf extract (Anwar et al. 2007; Galuppo et al. 2014). • Cardioprotective and anti-obesity activities of N, α-L-rhamnopyranosyl vincos amide, an alkaloid compound extracted from leaves (Waterman et al. 2015). • Coagulant lectins with flocculating properties from seeds (Beltran-Heredia et al. 2009).	Seeds

8. ***Zingiber officinale*** (Ginger)– Zingiberaceae family		
• Decoction or juice of ginger to treat cold, cough, asthma, headache, anorexia, flatulence and haemorrhoids (Bode and Dong 2011; Zhang et al. 2021). • Fresh ginger paste and ginger oil to treat arthritis and inflammations (Zhang et al. 2021). • Dried rhizome to treat sore throat and Hoarseness of voice (Bode and Dong 2011).	• Antioxidant, antidiabetic, anti-inflammatory, Antinausea and Antiemetic activities of phenolic compounds (6-shogaol, 6-gingerol) & phenylpropanoids from rhizome (Dugasani et al. 2010). • Antimicrobial potential of gingerenone-A and 6-shogaol from rhizome (Zhang et al. 2021). • Anticancer, anti-neuroinflammatory and antiobesity activities of ginger polyphenols such as 6-gingerol, 10-gingerol, 6-shogaol, and 10-shogaol (Bode and Dong 2011; Zhang et al. 2021).	Rhizomes
9. ***Momordica charantia*** (Bitter gourd) **–** Cucurbitaceae family		
• Juice of *M. charantia* to treat diabetes, dyspepsia, jaundice, fever, cold, malaria, cancer, high cholesterol, piles, cholera and psoriasis (Jia et al. 2017).	• Antioxidant, antidiabetic, immune enhancement, neuroprotective & antitumor activities of branched heteropolysaccharides (Jia et al. 2017). • Anti-tumour, anticancer, immunosuppressive and anti-microbial, hypoglycemic effect of *M. charantia* lectin, terpenoids and momorcharin (Joseph and Jini 2013; Jia et al. 2017). • Antihyperglycemic, hypolipidemic and antiviral activities of Saponins (Joseph and Jini 2013). • Antioxidant, anti-inflammation and immune enhancement activities of phenolics (Jia et al. 2017; Bortolotti et al. 2019).	Seeds
10. ***Catharanthus roseus*** (Periwinkle) – Apocynaceae family		
• Root and shoot extracts to treat diabetes, malaria, ulcer and Hodgkin's lymphoma (Kumar et al. 2022). • Infusion of the leaves to control hemorrhage and scurvy, as a mouthwash for toothache, and for the healing and cleaning of chronic wounds (Kumar et al. 2022). • Flower and leaf extracts as hypoglycemic and antibacterial agents (Kumar et al. 2022).	• Anticancer properties of alkaloids Vinblastine and Vincristine derived from methanolic stem and leaf extracts (Martino et al. 2018; Kumar et al. 2022). • Antimicrobial & hypoglycemic effect of alkaloids isolated from ethanol/chloroform/methanol extracts of leaves & flowers (Noble 1990; Martino et al. 2018). • Vincamine and Vindoline alkaloids from plant leaves showed cerebral vasodilatory, neuroprotective, hypotensive, anti-diarrheal and anti-ulcer properties (Noble 1990; Kumar et al. 2022).	Leaves

11. ***Hibiscus rosa-sinensis*** (China rose)**–** Malvaceae family		
• Hibiscus flowers and leaves to treat bronchitis, cough, menorrhagia, cardiac and nerve diseases and as a demulcent (Jadhav et al. 2009). • Leaves are used as emollients to treat burning sensations and skin disease (Jadhav et al. 2009). • Herbal tea prepared from leaves and flowers to treat hypertension and digestive diseases (Jadhav et al. 2009). • Flower and leaf preparations to promote hair growth (Jadhav et al. 2009).	• Antimicrobial activities of flavonoids, alkaloids, tannins, terpenoids, saponins, cardiac glycosides, anthraquinones, and phlobatannins from methanolic/aqueous flower and leaf extracts (Jadhav et al. 2009; Ngan et al. 2021). • Anticancer, neuroprotective and antioxidant activities of tannins, saponins & flavonoids extracted in acetone/methanol/aqueous flower & leaf extracts (Nade **et al. 2011;** Rengarajan et al. 2020). • Anti-diabetic and hypoglycaemic effects of glycosides from alcoholic flower & leaf extracts (Afiune et al. 2017). • Hair growth promoting activity of phytosterols & triterpenoids purified from petroleum ether/aqueous leaf extracts (Jadhav et al. 2009).	Leaves
12. ***Clitoria ternatea*** (Blue pea)– Fabaceae family		
• Seeds and roots used as a nerve tonic (Mukherjee et al. 2008). • Root extracts to treat severe asthma, remittent fever, bronchitis, whooping cough, goitre, epilepsy, rheumatism and ear disease (Mukherjee et al. 2008). • Seed powder to treat cough, digestive disorders, hepatic disorders, spleen and rheumatic infections (Mukherjee et al. 2008; Nithianantham et al. 2011). • Leaf juice to treat hepatic fever, inflammation and to mitigate toxins (Mukherjee et al. 2008; Nithianantham et al. 2011). • Infusion of flowers and stem to treat insect/snake bites and intestinal problems (Mukherjee et al. 2008).	• Antioxidant and hepatoprotective properties of flavonoids, tannins and phenolic compounds isolated from aqueous & ethanolic flower extracts (Nithianantham et al. 2011). • Antimicrobial activities of alkaloids, tannins, flavonoids and glucosides, extracted in methanol/chloroform/aqueous flower, leaf and pod extracts (Mukherjee et al. 2008; Pratap et al. 2012). • Antimicrobial and anticancer potential of cyclic proteins/peptides from seeds (Oguis et al. 2019). • Nootropic, anxiolytic, anti-depressant, anticonvulsant, anti-stress, diuretic, antihelmintic, anti-inflammatory properties of methanolic root extracts rich in glycosides, phytosterols & terpenoids (Mukherjee et al. 2008; Talpate et al. 2014). • Anti-diabetic and hypoglycaemic effects of glycosides & ternatins from ethanolic/aqueous flower & leaf extracts (Talpate et al. 2013).	Seeds

13. *Trigonella foenum-graecum* (Fenugreek)– Fabaceae family		
• Leaf extracts to alleviate cold, asthma, cough, splenomegaly, hepatitis and backache (Ahmad et al. 2016). • Seed powder to treat diabetes, skin diseases, cough and eye diseases (Gaddam et al. 2015; Ahmad et al. 2016). • The whole plant used as an emollient in treatment of pellagra, loss of appetite and gastrointestinal disorders (Ahmad et al. 2016).	• Antimicrobial & antidiabetic properties of saponin-rich ethanolic seed extracts (Gaddam et al. 2015; Ahmad et al. 2016). • Hypocholesterolemic effects of steroid and saponin purified from alcoholic seed extracts (Sowmya and Rajyalakshmi 1999). • Hypoglycaemic effects of trigonelline, fibre-galactose and mannose of seed extracts (Gaddam et al. 2015). • Antioxidant effects of flavonoids from organic solvent extracts of seeds (Dixit et al. 2005; Ahmad et al. 2016). • Anticancer potential and immunomodulatory effects of whole plant extracts (El Bairi et al. 2017).	Seeds
14. *Tabernaemontana divaricata* (Crape jasmine)– Apocyanaceae family		
• Root decoction to treat fever, diarrhoea, hypertension, headache, and various abdominal complaints (Pratchayasakul et al. 2008). • Infusion of leaves to treat influenza (Pratchayasakul et al. 2008). • The flowers, mixed with oil, as applied to sore eyes (Pratchayasakul et al. 2008). • The latex of the leaves to prevent inflammation (Pratchayasakul et al. 2008).	• Antimicrobial, anti-inflammatory and cardio-protective effects of alkaloids purified from organic solvent extracts of *T. divaricata* (Van Beek et al. 1984). • The anti-inflammatory activity of flavonoids and phenolic acids (Jain et al. 2013). • Antioxidative and anti-tumour effects of crude methanol extract of *T. divaricata* (Kumar and Selvakumar 2015). • Neuroprotective effects of bisindole alkaloids, conodurine, and tabernaelegantine A purified from *T. divaricata* root extracts (Pratchayasakul et al. 2008; Khongsombat et al. 2017).	Leaves
15. *Plumeria rubra* (Frangipani) – Apocyanaceae family		
• Flowers, leaf juice and latex to treat toothache, fractures and blisters (Bihani 2021). • Stem bark decoction to treat asthma (Dey and Mukherjee 2015). • Leaf and flower extracts to treat diabetes and enteric diseases (Dey and Mukherjee 2015).	• Anticancer potential of iridoids, fulvoplumierin, allamcin, allamandin and plumericins purified from *P. rubra* extracts (Ye et al, 2009; Bihani 2021). • Antiviral activities of fulvoplumierin, an iridoid, from *P. rubra* (Ye et al, 2009). • Antifungal activities of *P. rubra* latex proteins against fungal pathogens (Souza et al. 2011; Dey and Mukherjee 2015).	Leaves

• Bark and latex preparations to clean eye and tongue (Dey and Mukherjee 2015). • Bark paste used for wound healing and rabies treatment (Bihani 2021).	• Larvicidal activity of laticifer proteins of *P. rubra* against *Aedes aegypti* (Dey and Mukherjee 2015). Plumericin and isoplumericin isolated from the heartwood of *P. rubra* found to possess antibacterial as well as molluscicidal activities (Dey and Mukherjee 2015).	
16. *Phyllanthus niruri* (Seed-under-leaf plant) – Phyllanthaceae family		
• The whole plant used to treat jaundice, diabetes, gonorrhea, intermittent fevers, skin ulcers, sores, swelling, itchiness, ophthalmia and conjunctivitis (Bagalkotkar et al. 2006). • Shoots of the plant to treat various gastrointestinal disorders (Bagalkotkar et al. 2006). • Powdered leaves to treat Hepatitis B (Bagalkotkar et al. 2006).	• Antiviral and hepatoprotective effects of phyllanthin, hypophyllanthin and triacontanol purified from leaf extracts (Liu et al. 2014). • Anticancer, antibacterial and cellular protective actions of whole plant extracts (Araujo et al. 2012). • Anti-inflammatory activity, immune-modulatory effect and anti-ulcer activity of glycosides and lignan-rich fraction purified from organic solvent extracts (Mostofa et al. 2017). • Antioxidant activity of phenolic contents purified from methanolic extracts (Bagalkotkar et al. 2006).	Whole plant
17. *Eichhornia crassipes* (Water hyacinth) – Pontederiaceae family		
• An infusion of the inflated petioles used to treat fevers (Aboul-Enein et al. 2011). • The leaf petioles eaten as a treatment for diarrhoea (Ben Bakrim et al. 2022). • Leaves and petioles used as a carotene-rich table vegetable (Ben Bakrim et al. 2022).	• Antimicrobial activity of alkaloids and saponins from methanolic root and leaf extracts (Aboul-Enein et al. 2011; Ben Bakrim et al. 2022). • Antioxidant activity of polyphenols and flavonoids from ethanolic leaf and stem extracts (Ben Bakrim et al. 2022). • Wound-healing activities of aqueous, methanol and ethyl acetate extracts of leaves (Ben Bakrim et al. 2022). • Antitumor activity of methanolic leaf extracts. Larvicidal activity of crude root extracts prepared in acetone (Aboul-Enein et al. 2011; Ben Bakrim et al. 2022).	Leaves
18. *Salvinia molesta* (Kariba weed) – Salviniaceae family		

• *S. molesta* used for waste water phytoremediation and treatment of sewage effluent (Kumar and Deswal 2020).	• Antimicrobial activity of aqueous extracts of *S. molesta* (Verma et al. 2016). • Anticancer potential of organic solvent extracts (Li et al. 2013). • Antioxidant potential of two glycosides, 6'-O-(3,4-dihydroxy benzoyl)-beta-D glucopyranosyl ester, and 4-O-beta-d-glucopyranoside-3 hydroxymethyl benzoate purified from organic solvent extracts of *S. molesta* (Choudhary et al. 2008).	Whole plant
19. *Centella asiatica* (Indian pennywort) – Apiaceae family		
• The whole plant extract used to treat leprosy, lupus, varicose ulcers, eczema, psoriasis, fever, diarrhoea and amenorrhea (Gohil et al. 2010). • Leaf extracts for wound healing (Gohil et al. 2010).	• Wound-healing properties of asiaticosides, saponins purified from *C. asiatica* extracts (Somboonwong et al. 2012). • Centelloside and its derivatives effective in the treatment of venous hypertension (Cesarone et al. 2001). • Sedative and anxiolytic properties of pentacyclic triterpenoids including brahmoside and brahminoside constituents (Sun et al. 2020). • The antidepressant & antiepileptic properties of total triterpenes & steroids (Gohil et al. 2010; Sun et al. 2020). • Aqueous extract of *C. asiatica* found effective in inhibiting gastric lesions (Gohil et al. 2010).	Whole plant

2.2. MATERIALS AND METHODS

2.2.1. Plant extract preparation

Due to the basic nature of cationic peptides, an acetic acid–based extraction method was adopted to prepare the crude plant extracts (Cole and Ganz 2000; Liang et al. 2011). For initial screening, 10% homogenates of the plant samples were prepared in 0.5% acetic acid solution (pH 4.5) and incubated at 4°C for 24 h. The extracts were filtered and centrifuged (4°C, 30 min at 10,000 rpm). The supernatant was collected and subjected to ammonium sulfate precipitation (0–90% saturation). The pellet obtained was resuspended in 0.01M sodium acetate buffer (pH 5.6) and screened for antimicrobial properties.

2.2.2. Maintaining pathogen cultures

Bacterial as well as fungal pathogen cultures procured from the Microbial Type Culture Collection & Gene Bank (MTCC, India) were used as test cultures for the present study:

- Gram-negative strains – *Salmonella typhimurium* (MTCC 3231), *Pseudomonas aeruginosa* (MTCC 741), *Escherichia coli* (MTCC 443) & *Aeromonas hydrophila* (MTCC 1739)
- Gram-positive strain – *Staphylococcus aureus* (MTCC 3160)
- Human pathogenic yeast – *Candida albicans* (MTCC 227)
- Fish pathogens – *Aeromonas sobria* (MTCC 3613) & *Pseudomonas fluorescence* (MTCC 7200)

Stock cultures were routinely maintained at 4°C on slants of Nutrient Agar (*HiMedia*) for bacteria and Potato Dextrose Agar (*HiMedia*) for fungi.

2.2.3. Antimicrobial assay of plant extracts

Antimicrobial activity of the plant samples was tested against various pathogenic strains by the agar well diffusion method, using Mueller-Hinton agar or Mueller-Hinton broth (*HiMedia*) (Holder and Boyce 1994; Cole and Ganz 2000). Wells of 6 mm diameter were bored on agar plates seeded with pathogen cultures and 50µl of plant extract was added into the wells. The plates were then incubated at 37 °C for 24 h and 48 h for bacterial and fungal strains,

respectively, and observed for microbial growth inhibition due to the plant extracts. Controls of standard antibiotics as well as buffer controls were maintained in each experiment. The zone of inhibition was measured and the well diameter of 6 mm excluded during tabulations. All values are presented as Mean ± SD in the tables and figures.

2.2.4. Optimization of plant crude extraction

In order to facilitate maximal extraction of the cationic peptides, the plant crude extraction procedure was optimized by varying the strength of acetic acid solution used (Cole and Ganz 2000). A 20% homogenate of plant samples prepared in 5% or 10% acetic acid solution (pH 3.2 and 2.7, respectively) was therefore kept at 4°C for 24 h, followed by filtration and centrifugation at 4°C and 10,000 rpm for 30 min. The supernatant was precipitated at 0–90% ammonium sulfate saturation and evaluated for antimicrobial activity by the agar well diffusion assay.

2.2.5. Ammonium sulfate fractionation

Crude plant extracts prepared in 10% acetic acid solution were subjected to fractionation by ammonium sulfate precipitation at 0–30%, 30–60% and 60–90% salt saturation levels. The pellet obtained for each fraction was tested for antimicrobial activity by the agar well diffusion method. Protein/peptide estimation was carried out by the Bradford method (Bradford 1976) using Bovine Serum Albumin (BSA) as the standard.

2.2.6. Polyacrylamide Gel Electrophoresis (PAGE) analysis

Sodium dodecyl sulphate–PAGE (SDS-PAGE) was carried out using an 18% resolving gel under reducing conditions (Laemmli 1970). Low Molecular Weight protein markers (Sigma-Aldrich) in the mass range of 3–40 kDa served as standards. Coomassie Brilliant Blue G-250 was used to visualize the peptide/proteins bands (Candiano et al. 2004).

2.3. RESULTS AND DISCUSSION

2.3.1. Screening for antimicrobial activity of plant extracts

The distinct biochemical features of cationic peptides facilitate their selective extraction in acidic solvents. Crude extraction using acetic acid solution is an effective method to isolate antimicrobial cationic peptides from various biological samples (Cole and Ganz 2000; Liang et al. 2011). For the present study, an acetic acid–based extraction method was hence adopted to prepare crude plant extracts. The extracts of 19 plant samples prepared in 0.5% acetic acid solution were precipitated at 0–90% ammonium sulfate saturation and screened for antimicrobial activity against eight different pathogenic microbes by the agar well diffusion method; the results are summarized in Table 2.2. Among the 19 plants screened, five samples, *viz.*, *A. sativum, C. ternatea, M. oleifera, T. foenum-graecum* and *T. divaricata* exhibited comparatively high inhibition zones against one or more of the pathogenic strains including *C. albicans, S. aureus, E. coli* or *S. typhimurium*, whereas *P. amboinicus*, *C. gigantea, A. vera, A. cepa, Z. officinale, M. charantia, C. roseus, H. rosa-sinensis, P. rubra, C. asiatica, P. niruri, E. crassipes* and *S. molesta* showed only weak inhibitory effect on the growth of various test pathogens. *A. paniculata* preparations were found ineffective against all the tested pathogens. As none of the extracts were active against the fish pathogens *A. sobria* and *P. fluorescence* (data not shown), these two pathogenic strains were excluded from further studies.

Table 2.2: Antimicrobial activity of plant samples as examined by the agar well diffusion assay

Plant Samples	Zone of Inhibition (mm)*					
	S. aureus	***A. hydrophila***	***E. coli***	***P. aeruginosa***	***S. typhimurium***	***C. albicans***
P. amboinicus	-	-	-	-	5±0	-
C. gigantea	-	-	4±1	-	3±0.5	-

A. paniculata	-	-	-	-	-	-
A. vera var. chinensis	-	2±0.5	-	-	-	-
M. oleifera	**15±0.6**	-	-	-	**16±1**	12±1
A. cepa	-	-	-	-	3±0.6	8±0
A. sativum	**18±0.5**	8±0	**15±0.5**	-	13±0	**21**±1.5
Z. officinale	-	-	-	-	4±0	11±1
M. charantia	-	-	4±0.5	-	9±0	10±0
C. roseus	2±0	-	-	-	-	-
H. rosa-sinensis	2±0.6	-	-	-	-	-
C. ternatea	**17**±0	9±0.5	6±0.5	-	-	**23**±1
P. rubra	-	-	-	5±1	3±0.6	-
C. asiatica	-	3±0.6	5±0	-	-	3±1
P. niruri	-	-	7±0.5	-	5±0	2±0.6
T. divaricata	12±0	-	-	-	8±0.5	**15**±1.5
E. crassipes	-	-	6±0	-	-	-
S. molesta	-	-	-	-	-	2±0.5
T. foenum-graecum	**16**±1	-	-	5±0.6	5±0	10±1

*diameter of inhibition zone, excluding the well diameter

2.3.2. Optimization of the crude sample extraction protocol

Based on the results of primary screening (Table 2.2), five plant samples viz, *Allium sativum, Clitoria ternatea, Moringa oleifera, Trigonella foenum-graecum* and *Tabernaemontana divaricata,* that exhibited comparatively higher antimicrobial activity were selected for optimization studies. Cole and Ganz (2000) had demonstrated an effective method to

selectively solubilize and concentrate CAPs from the crude extracts of biological samples using acetic acid of 5-10% strength; during this process many other proteins in the extract would invariably precipitate out. Accordingly, in our search for maximal activity, extraction of the five selected plant samples was carried out in 5% as well as 10% acetic acid solutions, without affecting the further assay protocol. Antimicrobial activity of ammonium sulfate precipitates (0–90% saturation) of these crude extracts was then evaluated by the agar well diffusion method. For all five plant samples, 10% acetic acid extracts were more active than 5% acid extracts (Fig. 2.1–2.5). The crude plant extracts prepared in 10% acetic acid solution were therefore used for subsequent studies, being the optimized concentration for superior activity extraction of cationic antimicrobial molecules from these samples.

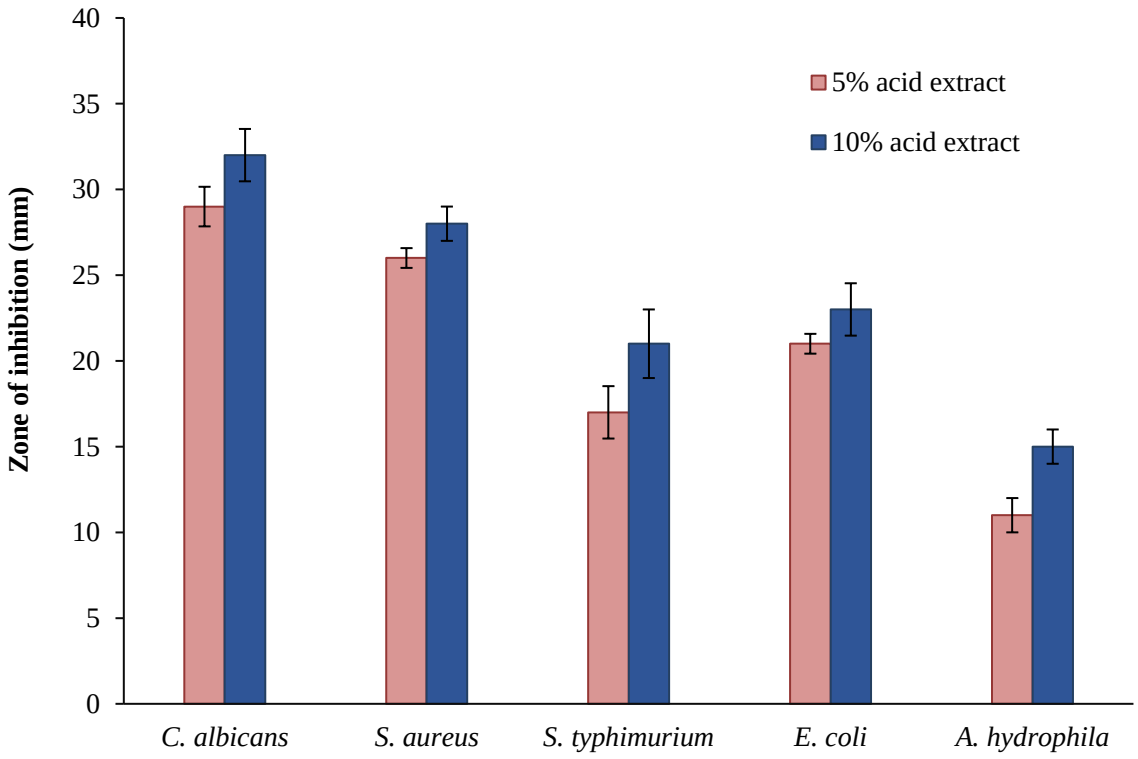


Fig. 2.1: Antimicrobial activity of 0-90% ammonium sulfate precipitate of *A. sativum* extracts.

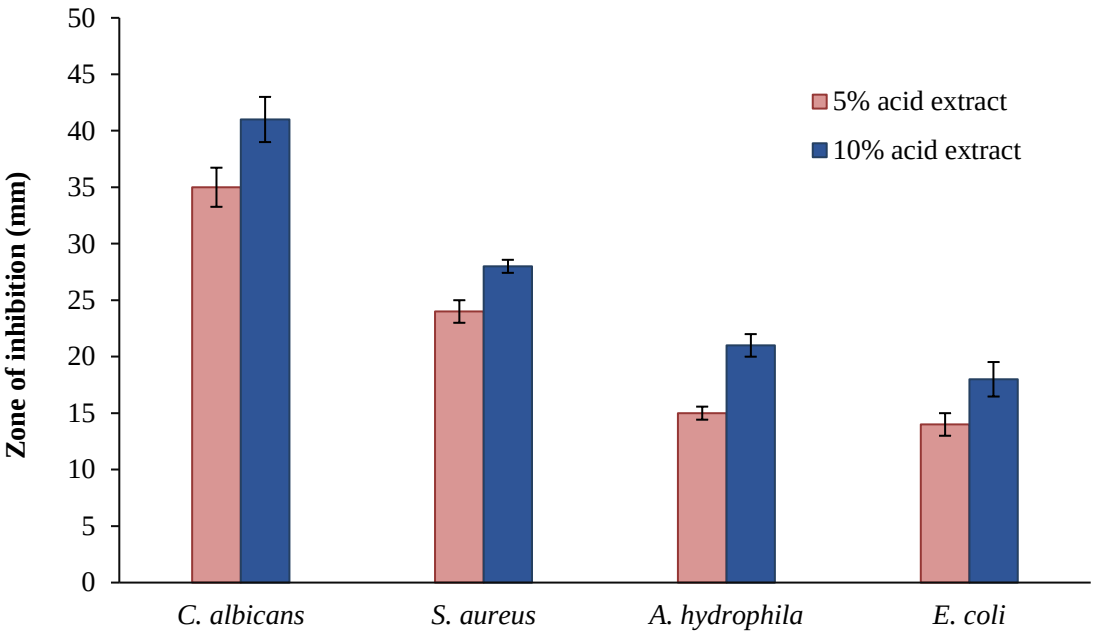


Fig. 2.2: Antimicrobial activity of 0-90% ammonium sulfate precipitate of *C. ternatea* seed extracts.

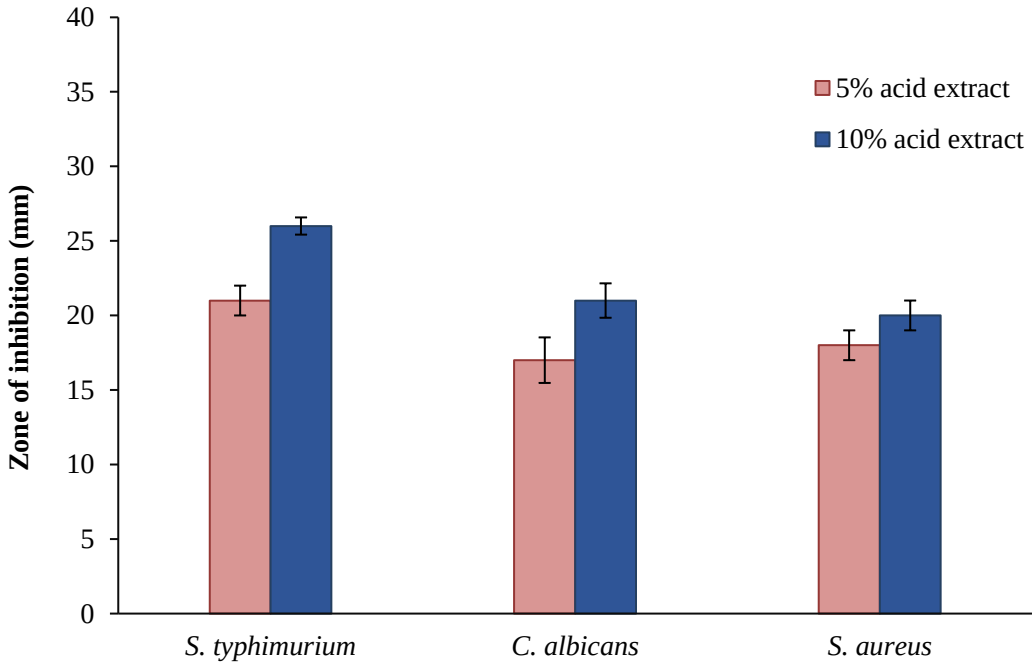


Fig. 2.3: Antimicrobial activity of 0-90% ammonium sulfate precipitate of *M. oleifera* seed extracts.

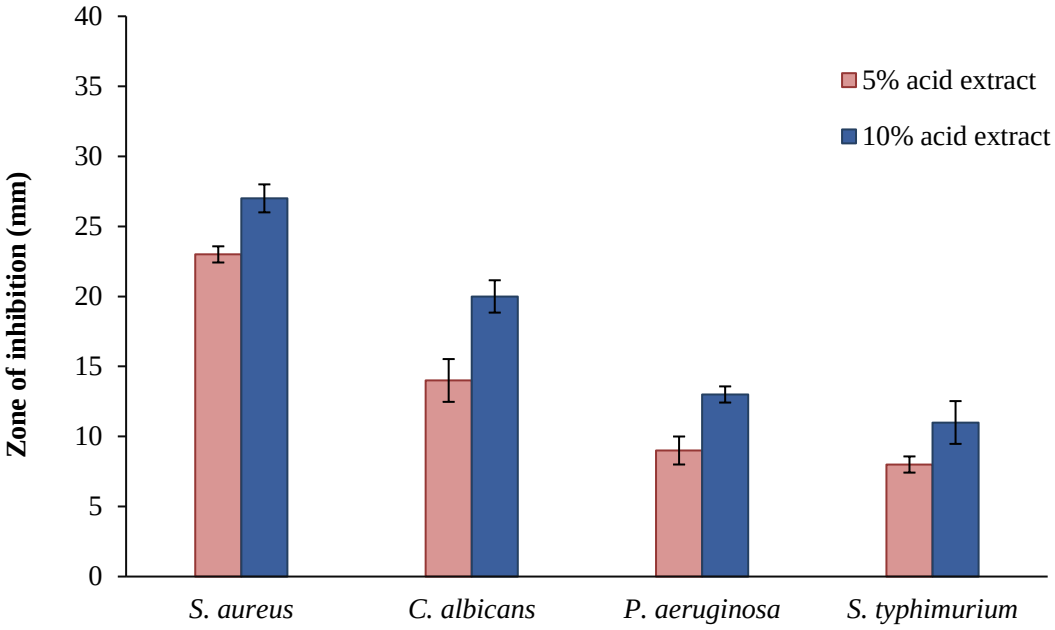


Fig. 2.4: Antimicrobial activity of 0-90% ammonium sulfate precipitate of *T. foenum-graecum* seed extracts.

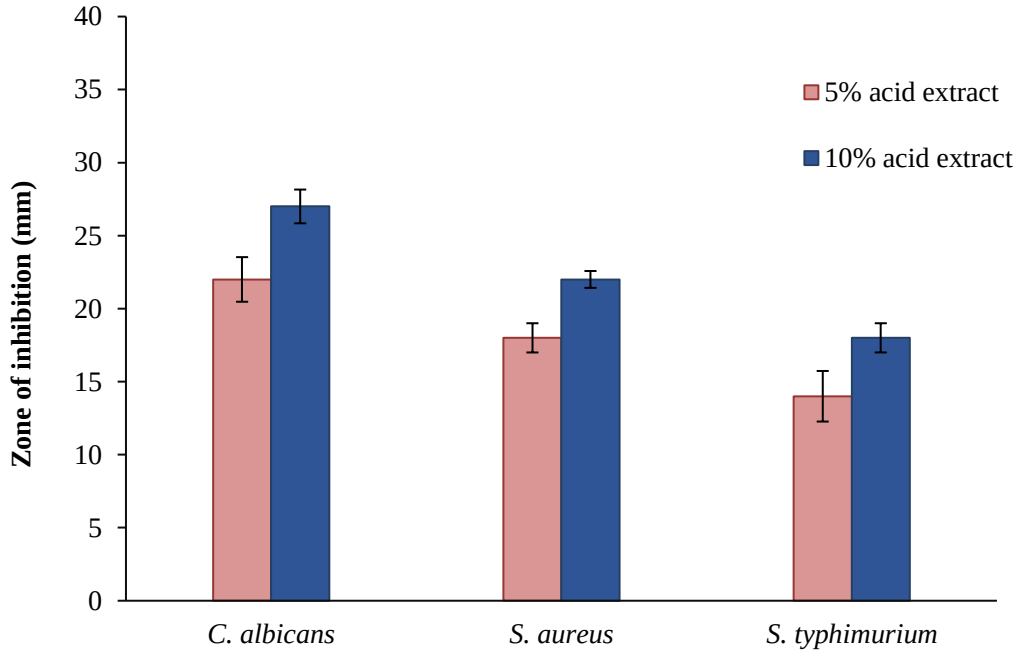


Fig. 2.5: Antimicrobial activity of 0-90% ammonium sulfate precipitate of *T. divaricata* leaf extracts.

2.3.3. Partial purification by ammonium sulfate fractionation

Crude extracts of *A. sativum*, *C. ternatea*, *M. oleifera*, *T. foenum-graecum* and *T. divaricata* were partially purified by ammonium sulfate fractionation. The fractions obtained at different saturation levels were analyzed by antimicrobial activity tests and by SDS-PAGE. The 0–30% and 30–60% ammonium sulfate fractions of *A. sativum* extracts exhibited growth inhibition against C. *albicans* (Fig. 2.7a), *S. aureus*, *E. coli*, *S. typhimurium* and *A. hydrophila* (Fig. 2.6). The SDS-PAGE analysis revealed a broad peptide band around 7 kDa in these fractions (Fig. 2.7b). Antimicrobial molecules from *C. ternatea* were comparatively more concentrated in the 0–30% fraction than the 30–60% fraction, the 60–90% fraction being inactive (Fig. 2.8; 2.9a). The SDS-PAGE results showed that a low MW peptide (> 7kDa) more concentrated in the 0–30% fraction formed a comparatively broad band on the gel, the same peptide being also visible as a narrow band in the 30–60% fraction (Fig. 2.9b). For *M. oleifera*, the active 0–30% ammonium sulfate fraction (Fig. 2.10; 2.11a) appeared as a peptide band around 7 kDa on SDS-PAGE analysis (Fig. 2.11b). Antimicrobial molecules from *T. foenum-graecum* were distributed in both 30–60% as well as 60–90 % fractions (Fig. 2.12; 2.13a), the SDS-PAGE profile of these fractions pointing to the presence of higher MW proteins (Fig. 2.13b). Antimicrobial activity of the 0–30% and 30–60% ammonium sulfate fractions (Fig. 2.14; 2.15a) of *T. divaricata* extract was also due to higher MW proteins that were concentrated in these fractions (Fig. 2.15b).

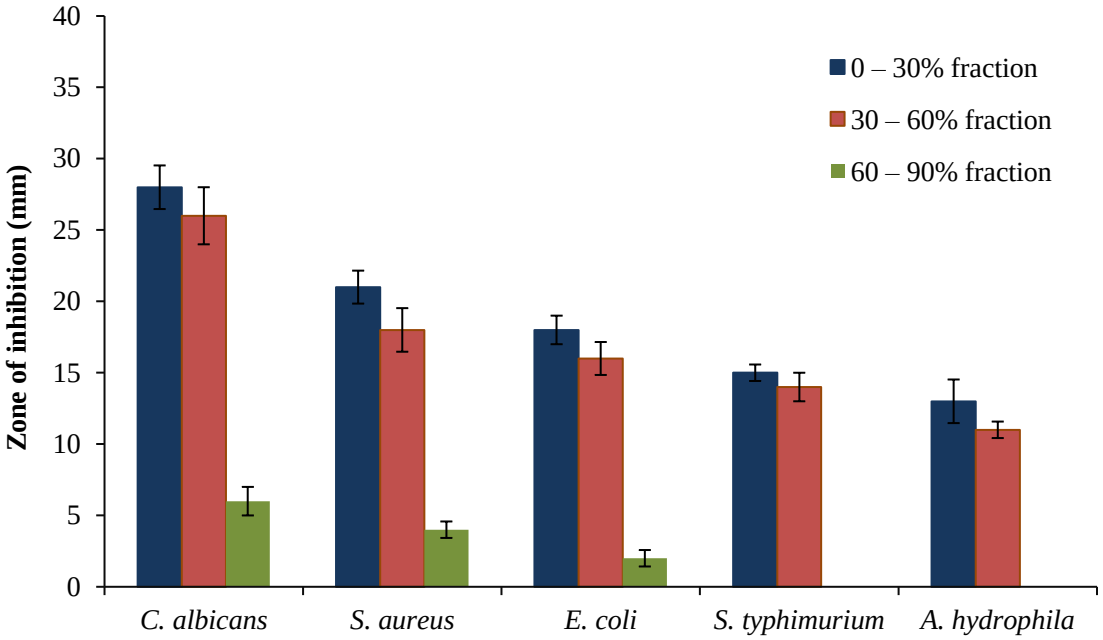
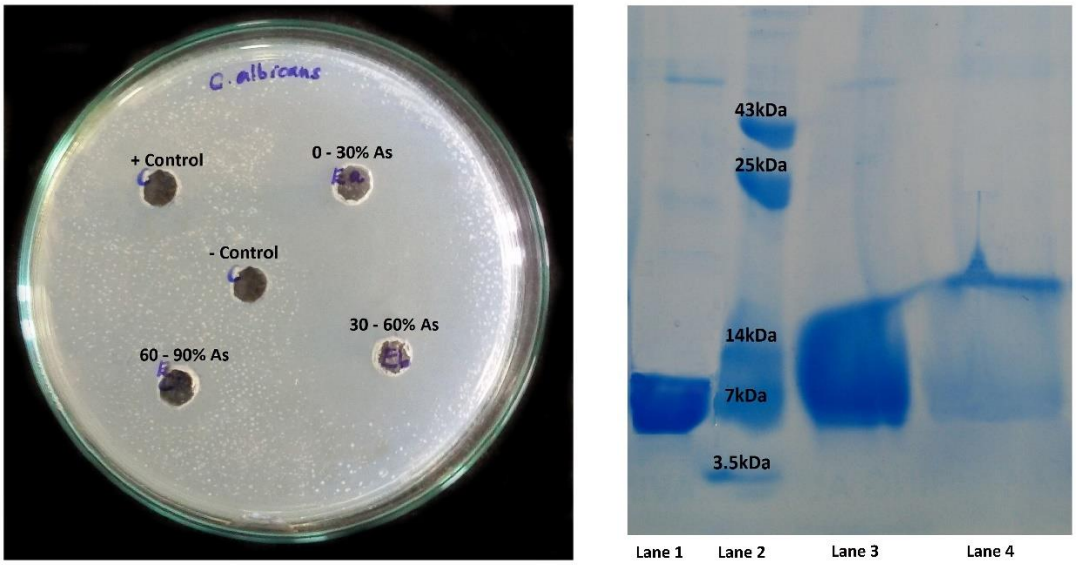


Fig. 2.6: Antimicrobial activity of ammonium sulfate fractions of *A. sativum* extracts.



(a) **(b)**

Fig. 2.7: (a) Activity of ammonium sulfate fractions of *A. sativum* against *C. albicans*. Amphotericin and 0.01 M sodium acetate buffer (without any added sample) served as positive and negative controls, respectively. **(b)** SDS-PAGE analysis, Lane 1: 0-30% fraction, Lane 2: LMW Protein marker, Lane 3: 30-60% fraction and Lane 4: 60-90% fraction.

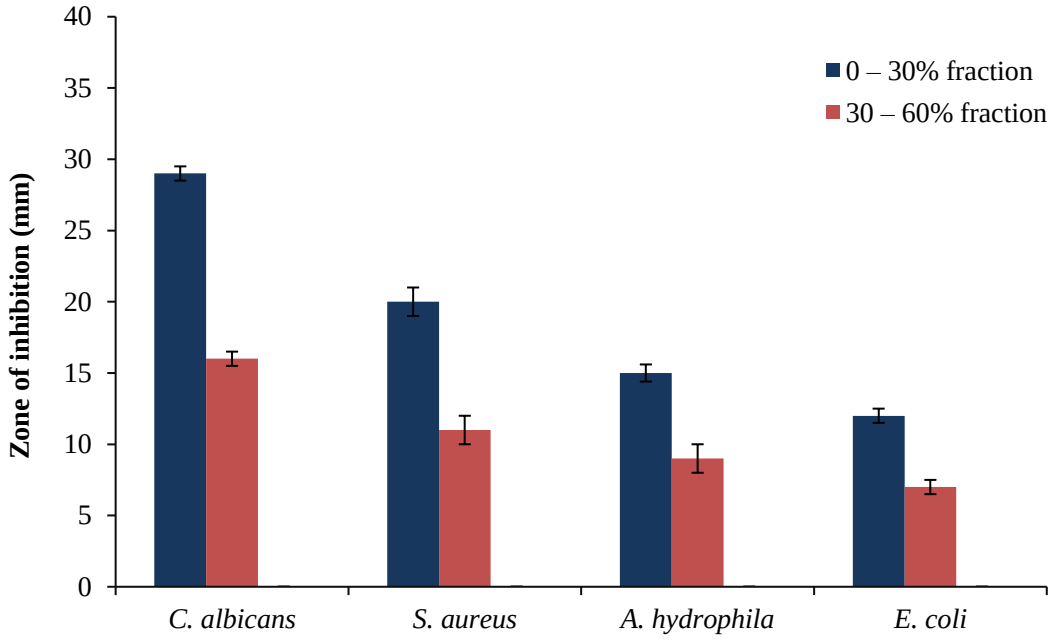
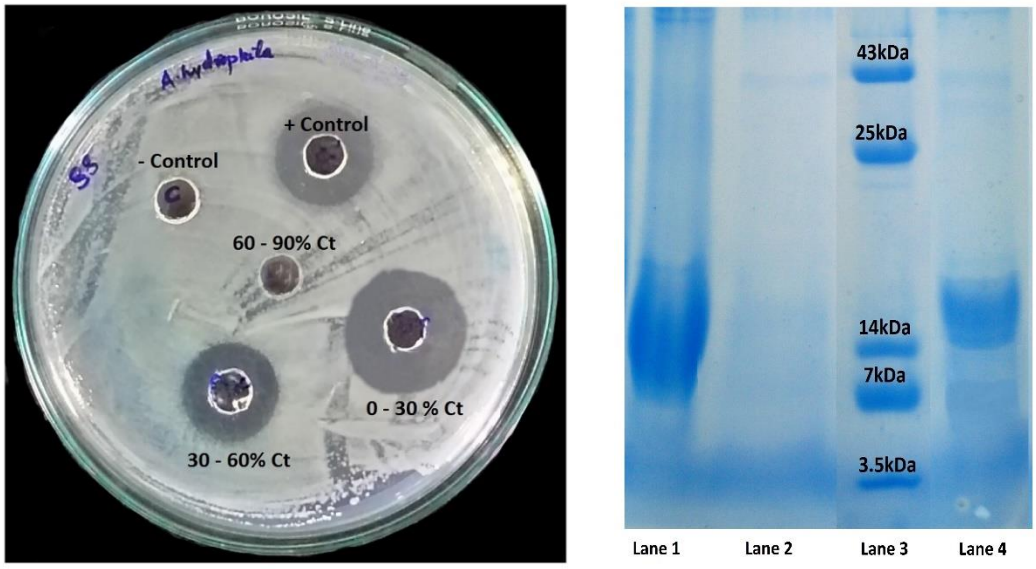


Fig. 2.8: Antimicrobial activity of ammonium sulfate fractions of *C. ternatea* extracts.



(a) **(b)**

Fig. 2.9: (a) Activity of ammonium sulfate fractions of *C. ternatea* against *A. hydrophila*. Tetracycline and 0.01 M sodium acetate buffer (without any added sample) served as positive and negative controls, respectively. (**b)** SDS-PAGE analysis, Lane 1: 0-30% fraction, Lane 2: 60-90% fraction, Lane 3: LMW Protein markers, and Lanes 4: 30-60% fraction.

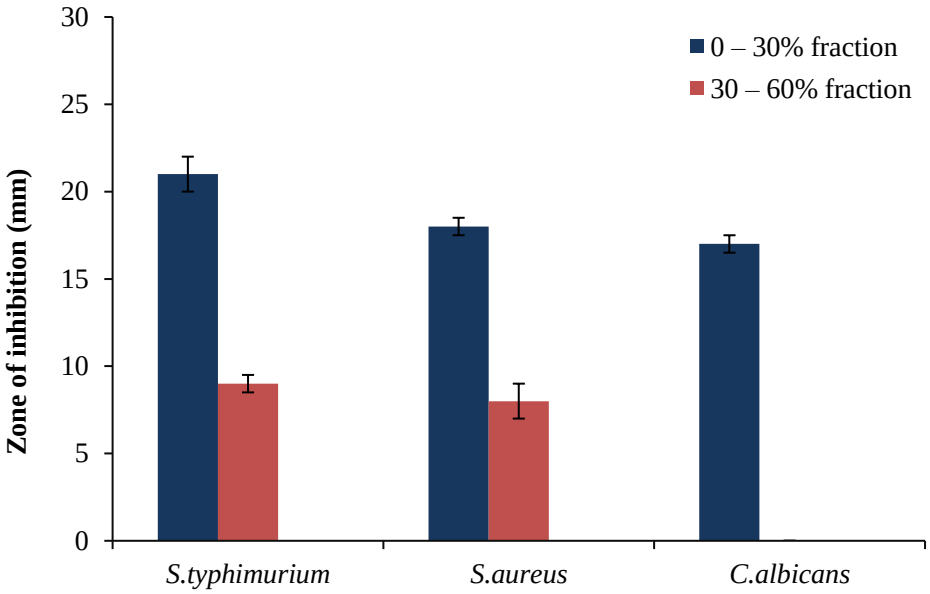


Fig. 2.10: Antimicrobial activity of ammonium sulfate fractions of *M. oleifera* extracts.

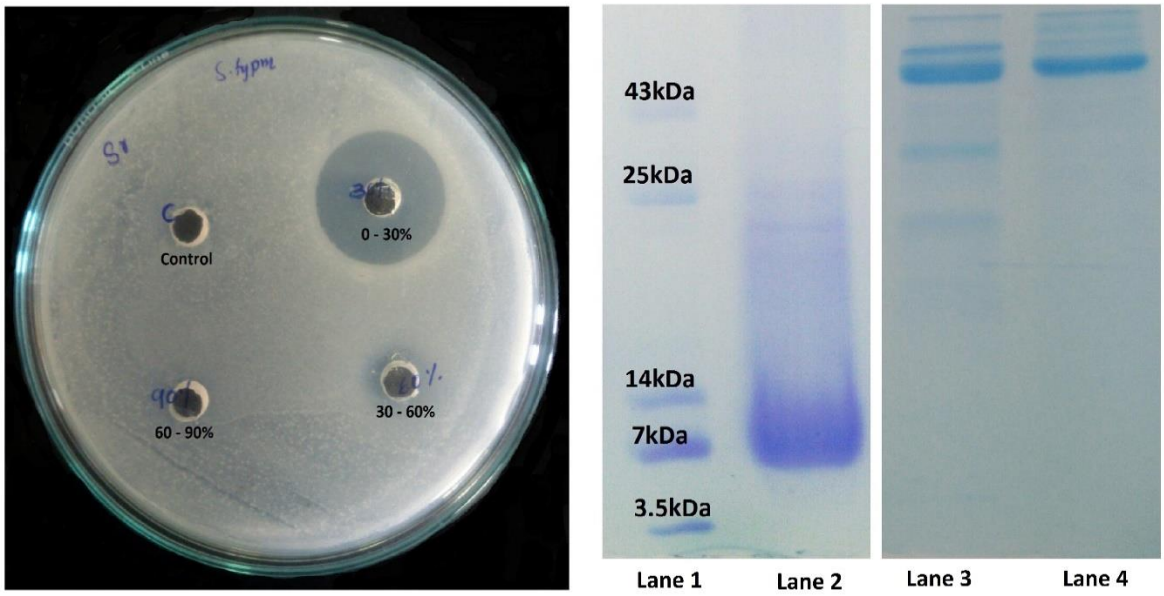


Fig. 2.11: (a) Activity of ammonium sulfate fractions of *M. oleifera* against *S. typhimurium*. 0.01 M sodium acetate buffer (without any added sample) served as control **(b)** SDS PAGE analysis, Lane 1: LMW Protein marker, Lane 2: 0-30% fraction, Lane 3: 30-60% fraction and Lane 4: 60-90% fraction.

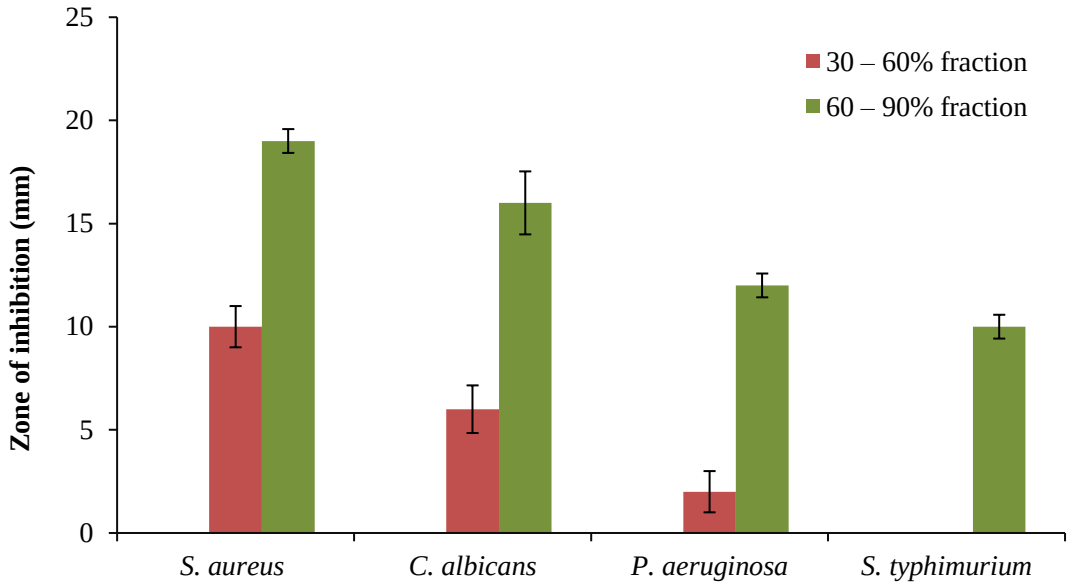
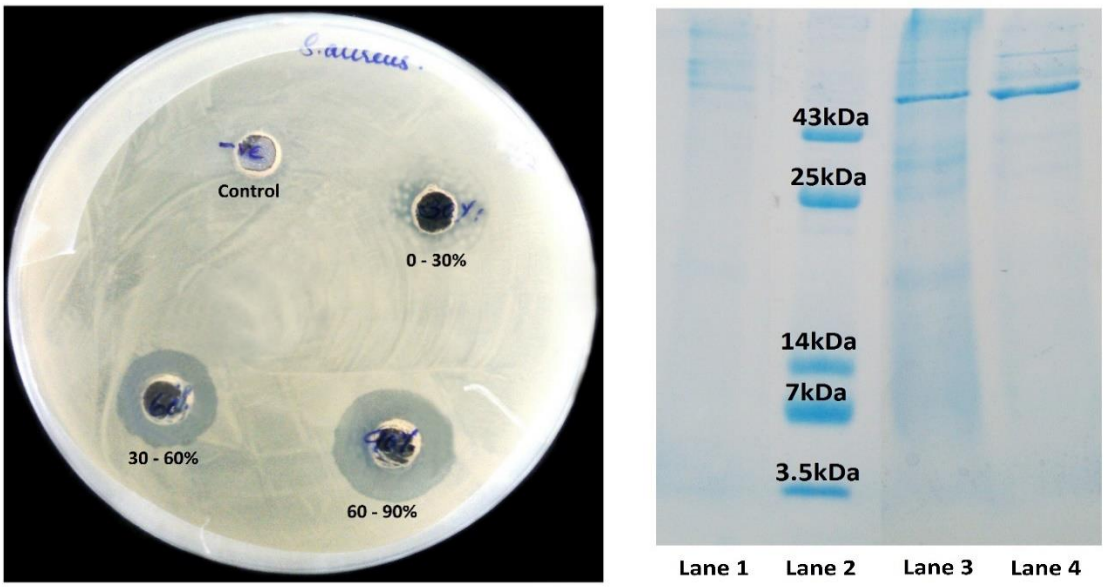


Fig. 2.12: Antimicrobial activity of ammonium sulfate fractions of *T. foenum-graecum* extracts.



(a) **(b)**

Fig. 2.13: (a) Activity of ammonium sulfate fractions of *T. foenum-graecum* against *S. aureus*. 0.01 M sodium acetate buffer (without any added sample) served as control **(b)** SDS PAGE analysis, Lane 1: 0-30% fraction, Lane 2: LMW Protein marker, Lane 3: 30-60% fraction and Lane 4: 60-90% fraction.

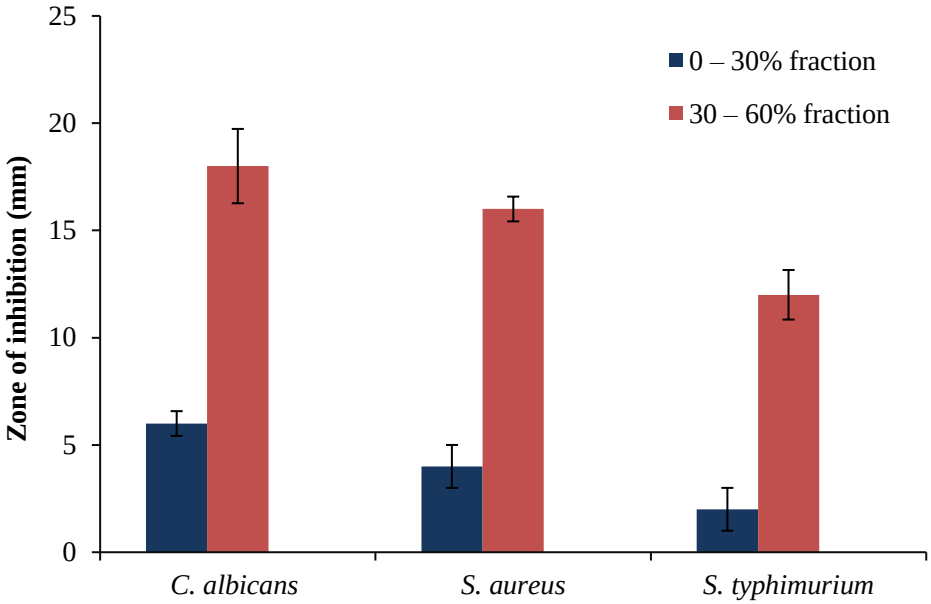


Fig. 2.14: Antimicrobial activity of ammonium sulfate fractions of *T. divaricata* extracts.

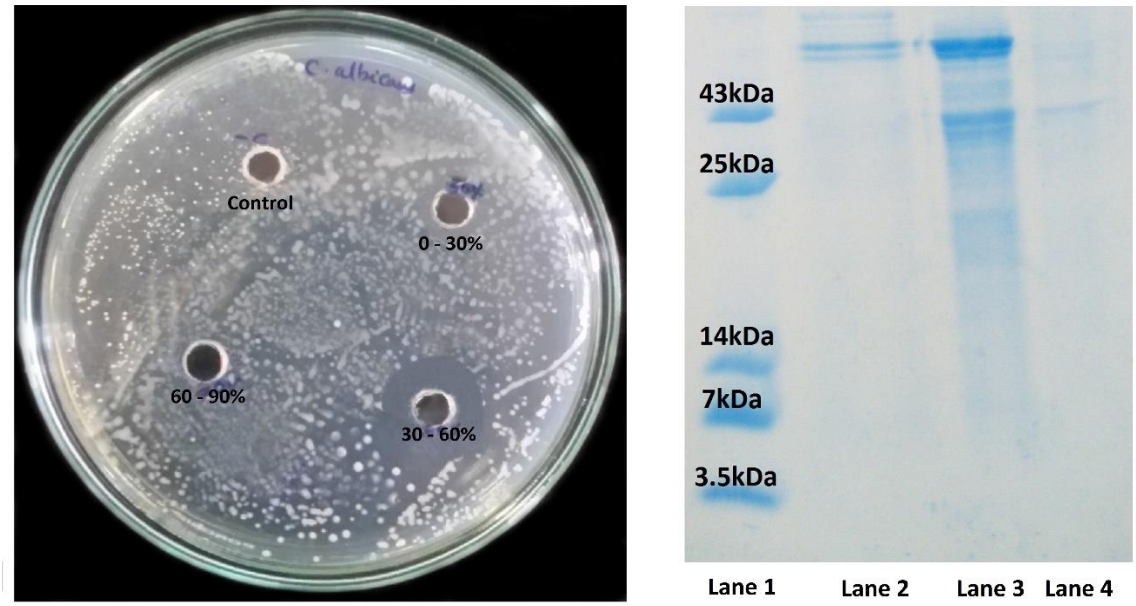


Fig. 2.15: (a) Activity of ammonium sulfate fractions of *T. divaricata* against *C. albicans*. 0.01 M sodium acetate buffer (without any added sample) served as control (**b**) SDS PAGE analysis, Lane 1: LMW Protein marker, Lane 2: 0-30% fraction, Lane 3: 30-60% fraction and Lane 4: 60-90% fraction.

From the above data based on agar well diffusion assays and electrophoretic analyses, antimicrobial activity due to low MW peptides (<10 kDa) appeared most promising from three plant samples *viz*, *A. sativum, C. ternatea* and *M. oleifera*. These were hence shortlisted for further purification and detailed characterization, with the aim to obtain at least one molecule of significant bioactivity, as per the objectives of the study listed out under Section 1.5.

Chapter 3

Purification and characterization of a cationic peptide from Moringa oleifera

3.1. INTRODUCTION

Moringa oleifera

Moringa oleifera or drumstick tree, a pan-tropical species of the Moringaceae family, is often lauded as a 'Miracle tree' on account of the exceptional nutritional values as well as health benefits that it offers (Olson et al. 2016; Keating et al. 2017). Many scientific studies have substantiated traditional folklore claims on the medicinal value of *M. oleifera* in the treatment of a variety of ailments such as digestive disorders, inflammations, skin diseases, asthma, heart complaints, rheumatism and gastroenteric diseases (Anwar et al. 2007; Amaglo et al. 2010; Fahey 2017). Every morphological part of *M. oleifera* is valued to have a profusion of pharmacological attributes such as antimicrobial, antidiabetic, cardio-protectant, hepato-protectant, antitumor, diuretic, anti-inflammatory, antioxidant, antineoplastic, and the like (Guevara et al. 1999; John and Chellappa 2005; Amaglo et al. 2010; Emmanuel et al. 2014; Ratshilivha et al. 2014; Jaja-Chimedza et al. 2017). A complex array of phytochemicals has

been identified from *M. oleifera* leaf, flower, stem, root and seed extracts, of which some have attracted special attention on account of their pharmaceutical applications (Haristoy et al. 2005; Galuppo et al. 2013; Onsare and Arora 2015).

The antimicrobial activity of seed extracts of *M. oleifera* has to date been ascribed to glucosinolates, glycosides, isothiocyanates and flocculating agents (Madsen et al. 1987; Haristoy et al. 2005; Galuppo et al. 2013). Most of the water-soluble proteins identified from *Moringa* seeds were categorized as agglutinins or lectins with flocculating properties, and were found effective in removing contaminants during primary wastewater treatment (Madsen et al. 1987; Santos et al. 2012). The present study aimed to address the purification and characterization of a distinct antimicrobial cationic peptide from *M. oleifera* seed and provide a lead for future investigation into more detailed structural and functional properties of the purified peptide.

3.2. MATERIALS AND METHODS

3.2.1. Purification of *M. oleifera* seed peptide

The precipitate obtained after ammonium sulfate fractionation at 0-30% saturation of *M. oleifera* seed extract (as discussed in Chapter 2, section 2.2.5) was subjected to size exclusion column chromatography on a Sephadex G–50 column (12 x 500 mm) pre-equilibrated with 0.01 M sodium acetate buffer, pH 5.6. The samples were eluted in 0.01 M sodium acetate buffer at a flow rate of 14 ml/h and their absorbance read at 220 and 280 nm. An aliquot of individual eluted fractions was assayed for antimicrobial activity by the agar well diffusion method (Holder and Boyce 1994). The active fractions were pooled and concentrated by lyophilization.

3.2.2. Polyacrylamide gel electrophoretic analyses

The purified *M. oleifera* seed fraction was analyzed by native-PAGE on a 15% resolving gel (under non-reducing conditions) and SDS-PAGE on an 18% resolving gel as discussed previously (Chapter 2, Section 2.2.6). To assess the cationic or basic nature of the purified samples, Acid-urea-PAGE analysis was performed on a 15% resolving gel containing 6 M

urea and 5% acetic acid (Panyim and Chalkley 1969) and Coomassie Brilliant Blue G-250 was used to locate the protein/peptide bands (Candiano et al. 2004).

3.2.3. Determination of molecular weight by mass spectral analyses

Molecular weight of the purified *M. oleifera* seed peptide was determined by Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS) on a Triple Quadrupole Mass spectrometer (Agilent 6460). The peptide solution was prepared in formic acid (0.1% v/v, in deionized distilled water) and 10 µl was loaded onto a High-Performance Liquid Chromatography (HPLC) C18 column coupled to a Triple Quadrupole MS system. Acetonitrile (20% v/v) in water was used as the elution buffer. The molecular ions were detected in positive mode as the function of *m/z*. Deconvolution of ESI-MS spectra was carried out using Agilent MassHunter Bioconfirm software. Intact molecular size of the peptide was detected by Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight (MALDI-TOF) mass spectrometry on a Bruker-UltrafleXtreme MALDI-TOF/TOF (Bruker Daltonics) MS system. The *m/z* spectrum was acquired in linear positive mode in a mass range of 5–20 kDa.

3.2.4. Agar well diffusion assay and broth microdilution methods

In the agar well diffusion method (Holder and Boyce 1994), purified *M. oleifera* seed peptide (~25 µg /well) was added to 6 mm wells on Mueller-Hinton agar plates seeded with microbial cultures, as discussed in Section 2.2.3. Minimum inhibitory concentration (MIC) of the purified peptide sample was examined as per the Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI 2012). In brief, 50 µl of microbial inoculum prepared in Mueller–Hinton broth was dispensed in each well of a sterile 96-well plate to attain a final density of 3-5 x 10^5 CFU/ml. Thereafter, 50 µl of peptide solution in twofold serial dilutions (1–128 µg/ml) was added to each well and the plates incubated as before. Microbial growth inhibition was evaluated by measuring the absorbance at 595 nm using Bio-Rad's iMark Microplate Absorbance Reader. The lowest peptide concentration required to inhibit microbial growth to 99.9% was gauged as the MIC (CLSI 2012; Bar et al. 2009). All experiments were repeated thrice and with samples in triplicate, in order to ensure maximum reproducibility of the results.

3.2.5. Heat, pH and protease treatment studies

Effect of physicochemical parameters such as heat and pH as well as of protease treatment, on the stability of the peptide antimicrobial activity was studied by standard methods. To evaluate the thermal stability, prior to the antimicrobial assay the purified peptide sample was incubated for 1h at temperatures ranging from 40–100°C, (Barboza-Corona et al. 2007; Ge et al. 2016). The sample incubated at room temperature (28 ^{o}C) served as a control. The pH sensitivity was evaluated based on antimicrobial activity of the peptide incubated at room temperature for 1h in 50 mM citrate buffer (for pH 5 and 6), PBS (for PH 7) or Tris-HCl (for pH 8) (Huang et al. 2016). Effect of proteolytic degradation on peptide activity was examined by treating the peptide with pronase (Sigma-Aldrich), trypsin (HiMedia) or proteinase K (HiMedia) at a ratio of 100:1 (w/w) for 3h at 37°C, prior to carrying out antimicrobial activity tests. Enzyme alone in buffer and untreated peptide in buffer served as controls (Ebbensgaard et al. 2015).

3.2.6. Salt sensitivity assay

Sensitivity of the *M. oleifera* seed peptide to various salts of monovalent, divalent and trivalent cations was evaluated based on a previously described method (Wang et al. 2015; Zhu et al. 2014). Microbial inocula was prepared in Mueller-Hinton broth and different dilutions of various salts were then added to attain final concentrations at 150 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$ or 4 µM $FeCl_3$. Antimicrobial activity of the treated peptide was evaluated by the broth microdilution method, with peptide activity in the absence of salt treatment serving as the control. All experiments were repeated thrice and with samples in triplicate, to ensure maximum reproducibility of the results.

3.3. RESULTS AND DISCUSSION

3.3.1. Purification of *M. oleifera* peptide

The most antimicrobially active fraction which was obtained at 0–30% ammonium sulfate saturation of the seed extract (Figs. 3.1 and 2.10) was further purified by Sephadex G-50 column chromatography. The eluted fractions were assayed for activity against *S. typhimurium*, *C. albicans* and *S. aureus*, and the typical effect on *S. typhimurium* is depicted in Fig. 3.2. Although the pattern of protein elution based on the absorbance at 280 nm perfectly paralleled that at 220 nm, the latter values were used while plotting the profile; the 280 nm values were generally low, probably on account of the low aromatic amino acid content, especially at the dilution of the eluting fractions. The antimicrobially active peak fractions were then pooled, concentrated by lyophilization and used for further characterization.

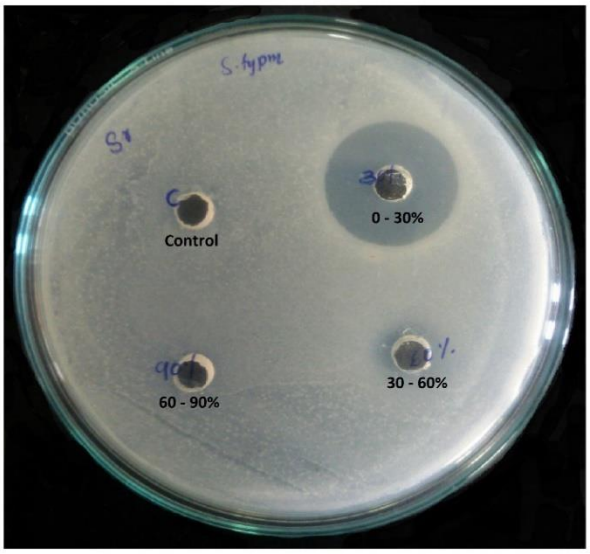


Fig. 3.1: Effect of ammonium sulphate fractionated samples of *M. oleifera* seed extract on *S. typhimurium* in culture; sodium acetate buffer (0.01 M), pH 5.6 (without any added sample) served as control.

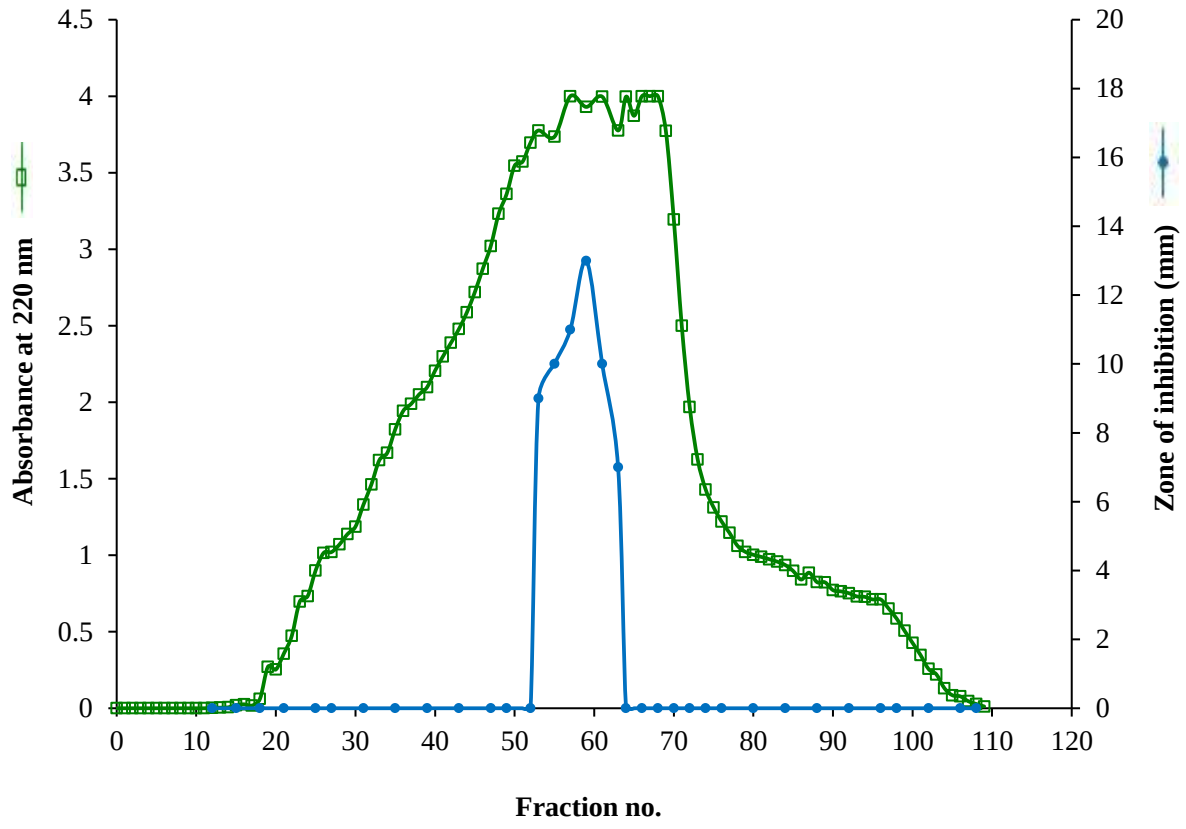


Fig. 3.2: Gel filtration profile of the 0–30% ammonium sulphate fraction of *M. oleifera* seed extract on a Sephadex G-50 column matrix; antimicrobial activity of eluted fractions was as tested against *S. typhimurium* by the agar well diffusion assay.

3.3.2. Electrophoresis and mass spectral analyses

Native-PAGE of the purified *M. oleifera* seed peptide revealed a single band (Fig. 3.3a) which was of molecular weight ~ 6.5 kDa, as per SDS-PAGE analysis (Fig. 3.3b, 3.4). The cathodal migration of the peptide on an AU gel (Fig. 3.3c) confirmed its cationic or basic nature. The HPLC elution profile of the purified seed peptide showed a single peak (Fig. 3.5a), and ESI-MS analysis gave the accurate molecular weight of the peptide as 6707.16 Da (Fig. 3.5b & 3.5c). Further, MALDI-TOF-MS analysis confirmed the intact molecular mass of the peptide as 6692.485 Da (Fig. 3.6).

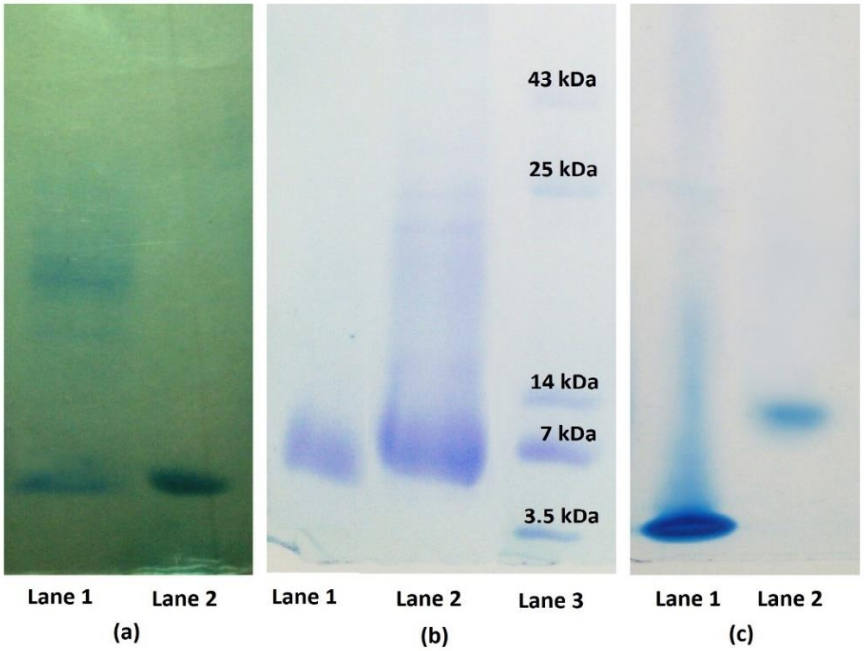


Fig. 3.3: PAGE analyses of *M. oleifera* seed samples. (a) Native-PAGE: Lane 1 – 0-30% ammonium sulphate fraction, Lane 2 – purified *M. oleifera* seed peptide; (b) SDS-PAGE: Lane 1 – the purified *M. oleifera* peptide, Lane 2 – 0-30% ammonium sulphate fraction, Lane 3 – standard protein markers; (c) AU-PAGE: Lane 1 – lysozyme as a 'basic protein' marker, Lane 2 – the purified *M. oleifera* peptide.

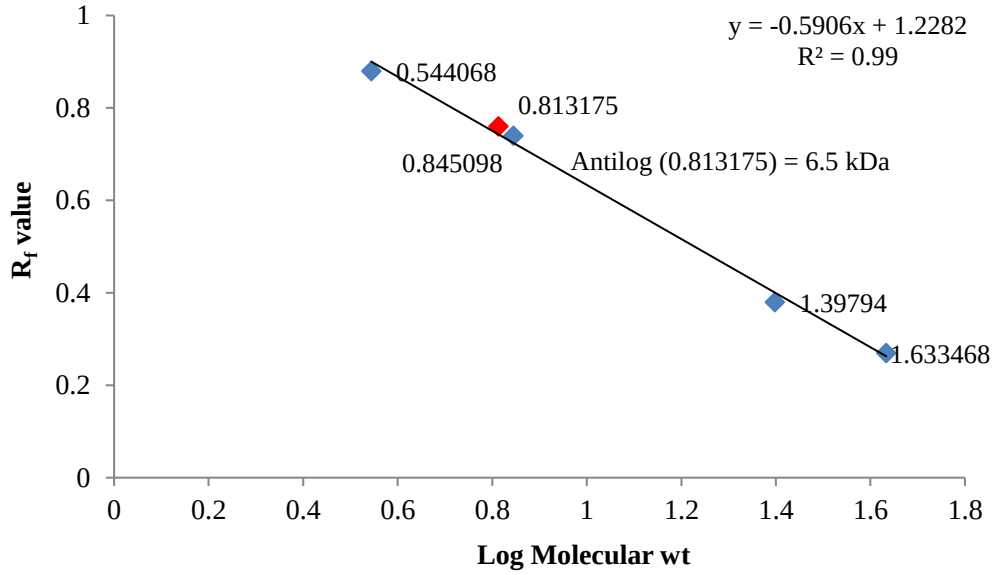


Fig. 3.4: Estimation of molecular weight of *M. oleifera* seed peptide based on SDS-PAGE data; the standard curve was plotted using R_f values of low MW protein markers run simultaneously.

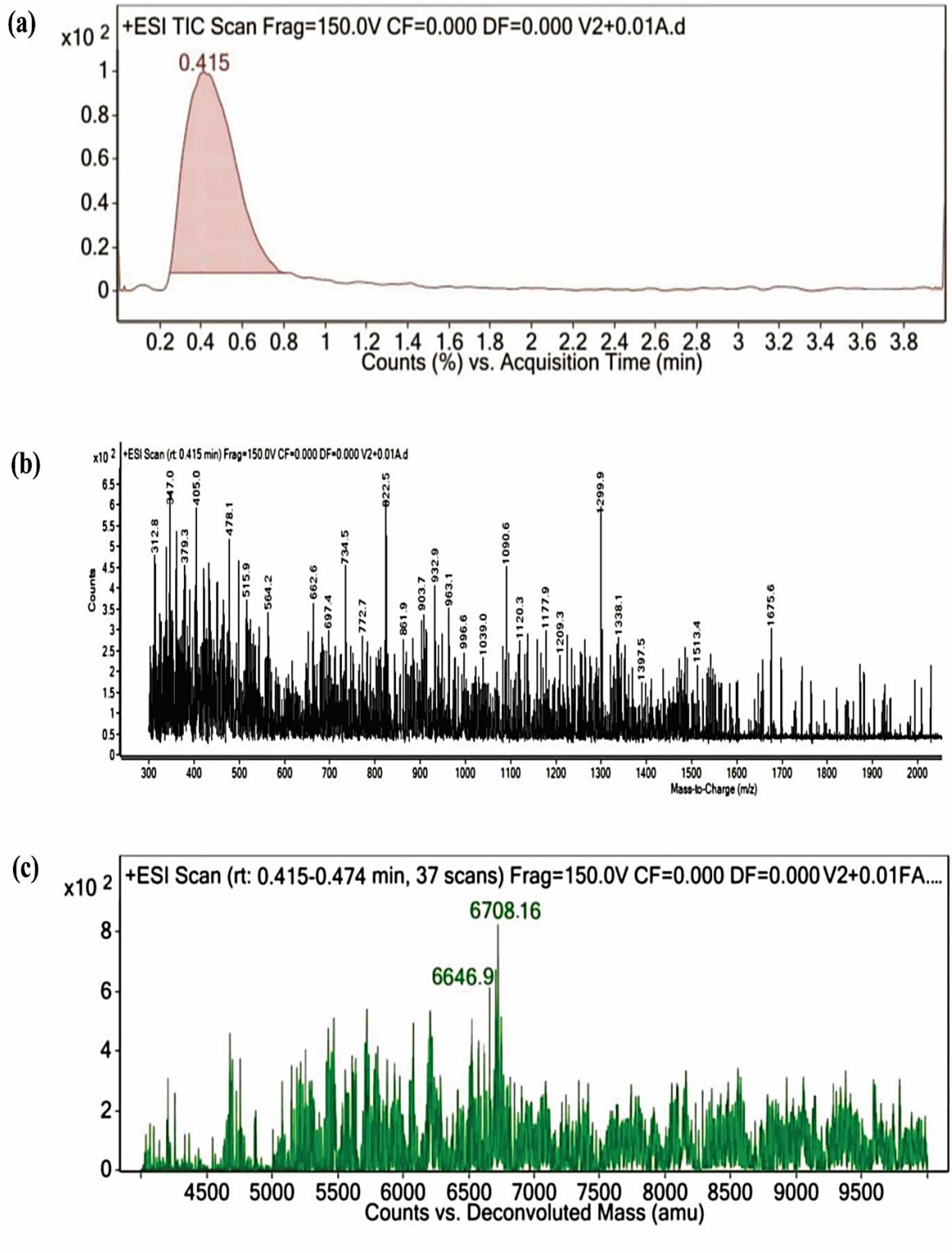


Fig. 3.5: LC–ESI MS analysis of the *M. oleifera* seed peptide: (a) LC elution profile; (b) ESI mass spectrum; (c) Deconvoluted mass spectrum.

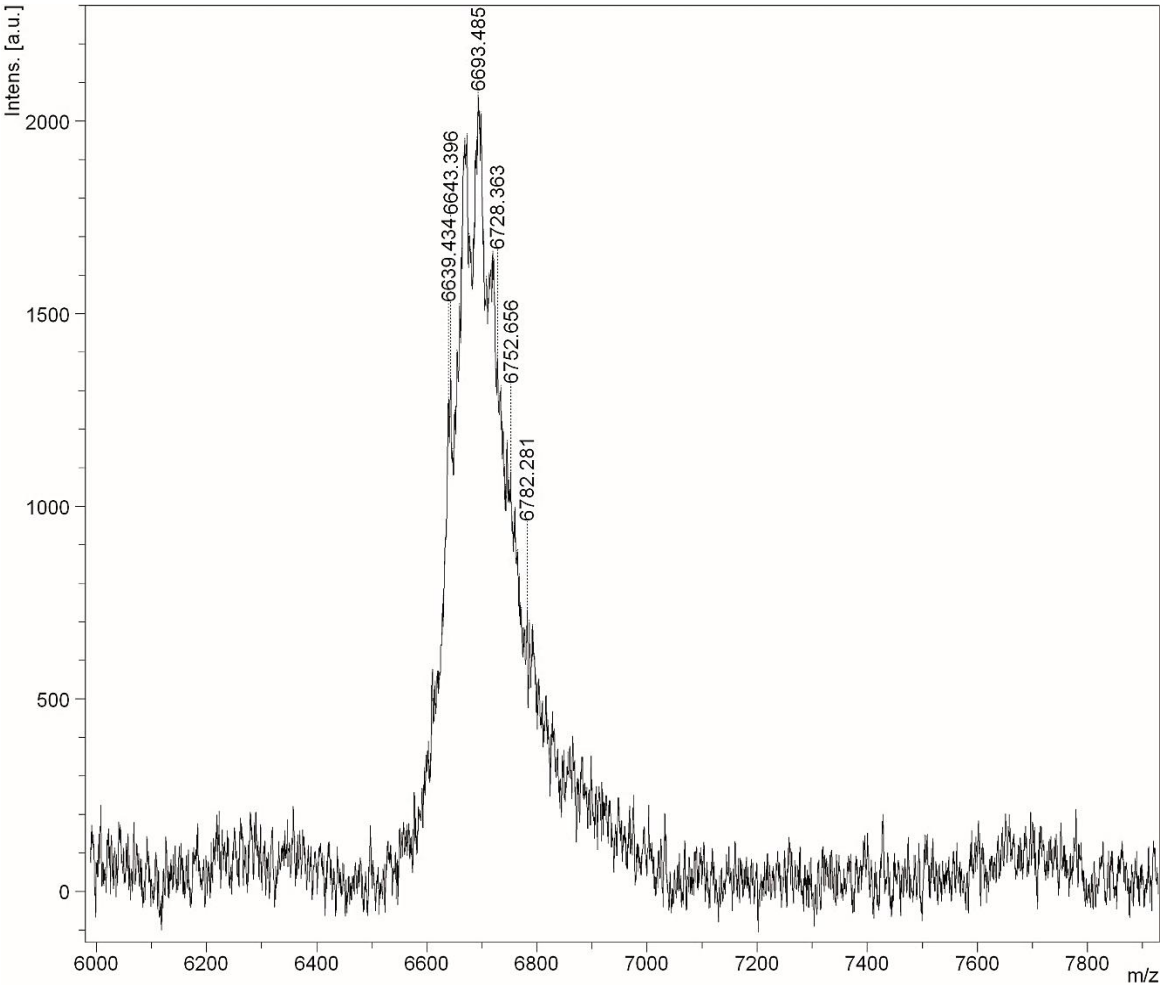


Fig. 3.6: MALDI-TOF Mass spectrum of the *M. oleifera* peptide.

3.3.3. Antimicrobial profile and MIC analysis

The broth microdilution method and the agar well diffusion method were adopted to gauge the pathogenic potential of the purified peptide. As summarized in Fig. 3.7, the peptide exhibited remarkable inhibitory effect against *S. typhimurium, C. albicans* and *S. aureus***,** with MIC values ranging from 16 – 32 µg/ml. The other microbial strains used, *viz., E. coli*, *P. aeruginosa* and *A. hydrophila*, were resistant to this peptide activity.

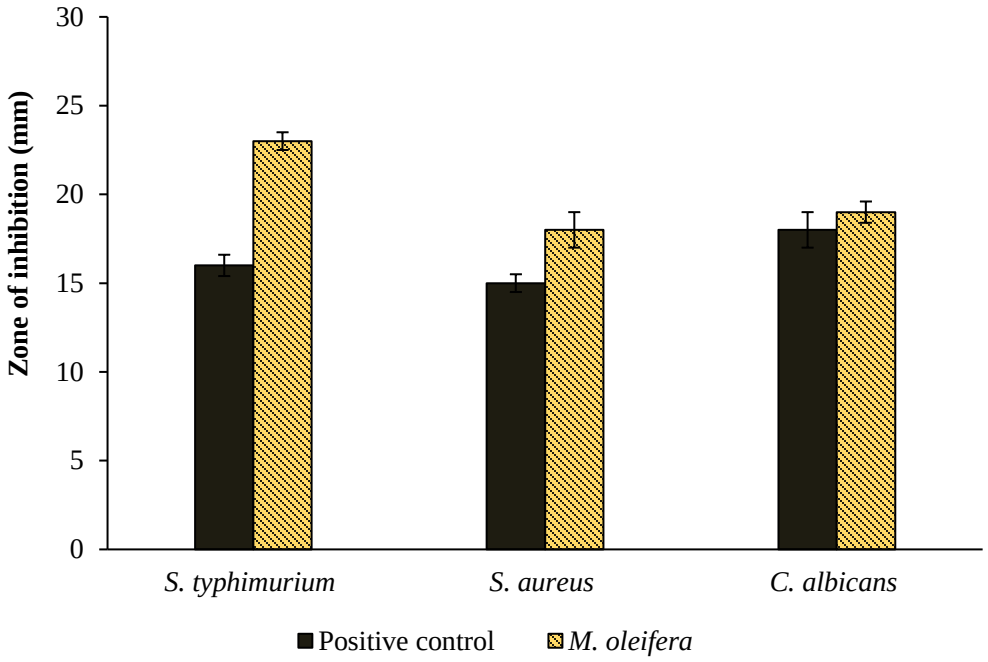


Fig. 3.7: Antimicrobial activity of the purified *M. oleifera* peptide as determined by agar well diffusion assay (~25 µg peptide/well); Chloramphenicol, gentamicin and amphotericin served as positive controls (25µg/well) against *S. typhimurium*, *S. aureus* and *C. albicans*, respectively.

Microbicidal and insecticidal properties of several flocculant or coagulant proteins of the lectin family have been reported in previous studies (Suarez et al. 2003, 2005; Santos et al. 2005, 2009, 2012; Ghebremichael et al. 2005; Luz et al. 2013). Detailed characterization of antimicrobial properties of water-soluble lectins purified from *Moringa* seeds had revealed their efficiency in removing contaminants during waste water treatment; Suarez et al (2003) characterized a cationic protein from heat stable fractions of *M. oleifera* seeds and designated it as 'Flo'. This had a molecular size ~6.5 kDa and showed bacteriostatic effect against *Streptococcus pneumonia, Streptococcus pyogenes, Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus,* in the MIC range of 5–50 mg/ml. Shortly thereafter, bactericidal or bacteriostatic effects of Flo-derived peptides or synthetic Flo was demonstrated against *S. pneumonia, S. pyogenes, P. aeruginosa, E. coli* and *S. aureus*, with MIC values of 2–10 mg/ml, while *C. albicans* exhibited complete resistance against Flo or Flo-derived peptides (Suarez et al. 2005). A bactericidal protein termed *M. oleifera* Coagulant Protein (MOCP; MW <6.5 kDa) purified from heat-treated seed fraction was found active against *B.*

thuringiensis, E. coli and *P. aeruginosa* and displayed high sequence similarity to Flo protein upon mass spectral analysis (Ghebremichael et al. 2005). Different isoforms of anionic chitin-binding glycoproteins (Mo-CBP3; MW >11.5 kDa) purified from *M. oleifera* seeds exhibited antifungal activity against various phytopathogens (Gifoni et al. 2012; Freire et al. 2015). Significantly, the antimicrobial profile of the 6.7 kDa peptide purified in our study was quite different from those exhibited by these previously explored *M. oleifera* coagulant or flocculant proteins of similar molecular size.

3.3.4. Effect of temperature, pH and proteases

Typical characteristics of the *M. oleifera* seed peptide were evaluated by assessing the effects of temperature, pH and proteolytic treatment on its antimicrobial ability. Temperature sensitivity assays were carried out by heating the peptide sample at 40–100 ℃ for 1 h prior to the activity test. As shown in Fig. 3.8, the activity of the peptide against *S. aureus*, *C. albicans* and *S. typhimurium* remained intact even after being subjected to 60–70 ℃ for 1h, a drastic decline or complete loss of activity being observed only after incubation at 80 ℃ or above.

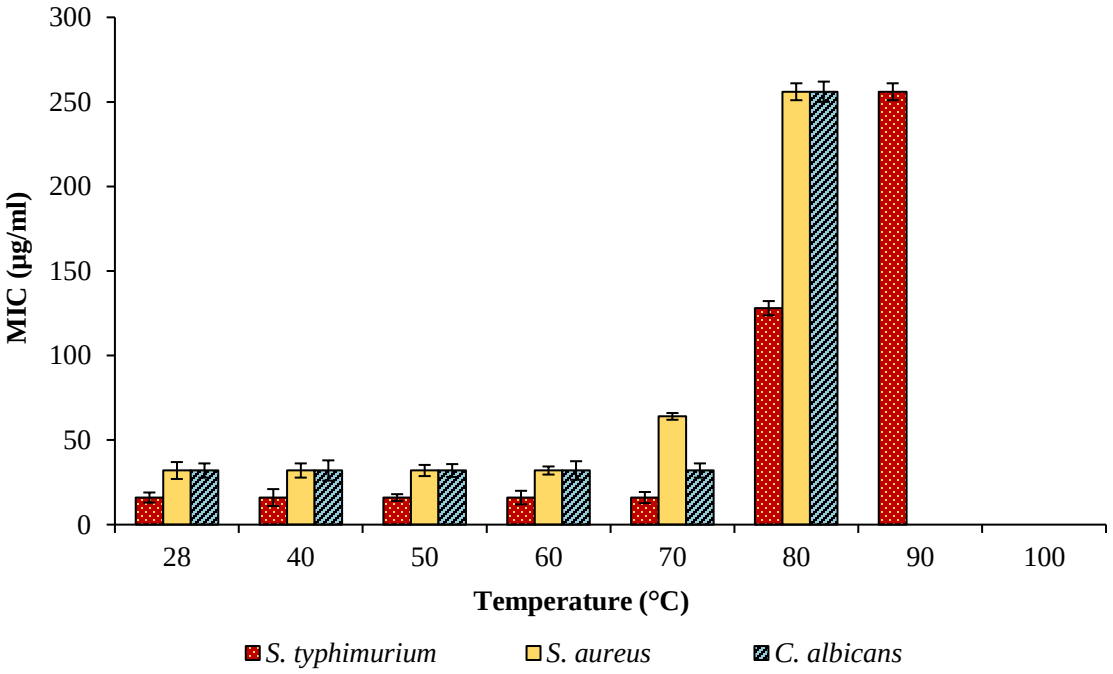


Fig. 3.8: Effect of heat treatment on antimicrobial activity of the *M. oleifera* peptide. The peptide treated at room temperature (28°C) was used as control. Experiments were carried out in triplicate and the mean ± SD values were plotted.

Heat stability is a common attribute of many coagulant or flocculant proteins characterised from *M. oleifera* seeds (Luz et al. 2013). Flo or Flo-derived peptides (MW ≤6.5 kDa) with remarkable antibacterial and coagulant properties were purified from *M. oleifera* seed extracts preheated at 80 ºC (Suarez et al. 2003). Ghebremichael et al (2005) reported high thermostability of *M. oleifera* Cationic Polypeptide (MW <6.5 kDa) which retained significant coagulation efficiency after heat treatment for 5 h at 95 ºC. Katre et al (2008) demonstrated the heat-stable nature of hemagglutination properties of *M. oleifera* Lectin (MW ~14 kDa) after being treated for 30 min at 85 ºC. The structural elucidation of heat-treated (30 min at 80°C) Coagulant *M. oleifera* Lectin of ~30 kDa revealed the presence of more than eight cysteine residues involved in disulfide bonding (Santos et al. 2009; Luz et al. 2013).

The pathogenic susceptibility of our *M. oleifera* seed peptide was stable in the pH range of 5–8, with MIC values of 16, 32 and 32 µg/ml, respectively, against *S. typhimurium*, *S. aureus* and *C. albicans*. The sensitivity to protease treatment was evaluated by testing the antimicrobial effect of the *M. oleifera* seed peptide incubated for 3 h at 37 °C with trypsin, pronase or proteinase K. As captured in Fig. 3.9, the peptide activity against all the tested pathogens was completely abolished after protease treatment, clearly indicating that the intact structural conformation of the peptide was a requisite for stable antimicrobial activity. This feature stood out as being markedly different from those of all previously reported molecules from *M. oleifera* seeds.

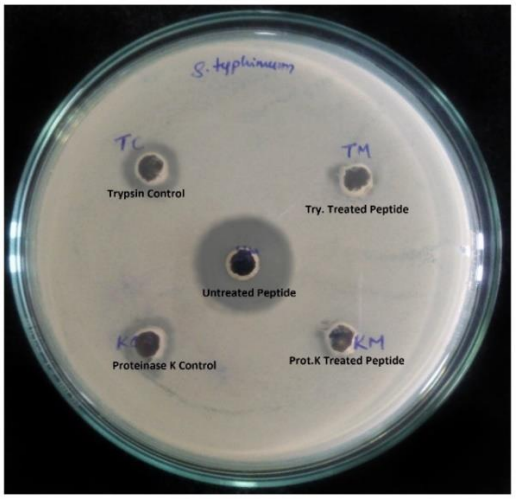


Fig. 3.9: Effect of protease-treated *M. oleifera* peptide on *S. typhimurium* in culture. Untreated peptide in buffer and enzyme alone in buffer served as controls.

3.3.5. Effect of salt treatment

Most cationic peptides exert microbicidal effects primarily by interacting with the negatively membrane components of microbial cells. Many studies indicated that high concentration of salts containing mono-, di- or tri-valent cations could impede the peptide interaction with microbial membranes, which could diminish the antimicrobial potential (Matsuzaki et al. 1991). In this perspective, the salt sensitivity of the *M. oleifera* seed peptide was examined by testing the antimicrobial activity in presence of various salts at physiological concentrations. As indicated in Fig. 3.10, while divalent Mg^{2+}, Ca^{2+} and trivalent Fe^{3+} ions weakly diminished the peptide activity against *S. typhimurium*, *S. aureus* and *C. albicans*, the monovalent Na^{+} and K^{+} ions could not bring any noticeable change in the MICs against all tested pathogens.

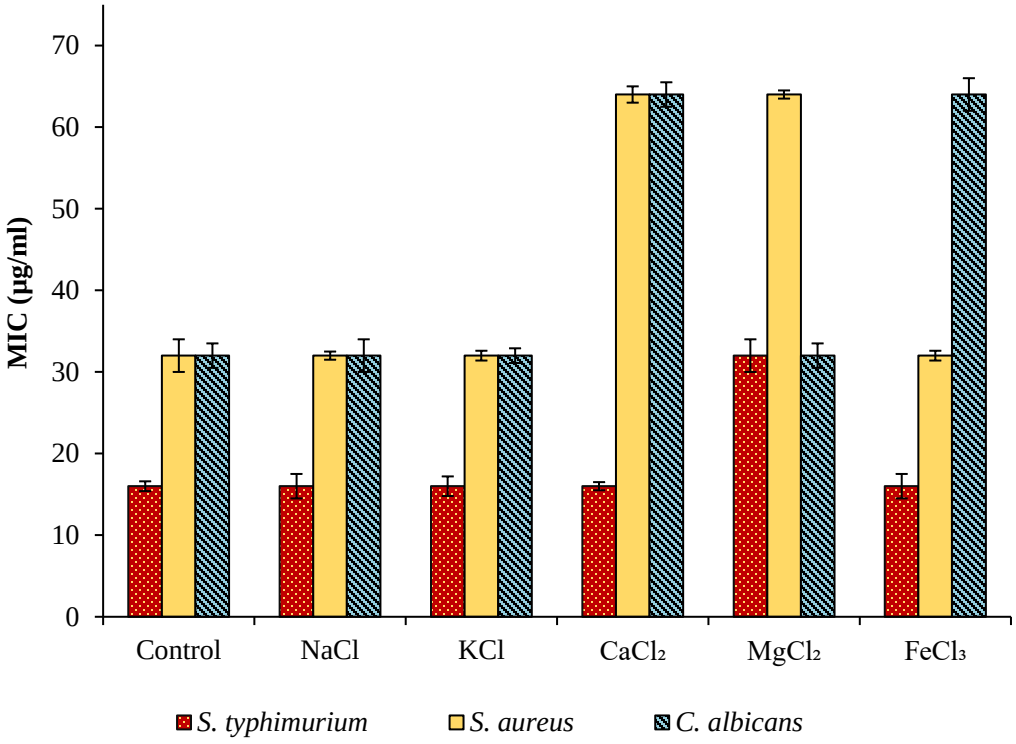


Fig. 3.10: Effect of different salts on antimicrobial activity of the *M. oleifera* peptide. Activity in the absence of salts was taken as the control. Experiments were carried out in triplicate and the mean ± SD values were plotted.

In the light of available literature to date, it is quite obvious that in terms of antimicrobial characteristics as well as physicochemical properties, the *M. oleifera* cationic peptide reported in the present work clearly differs from previously described *Moringa* seed peptides/proteins of the same range of molecular size.

Chapter 4

Purification and characterization of a cationic peptide from Clitoria ternatea

4.1. INTRODUCTION

Clitoria ternatea seeds

Clitoria ternatea, commonly called blue pea or butterfly pea, is a perennial herbaceous climber of the family Fabaceae. While acclaimed for its medicinal properties, this plant has also found place as a garden ornamental, widely featuring in agroforestry systems in tropical and subtropical regions of the world. It has been used since ancient times in traditional herbal medicine, functioning as a nootropic, tranquilizing and sedative agent (Mukherjee et al. 2008; Morris 2009). Different parts of *C. ternatea* have been attributed with promising bioactivities such as anti-inflammatory (Devi et al. 2003), antimicrobial (Kamilla et al. 2009; Ponnusamy et al. 2010; Pratap et al. 2012), anticancer (Kumar and Bhat 2011), hepatoprotective (Nithianantham et al. 2011), antidiabetic (Daisy and Rajathi 2009; Chusak et al. 2018),

cardioprotective (Maneesai et al. 2021), antioxidant (Chusak et al. 2018), antiasthmatic (Taur and Patil 2011), antihyperlipidemic (Solanki and Jain 2010) and nootropic (Malik et al. 2011). Various phytochemical constituents including anthocyanins, flavonoids, tannins, steroids, saponins, alkaloids, glycosides, *p*-hydroxycinnamic acid and pentacyclic triterpenoids such as taraxerols and taraxerones with known bioactivities have been identified from roots, seeds, stem, leaves and flowers of *C. ternatea* (Banerjee and Chakravarti 1963; Kulshreshtha and Khare 1967; Terahara et al. 1989, 1990; Kazuma et al. 2003). Methanolic extracts of *C. ternatea* had demonstrated antidepressant, anxiolytic, anti-stress and anticonvulsant activity in *in vivo* studies (Jain et al. 2003; Kamilla et al. 2014). Literature reports on preliminary phytochemical analysis of *C. ternatea* extracts with potential bioactivities reveal more peptide and protein content rather than of other secondary metabolites (Kulshreshtha and Khare 1967; Barro and Ribeiro 1983; Oguis et al. 2019). Kelemu *et al.* (2004) described a *C. ternatea* seed protein named finotin (molecular size 20 kDa) that displayed insecticidal and antifungal activity against various plant pathogens. Poth *et al*. (2011) have been credited with providing the first insights into the distribution and evolution of cyclic peptides (cyclotides) within the Fabaceae plant family. Not long after, Nguyen *et al.* (2011) reported the isolation of 15 cysteine-rich peptides (CRPs) from heat-stable fractions of whole plant and flower extracts of *C. ternatea*. The 12 novel sequences from among these were termed 'cliotides' and projected to have the potential to be developed as anti-cancer and anti-infective agents (Gould et al. 2011; Oguis et al. 2019). Structural features and broad-spectrum bioactivities of various biologics from *C. ternatea* have been intensively studied, but no reports could be traced on antimicrobial cationic peptides from seeds of *C. ternatea*, other than cyclotides (<3.7 kDa). The present study aimed to uncover the molecular characteristics as well as the bioactive potential of likely novel cationic peptides from seeds of *C. ternatea*.

4.2. MATERIALS AND METHODS

4.2.1. Purification of *C. ternatea* seed peptide

The precipitate obtained in the selected active ammonium sulfate fraction of *C. ternatea* seed extract was subjected to size exclusion column chromatography and further procedures, as discussed in Chapter 3, Section 3.2.1. Sodium acetate buffer (0.01 M, pH 5.6) was used for

the elution at a flow rate of 12 ml/h. The active fractions in the eluate were pooled and concentrated by lyophilization.

4.2.2. Polyacrylamide gel electrophoretic analyses

Purified *C. ternatea* seed samples were analyzed by native and SDS-PAGE as previously detailed (Section 3.2.2.) As before, all gels were stained with Coomassie Brilliant Blue G-250 to visualize the protein/peptide bands.

4.2.3. Determination of molecular weight by mass spectral analyses

The purified *C. ternatea* seed peptide was subjected to LC-ESI MS, and its MALDI-TOF analyses for molecular weight determination was carried out as described previously (Section 3.2.3).

4.2.4. Antimicrobial activity tests

The agar well diffusion method followed for antimicrobial activity assessment of the purified *C. ternatea* seed peptide and the protocol for MIC determination were as detailed earlier (Section 3.2.4).

4.2.5. Effect of heat, pH, protease and salt treatment

Stability of the *C. ternatea* peptide samples to heat, pH and proteases was examined as per the procedures described under Section 3.2.5. Sensitivity of this seed peptide to various salts was evaluated as described before, in Section 3.2.6.

4.2.6. Tandem mass spectrometry and sequence database search

Shotgun proteomics strategy was adopted to identify the peptide sequence from MS/MS spectra. The tryptic peptides of the purified samples were separated using a nanoAcquity UPLC (Ultra-Performance Liquid Chromatography) system and MS/MS analysis was performed on a SYNAPT G2-Si High Definition MS system (HDMS; Waters Corporation). The acquired ion mobility enhanced MS/MS spectra were analyzed by Progenesis QI for proteomics software (Nonlinear Dynamics, Waters Corporation). Peptide identification from the acquired MS/MS spectra was achieved by searching against the database of *C. ternatea*

from UniProt repository. The search parameters applied were as follows: trypsin as the digesting agent, one missed cleavage allowed, false discovery rate (FDR) for peptide and protein identification set to less than 4%, carbamidomethylation of cysteine and oxidation of methionine set as fixed and variable modifications, respectively.

4.3. RESULTS AND DISCUSSION

4.3.1. Purification of *C. ternatea* peptide

The details of ammonium sulfate fractionation of *C. ternatea* seed extracts were described in Section 2.3.3. Further refining of this fractionation confirmed that maximal antimicrobial activity could be concentrated at 0-50% saturation (Fig. 4.1). This preparation was hence loaded on a Sephadex G-50 gel filtration column and the eluted fractions assayed for antimicrobial activity against various pathogens. Those that showed highest activity against a test pathogen (Fig. 4.2) were pooled together, lyophilized and used for subsequent analysis.

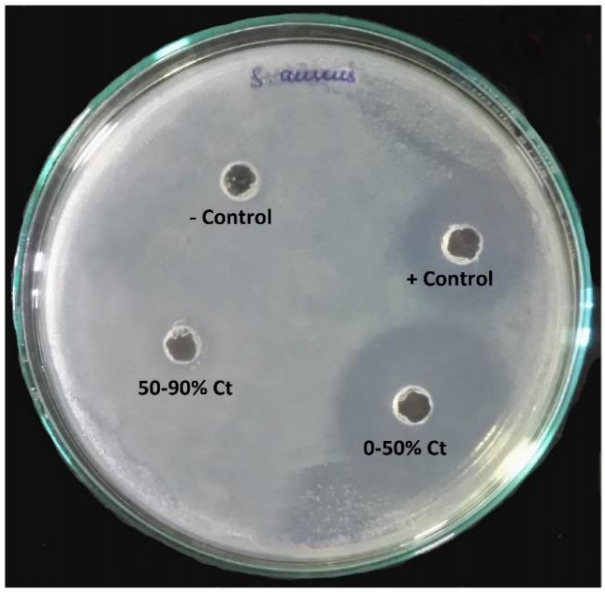


Fig. 4.1: Activity of ammonium sulfate precipitated fractions of *C. ternatea* seed extract against *S. aureus*. Gentamicin was used as positive control. 0.01 M sodium acetate buffer (without any added sample) served as negative control.

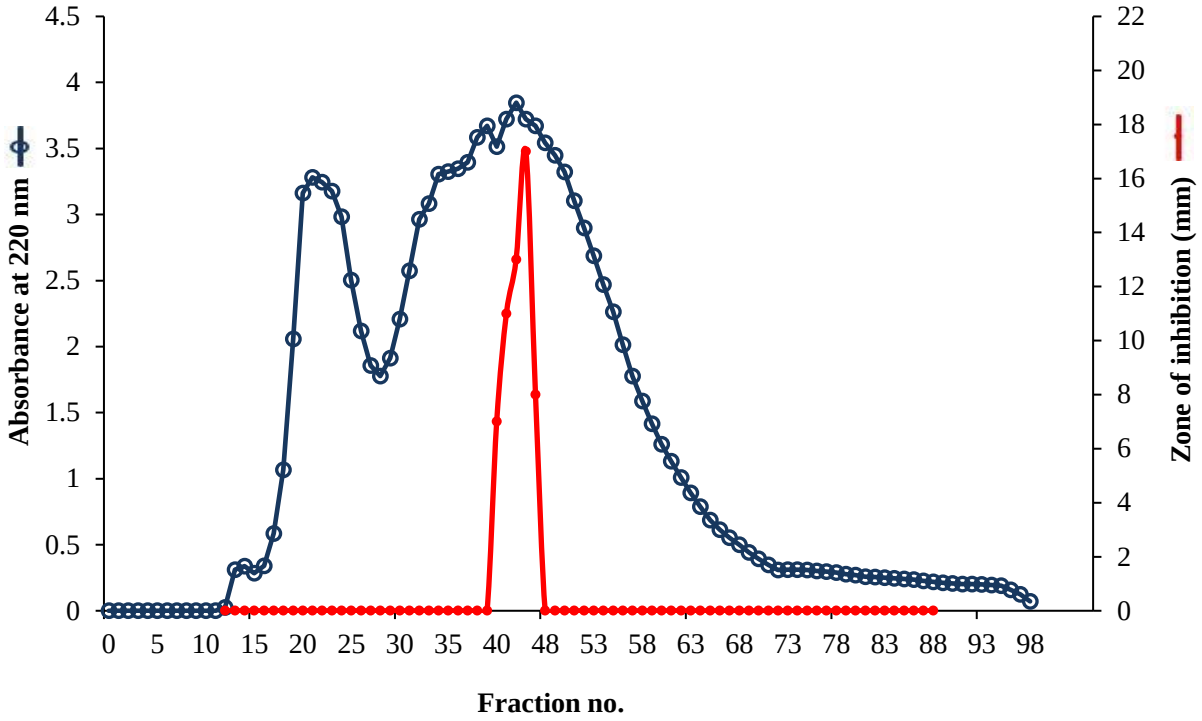


Fig. 4.2: Elution profile of the 0–50% ammonium sulfate fraction of *C. ternatea* seed extract on a Sephadex G-50 column; antimicrobial activity of eluted fractions was tested against *C. albicans* by the agar well diffusion assay.

4.3.2. PAGE and MS analyses

The native and SDS-PAGE analyses of the purified *C. ternatea* seed fraction proved the homogeneity of the sample (Fig. 4.3a) which lighted up as a single peptide band (Fig. 4.3b) of molecular weight ~8.3 kDa (Fig. 4.4). The peptide migrated cathodally on an AU gel (Fig. 4.3c) confirming its cationic or basic nature. The HPLC fractionation prior to MS analysis showed a single peak in the elution profile (Fig. 4.5a). The deconvoluted mass spectrum derived from ESI-MS analysis exhibited a major peak at 8464.61 Da (Fig. 4.5b & 4.5c) and the intact molecular weight of the peptide was determined to be 8455.285 Da by MALDI-TOF MS analysis (Fig. 4.6).

Previous reports have detailed molecular characteristics of cysteine-rich cyclic peptides named cyclotides or cliotides but typically smaller than 3.7 kDa, as purified from aqueous or organic solvent extracts of flowers, leaves, stem, root nodules, seeds and pods of *C. ternatea* (Poth et al. 2011; Nguyen et al. 2011, 2016).

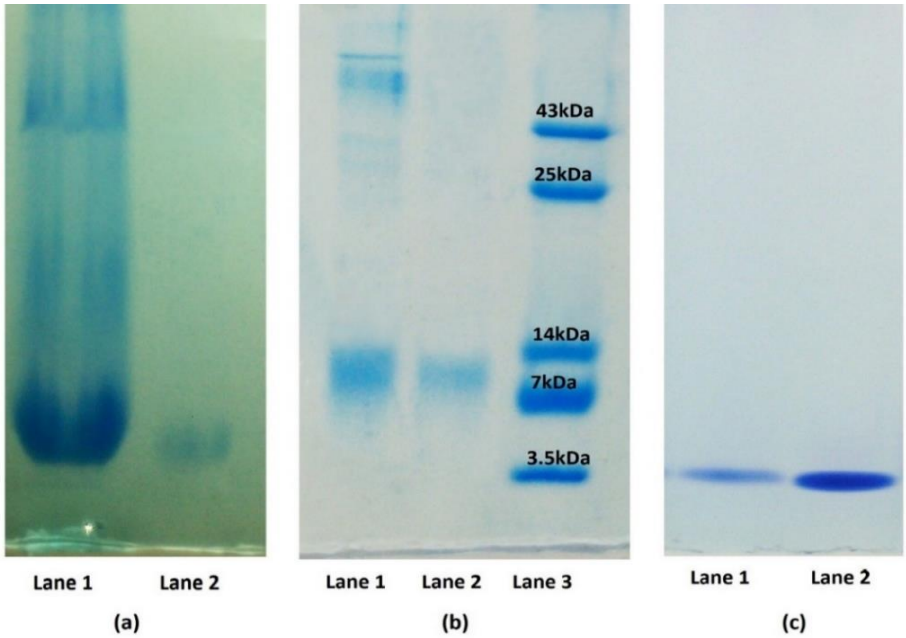


Fig. 4.3: PAGE analyses of *C. ternatea* seed samples. (a) Native-PAGE: Lane 1 - 0-50% ammonium sulfate fraction, Lane 2 - purified *C. ternatea* seed peptide; (b) SDS-PAGE : Lane 1 - 0-50% ammonium sulfate fraction, Lane 2 - purified *C. ternatea* seed peptide and Lane 3 – standard protein markers; (c) AU-PAGE: Lane 1 - purified *C. ternatea* seed peptide, Lane 2 – Lysozyme, as a marker for basic proteins.

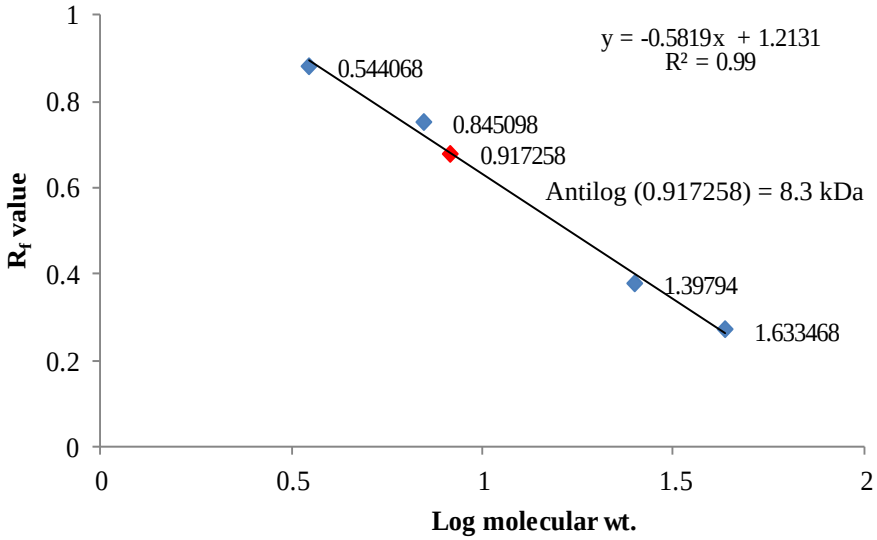


Fig. 4.4: Estimation of molecular weight of *C. ternatea* seed peptide based on SDS-PAGE data; the standard curve was plotted using R_f values of low molecular weight protein markers run simultaneously.

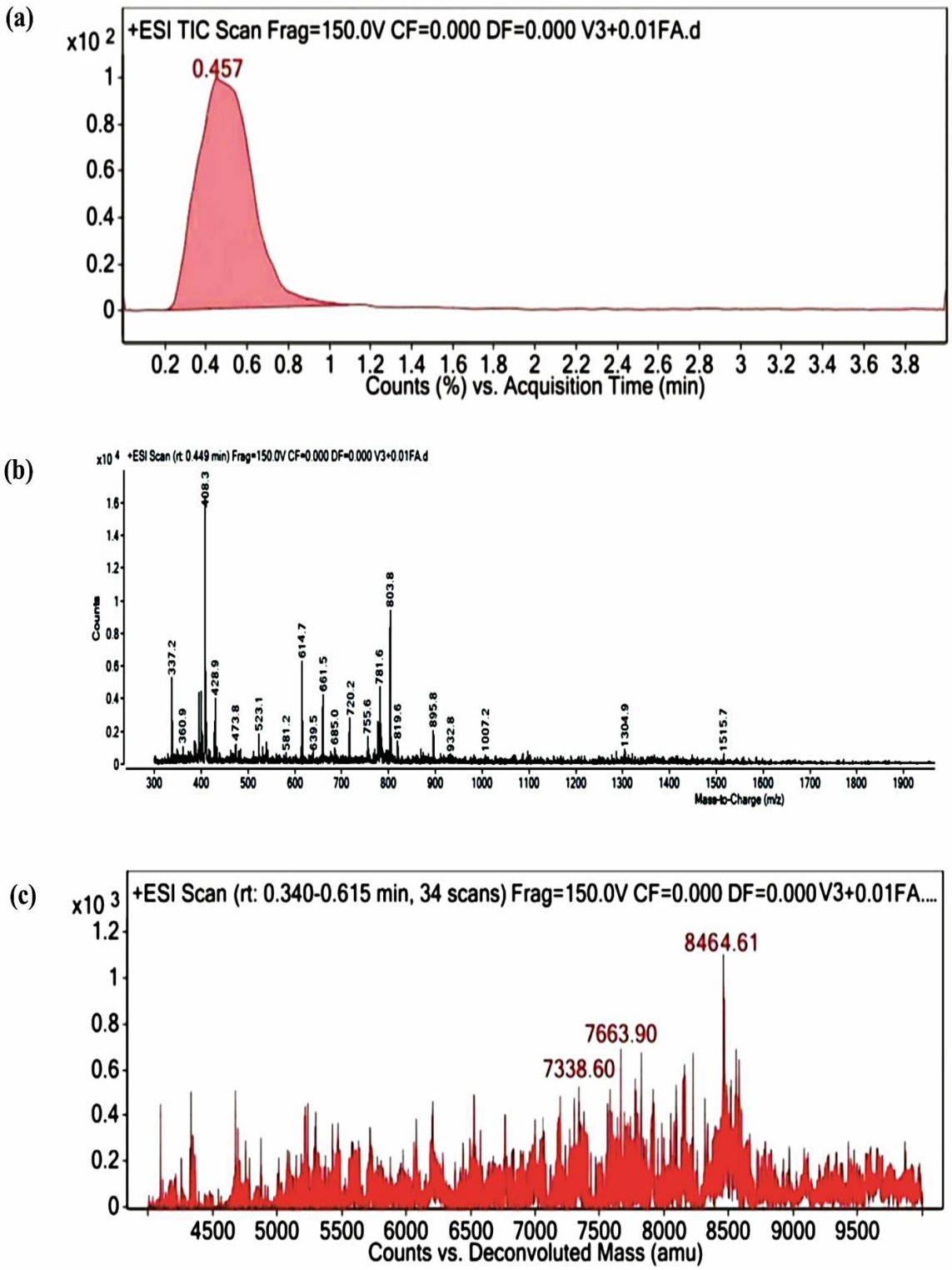


Fig. 4.5: LC-ESI-MS analysis of *C. ternatea* seed peptide: (a) LC elution profile (b) ESI mass spectrum (c) Deconvoluted mass spectrum

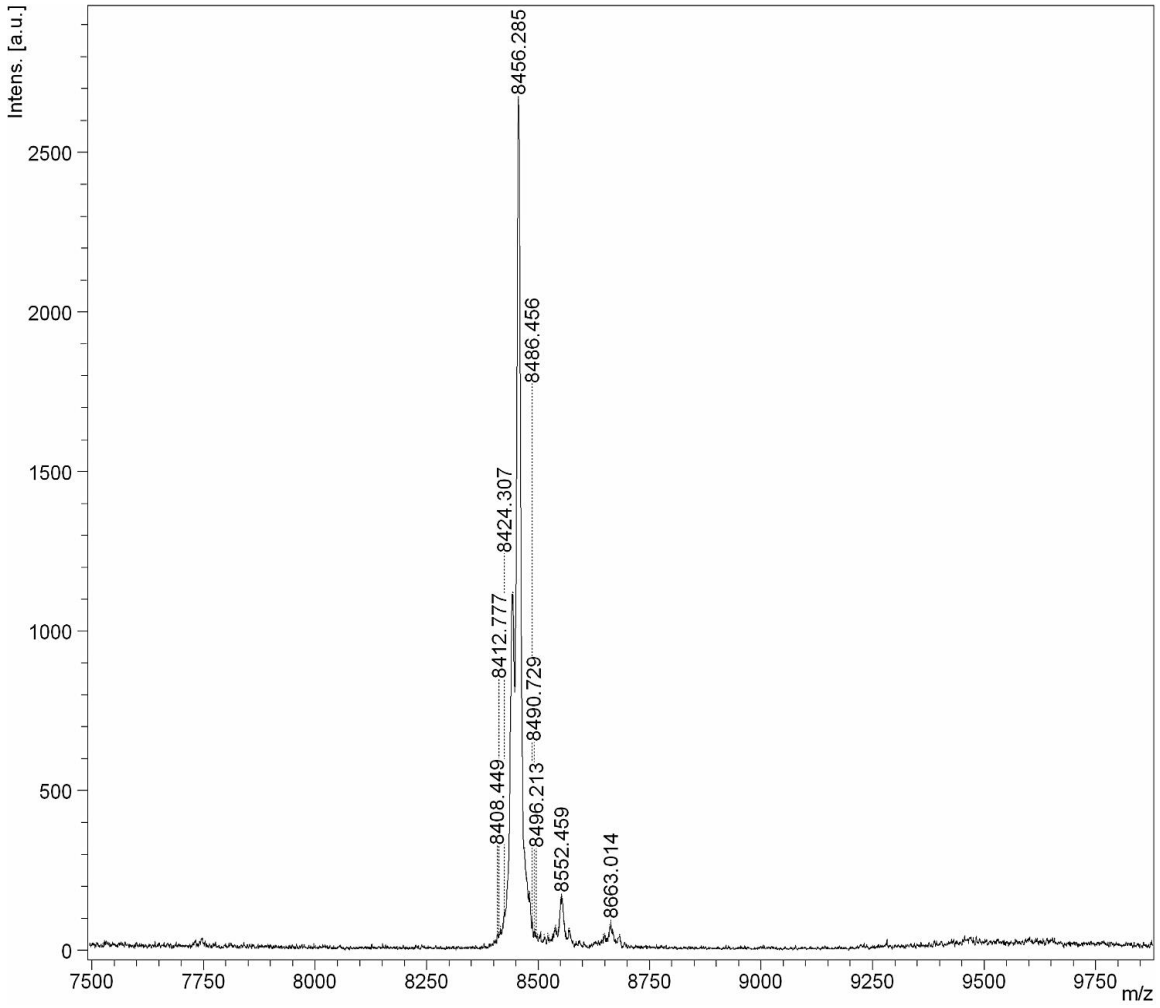


Fig. 4.6: MALDI-TOF Mass spectrum of *C. ternatea* seed peptide

4.3.3. Antimicrobial activity and MIC

Antimicrobial susceptibility of the purified *C. ternatea* seed peptide was assessed by both agar well diffusion method and broth microdilution method. As illustrated in Fig. 4.7, the peptide exhibited comparatively higher activity against *C. albicans*, with an MIC value of 8 µg/ml and moderate activity against *S. aureus*, *A. hydrophila* and *E. coli* with MIC values of 16, 32 and 32 µg/ml, respectively. It was, however, relatively ineffective against *S. typhimurium* and *P. aeruginosa*, as also observed when tested on the partially purified ammonium sulfate precipitated sample (data not shown). This clearly differs from the reported antimicrobial activity of cyclic peptides designated cliotides T1 and T4 from flowers, pods, nodules and shoots of *C. ternatea*, which had demonstrated strong bactericidal effect against *K.*

pneumoniae, *E. coli* and *P. aeruginosa* at MIC values <5 µM, while being ineffective against the Gram-positive *S. aureus* (Nguyen et al. 2011, 2016).

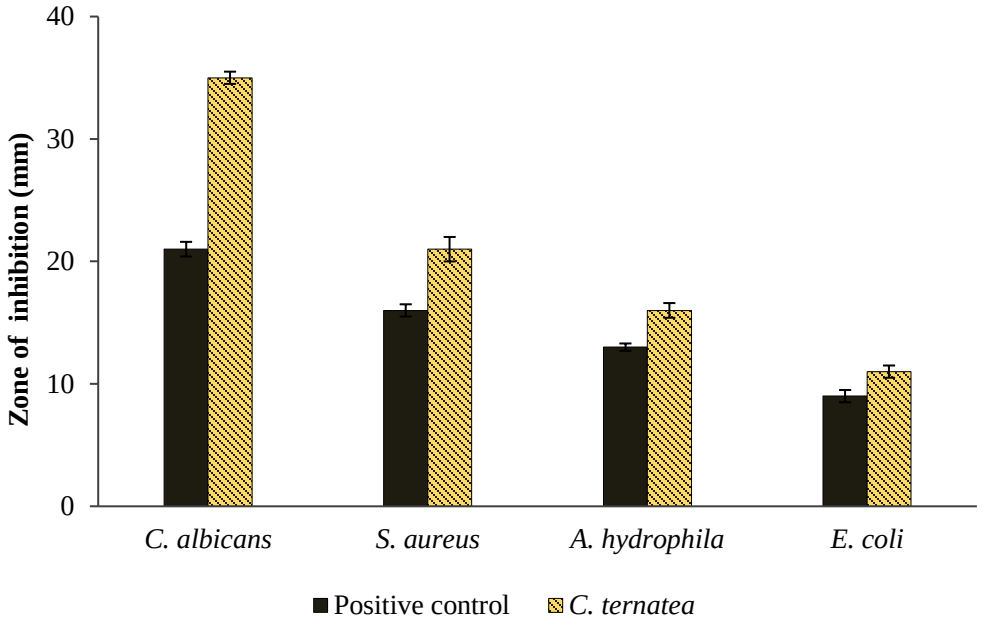


Fig. 4.7: Activity of purified *C. ternatea* seed peptide as estimated by the agar well diffusion method (~25 µg peptide/well). Amphotericin, gentamicin, tetracycline and chloramphenicol served as positive controls (25µg/well) against *C. albicans*, *S. aureus*, *A. hydrophila* and *E. coli*, respectively.

4.3.4. Sensitivity to heat, pH and proteases

Thermostability of the *C. ternatea* seed peptide was evaluated by testing the antimicrobial activity of the heat-treated peptide. As summarized in Fig. 4.8, the purified peptide preparation was incubated at various temperatures ranging from 40 to 100 ºC for 1 h and the antimicrobial activity then assayed by broth micro-dilution method, the peptide incubated at room temperature (28 °C) being considered as 'control'. The activity of the peptide against *S. aureus*, *A. hydrophila*, *E. coli* and *C. albicans* was retained up to 70-80 ºC, but treatment at temperatures of 90 ºC and above resulted in gradual reduction or complete loss of activity against all the four tested pathogens. This was in marked contrast to the extreme thermostability exhibited by cysteine-rich cliotides isolated from flowers, stem, root and seeds of *C. ternatea* wherein the homogenates were incubated at 100 °C for 1 h; the detailed

structural analysis of *C. ternatea* leaf and flower cyclotides before and after heat treatment at 95-100 °C for 30 min revealed their high stability to heat denaturation (Poth et al. 2011; Nguyen et al. 2011). In addition, their MS analysis had indicated peptides with *m/z* intensity of around 3 kDa.

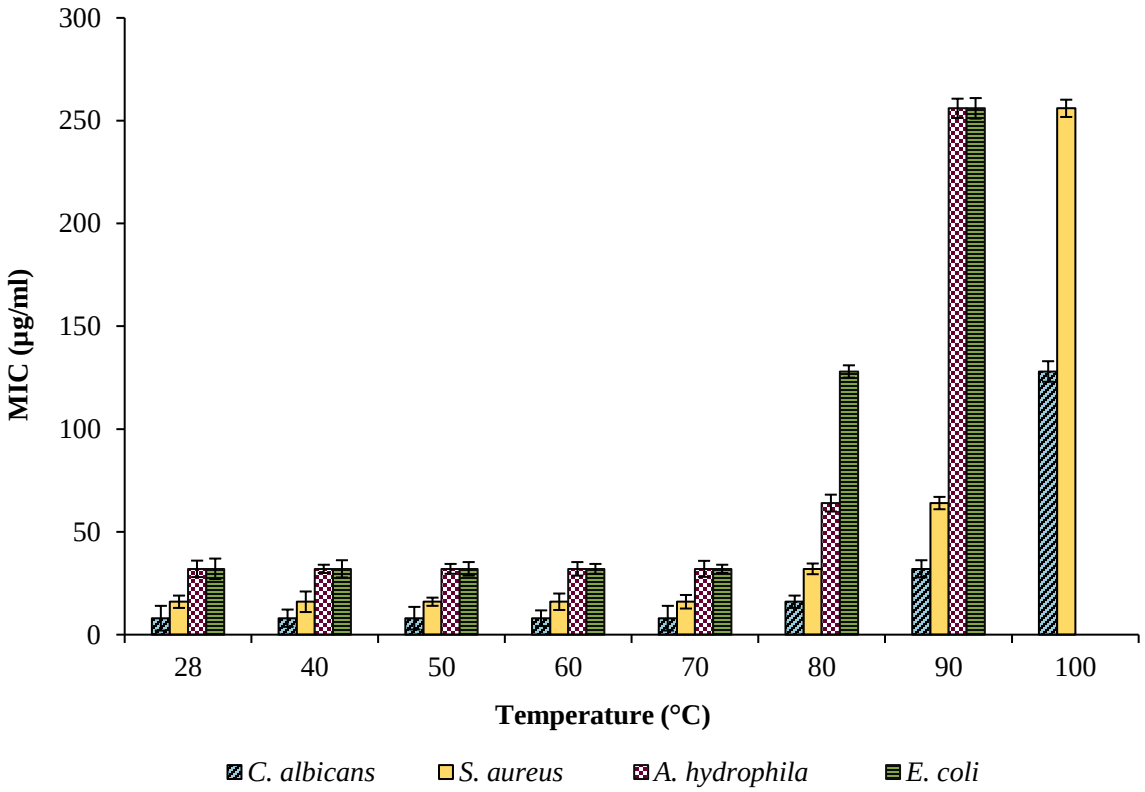


Fig. 4.8: Antimicrobial activity of heat-treated *C. ternatea* seed peptide as determined by broth micro-dilution method. Experiments were carried out in triplicate and the mean ± SD values were plotted.

Influence of pH on antimicrobial activity of the purified peptide was determined after incubating it at different pH values. Activity against *C. albicans, S. aureus, A. hydrophila* and *E. coli* was not affected by pH variations from 5–8, the calculated MIC values remaining unchanged at 8, 16, 32 and 32 µg/ml, respectively.

Susceptibility of the *C. ternatea* seed peptide to proteolytic enzymes was examined by treating the purified peptide with trypsin, proteinase K or pronase for 3 h at 37 ºC, prior to the antimicrobial assay. Complete loss of activity was observed after protease treatment (Fig. 4.9),

while the untreated peptide (control) showed clear zones of inhibition of diameter 33±1, 22±0.5, 16±0.5 and 12±1 mm, respectively, against *C. albicans*, *S. aureus, A. hydrophila* and *E. coli*. This was inconsistent with the remarkably resilient nature of *C. ternatea* cyclotides to proteolytic degradation even after treating with trypsin or chymotrypsin for 8 h at 37 °C (Poth et al. 2011). Previously described features of cyclotides (Poth et al. 2011; Nguyen et al. 2011,2016) are thus incongruous with the main characteristics of the *C. ternatea* seed peptide elaborated in this study, suggesting that the latter would not find a place among the cyclotide class of peptides.

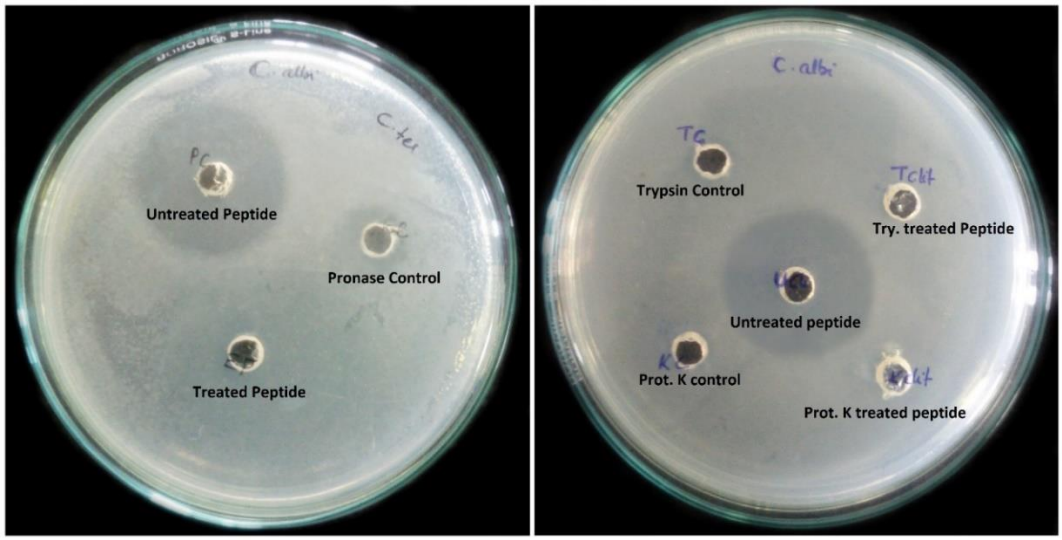


Fig. 4.9: Activity of protease-treated (pronase, trypsin and proteinase K) *C. ternatea* seed peptide against *C. albicans* as tested by agar well diffusion method (~25 µg peptide/well). Untreated peptide in buffer and protease in buffer were used as controls.

4.3.5. Salt sensitivity assays

Sensitivity of the *C. ternatea* seed peptide to salt treatment was studied by assessing its pathogenic susceptibility in presence of different salts at physiological concentrations (Fig. 4.10). The peptide exhibited stable antimicrobial activity in presence of monovalent Na^+ and K^+ ions while divalent Mg^{2+}, Ca^{2+} and trivalent Fe^{3+} ions caused partial decrease in activity against *C. albicans, A. hydrophila*, *E. coli* and *S. aureus*, respectively.

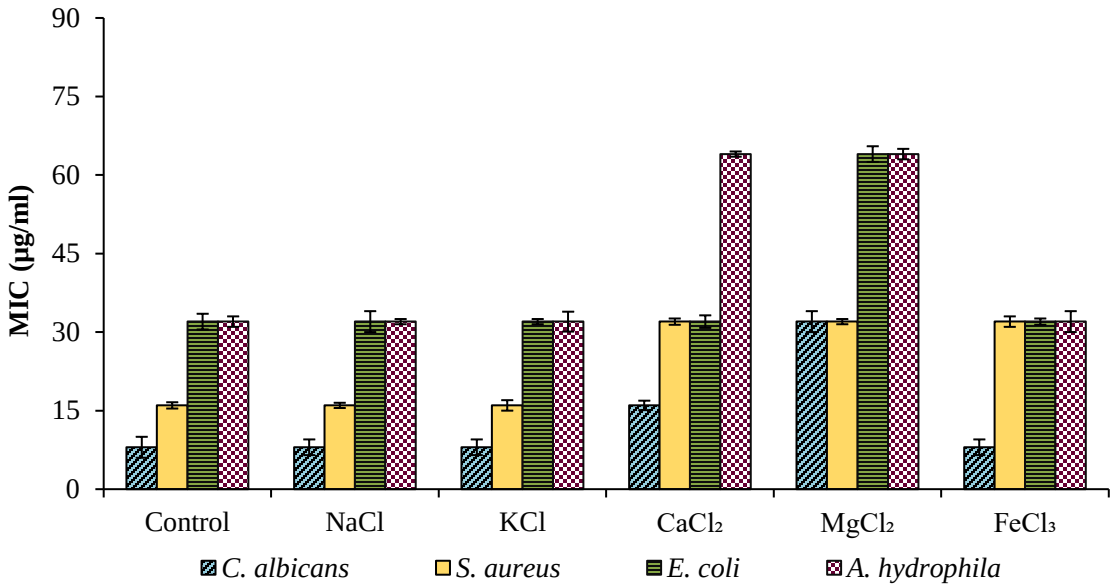


Fig. 4.10: Effect of different salts on antimicrobial activity of *C. ternatea* seed peptide. Activity of peptide in the absence of salts was taken as the control. Experiments were carried out in triplicate and the mean ± SD values were plotted.

4.3.6. Tandem MS and sequence database search

The trypsin-digested *C. ternatea* seed peptide was analyzed by Tandem MS (UPLC-MS/MS), following which protein database search (Progenesis QI software) was performed based on shotgun proteomics strategy. Peptide sequence identification was carried out by correlating the fragmentation pattern encoded by the experimental MS/MS spectra of *C. ternatea* seed peptide with theoretical spectra constructed for peptides contained in the protein database of *C. ternatea* from UniProt repository. As summarised in Table 4.1 (a and b), tryptic digestion of the seed peptide produced only four detectable matching peptides with one or two cysteine residues, and showed 17%, 16% and 7% sequence coverage with Cliotide T30 (fragment), Cyclotide Cter-C and *C. ternatea* Albumin 1, respectively, with confidence score ≥6. Of these, Albumin 1 (15.2 kDa) and Cliotide T30 (11.6 kDa) belong to the family of linear cysteine-rich proteins which exhibit high degree of sequence homology, heat stability and ability to survive acid or proteolytic degradation; more detailed characterization had revealed that they originated from common precursor sequences (Serra et al. 2016; Gilding et al. 2016; Kalmankar et al. 2020). These proteins are found to be distributed in flowers, nodules and seeds of *C. ternatea* and known for their insecticidal and hormonal functions (Rahioui et al.

2007; Oguis et al. 2020). Cyclotides of cyclic cysteine-rich peptide family usually have a molecular weight less than 3.7 kDa, and are derived from chimeric precursors consisting of cyclotide domain and Albumin 1 chain a domain (Nguyen et al. 2011; Kalmankar et al. 2020). Cyclotide family of peptides display a relatively high sequence similarity, with six cysteine residues which form a stable circular peptide backbone resistant to chemical, thermal and enzymatic degradation (Poth et al. 2011; Nguyen et al. 2011; Gilding et al. 2016). Cyclotides expressed in flowers, pods, seeds and roots of *C. ternatea* are shown to possess anti-infective and anti-tumorigenic activities (Poth et al. 2011; Nguyen et al. 2011). Since the *C. ternatea* seed peptide purified in this study exhibited comparatively low percent sequence coverage (≤17%) to known sequences in the *C. ternatea* protein database (UniProt), it is unlikely to belong to known cyclotide or cliotide protein families of *C. ternatea.*

Table 4.1: a) List of tryptic peptides identified by UPLC-MS/MS analysis of *C. ternatea* seed peptide

Retention time (min)	Identified Peptide sequence	Modifications	Ions	Deconvoluted charges	Neutral mass	Score	Accession
27.5203	CGESCFAGK	C1 Carbamidomethyl C5 Carbamidomethyl	1	2	1014.4021	4.749	A0A126TRF4
36.2222	TEGGDPLK		1	1	815.4266	4.1719	
32.2191	VCYLD	C2 Carbamidomethyl	1	1	668.2901	6.7762	P86843
29.8543	CGESCLLGK	C1 Carbamidomethyl C5 Carbamidomethyl	1	2	1022.4616	5.6218	A0A0S1RUB2

Table 4.1: b) List of master proteins obtained by *C. ternatea* protein database (UniProt) search using Progenesis QI for proteomics software.

Accession	Description	Mass (Da)	Amino acids	Coverage [%]	Peptide count	Confidence score
A0A126TRF4	Cliotide T30 (Fragment) OS=Clitoria ternatea OX=43366 PE=2 SV=1	11627.2186	100	17	2	8.9209
P86843	Cyclotide Cter-C OS=Clitoria ternatea OX=43366 PE=1 SV=1	3620.0735	31	16	1	6.7762
A0A0S1RUB2	Albumin 1 OS=Clitoria ternatea OX=43366 PE=2 SV=1	15425.4853	133	7	1	5.6218

Taken as a whole, it is obvious that our purified *C. ternatea* seed peptide differs significantly from hitherto documented *C. ternatea* peptides/proteins, be it based on antimicrobial properties or thermal and proteolytic stabilities. Besides, the peptide exhibited low percent sequence coverage (≤17%) to known cyclotide or cliotide protein families of *C. ternatea.* The novelty of the purified 8.5 kDa seed peptide with regards to the specifics of various peptides/proteins from *C. ternatea* documented so far is highlighted the data summarised in Table 4.2.

Table 4.2: Comparison of the purified seed peptide with those of reported finotin, cliotides/cyclotides from *C. ternatea*

Source / Characteristics	**Finotin**	**Cyclotide or Cliotides**	**The Peptide in the present study**
Tissue of origin	Seeds	Flowers, leaves, stem, root, pods or seeds	Seeds
Extraction biochemistry	Aqueous extracts (Kelemu et al. 2004)	Aqueous and organic solvent extracts (Poth et al. 2011a, b; Nguyen et al. 2011, 2016)	Acetic acid extracts
Molecular mass	20 kDa (Kelemu et al. 2004)	<3.7 kDa (Poth et al. 2011a, b; Nguyen et al. 2011, 2016)	8.5 kDa
Heat stability	*NA	Resistant to heat denaturation (95-100 °C for 30 min) (Poth et al. 2011b; Nguyen et al. 2011)	Resistant to heat treatment up to 1 h at 70-80 °C
Proteolytic stability	*NA	Resistant to proteolytic degradation after treating with trypsin or chymotrypsin for 8 h (Poth et al. 2011)	Sensitive to trypsin, proteinase K and pronase treatment for 3 h

*NA = Information not available

Chapter 5

Purification and characterization of a cationic peptide from Allium sativum

5.1. INTRODUCTION


Allium sativum

The bulbous plant *Allium sativum* or garlic, a member of the Amaryllidaceae family, has gained reputation worldwide as an edible spice with enormous health-enhancing effects. It has been used for centuries as a traditional herbal remedy to treat many health disorders (Butt et al. 2009; Rana et al. 2011; Mikaili et al. 2013*).* Many studies have attributed the potential bioactivities of garlic extracts to the presence of allicin or organosulfur derivatives, and attempts to uncover the contribution of other bioactive constituents are limited (Rana et al. 2011; Ansary et al. 2020).

The combined action of several bioactive constituents such as allicin (S-allyl 2-propene-1-sulfinothioate), alliin (S-allyl cysteine sulfoxide), diallyl sulfides, alliinase, agglutinins or lectins and other obscure substances are likely to account for the curative effect of *A. sativum* extracts (Butt et al. 2009*;* Mikaili et al. 2013; *Rana* et al. 2011; Ansary et al. 2020). A number of garlic products have been evaluated for potential clinical applications including the treatment of diabetes, tumours, atherosclerosis, hypertension, hypercholesterolemia and arthritis (Mikaili et al. 2013; Shan et al. 2016; Wang et al. 2017; El-Saber Batiha et al. 2020).

Depending on the crude extraction procedures or the processing methods, the phytochemical composition as well as degrees of efficacy may vary among garlic preparations sold for therapeutic purposes (Rana et al. 2011; Mikaili et al. 2013; Ansary et al. 2020; El-Saber Batiha et al. 2020). Much earlier, Cavallito and Bailey (1944) had explored a host of methods for the extraction of major organosulfur compounds from garlic bulbs, and they inferred that the antimicrobial and antioxidant properties of allicin or diallyl disulfide were more stable when extracted in organic solvents. Due to its volatile nature, allicin has an extremely short half-life, and exhibits poor solubility in aqueous garlic extracts (Ankri and Mirelman 1999; Wallock-Richards et al. 2014; Salehi et al. 2019). Several studies have documented that the garlic products obtained without allicin or organosulfur compounds have strong immunomodulatory effects and that they could be used to treat microbial infections, cancer, cardiovascular diseases, liver diseases, etc. (Clement et al. 2010; Arreola et al. 2015).

Many reports available on therapeutic effects of garlic extracts indicate the possible existence of antimicrobial, antiparasitic and antitumor compounds which require more detailed explorations (Mikaili et al. 2013; Ansary et al. 2020; El-Saber Batiha et al. 2020). The present study specifically investigates potential bioactivities as well as biochemical characteristics of cationic peptides as an unexplored class of compounds from *A. sativum* extracts.

5.2. MATERIALS AND METHODS

5.2.1. Size exclusion column chromatography

The ammonium sulfate fraction of *A. sativum* sample was loaded on a Sephadex G-50 column and purification was effected as described earlier (Section 4.2.1).

5.2.2. Antimicrobial activity estimation

The agar well diffusion method followed for antimicrobial activity assessment of the purified *A. sativum* peptide and the protocol for MIC determination were as detailed earlier (Section 3.2.4).

5.2.3. Electrophoretic analysis

Purified peptide samples were analyzed by native-, SDS- and AU-PAGE as previously detailed (Section 3.2.2.)

5.2.4. Molecular weight determination by MS analysis

Accurate molecular weight of purified peptide was determined by LC-ESI MS and MALDI-TOF MS, as described previously (Section 3.2.3).

5.2.5. Effect of heat, pH, protease and salt treatment

Stability of the *A. sativum* peptide samples to heat, pH, proteases and salt treatment was examined as per the procedures described under Section 3.2.5. and Section 3.2.6.

5.2.6. LC-MS/MS and database search

The tryptic digest of purified *A. sativum* peptide was injected on a Thermo Fisher EASY-nano-HPLC coupled to a Q Exactive Plus Biopharma-HR Orbitrap MS. Elution was achieved over 60 min using a linear gradient of acetonitrile/water (0–100% v/v) at a flow rate of 300 nl/min. The MS analysis was performed in positive ionization mode for 60 min. the resolution being set to 70000 for survey scan within a mass range of 350–2000 *m/z*. The maximum Ion injection Time (IT) was 60 ms and the Automatic Gain Control (AGC) target was 3e6. Based on the survey scan, the most abundant precursor ions were selected and fragmented *via* higher energy collisional dissociation (HCD) to acquire MS/MS data. For MS/MS scans, the dynamic exclusion of the precursor ions was adjusted to 60 s and the resolution was set to 17500 with a mass range of 200–2000 *m/z*. The MS/MS raw data of *A. sativum* peptide was then processed and searched against the protein database of *A. sativum* by using SEQUEST algorithm. The parameters applied were: Trypsin as the enzyme, two missed cleavages allowed; carbamidomethylation of cysteine and acetylation of protein N-term were as set as fixed modifications while oxidation of methionine was the variable modification. False discovery rate for peptide and protein identification was set as 1%.

5.3. RESULTS AND DISCUSSION

5.3.1. Purification of the *A. sativum* peptide

Optimization of ammonium sulfate fractionation of crude *A. sativum* extracts confirmed that the highest antimicrobial activity was obtained in the precipitate at 0–60% saturation (Fig. 5.1). This fraction was therefore subjected to molecular sieving on a pre-equilibrated Sephadex G-50 column. The elution profile showed two peaks of protein/peptide absorbance, whether at 220 nm (Fig. 5.2) or 280nm (data not shown, the peak positions being superimposable on the 220 nm profile). The fractions collected were also tested for antimicrobial activity by the agar well diffusion method. Based on the activity profile, "Peak 2" fractions were pooled and concentrated for further analysis.

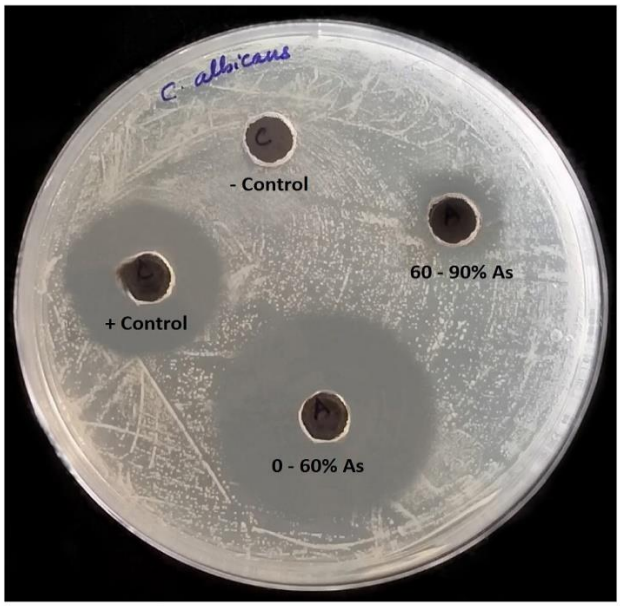


Fig. 5.1: Activity of ammonium sulfate precipitated fractions of *A. sativum* extract against *C. albicans.* Amphotericin was used as positive control. 0.01 M sodium acetate buffer (without any added sample) served as negative control.

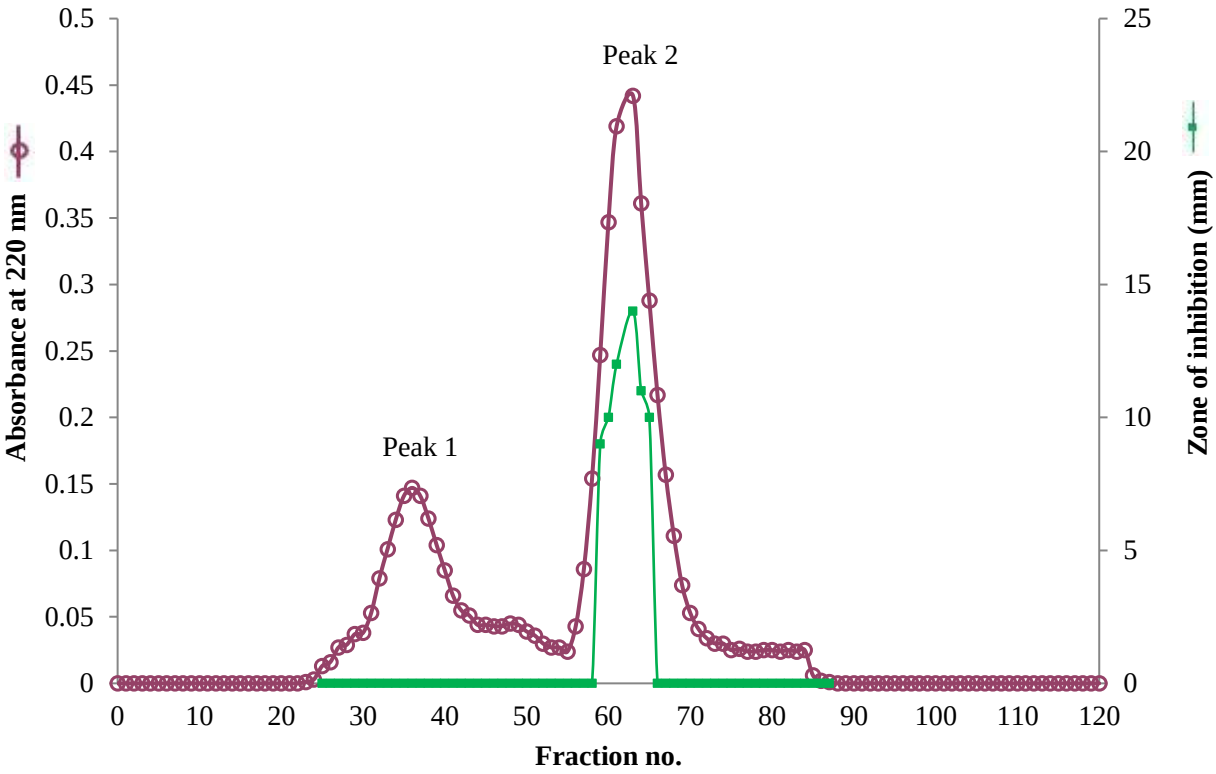


Fig 5.2: Gel filtration profile of the 0–60% ammonium sulphate fraction of *A. sativum* extract on a Sephadex G-50 column matrix; antimicrobial activity of eluted fractions was tested against *C. albicans* by the agar well diffusion assay.

5.3.2. PAGE and MS analyses

The purified *A. sativum* fraction resolved as a single peptide band on native-PAGE (Fig. 5.3a), and on the basis of SDS-PAGE analysis (Fig. 5.3b), the molecular weight of the peptide was gauged as 5.9 kDa (Fig. 5.4). The peptide had shown cathodal migration on an AU gel (Fig. 5.3c), which reiterated its cationic or basic nature.

During LC-ESI-MS analysis, the *A. sativum* peptide eluted as a single peak through the HPLC column (Fig. 5.5a) coupled to an ESI-MS system, and the deconvoluted ESI-MS spectrum shown a major peak at 6124.48 Da (Fig. 5.5b & 5.5c). Congruous with this, MALDI-TOF MS analysis revealed the intact molecular weight of this peptide as 6117.12 Da (Fig. 5.6).

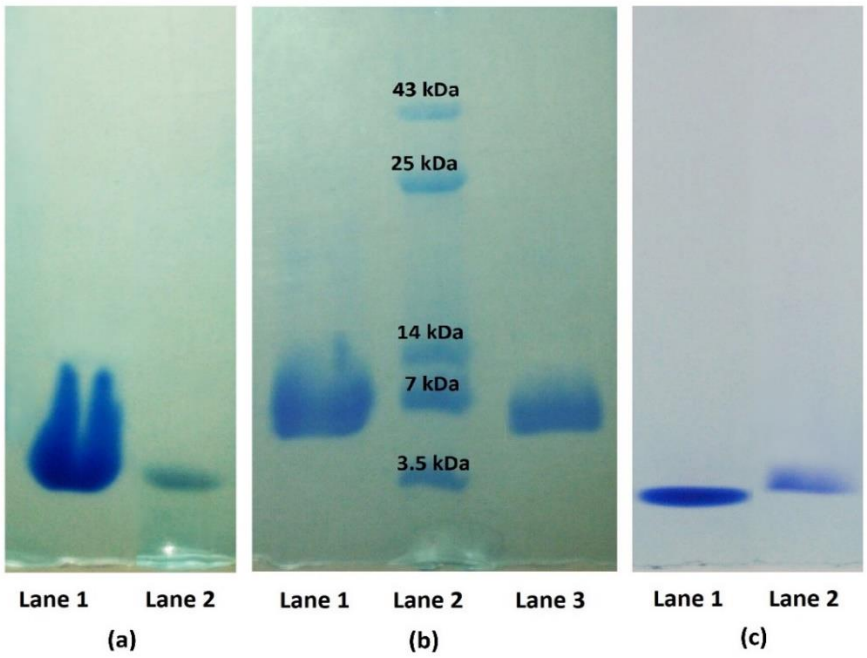


Fig. 5.3: PAGE analyses of *A. sativum* samples. (a) Native-PAGE: Lane 1 – 0-60% ammonium sulphate fraction, Lane 2 – purified *A. sativum* peptide (b) SDS PAGE: Lane 1 – 0-60% ammonium sulphate fraction, Lane 2 - LMW protein markers and Lane 3 - purified *A. sativum* peptide (c) AU-PAGE: Lane 1 – lysozyme as a 'basic protein' marker, Lane 2 - purified *A. sativum* peptide.

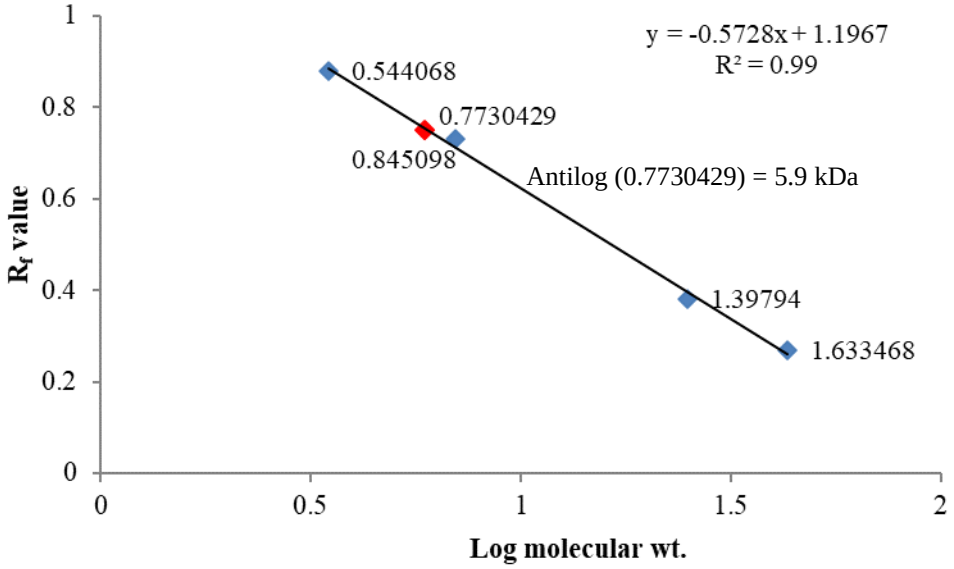


Fig. 5.4: Determination of molecular weight of the *A. sativum* seed peptide based on SDS-PAGE data; the standard curve was plotted using R_f values of low molecular weight protein markers run simultaneously.

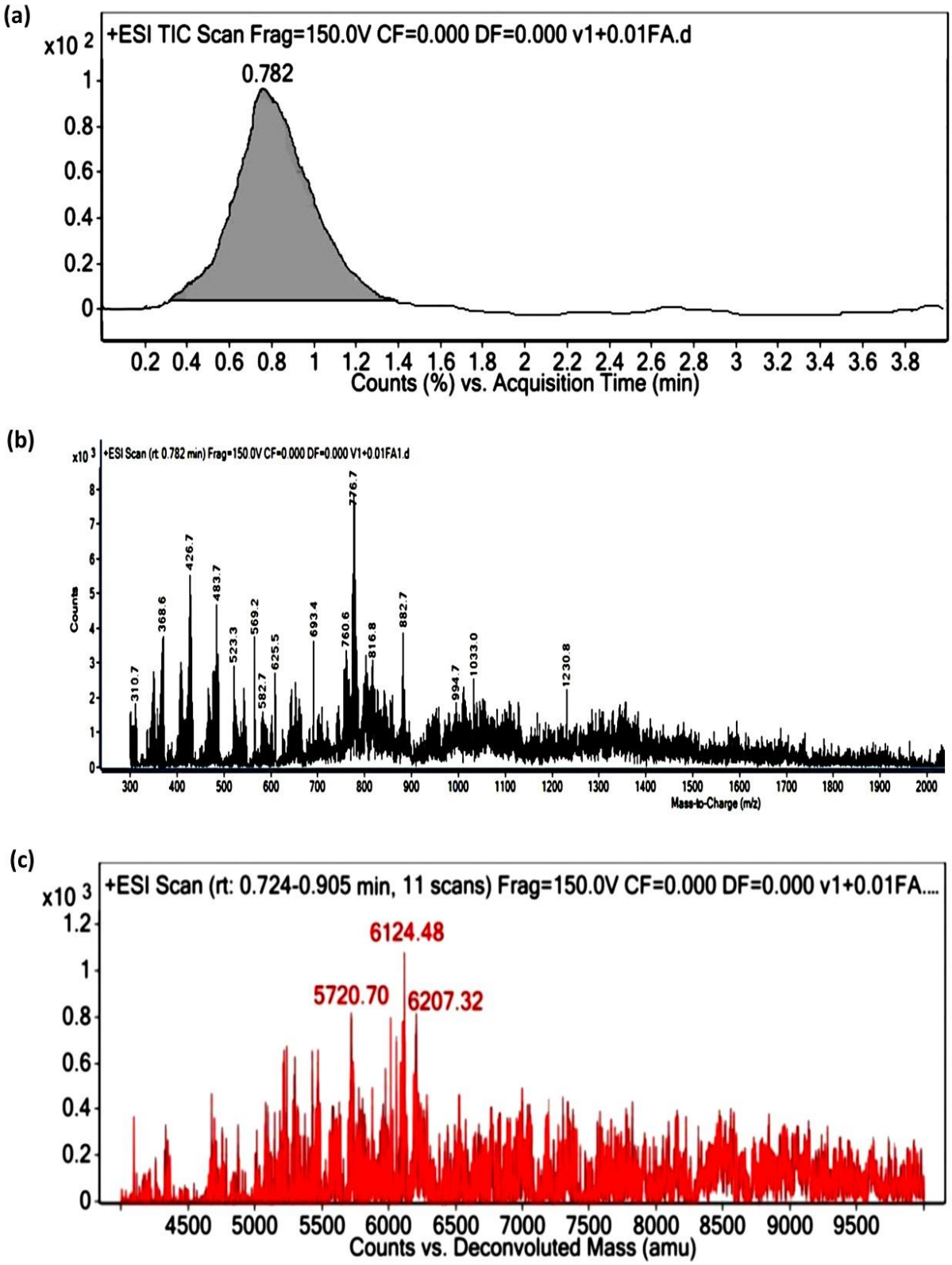


Fig. 5.5: LC–ESI MS analysis of the *A. sativum* peptide: (a) LC elution profile; (b) ESI mass spectrum; (c) Deconvoluted mass spectrum.

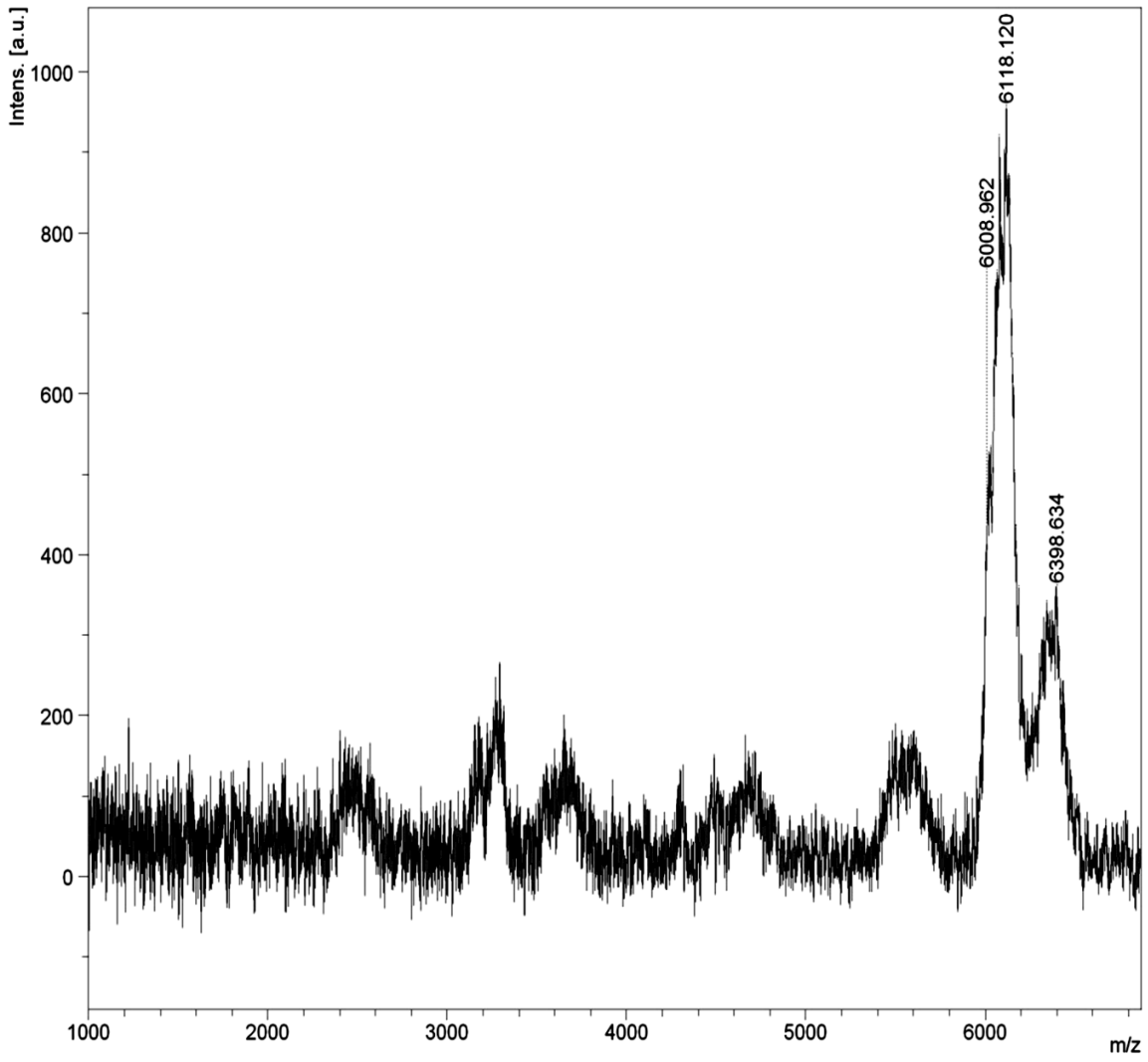


Fig. 5.6: MALDI-TOF mass spectrum of the *A. sativum* peptide.

5.3.3. Antimicrobial activity and MIC of the *A. sativum* peptide

Antimicrobial potential of the purified *A. sativum* peptide was determined by agar well diffusion assay as well as the broth microdilution method. As indicated in Fig. 5.7, the *C. albicans* strain showed high susceptibility to the *A. sativum* peptide, with an MIC gauged at 16 µg/ml, whereas *S. aureus*, *S. typhimurium*, *E coli* and *A. hydrophila* exhibited intermediate susceptibility to the peptide, with MICs of 32, 32, 32 and 64 µg/ml, respectively. The *P. aeruginosa* strain showed resistance to this peptide activity even at higher concentrations (data not shown).

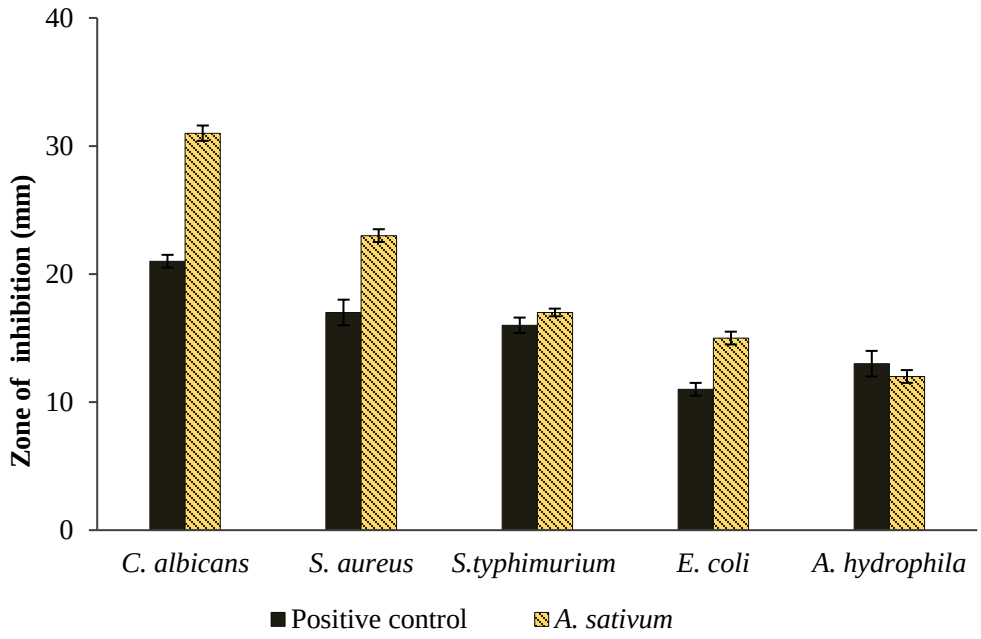


Fig. 5.7: Antimicrobial potential of the purified *A. sativum* peptide as determined by agar well diffusion assay (~25 µg peptide/well); Amphotericin, gentamicin, chloramphenicol and tetracycline served as positive controls (25µg/well) against *C. albicans*, *S. aureus*, *S. typhimurium*, *E. coli* and *A. hydrophila*, respectively.

In the early 1940s, organosulfur compounds extracted from garlic bulbs were examined for their antimicrobial properties (Cavallito and Bailey 1944). The S-allyl 2-propene-1-sulfinothioate or allicin isolated from organic solvent extracts of *A. sativum* exhibited antifungal, bactericidal or bacteriostatic effects against various human pathogens, at minimal concentrations in the range of 0.5–80 µM (Cavallito and Bailey 1944; Borlinghaus et al. 2014). Pure allicin is a volatile, transient substance which decomposes rapidly to other sulfur-containing compounds when exposed to heat, water or air (Ankri and Mirelman 1999; Salehi et al. 2019). Furthermore, alliinase, an enzyme which converts alliin or S-allylcysteine sulfoxide to allicin, is irreversibly deactivated at extremely acidic conditions (Colin-Gonzalez and Santamaria 2017), indicating that allicin could not be produced when garlic pod crude extraction was carried out in acidic solvents of pH below 3. The water solubility of allicin is as low as 0.8% (Jiang et al. 2020). Besides, antimicrobial action of allicin was more pronounced and stable when the crude extraction was carried out in alcoholic solvents such as ethanol, methanol or chloroform. (Cavallito and Bailey 1944; Jiang et al. 2020). Allicin loses its thiosulfinate group when broken down into diallyl disulfides and this drastically reduces the

antimicrobial capability (Cavallito and Bailey 1944; Borlinghaus et al. 2014). The diallylpolysulfanes or diallyl sulfides formed during the degradation of allicin have not shown any noteworthy antimicrobial activity, except when used at very high concentrations (> 6.15 mM). Gao et al (2019) studied the antimicrobial properties of three peptides with molecular mass of ≤600 Da from a traditional Chinese medicinal preparation of garlic known as Laba garlic. These peptides exhibited strongest microbicidal effect against *E. coli* and *S. aureus* at an MIC of 100 µM. In the light of all of the above, the *A. sativum* peptide isolated in the current study stands out with markedly distinct molecular and antimicrobial properties.

5.3.4. Sensitivity to temperature, pH and protease treatment

Heat stability of the *A. sativum* peptide was examined by analyzing its antimicrobial effect after being incubated for 1 h at 40–100 °C. As depicted in Fig. 5.8, the peptide could retain stable activity against *A. hydrophila, E coli, S. typhimurium*, *S. aureus*, and *C. albicans* even after 1 h treatment at 70 or 80 °C. Nevertheless, incubation at temperatures above 80 °C substantially reduced the peptide activity against all five tested pathogens.

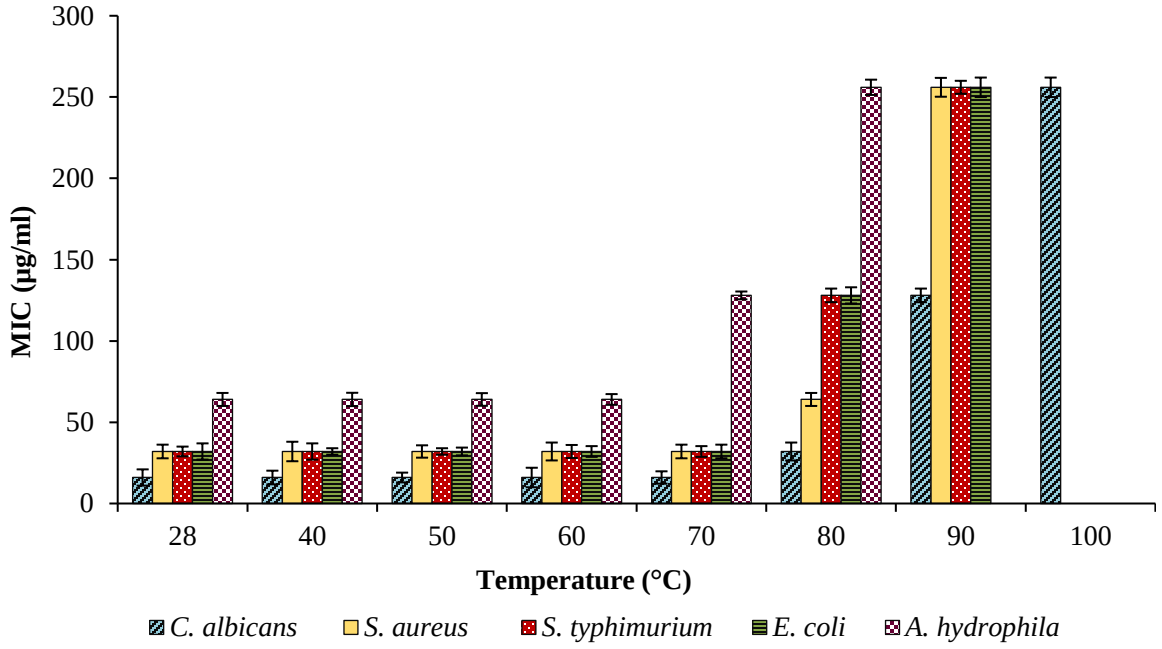


Fig. 5.8: Effect of heat treatment on antimicrobial activity of the *A. sativum* peptide. The peptide treated at room temperature (28°C) was used as the control. Experiments were carried out in triplicate and the mean ± SD values were plotted.

The activity of the peptide remained unchanged in the pH range of 5–8, with MIC values of 16, 32, 32, 32 and 64 µg/ml, respectively, against *C. albicans*, *S. aureus*, *S. typhimurium*, *E coli* and *A. hydrophila*. Previous reports have described temperature and pH stability of various bioactive compounds from garlic bulbs. The allicin in garlic extracts readily gets decomposed when the temperature exceeds 37 ºC or at extremely lower or higher pH; high concentration of allicin in solution was marginally more stable than lower amounts (Ankri and Mirelman 1999; Wang et al. 2015). Marzouki et al. (2005) characterized two isoforms of peroxidase enzyme from garlic bulbs (designated as POX1 and POX2, and of molecular mass ~36.5 kDa), which appeared heat resistant and exhibited consistent activity at 50 ºC for 5 h; isoform POX2 was highly stable over a broad pH range of 3.5–11. A garlic agglutinin abbreviated as ASA II, a homodimer of 12 kDa subunits, exhibited consistent immunomodulatory effect after heat treatment for 30 min at 80 °C. The ASA I, a heterodimer of 11.5 kDa and 12.5 kDa subunits, could retain 100% activity at pH values in the range of 5–8 (Clement et al. 2010).

Susceptibility of the *A. sativum* peptide to proteolytic degradation was examined by incubating the purified peptide with trypsin, pronase or proteinase K for 3 h (37 °C) and then assaying for antimicrobial activity. It showed high sensitivity to such enzymatic treatment, all five pathogens exhibiting resistance to the protease-treated peptide (Fig. 5.9). This indicated that intactness of the peptide structure was a crucial factor for its antimicrobial activity.

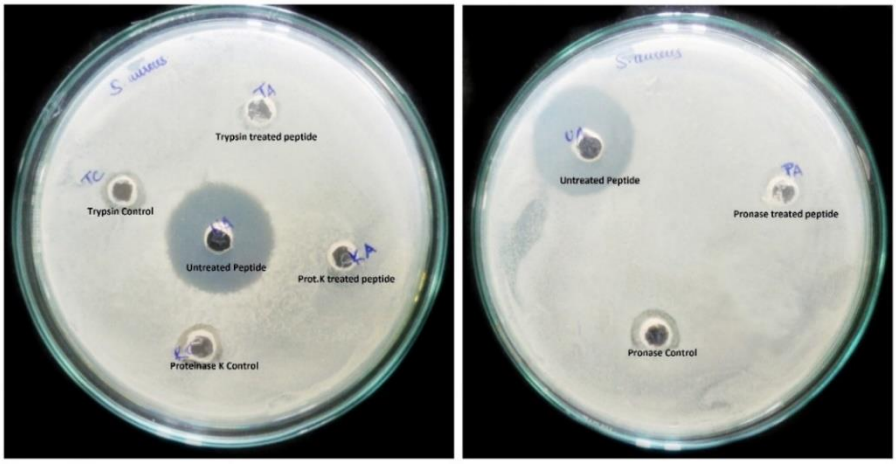


Fig. 5.9: Activity of protease-treated (trypsin, proteinase K or pronase) *A. sativum* peptide against *S. aureus,* as tested by the agar well diffusion method (~25 µg peptide/well). Untreated peptide in buffer and protease in buffer were used as controls.

5.3.5. Salt sensitivity assay

Antimicrobial activity of the *A. sativum* peptide in presence of different salts at physiological concentrations was assayed by broth microdilution method. The sensitivity of different pathogenic strains to the peptide was unaffected by the presence of the monovalent ions Na^+ and K^+ and the trivalent Fe^{3+} (Fig. 5.10), however, the divalent Ca^{2+} and Mg^{2+} ions noticeably diminished the activity against *A. hydrophila*, and they also had a mild inhibitory effect on activity against *C. albicans, S. aureus, S. typhimurium* and *E. coli*. Thus, in general, antimicrobial activity of the *A. sativum* peptide on most of the tested pathogens appeared to be minimally affected by different salts at their physiological concentrations.

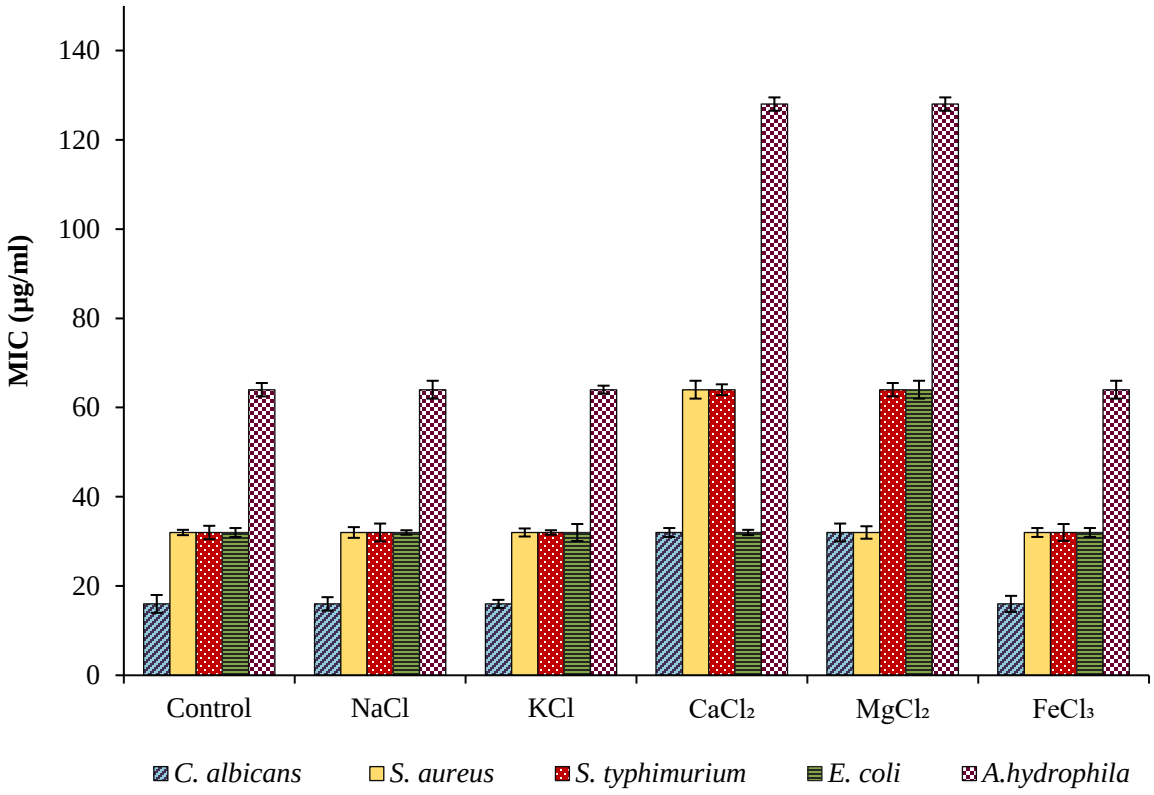


Fig. 5.10: Effect of different salts on antimicrobial activity of the *A. sativum* peptide. Activity in the absence of salts was taken as the control. Experiments were carried out in triplicate and the mean ± SD values were plotted.

5.3.6. Tandem MS and Database search

The trypsin treated *A. sativum* peptide was subjected to HR-LC-MS/MS along with sequence database search against the protein database of *A. sativum* (UniProt) using SEQUEST algorithm. The tryptic digestion of *A. sativum* peptide could produce several matching peptides (Table 5.1a) with cysteine residues or hydrophobic amino acid residues and showed 20%, 20% 17%, 9% and 6% sequence coverage to Lectin, Mannose-specific lectin, I lectin, Alliinase and Alliin lyase, respectively, with XCorr value >2 (Table 5.1b). Since the peptide had comparatively low sequence coverage (≤20%) to known peptide/protein sequences in the *A. sativum* database (UniProt), it is unlikely to belong to previously documented peptide/protein families of *A. sativum.*

Considered as a whole, the results clearly indicate that the cationic peptide purified in this study had distinctly different molecular as well as functional characteristics from those proteinaceous molecules previously reported from garlic bulbs. It has also been shown for the first time that besides the much-documented allicin and related compounds, significant antimicrobial activity from garlic pods was also contributed by a totally different kind of molecule, the hitherto unreported 6.1 kDa cationic peptide.

Table 5.1: a) List of tryptic peptides identified by HRLC-MS/MS analysis of the purified *A. sativum* peptide

Retention time (min)	Identified Sequence	Modifications	Missed Cleavages	Charge	m/z [Da]	MH+ [Da]	XCorr	Accession
35.9234	AVMQADGNFVVYDANGHPVWASNSVR	0	0	3	935.44732	2804.32742	8.63	K4P0T2
23.724	VGTLTRNILR	0	1	3	381.5713	1142.69935	2.51	
34.256	AVLQSDGNFVVYDAEGRSLWASHSVR	0	1	4	716.60995	2863.41798	5.7	
34.8783	AVLQSDGNFVVYDADGRPLWASHSVR	0	0	4	715.60983	2859.41749	7.78	Q38787
34.256	AVLQSDGNFVVYDAEGRSLWASHSVR	0	1	4	716.60995	2863.41798	5.7	
36.1411	DGNFVVYDVNGRPVWASNSVR	0	0	3	784.38655	2351.14511	4.91	P83886
37.8307	NVVIYGSDIWSTGTYR	0	0	2	915.95569	1830.9041	4.8	
19.9234	CECNTCYTGPDCSEK	4xCarbamidomethyl [C1; C3; C6; C12]	0	2	940.83136	1880.65544	3.84	Q01594
35.4817	AFLDGIISEGSPK	0	0	2	667.35288	1333.69848	3.27	
47.730	SEKIQGCSADVASGDGLFLEEYWK	1xCarbamidomethyl [C7]	1	4	673.07169	2689.26492	3.7	D2CXG4
35.4817	AFLDGIISEGSPK	0	0	2	667.35288	1333.69848	3.27	

Table 5.1: b) List of master proteins as obtained from the *A. sativum* protein database (UniProt) search using SEQUEST algorithm

Accession	Description	Mass (kDa)	Amino acids	Coverage [%]	Peptide count	Unique peptides	Score Sequest HT: Sequest HT
K4P0T2	Lectin (Fragment) OS=Allium sativum OX=4682 GN=LECASAI PE=4 SV=1	32.4	303	20	3	2	16.84
Q38787	I lectin (Fragment) OS=Allium sativum OX=4682 GN=LECASAI1 PE=2 SV=1	34	313	17	2	1	13.48
P83886	Mannose-specific lectin OS=Allium sativum OX=4682 GN=LECASAL PE=1 SV=3	19.3	181	20	2	2	9.71
Q01594	Alliin lyase 1 OS=Allium sativum OX=4682 PE=1 SV=1	55.6	486	6	2	1	7.11
D2CXG4	Alliinase (Fragment) OS=Allium sativum OX=4682 PE=4 SV=1	45.7	400	9	2	1	6.97

Chapter 6

Other potential bioactivities of the purified peptides

6.1. INTRODUCTION

Besides their antimicrobial properties, some cationic peptides can also function as potent antioxidants (Lopez-Garcia et al. 2022; Tadesse and Emire 2020). Small peptides containing basic amino acid residues as well as hydrophobic amino acid residues have exhibited strong antioxidant capacities (Zou et al. 2016). Most recently, such antioxidant peptides have attracted enormous interest from nutraceutical and food industry researchers for their promising applications in maintaining the safety and quality of health-promoting functional foods (Lopez-Garcia et al. 2022; Tadesse and Emire 2020; Zaky et al. 2022).

Cationic peptides effective against different cultured stages of the malarial parasites have been the subject of several reports (Wegscheid-Gerlach et al. 2010; Vale et al. 2014). The small molecular size (<10 kDa), amino acid composition, cationic nature, diverse secondary structures, and the distinct mode of action of such broad-spectrum peptides are highly impressive traits favoring their rise as a potential novel class of antimalarials (Zasloff 2002; Boman 2003). *Plasmodium* is a unicellular eukaryotic parasite, which may seem to contradict the idea that AMPs selectively target negatively charged membrane components in prokaryotes; however, RBCs, the host eukaryote cells, undergo major membrane modifications after being infected by *P. falciparum*, which causes them to resemble the parasite membrane in composition and have higher phosphatidylinositol and phosphatidic acid content than healthy RBCs (Hsiao et al. 1991; Vale et al. 2014). This obviously relates to how the cationic AMPs solely target *P. falciparum* infected RBCs and impede the growth of intracellular parasites, yet barely affect healthy RBCs (Gelhaus et al. 2008). In fact, a variety of natural AMPs from plant or animal sources have been shown to have varying degrees of antiplasmodial effects, demonstrating that modulation of innate immune response is a promising strategy to develop novel peptide-based antimalarial agents (Bell 2011; Haney and Hancock 2013). This Chapter addresses the antioxidant as well as antiplasmodial potentials of the purified cationic peptides from *M. oleifera*, *C. ternatea* and *A. sativum* extracts.

6.2. MATERIALS AND METHODS

6.2.1. Antioxidant assays

The DPPH (2,2-diphenyl-1-picrylhydrazyl) method and the FRAP (Ferric Reducing Antioxidant Power) method were adopted to evaluate antioxidant activity. Ascorbic acid was used as a standard antioxidant. In the DPPH method (Brand-Williams et al. 1995) the free radical scavenging activity (expressed as %) was estimated based on the reduction of DPPH radical, as it converts to a non-radical form in the presence of antioxidants, leading to a decreased absorbance at 517 nm. Briefly, 190 µl of 0.05 mM DPPH (Sigma) solution in methanol was mixed with 10 µl of sample solution at various concentrations from 1- 250 µg/ml. The mixture was incubated in the dark for 30 min at room temperature and the absorbance then measured at 517 nm against a blank. A 0.05 mM DPPH solution (without any sample) served as negative control. The DPPH scavenging activity of the sample was calculated using the formula:

$$\text{DPPH Scavenging activty (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \text{ x } 100$$

In the FRAP method (Benzie and Strain 1996), presence of antioxidants would cause reduction of the Fe^{3+} complex of TPTZ (2,4,6-Tripyridyl-S-triazine) to a blue colored Fe^{2+} complex at acidic pH. Samples (50 µl) at concentrations in the range of 1- 25 µg/ml were mixed with 100 µl FRAP reagent and incubated in the dark for 30 min at room temperature. Absorbance of the mixture was then measured against a blank at 593 nm and the FRAP value (µM) of the sample determined based on the standard curve ($R^2 = 0.998$) of absorbance (at 593 nm) *vs* Fe^{2+} concentration (µM).

At least three independent experiments were carried out for the antioxidant assays, with not less than 5 concentrations of samples, each being estimated in triplicate.

6.2.2. Antiplasmodial activity

Antiplasmodial activity of the samples was tested against a Chloroquine–sensitive *Plasmodium falciparum* strain (3D7) maintained at the ICMR-NIMR (National Institute of Malaria Research), New Delhi. An RPMI (Roswell Park Memorial Institute) medium supplemented with 10% AB^{+ve} human serum and A^{+ve} erythrocytes (5% hematocrit) was used

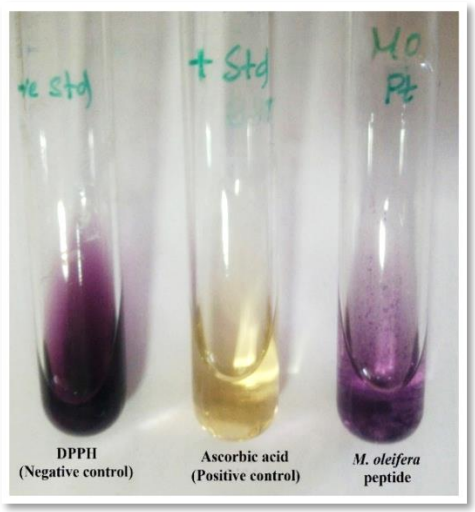
to culture the *P. falciparum* (3D7) strain. The parasite culture was synchronized at early trophozoite stage by using sorbitol (5%) (Lambros and Vanderberg 1979): briefly, the sorbitol and pellets at 1:9 ratio were maintained for 5 min at room temperature and then centrifuged to remove the supernatant. The pellet thus obtained was washed thrice using complete media (RPMI-1640 media supplemented with 10% AB^{+ve} human serum). To the synchronized culture, the complete medium was added at 1:9 ratio to attain 5% parasitemia (10% hematocrit). Antiplasmodial potential of the peptides was evaluated as described by the modified WHO MARKIII protocol (Sharma et al. 2016). Briefly, the peptide stock solutions (1 mg/ml) prepared in DMSO were serially diluted with RPMI-1640 media to obtain final concentrations of 0.8–50 µg/ml and dispensed into sterile 96-well (flat bottom) microplates. Synchronized *P. falciparum* (3D7) culture (5% parasitemia) was then added to each well and the plates incubated in a gas mixture (90% N, 5% CO_2 and 5% O_2) for 24 h at 37 ºC. Appropriate positive and negative controls were maintained in each experiment. The incubation continued until the schizont growth reached 10 % in the control and then the blood smear of each well was prepared. Giemsa stain was used to stain the blood smear. Those schizonts with at least three merozoites were counted and the peptide concentration required for half-maximal activity (EC_{50}) determined according to the dose-response curve, by HN-NonLin Reg Analysis (Noedl et al. 2002).

6.3. RESULTS AND DISCUSSION

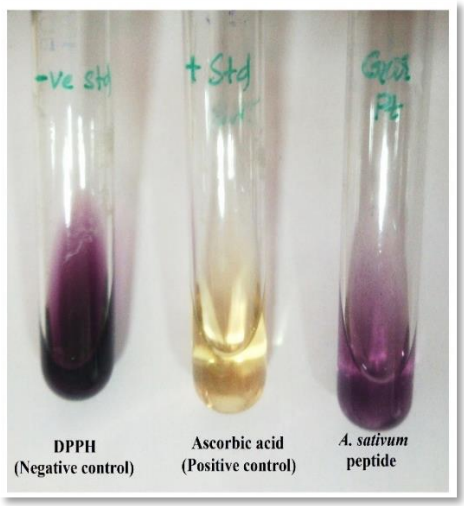
6.3.1. Antioxidant activity

When the purified peptides were screened for antioxidant properties, *M. oleifera* and *A. sativum* peptides did not show any activity (Fig. 6.1a; 6.1b), while the *C. ternatea* peptide demonstrated remarkably high antioxidant ability (Fig. 6.1c), which was further subjected to quantification by the DPPH and FRAP methods (Fig. 6.2). Ascorbic acid was used as a positive standard in both the assays. The DPPH scavenging activity was concentration-dependent, with an IC_{50} value of 126 µg/ml, as against that of ascorbic acid measured as 19 µg/ml (Fig. 6.2a). The FRAP method recorded an increase in Ferric Reducing Capacity (FRAP value) with increasing concentration of the peptide, indicating strong antioxidant potential (Fig. 6.2b). Kamkaen and Wilkinson (2009) had reported antioxidant activity of

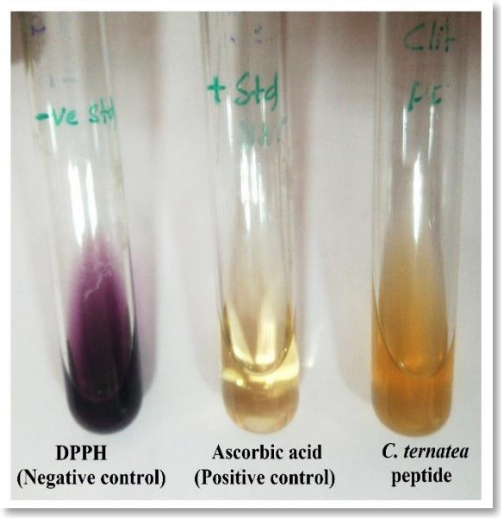
aqueous extracts of *C. ternatea* with an IC_{50} value 2 mg/ml, which was interpreted as mainly due to their phenolic content (Jaafar et al. 2020). Experimental data from the present study would suggest that the peptide component has a much more significant contribution to the antioxidant activity observed in *C. ternatea* seed extracts.



(a)



(b)



(c)

Fig. 6.1: Screening for Antioxidant activity of (**a)** *M. oleifera* peptide, (**b)** *A. sativum* peptide and (**c)** *C. ternatea* peptide, as examined by DPPH method. Ascorbic acid was used as the positive control. The DPPH solution (0.05 mM) without any sample served as a negative control.

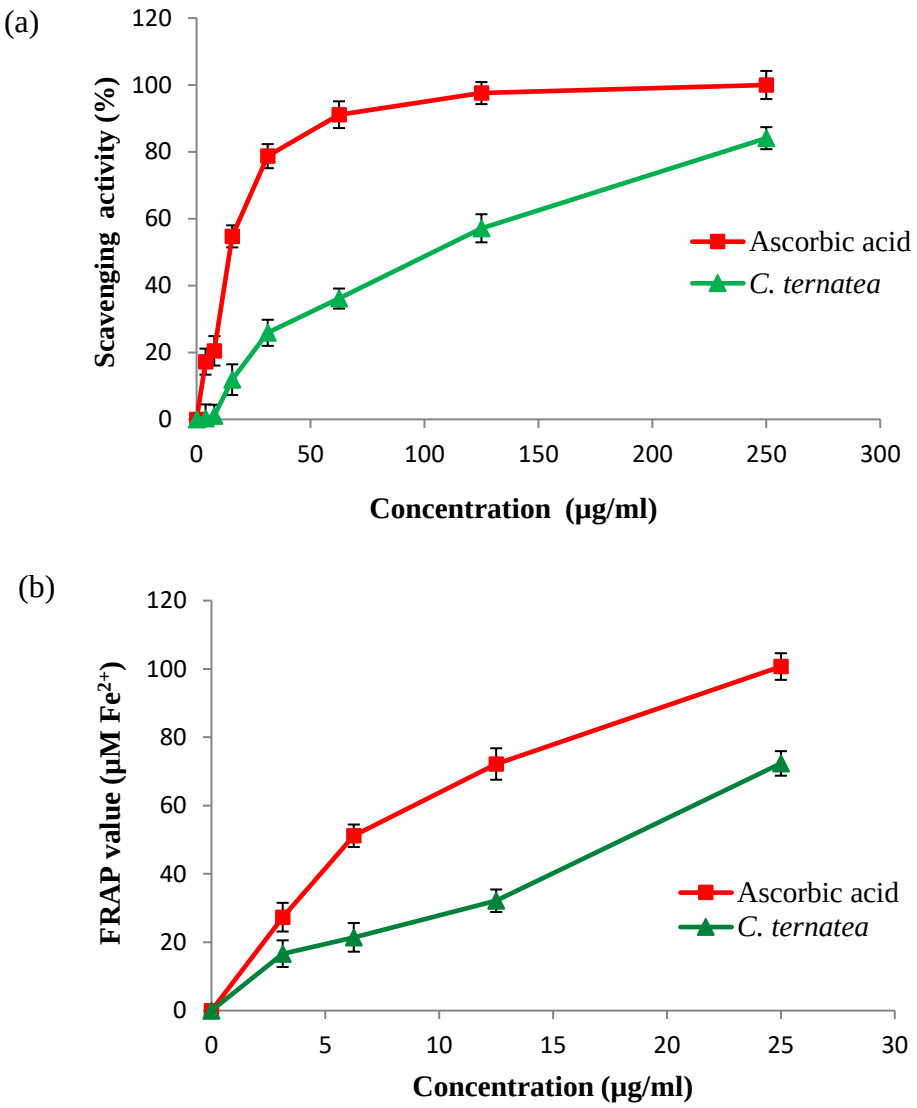


Fig. 6.2: Antioxidant activity of *C. ternatea* seed peptide based on: (**a)** DPPH radical scavenging activity and (**b)** Ferric reducing antioxidant power. Ascorbic acid was used as the positive control.

6.3.2. Antiplasmodial activity

The *in vitro* antiplasmodial activity of purified peptide samples was evaluated against the human malarial parasite *P. falciparum* in accordance with the modified WHO MARKIII protocol. The peptides from *A. sativum*, *C. ternatea* and *M. oleifera* demonstrated remarkable effectiveness against *P. falciparum* 3D7 strains, with half-maximal effective concentrations (EC_{50}) as 1.911, 2.171 and 2.42 µg/ml, respectively (Fig. 6.3); the EC_{50} value of the standard antimalarial drug chloroquine was 0.998 µg/ml.

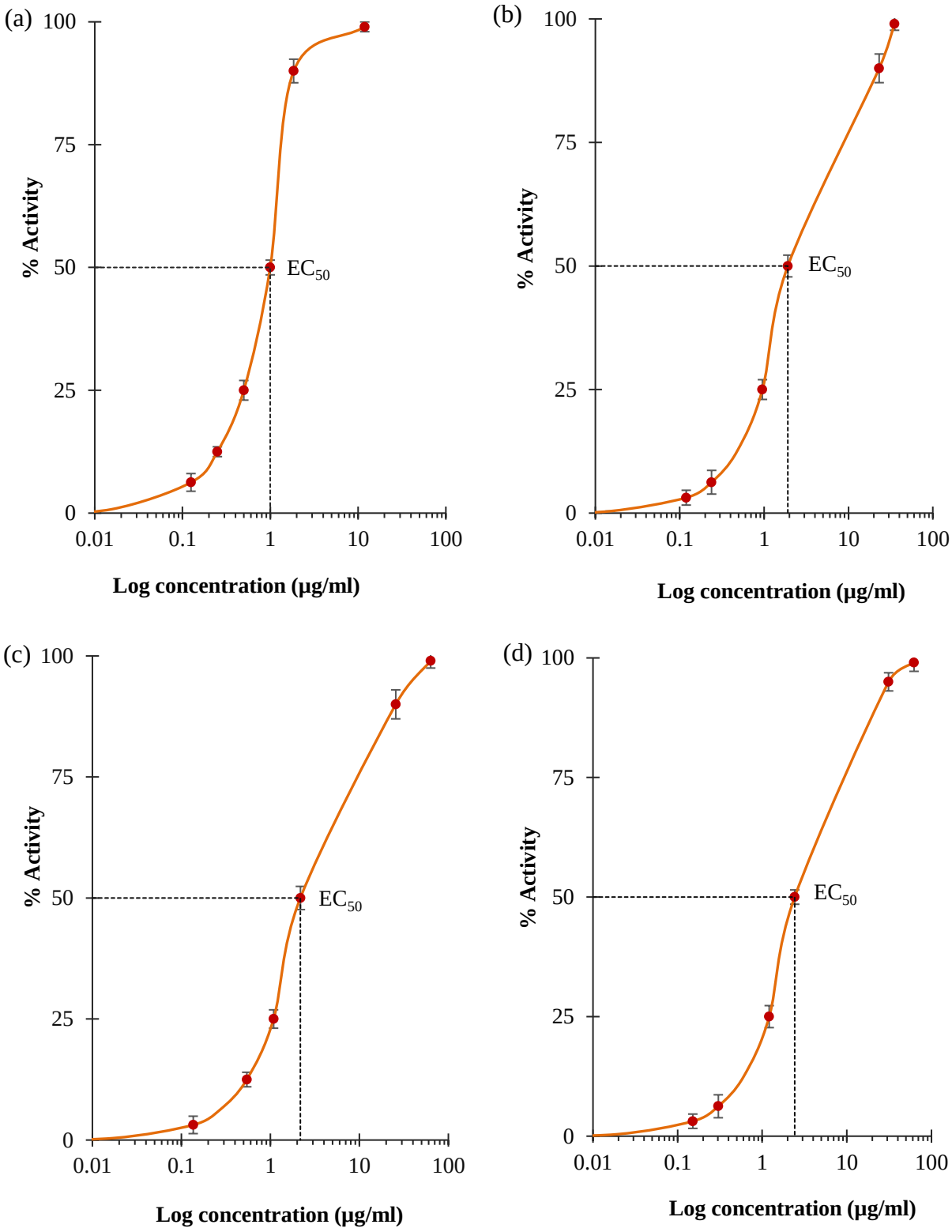


Fig. 6.3: Dose-response curves of the antiplasmodial activity of (**a)** Chloroquine, (**b)** purified *A. sativum* peptide, (**c)** purified *M. oleifera* peptide and (**d)** purified *C. ternatea* peptide against *Plasmodium falciparum* 3D7 strain.

The antimalarial properties of several bioactive molecules from *A. sativum* as well as *M. oleifera* extracts have been investigated previously by other workers (Perez et al. 1994; Coppi et al. 2006; Somsak et al. 2016). Several lines of evidence suggested that allicin or other thiosulfinates were predominantly responsible for the antiparasitic properties of *A. sativum* extracts, although the exact mode of action had not been established in many instances (Mirelman et al. 1987; Rabinkov et al.1998). Perez et al. (1994) elaborated the antiparasitic activity of ajoene, an organosulfur compound from *A. sativum* in a mouse model infected with *Plasmodium berghei* (which mimics *P. falciparum* infection in humans), which showed a half-maximal effective dose (ED_{50}) of 50 mg/kg. Coppi et al (2006) studied the inhibitory effect of allicin on the erythrocytic stage of *Plasmodium* and inferred that longer exposure to allicin at higher concentrations (≥50 μM) could effectively kill *P. berghei* sporozoites, while at lower concentrations (≤10 μM) viability of the sporozoites remained unaffected. When used in combination with artesunate, the antiplasmodial activity of allicin increased in a dose-depended manner, with an ED_{50} of 14 mg/kg (Ounjaijean and Somsak 2022).

M. oleifera has been a traditional herbal remedy to treat severe malaria-related symptoms, and reported to have strong and curative antimalarial effects (Eilert et al. 1981; Ranasinghe et al 2015). Somsak et al (2016) reported *in vivo* antiplasmodial activity of aqueous dried *M. oleifera* leaf extracts against *P. berghei*. More recently, Obediah and Obi (2020) described the *in vivo* inhibitory effect of *M. oleifera* seed ethanolic extracts of high alkaloid and phenolic content, against *P. berghei*. The potential of cationic peptides from *A. sativum*, *M. oleifera* or *C. ternatea* as antimalarials or antiplasmodials has, however, not yet been investigated. The current study thus reports for the first time, three novel plant-derived molecules with antiplasmodial potential.

Summary and Conclusions

Propelled by the fascinating characteristics of plant-derived CAPs such as low molecular size, broad-spectrum antimicrobial activity, distinct mode of action, target specificity, high efficacy at lower concentrations, low propensity towards antibiotic resistance, synergistic actions with classical drugs, heat-stable nature, chemical and enzymatic stability, immunomodulatory effects and biodegradability, there has been a surge in their demand as novel cationic peptide drugs with extensive applications in pharmaceutical, agricultural and food industries. The present study was aimed to uncover potential cationic antimicrobial peptides from selected plant sources, with detailed characterisation of molecular as well as biochemical properties. The search for other potential bioactivities of the peptides was also initiated.

Screening of plants for cationic peptides with antimicrobial activity and optimization of crude extraction procedures

- Extracts from 19 plant species which had earned a distinct space in traditional/indigenous systems of medicine were screened for cationic peptides with antimicrobial activity.
- On account of the basic nature of cationic peptides, an acetic acid - based extraction method was adopted to prepare the crude extracts.
- Antimicrobial activity assays were carried out using Gram-positive and Gram-negative bacterial strains and fungal test pathogens procured from the MTCC, India.
- A set of five plant samples with comparatively high antimicrobial activity, *viz.*, *Clitoria ternatea*, *Allium sativum*, *Moringa oleifera*, *Trigonella foenum-graecum* and *Tabernaemontana divaricata*, was singled out for subsequent investigations.
- The acetic acid concentration for optimal extraction of antimicrobial activity was worked out and adopted for further peptide extractions from the selected plants.
- Antimicrobial assays coupled with PAGE analysis partially confirmed the presence of CAPs of molecular size <10 kDa from *M. oleifera*, *C. ternatea* and *A. sativum* extracts, which were hence selected for purification and further characterization.

Purification and characterization of a cationic peptide from Moringa oleifera

Moringa oleifera, a plant of high nutritional value, is a widely cultivated species of the Moringaceae family. Different parts of this plant possess a plethora of bioactivities, carving out a conspicuous role for it in indigenous systems of medicine. Although many bioactive compounds from *M. oleifera* have been described, identities of some potentially active

compounds are still elusive. The present research discusses the isolation and characterization of antimicrobial cationic peptide(s) from *M. oleifera* seeds.

- Peptide components of acetic acid extracts of the *M. oleifera* seeds were separated by ammonium sulfate fractionation and purified by size exclusion chromatography.
- The native- as well as SDS- PAGE analyses detected a small peptide of molecular mass ~6.5 kDa which resolved cathodally on an acid-urea gel.
- The mass spectra obtained by LC-ESI MS and MALDI-TOF MS analyses established the intact molecular weight of the peptide as 6707.16 Da and 6692.485 Da, respectively.
- Pathogenic strains such as *Salmonella typhimurium, Candida albicans* and *Staphylococcus aureus* exhibited remarkable susceptibility to the peptide activity at minimum inhibitory concentration (MIC) values of 16–32 µg/ml.
- The temperature and pH sensitivity assay results evidenced the functional stability of the peptide upon heating for 1h at 40–70 ºC or being subjected to pH ranging from 5–8. The activity was, however, extremely sensitive to trypsin, proteinase K and pronase treatments.
- A salt sensitivity assay conducted to evaluate the effect of monovalent, divalent and trivalent salts on the peptide antimicrobial activity showed moderate salt tolerance of the *M. oleifera* seed peptide to various salts at physiological concentrations.

Purification and characterization of a cationic peptide from Clitoria ternatea

Clitoria ternatea, a herbaceous climber of the family Fabaceae, is well recognised in traditional system of medicines, especially in tropical Asian countries. While several bioactivities have been reported from different parts of this plant, specific production of cationic peptides other than cyclotides (<3.7 kDa) has barely been investigated or their antimicrobial potential looked into.

- The present research reports for the first time, isolation and purification of an antimicrobial cationic peptide from seed extracts of *C. ternatea* by a simple procedure involving ammonium sulphate fractionation followed by Sephadex G–50 column chromatography.
- The homogeneity of the purified *C. ternatea* seed peptide was indicated by native-PAGE and SDS-PAGE analysis estimated its molecular mass as ~8.3 kDa. Acid-Urea-PAGE profiles reflected the basic or cationic nature of the peptide.

- The deconvoluted mass spectrum obtained by LC-ESI-MS analysis showed a major peak at 8464.61 Da. The intact molecular mass of the peptide was determined as 8455.285 Da by MALDI-TOF MS.
- When evaluated for antimicrobial potential against Gram positive, Gram negative and fungal pathogens, distinctive activity was observed against *C. albicans*, *S. aureus*, *A. hydrophila* and *E. coli*, with MIC values of 8, 16, 32 and 32 µg/ml, respectively. This activity was totally uncompromised for 1h at 70-80ºC or in a pH range of 5-8 but was sensitive to pronase, proteinase K and trypsin treatment.
- The *C. ternatea* seed peptide activity could withstand the effects of various salts at physiological concentrations.
- Tandem MS analysis of the trypsin-digested *C. ternatea* peptide based on a shotgun proteomics approach detected matching peptide sequences with one or two cysteine residues but showed only 17%, 16% and 7% sequence coverage, respectively, in relation to other peptides/proteins documented from *C. ternatea, viz.,* Cliotide T30, Cyclotide cter-C and albumin.

Purification and characterization of a cationic peptide from Allium sativum

Allium sativum (garlic), a strongly aromatic bulbous crop of the family Amaryllidaceae, has been used since ancient times as a valuable spice and remains a popular herbal remedy for various physiological disorders. Numerous scientific reports have detailed the characteristics of several putative active compounds, but specific production of an antimicrobial cationic peptide and its bioactive potential have been demonstrated for the first time through the present study.

- Peptide components of acid extracts of *A. sativum* bulbs were purified by ammonium sulfate fractionation followed by size exclusion chromatography.
- The purified active peptide resolved as a single band of molecular mass ~5.9 kDa on SDS-PAGE. Cathodal migration of the peptide on an AU-gel confirmed its cationic nature.
- The intact molecular weight of the peptide was determined as 6123.48 Da by LC-ESI MS analysis and 6117.120 Da by MALDI-TOF MS analysis.
- The peptide was most effective against *Candida albicans*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli* and *Aeromonas hydrophila*, at an MIC range of 16 – 64 µg/ml. It

- was totally stable after 1h of treatment at 40–80 ºC or at a pH range of 5–8, but showed high susceptibility to proteolytic degradation.
- Antimicrobial activity of the *A. sativum* peptide did not appear to be significantly affected by different salts at their physiological concentrations.
- Sequence database search based on LC-ESI MS/MS spectra of the *A. sativum* peptide using SEQUEST algorithm detected matching peptide sequences with several hydrophobic amino acid residues or cysteine residues but had low sequence coverage (≤ 20%) to known protein sequences in the *A. sativum* protein database (UniProt).

Other potential bioactivities of the purified peptides

- The purified peptides from *A. sativum*, *M. oleifera* and *C. ternatea* were screened for antioxidant properties by the DPPH method.
- Antioxidant activity of the *C. ternatea* seed peptide was quantified by the DPPH and FRAP methods using ascorbic acid as a reference molecule. The ferric-reducing capacity as well as DPPH-scavenging activity of this peptide was concentration-dependent, with an IC_{50} value of 126 µg/ml.
- The *in vitro* antiplasmodial activity of the peptides from *A. sativum*, *M. oleifera* and *C. ternatea* was examined by modified WHO MARKIII protocol, with the standard antimalarial drug chloroquine being used as a reference molecule.
- *The dose-response curves indicated remarkable* antiplasmodial activity in the purified *A. sativum, M. oleifera* and *C. ternatea* peptides, with EC_{50} values of 1.911, 2.171 and 2.42 µg/ml, respectively.

To summarise, the current investigation has shed light on the molecular as well as functional characteristics of what appear to be novel bioactive cationic peptides from *M. oleifera*, *C. ternatea* and *A. sativum* extracts. The antimicrobial, antioxidant and antimalarial properties of the peptides were evidenced through various *in vitro* assays. The molecular mass as well as the sensitivity of these peptides to heat, pH, protease and salt treatments stood out as clearly distinct from those of previously reported peptide/protein families of *M. oleifera*, *C. ternatea* and *A. sativum*. Besides, the sequence database search based on MS/MS spectra of the *C. ternatea* and *A. sativum* peptides demonstrated relatively low sequence coverage to known peptide/protein families. As a whole, the findings of the present study have laid firm

foundations for future research into more specific structural and functional aspects of three hitherto unreported bioactive cationic peptides with potential practical applications.

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Appendix

APPENDIX – I

Nutrient Agar

Nutrient agar (HiMedia)	28 g
Distilled water	1000 ml

Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 121 ºC, 15 lb pressure for 15 min. Cool to 45–50 ºC. Mix well and pour into sterile petri plates.

Nutrient Broth

Nutrient agar (HiMedia)	13 g
Distilled water	1000 ml

Heat to boiling to dissolve the medium completely. Mix well and dispense into test tubes/flasks. Sterilize by autoclaving at 121 ºC, 15 lb pressure for 15 min.

Mueller-Hinton Broth

Mueller Hinton broth (HiMedia)	21 g
Distilled water	1000 ml

Heat to boiling to dissolve the medium completely. Mix well and dispense into test tubes/flasks. Sterilize by autoclaving at 121 ºC, 15 lb pressure for 15 min.

Mueller-Hinton Agar

Mueller Hinton agar (HiMedia)	38 g
Distilled water	1000 ml

Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 121 ºC, 15 lb pressure for 15 min. Cool to 45–50 ºC. Mix well and pour into sterile petri plates.

APPENDIX – II

Sodium acetate buffer - 0.2 M Stock (pH 5)

Sodium acetate	27.21 g
Glacial acetic acid	6 ml
Distilled water	994 ml

The buffer can be stored at RT or 4 °C.

Bradford reagent

Coomassie-brilliant blue G-250	10 mg
95% (v/v) Methanol	5 ml
85% (v/v) Phosphoric acid	10 ml
Glycerol	10 ml
Distilled water	75 ml

Filter the reagent before using for the assay. The reagent can be used stored at 4 °C.

SDS-PAGE reagents

18% Resolving gel

1.5 M Tris-HCl (pH 8.8)	2.6 ml
Deionized distilled water	1 ml
10% (w/v) SDS	100 µl
30% (w/v) Acrylamide/bisacrylamide	6.2 ml
10% (w/v) APS	100 µl
TEMED	10 µl

4% Stacking gel

0.5 M Tris-HCl (pH 6.8)	1.25 ml
Deionized distilled water	2.975 ml
10% (w/v) SDS	50 µl
30% (w/v) Acrylamide/bisacrylamide	0.67 ml

10% (w/v) APS	50 µl
TEMED	5 µl

Tank buffer (5X Stock, pH 8.3)

Tris base	15 g
Glycine	72 g
SDS	5 g
Deionized D/W	1000 ml

Sample loading dye (5X Stock)

0.5% (w/v) Bromophenol blue	0.6 ml
0.5 M Tris-HCl (pH 6.8)	1 ml
10% (w/v) SDS	1.6 ml
β-mercaptoethanol	0.4 ml
Glycerol	8 ml
Deionized distilled water	4 ml

Store the sample loading dye in a light proof container at 4 °C.

Coomassie Brillant Blue stain

Ammonium sulfate	50 g
Deionized distilled water	4 ml
5% (w/v) Coomassie blue G-250	12 ml
Orthophosphoric acid	50 ml
100% (v/v) Methanol	100 ml

Filter the solution using Whatman filter paper and store at 4 °C.

<u>Native-PAGE reagents</u>

15% Resolving gel

30% (w/v) Acrylamide/Bisacrylamide	5 ml
0.375 M Tris–HCl (pH 8.8)	4.89 ml
10% (w/v) APS	100 µl

TEMED	10 µl

4% Stacking gel

30% (w/v) Acrylamide/Bisacrylamide	0.67 ml
0.375 M Tris–HCl (pH 8.8)	4. 275 ml
10% (w/v) APS	50 µl
TEMED	5 µl

Tank buffer (5X Stock)

Tris base	15 g
Glycine	72 g
Deionized distilled water	1000 ml

Sample loading dye (2X Stock)

62.5 Mm Tris–HCl (pH 6.8)	250 µl
Glycerol	500 µl
1% Bromophenol Blue	40 µl
Deionized distilled water	1.210 ml

Acid-Urea PAGE reagents

15% Resolving gel

Urea	3.6 g
30% (w/v) Acrylamide/bisacrylamide	5 ml
Glacial acetic acid	500 µl
Deionized distilled water	4.1 ml
10% (w/v) APS	140 µl
TEMED	60 µl

Sample buffer (1X)

Urea	3.6 g
Glacial acetic acid	50 µl
0.2% (w/v) Methylene blue	100 µl
Glycerol	300 µl
Deionized distilled water	300 µl

Running buffer

5% (v/v) Acetic acid

DPPH assay reagents

DPPH Solution (5 Mm stock)

DPPH (1,1-diphenyl-2-picrylhydrazyl)	1.9716 mg
Methanol	1 ml

FRAP assay reagents

10 mM TPTZ

TPTZ (2,4,6-Tripyridyl-S-triazine)	3.123 g
40 mM HCl	1000 ml

20 mM $FeCl_3.6H_2O$

Ferric chloride ($FeCl_3$)	5.406 g
Deionized distilled water	1000 ml

0.001M $FeSO_4$ Standard

$FeSO_4.7H_2O$	2.78 g
Deionized distilled water	1000 ml

Abbreviations

FREQUENTLY USED ABBREVIATIONS

WHO	World Health Organization
CAPs	Cationic Antimicrobial Peptides
AMP	Antimicrobial peptide
HNP	Human defensins
MTCC	Microbial Type Culture Collection & Gene Bank
SD	Standard deviation
BSA	Bovine Serum Albumin
PAGE	Polyacrylamide Gel Electrophoresis
SDS	Sodium dodecyl sulfate
MW	Molecular weight
LMW	Low molecular weight
AU-PAGE	Acid-Urea Polyacrylamide Gel Electrophoresis
LC	Liquid Chromatography
MS	Mass Spectrometry
LC-ESI-MS	Liquid Chromatography-Electrospray Ionization-Mass Spectrometry
HPLC	High-Performance Liquid Chromatography
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight
MIC	Minimum inhibitory concentration
CLSI	Clinical and Laboratory Standards Institute
CFU	Colony forming unit
PBS	Phosphate buffered saline
MOCP	*M. oleifera* Coagulant protein
UPLC	Ultra-Performance Liquid Chromatography

HR-LC-MS	High-Resolution-Liquid Chromatography-Mass Spectrometry
FDR	False discovery rate
HCD	Higher energy collisional dissociation
DPPH	1,1-diphenyl-2-picrylhydrazyl
FRAP	Ferric reducing antioxidant power
RBC	Red blood cell
RPMI	Roswell Park Memorial Institute
DMSO	Dimethyl sulfoxide
EC_{50}	Half maximal effective concentration
ED_{50}	Half maximal effective dose
APS	Ammonium persulfate
TEMED	Tetramethylethylenediamine
DW	Distilled water
TPTZ	2,4,6-Tripyridyl-S-triazine

Publications

Published in refereed journal:

- **Sreekala, S.,** & Muraleedharan, U. D. (2021). Cationic *Clitoria ternatea* seed peptide as a potential novel bioactive molecule. *Protein Peptide Letters***,** *28* (11), 1259-1271. doi: 10.2174/0929866528666210922124735

Manuscripts communicated / in preparation:

- **Sreekala, S.,** & Muraleedharan, U. D. Antimicrobial cationic peptide activity from medicinal plants.
- **Sreekala, S.,** & Muraleedharan, U. D. A 6.7 kDa cationic antimicrobial peptide from *Moringa oleifera* seeds: purification and characterization.
- **Sreekala, S.,** & Muraleedharan, U. D. Bioactive cationic peptide purified from *Allium sativum*.
- **Sreekala S.,** et al. Antiplasmodial potential of cationic peptides from traditional medicinal plants.

Papers presented at Conferences:

- **Sreekala, S.,** & Muraleedharan, U. D. (2019). *Moringa oleifera* as a source of antimicrobial compounds**.** *3rd International Conference on In Sync-with Next Generation Biosciences*. Scire Science. Book of abstracts, p.53. [won Best Paper Presentation Award]
- **Sreekala, S.,** Muraleedharan, U.D. (2017) In vitro screening for antimicrobial peptide activity from plant sources. *National Conference of Young Researchers on New frontiers in Life Sciences & Environment*. Book of abstracts, p.21.

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RESEARCH ARTICLE

Cationic *Clitoria ternatea* Seed Peptide as a Potential Novel Bioactive Molecule

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Abstract: ***Background*:** While several biologics have been reported from different parts of *Clitoria ternatea,* a herbaceous climber of the family Fabaceae, specific production of cationic peptides other than cyclotides (<3.7 kDa) has barely been investigated, or their bioactive potential been looked into.

***Objective*:** The study aims to uncover potential bioactivities and characteristics of novel cationic peptides from *C. ternatea* seeds.

***Methods*:** *C. ternatea* seed cationic peptide purified by simple and cost-effective procedures was analyzed by electrophoresis and mass spectrometry. Antimicrobial efficacy was evaluated against bacterial and fungal pathogens. Antioxidant potential was quantified by *in vitro* antioxidant assays. Physicochemical characterization and Tandem mass spectrometry were performed.

***Results*:** An 8.5 kDa cationic peptide purified from *C. ternatea* seeds was active against *Candida albicans*, *Staphylococcus aureus*, *Aeromonas hydrophila* and *Escherichia coli* at a minimum inhibitory concentration in the range of 8-32 µg/ml. This activity was totally uncompromised at pH 5-8 or after 1 h of heat treatment at 70-80ºC, but was sensitive to protease treatment. Concentration-dependent free-radical scavenging activity and ferric-reducing capacity demonstrated the antioxidant potential of the peptide. Tandem MS analysis of trypsin-digested peptide based on shotgun proteomics detected matching peptide sequences with one or two cysteine residues but had low sequence coverage (≤17%) to known sequences in the *C. ternatea* protein database. Taken together, the distinct characteristics of this novel 8.5 kDa peptide clearly distinguish it from known cyclotides of *C. ternatea*.

***Conclusions*:** Insights have been obtained into the functional characteristics of what appears to be a novel cationic peptide from *C. ternatea* seeds, exhibiting significant antimicrobial and antioxidant activities.

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1. INTRODUCTION

Over the past few decades, antimicrobial peptides (AMPs) have gained increasing importance as a promising template for the development of novel therapeutic agents to fight the rapidly growing problem of pathogen resistance to conventional antibiotics [1-5]. Irrespective of their source and structural dissimilarities, AMPs display many common characteristic features such as net positive charge at physiological pH, being made up of <100 amino acid residues, amphipathic nature, and broad-spectrum activity against bacterial and fungal pathogens [6-10]. Most of the AMPs isolated from plant species are cysteine-rich peptides (CRPs) which have multiple disulfide bonds forming cross-braced structures that confer chemical, thermal and even proteolytic stability [11-14]. Defensins, knottins, leginsulins (A1bs) and cyclotides are the major classes of CRPs identified from various plant sources [13-17].

Clitoria ternatea, commonly called blue pea or butterfly pea, is a perennial herbaceous climber of the family Fabaceae. While acclaimed for its medicinal properties, this plant has also found its place as a garden ornamental, widely featuring in agroforestry systems in tropical and subtropical regions of the world. It has been used since ancient times in traditional herbal medicine, functioning as a nootropic, tranquilizing and sedative agent [18-20]. Different parts of *C. ternatea* have been attributed with promising bioactivities such as anti-inflammatory [21], antimicrobial [22-24], anticancer [25, 26], hepatoprotective [27], antidiabetic [28, 29], cardioprotective [30], antioxidant [31], antiasthmatic [32], antihyperlipidemic [33] and nootropic [34, 35]. Va-

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rious phytochemical constituents, including anthocyanins, flavonoids, tannins, steroids, saponins, alkaloids, glycosides, *p*-hydroxycinnamic acid and pentacyclic triterpenoids such as taraxerols and taraxerones with known bioactivities have been identified from root, seeds, stem, leaves and flowers of *C. ternatea* [36-42]. Methanolic extracts of *C. ternatea* demonstrated antidepressant, anxiolytic, anti-stress and anticonvulsant activity in *in vivo* studies [43, 44]. Literature reports on a preliminary phytochemical analysis of *C. ternatea* extracts with potential bioactivities reveal more peptide and protein content than other secondary metabolites [45-47]. Kelemu *et al.* (2004) described a *C. ternatea* seed protein named finotin (with molecular size 20 kDa) that displayed insecticidal and antifungal activity against various plant pathogens [48]. Poth *et al*. (2011) have been credited with providing the first insights into the distribution and evolution of cyclic peptides (cyclotides) within the Fabaceae plant family [49, 50]. Not long after, Nguyen *et al.* (2011) reported the isolation of 15 cysteine-rich peptides (CRPs) from heat-stable fractions of the whole plant and flower extracts of *C. ternatea* [51-53]. The 12 novel sequences from among these were termed 'cliotides' and projected to have the potential to be developed as anti-cancer and anti-infective agents [54-56]. Structural features and broad-spectrum bioactivities of various biologics from *C. ternatea* have been intensively studied, but no reports could be traced on antimicrobial cationic peptides from seeds of *C. ternatea*, other than cyclotides (<3.7 kDa). The present study uncovers a distinctly different cationic peptide from *C. ternatea* seeds, with antimicrobial as well as antioxidant potential.

2. MATERIALS AND METHODS

2.1. Isolation and Purification of Peptides

C. ternatea seeds were collected from Zuarinagar (South Goa, India), cleaned and a 10% (w/v) homogenate prepared in 10% acetic acid solution, pH 2.9 [57, 58]. The crude preparation was incubated at 4°C for 24 h and filtered using a muslin cloth. The filtrate was centrifuged at 10,000 rpm for 30 min at 4°C to obtain a clear supernatant which was then subjected to ammonium sulfate fractionation at various levels of saturation. The precipitate obtained was suspended in 0.01 M sodium acetate buffer (pH 5.6) for further analyses. Chromatography was carried out on a Sephadex G–50 column (12 x 500 mm) equilibrated with the buffer; fractions were eluted in the same buffer at a flow rate of 12 ml/h and their absorbance was monitored at 220 nm.

2.2. Polyacrylamide Gel Electrophoresis (PAGE) Analysis

Samples were subjected to native-PAGE (15% resolving gel) as well as Sodium Dodecyl Sulfate-PAGE (SDS-PAGE; 18% resolving gel, under reducing conditions) analyses [59]. Acid-Urea-PAGE (AU-PAGE; 15% resolving gel containing 5% acetic acid and 6 M urea) was carried out to assess the basic/cationic nature of samples with antimicrobial activity [57, 60, 61]. Coomassie Brilliant Blue G-250 was used to stain the gels [62].

2.3. Mass Spectrometry Analysis

LC-ESI-MS (Liquid Chromatography-Electrospray Ionization-Mass Spectrometry) analysis was performed on an Agilent 6460 Triple Quadrupole MS system. The purified peptide sample was dissolved in deionized distilled water with 0.1% (v/v) formic acid and 10 µl was loaded onto an HPLC (High-Performance Liquid Chromatography) C18 column coupled to a mass spectrometer. Elution was effected using 20% (v/v) acetonitrile/water. The molecular ions were detected in positive ionization mode as a function of *m/z*. Agilent MassHunter Bioconfirm software was used for the deconvolution of *m/z* spectra. Analysis by MALDI-TOF (Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight) was carried out using a Bruker-UltrafleXtreme MALDI-TOF/TOF (Bruker Daltonics) instrument. The mass spectrum was acquired in a linear positive ionization mode in the range of 5–20 kDa.

2.4. Antimicrobial Activity and MIC (Minimal Inhibitory Concentration)

Fungal as well as bacterial (Gram-positive and Gram-negative) strains obtained from MTCC (Microbial Type Culture Collection & Gene Bank, India), namely, *Candida albicans* MTCC 227, *Staphylococcus aureus* MTCC 3160, *Aeromonas hydrophila* MTCC 1739, *Salmonella typhimurium* MTCC 3231, *Pseudomonas aeruginosa* MTCC 741 and *Escherichia coli* MTCC 443 were used as test cultures. All pathogenic strains were cultured in Mueller-Hinton agar or Mueller-Hinton broth for susceptibility testing [57] by agar well diffusion method [63]; the zone diameter values projected do not include the well diameter. Amphotericin, ampicillin, chloramphenicol, tetracycline and gentamicin at concentrations of 25 µg/well served as positive controls against *C. albicans*, *S. typhimurium*, *E. coli*, *A. hydrophila*, *P. aeruginosa* and *S. aureus,* respectively. The MIC was evaluated by broth microdilution method as per CLSI (Clinical and Laboratory Standards Institute) guidelines [64]. In brief, microbial inocula prepared in Mueller–Hinton broth were dispensed into a sterile microwell plate at a final density of 3-5 x 10^5 CFU/ml. The peptide preparations were added to the wells at various concentrations of 1-128 µg/ml, followed by incubation at 37°C for 24 h or 48 h, respectively, for bacterial or fungal strains. Microbial growth inhibition was assessed by the absorbance at 595 nm using an iMark Microplate Absorbance Reader (Bio-Rad). The MIC was evaluated as the lowest peptide concentration which inhibited microbial growth to 99.9% [64, 65]. All experiments were repeated at least thrice to ensure reproducibility.

2.5. Effect of Temperature, pH, and Proteases

Temperature sensitivity was evaluated [66, 67] by incubating the peptide sample for 1 h at different

temperatures from 40 to 100°C, preceding the antimicrobial activity test; a sample incubated at room temperature (28°C) was used as control. For pH sensitivity studies [67], the activity of lyophilized samples was estimated after incubation at room temperature for 1 h in the respective buffers at 50 mM: citric acid (pH 5 and 6), PBS (pH 7) and Tris–HCl (pH 8). The effect of proteases was assessed [68] by incubating the sample with pronase (Sigma), proteinase K (HiMedia) or trypsin (HiMedia) at a 100:1 (w/w) ratio for 3 h at 37ºC, prior to the assay.

2.6. Antioxidant Activity Assays

The DPPH (2,2-diphenyl-1-picrylhydrazyl) method and the FRAP (Ferric Reducing Antioxidant Power) method were adopted to evaluate antioxidant activity. Ascorbic acid was used as a standard antioxidant. In the DPPH method [69] the free radical scavenging activity (expressed as %) was estimated based on the reduction of DPPH radical, as it converts to a non-radical form in the presence of antioxidants, leading to a decreased absorbance at 517 nm. Briefly, 190 µl of 0.05 mM DPPH (Sigma) solution in methanol was mixed with 10 µl of sample solution at various concentrations from 1- 250 µg/ml. The mixture was incubated in the dark for 30 min at room temperature and the absorbance was then measured at 517 nm against a blank. A 0.05 mM DPPH solution (without any sample) served as negative control. The DPPH scavenging activity of the sample was calculated by the formula:

$$\text{DPPH Scavenging activty (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

In the FRAP method [70], the presence of antioxidants would cause reduction of the Fe^{3+} complex of TPTZ (2,4,6-Tripyridyl-S-triazine) to a blue-colored Fe^{2+} complex at acidic pH. Samples (50 µl) at concentrations in the range of 1- 25 µg/ml were mixed with 100 µl FRAP reagent and incubated in the dark for 30 min at room temperature. The absorbance of the mixture was then measured against a blank at 593 nm and the FRAP value (µM) of the sample was determined based on the standard curve ($R^2 = 0.998$) of absorbance (593 nm) *vs* Fe^{2+} concentration (µM).

At least three independent experiments were carried out for the antioxidant assays, with not less than 5 concentrations of sample, each estimated in triplicate.

2.7. Tandem Mass Spectrometry and Sequence Database Search

Shotgun proteomics strategy was adopted to identify the peptide sequence from MS/MS spectra. The tryptic peptides of the purified samples were separated using a nanoAcquity UPLC (Ultra-Performance Liquid Chromatography) system and MS/MS analysis was performed on an SYNAPT G2-S High Definition MS system (HDMS; Waters Corporation). The acquired ion mobility enhanced MS/MS spectra were analyzed by Progenesis QI for proteomics software (Nonlinear Dynamics, Waters). Peptide identification from the acquired MS/MS spectra was achieved by searching against the database of *C. ternatea* from the UniProt repository. The search parameters applied were as follows: trypsin as the digesting agent, one missed cleavage allowed, false discovery rate (FDR) for peptide and protein identification set to less than 4%, carbamidomethylation of cysteine and oxidation of methionine set as fixed and variable modifications, respectively.

3. RESULTS AND DISCUSSION

3.1. Isolation and Purification of Peptide from *Clitoria ternatea* Seeds

Acetic acid extracts of *C. ternatea* seeds were subjected to precipitation with ammonium sulfate at various saturation levels and the fractions were screened for antimicrobial activity (by agar well diffusion assay) against six pathogenic isolates. The procedures were found to be highly reproducible for five independent preparations. Activity against *S. aureus, C. albicans, A. hydrophila* and *E. coli* was distributed over the 0-30% and 30-60% ammonium sulfate saturated fractions (Table **1**), the positive controls used being as described under Materials and Methods Section 2.4); there was no inhibition of growth of *S. typhimurium* or *P. aeruginosa*. Further refining of the fractionation confirmed that maximal antimicrobial activity could be concentrated at 0-50% saturation (Figure **1**). This preparation was hence loaded on a Sephadex G-50 gel filtration column and the eluted fractions assayed for antimicrobial activity. Those that showed the highest activity (Figure **2**) were pooled together, lyophilized and used for further studies.

Table 1. Antimicrobial activity of ammonium sulfate fractions of *Clitoria ternatea* seed extract (based on zone of inhibition; well diameter subtracted).

Test Culture	Zone of Inhibition (mm)			
	0-30% Fraction	30-60% Fraction	60-90% Fraction	Positive Control*
C. albicans	29±0.5	16±0.5	0	21±0.6
S. aureus	20±1.0	11±1.0	0	18±0.5
A. hydrophila	15±0.6	9±1.0	0	16±1.0
E. coli	12±0.5	7±0.5	0	10±0.5

*Amphotericin, gentamicin, tetracycline and chloramphenicol served as positive controls against *C. albicans, S. aureus, A. hydrophila* and *E. coli*, respectively.

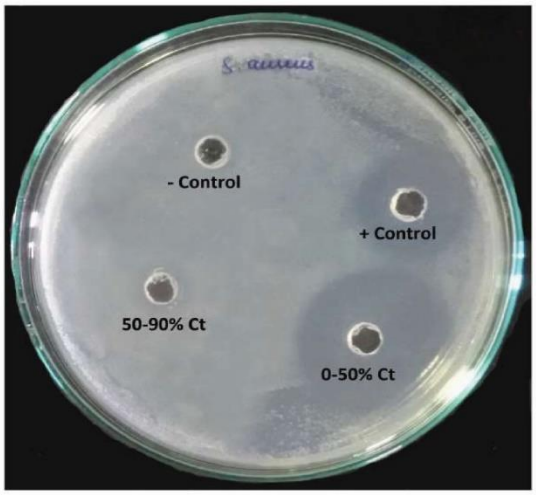

Figure 1. Activity of 0-50% ammonium sulfate precipitated fraction of *C. ternatea* seed extract against *S. aureus.* Gentamicin was used as positive control. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.2. PAGE Analyses

The concentrated active *C. ternatea* seed fraction, when analyzed by native-PAGE (Figure **3a**) as well as SDS-PAGE revealed only a single peptide band of molecular mass ~8.3 kDa (Figure **3b**). Acid-Urea PAGE (AU-PAGE) analysis on a 15% resolving gel containing 5% acetic acid and 6M urea showed cathodal migration of the purified peptide (Figure **3c**), which confirmed its basic or cationic nature.

3.3. LC-MS and MALDI-TOF MS Analyses

The HPLC (C18 column) separation of the *C. ternatea* seed peptide prior to mass spectral analysis exhibited a single peak in the chromatogram (Figure **4a**). The deconvoluted mass spectrum showed a major peak at 8464.61 Da (Figure **4b** and **4c**). The intact molecular weight of the peptide was determined to be 8455.285 Da by MALDI-TOF MS analysis (Figure **5**). Previous reports have detailed molecular characteristics of cysteine-rich cyclic peptides named cyclotides or cliotides but typically smaller than 3.7 kDa, purified from aqueous or organic solvent extracts of flowers, leaves, stems, root nodules, seeds, and pods of *C. ternatea* [49-53].

3.4. Antimicrobial Activity and MIC of *C. ternatea* Seed Peptide

Antimicrobial activity test results of the purified *C. ternatea* seed peptide as assayed by the agar well diffusion method are summarised in Figure (**6**). The peptide exhibited comparatively higher activity against *C. albicans*, with a MIC value of 8 µg/ml and moderate activity against *S. aureus, A. hydrophila* and *E. coli,* with MIC values of 16, 32 and 32 µg/ml, respectively. It was, however, relatively ineffective against *S. typhimurium* and *P. aeruginosa*, as also observed when tested on the partially purified ammonium sulfate precipitated sample (data not shown). This clearly differs from the reported antimicrobial activity of cyclic peptides designated cliotides T1 and T4 from flowers, pods, nodules and shoots of *C. ternatea,* which had demonstrated strong bactericidal effect against *K. pneumoniae, E. coli* and *P. aeruginosa* at MIC values <5 µM, while being ineffective against the Gram-positive *S. aureus* [51, 52].

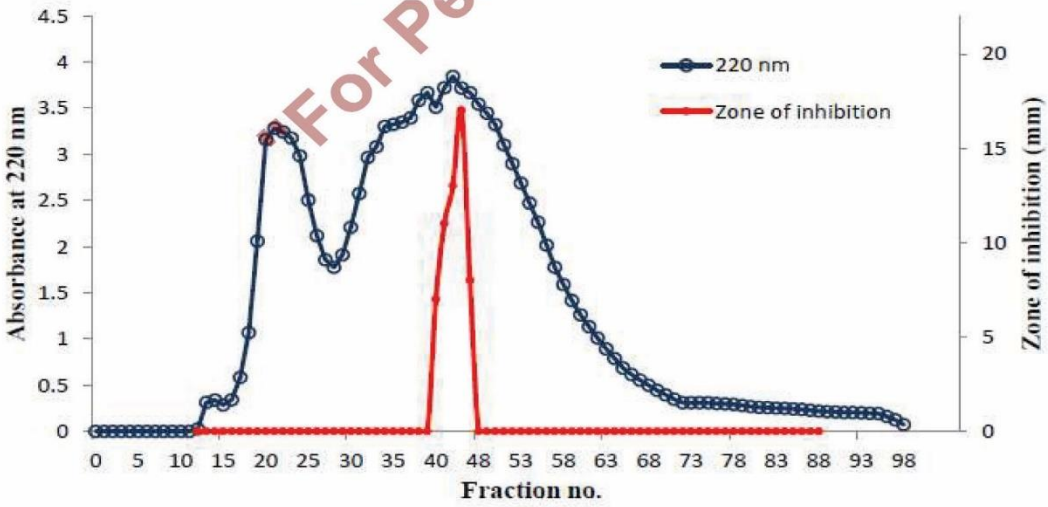

Figure 2. Elution profile of the 0–50% ammonium sulfate fraction of *C. ternatea* seed extract on a Sephadex G-50 column; antimicrobial activity of eluted fractions was tested against *Candida albicans* by the agar well diffusion assay. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

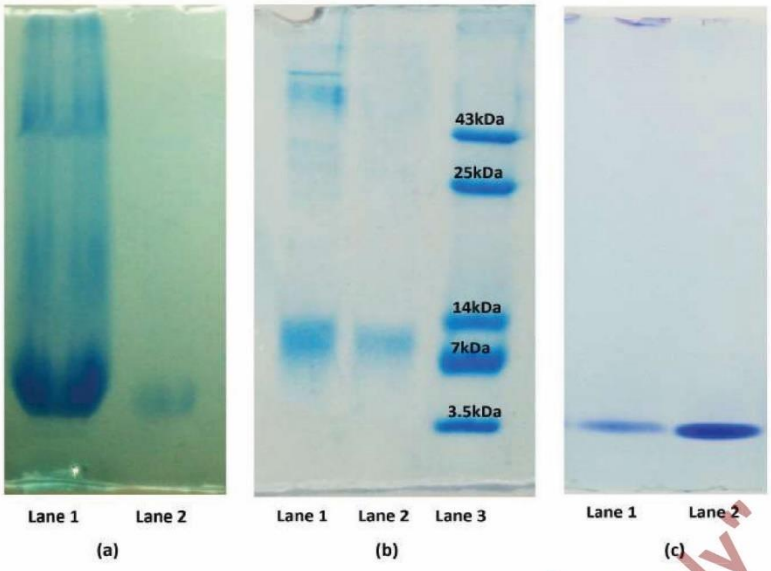

Figure 3. (**a**) Native-PAGE analysis of *C. ternatea* seed samples: Lane 1 - 0-50% ammonium sulfate fraction; Lane 2 - purified peptide (**b**) SDS-PAGE analysis: Lane 1 - 0-50% ammonium sulfate fraction; Lane 2 - purified peptide; Lane 3 - Low MW protein markers (**c**) AU-PAGE analysis: Lane 1 - purified peptide, Lane 2 – Lysozyme, as a basic protein marker. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

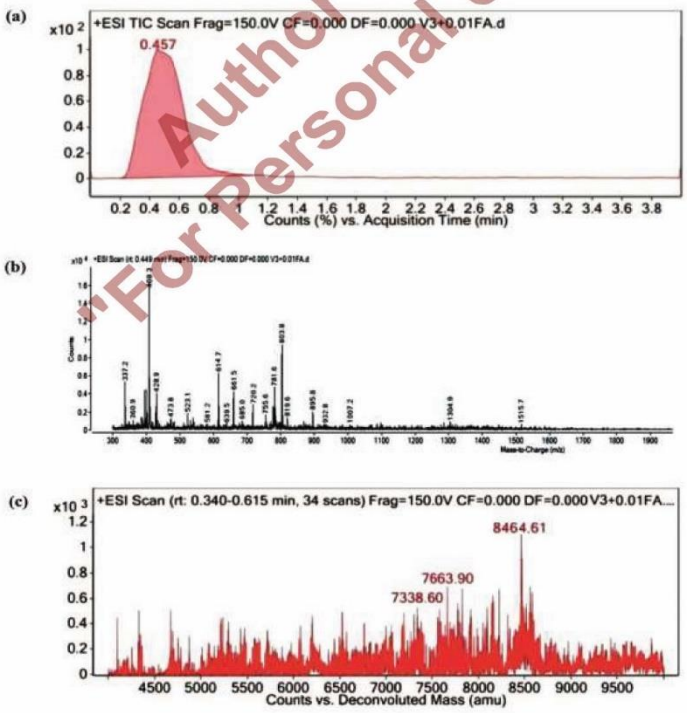

Figure 4. LC-ESI-MS analysis of *C. ternatea* seed peptide: (**a**) LC elution profile (**b**) ESI mass spectrum (**c**) Deconvoluted mass spectrum. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

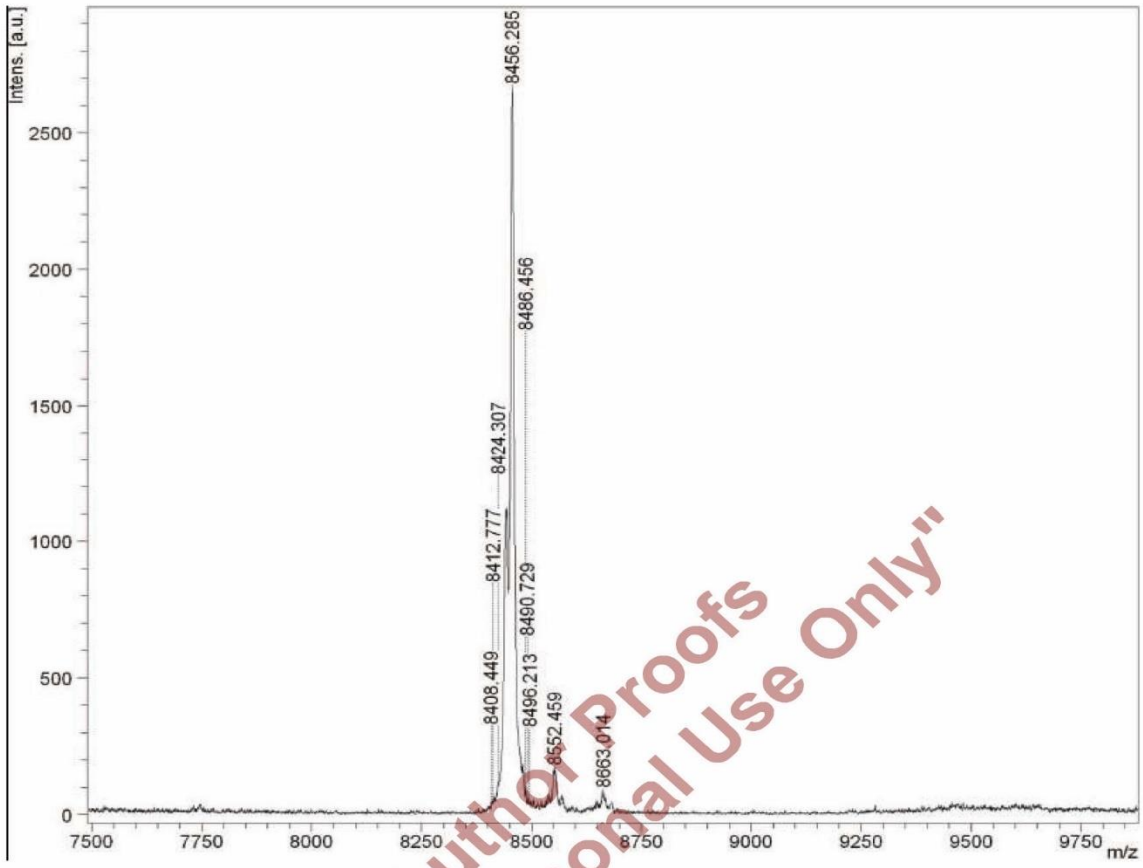

Figure 5. MALDI-TOF Mass spectrum of *C. ternatea* seed peptide.

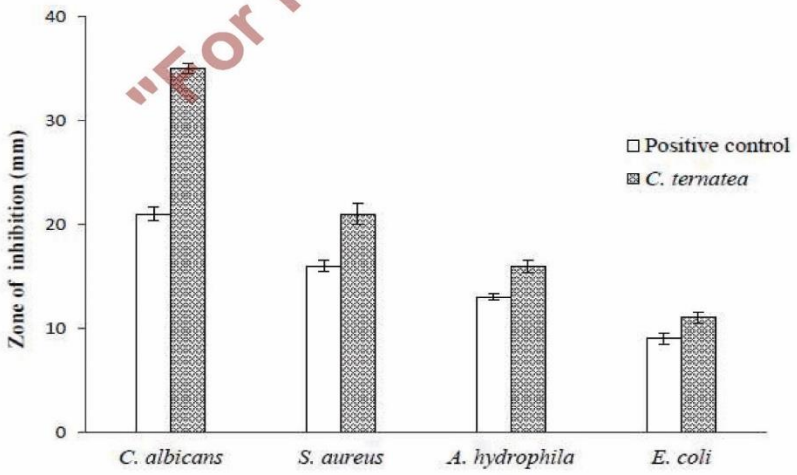

Figure 6. Activity of purified *C. ternatea* seed peptide as estimated by the agar well diffusion method (~25 µg peptide/well). Amphotericin, gentamicin, tetracycline and chloramphenicol served as positive controls (25 µg/well) against *C. albicans*, *S. aureus, A. hydrophila* and *E. coli*, respectively. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

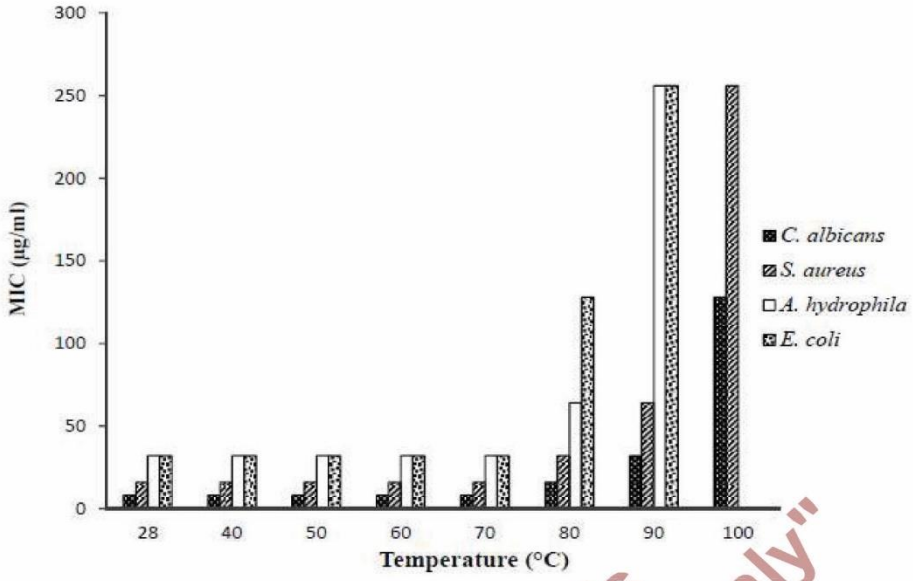

Figure 7. Antimicrobial activity of heat-treated *C. ternatea* seed peptide as determined by broth micro-dilution method. Experiments were carried out in triplicate and mean values plotted.

3.5. Effect of Temperature, pH and Proteases on the Antimicrobial Activity

The effect of parameters such as temperature, pH and protease treatment on antimicrobial activity of the seed peptide was evaluated. The purified peptide preparation was incubated at various temperatures ranging from 40 to 100°C for 1 h and the antimicrobial activity was then assayed by broth micro-dilution method, the peptide incubated at room temperature (28°C) being considered as 'control'. The activity of the peptide against *S. aureus, A. hydrophila, E. coli* and *C. albicans* was retained up to 70-80°C, but treatment at temperatures of 90°C and above resulted in gradual reduction or complete loss of activity against all the four tested pathogens (Figure **7**). This was in marked contrast to the extreme thermostability exhibited by cysteine-rich cliotides isolated from flowers, stems, roots and seeds of *C. ternatea,* wherein the homogenates were incubated at 100°C for 1 h [51]. The detailed structural analysis of *C. ternatea* leaf and flower cyclotides before and after the heat treatment at 95-100°C for 30 min revealed their high stability to heat denaturation [50, 51]. In addition, their MS analysis indicated peptides with *m/z* intensity at around 3 kDa [49-51].

The influence of pH on the peptide's antimicrobial activity was determined after incubating the purified peptide at different pH values. Activity against *C. albicans, S. aureus, A. hydrophila* and *E. coli* was not affected by varying pH from 5–8; the MIC values remained unchanged at 8, 16, 32 and 32 µg/ml, respectively.

Susceptibility of the *C. ternatea* seed peptide to proteolytic enzymes was examined by treating the purified peptide with trypsin, proteinase K or pronase for 3 h at 37°C, prior to the antimicrobial assay. Complete loss of activity was observed after protease treatment (Figure **8**), while the untreated peptide control showed clear zones of inhibition of diameter 33±1, 22±0.5, 16±0.5 and 12±1 mm, respectively, against *C. albicans*, *S. aureus, A. hydrophila* and *E. coli*. This was inconsistent with the remarkably resilient nature of *C. ternatea* cyclotides to proteolytic degradation even after treating with trypsin or chymotrypsin for 8 h at 37°C [50]. Thus, previously described features of cyclotides [49-52] are incongruous with the main characteristics of the *C. ternatea* seed peptide elaborated in this study (Table **2**), suggesting that the latter would not find a place among the cyclotide class of peptides.

The novelty of the purified *C. ternatea* seed peptide in relation to similar compounds reported to date is evident from the information summarized in Table **2**.

3.6. Antioxidant Activity of *C. ternatea* Seed Peptide

Antioxidant effects of the purified peptide were evaluated by the DPPH method and the FRAP method, with ascorbic acid being used as a positive control. The DPPH scavenging activity was concentration-dependent, with an IC_{50} value of 126 µg/ml, against that of ascorbic acid measured as 19 µg/ml (Figure **9a**). The FRAP method recorded an increase in Ferric reducing capacity (FRAP value) with increasing concentration of the peptide, indicating strong antioxidant potential (Figure **9b**). Kamkaen and Wilkinson (2009) had reported the antioxidant activity of aqueous extracts of *C. ternatea* with an IC_{50} value of 2 mg/ml [71], which was interpreted as mainly due to the

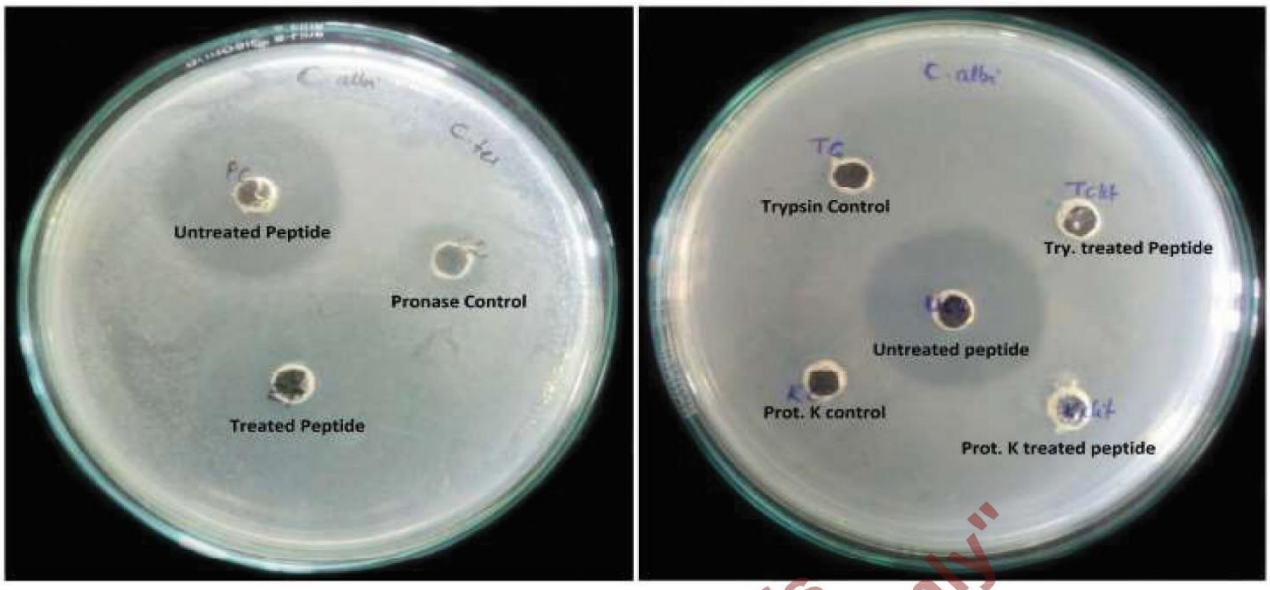

Figure 8. Activity of protease-treated (pronase, trypsin and proteinase K) *C. ternatea* seed peptide against *C. albicans* as tested by agar well diffusion method (~25 µg peptide/well). Untreated peptide in buffer and protease in buffer were used as controls. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Comparison of the *C. ternatea* seed peptide characteristics with those of reported finotin, cliotides / cyclotides.

Characteristics	Finotin	Cyclotide or Cliotides	The 8.5 kDa Peptide
Tissue of origin	Seeds	Flowers, leaves, stem, root, pods or seeds	Seeds
Extract type	Aqueous extracts [48]	Aqueous and organic solvent extracts [49-52]	Acetic acid extracts
Molecular mass	20 kDa [48]	< 3.7 kDa [49-52]	8.5 kDa
Heat stability	*N.A.	Resistant to heat denaturation (95-100°C for 30 min) [50, 51]	Stable at 70-80°C for 1 h
Proteolytic stability	*N.A.	Resistant to proteolytic degradation after treating with trypsin or chymotrypsin for 8 h [50]	Sensitive to trypsin, proteinase K and pronase treatment for 3 h

*N.A. = not available; References appear within [ ].

phenolic content [71-73]. Experimental data from the present study would suggest that the peptide component has a much more significant contribution to the antioxidant activity in *C. ternatea* seed extracts.

3.7. Tandem MS and Sequence Database Search

The trypsin-digested *C. ternatea* seed peptide was analyzed by Tandem MS (UPLC-MS/MS), following which protein database search (Progenesis QI software) was performed based on shotgun proteomics strategy. Peptide sequence identification was carried out by correlating the fragmentation pattern encoded by the experimental MS/MS spectra of *C. ternatea* seed peptide with theoretical spectra constructed for peptides contained in the protein database of *C. ternatea* from UniProt repository. As summarised in (Table **3a** and **b**), tryptic digestion of the seed peptide produced only four detectable matching peptides with one or two cysteine residues and showed 17%, 16% and 7% sequence coverage with Cliotide T30 (fragment), Cyclotide Cter-C and *C. ternatea* Albumin 1, respectively, with confidence score ≥6. Of these, Albumin 1 (15.2 kDa) and Cliotide T30 (11.6 kDa) belong to the family of linear cysteine-rich proteins, which exhibit a high degree of sequence homology, heat stability and ability to survive acid or proteolytic degradation; more detailed characterization had revealed that they originated from common precursor sequences [74-76]. These proteins were found to be distributed in flowers, nodules and seeds of *C. ternatea* and known for their insecticidal and hormonal functions [77, 78]. Cyclotides of cyclic cysteine-rich peptide family usually have a molecular weight less than 3.7 kDa, and are derived from chimeric precursors consisting of cyclotide domain and Albumin 1 chain a domain [51, 74]. Cyclotide family of peptides display a relatively high sequence similarity, with six cysteine residues which form a stable circular peptide backbone resistant to chemical, thermal and enzymatic degradation [49, 76]. Cyclotides expressed in flowers, pods, seeds and roots of *C. ternatea* are shown to possess anti-infective and anti-tumorigenic activities [49, 51]. Since the *C. ternatea* seed peptide purified in this study exhibited comparatively low percent sequence coverage

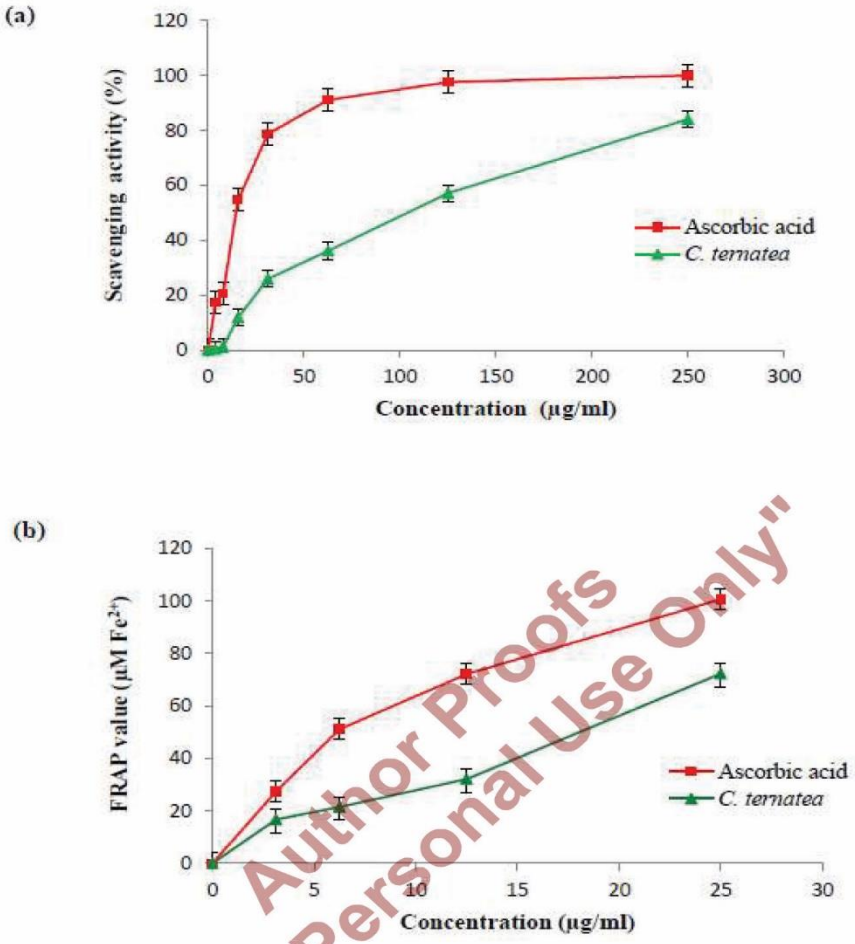

Figure 9. Antioxidant activity of *C. ternatea* seed peptide based on: (**a**) DPPH radical scavenging activity and (**b**) Ferric reducing antioxidant power. Ascorbic acid was used as the positive control. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 3. a) List of tryptic peptides identified by UPLC-MS/MS analysis of *C. ternatea* seed peptide.

Retention Time (min)	Identified Peptide Sequence	Modifications	Ions	Deconvoluted Charges	Neutral Mass	Score	Accession
27.5203	CGESCFAGK	C1 Carbamidomethyl C5 Carbamidomethyl	1	2	1014.4021	4.749	A0A126TRF4
36.2222	TEGGDPLK	-	1	1	815.4266	4.1719	
32.2191	VCYLD	C2 Carbamidomethyl	1	1	668.2901	6.7762	P86843
29.8543	CGESCLLGK	C1 Carbamidomethyl C5 Carbamidomethyl	1	2	1022.4616	5.6218	A0A0S1RUB2

3(b) List of master proteins obtained by *C. ternatea* protein database (UniProt) search using Progenesis QI for proteomics software.

Accession	Description	Mass (Da)	Amino Acids	Coverage [%]	Peptide Count	Confidence Score
A0A126TRF4	Cliotide T30 (Fragment) OS=Clitoria ternatea OX=43366 PE=2 SV=1	11627.2186	100	17	2	8.9209
P86843	Cyclotide Cter-C OS=Clitoria ternatea OX=43366 PE=1 SV=1	3620.0735	31	16	1	6.7762
A0A0S1RUB2	Albumin 1 OS=Clitoria ternatea OX=43366 PE=2 SV=1	15425.4853	133	7	1	5.6218

(≤17%) to known sequences in the *C. ternatea* protein database (UniProt), it is unlikely to belong to known cyclotide or cliotide protein families of *C. ternatea.*

CONCLUSION

In conclusion, the present study has provided insights into the functional characteristics of what appears to be a novel cationic 8.5 kDa peptide from seeds of *C. ternatea,* with significant antimicrobial as well as antioxidant activities. The peptide was active against *C. albicans*, *S. aureus*, *A. hydrophila* and *E. coli,* with MIC values of 8, 16, 32 and 32 µg/ml, respectively. The ferric-reducing capacity as well as DPPH-scavenging ability demonstrated the antioxidant potential of the peptide, with an IC_{50} value of 126 µg/ml. The peptide was resistant to heat treatment at 70-80°C for 1 h and exhibited stable activity in a pH range of 5-8. Tandem mass spectral analysis of trypsin-digested *C. ternatea* seed peptide based on shotgun proteomics approach identified matching peptide sequences with one or two cysteine residues. The protein database search based on experimental MS/MS spectra of this peptide revealed comparatively low percent sequence coverage (≤17%) to known cyclotide or cliotide protein families of *C. ternatea.* Moreover, the sensitivity of the 8.5 kDa peptide to high temperatures (90-100°C) and proteolytic degradation was markedly distinct from the extreme thermal and proteolytic stability of cliotides or the cyclotide family of proteins characterized from different parts of this plant. Ongoing research in our laboratory is being directed to more detailed structural characterization and applications of the purified seed peptide.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used in the study that forms the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTERESTS

The authors declare no conflict of interest, financial or otherwise.

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