Functional characterization of a novel ribosome inactivating protein from *Momordica balsamina*.

by

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*from Momordica balsamina*

submitted for the degree of **Doctor of Philosophy**

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Abstract

Cancer remains one of the leading causes of death worldwide. Many novel anticancer drugs have been identified to improve cancer treatment. However, demerits such as toxic side effects and development of resistance to chemical agents limit its effectiveness. Hence, development and identification of a safer and more effective therapeutic drug is a need of an hour. Nowadays, majority of research focuses on discovering natural therapeutics such as plant derived products with minimal side effects as effective anticancer drugs.

Ribosome inactivating proteins (RIPs) are RNA N-glycosidases that inactivate ribosomes in an irreversible manner through depurination of specific adenine residues found in a highly conserved SR (α-sarcin/ricin) loop on the large ribosomal subunit. The inactivation of ribosomes prevents binding to elongation factor-2 (eF-2), thus arresting protein synthesis. RIPs are widely distributed in plants, and certain bacteria, fungi and algae. In recent years, RIPs have received considerable attention as they target conserved host protein synthesis machinery, which makes it difficult to develop drug resistance, a common problem observed during chemotherapy. In addition, RIPs exhibit low or no detectable side effect on normal cells as they recognize specific receptors expressed on the surface of tumor cells, which makes them a promising tool for cancer treatment.

Balsamin, a type I RIP, has been purified from Momordica balsamina. Previous studies from our group have demonstrated that balsamin exhibits potent growth inhibitory activity against human immunodeficiency virus (HIV). In this study, we investigated other biological and therapeutic activities of balsamin. Balsamin purified from Momordica balsamina (nBalsamin) exhibited DNase-like activity, converting the supercoiled form of a plasmid DNA into the linear form in a concentration-dependent manner. DNase-like activity of nBalsamin towards plasmid DNA was pH, incubation time and temperature dependent. In addition, nBalsamin exhibited antioxidant and broad-spectrum antimicrobial activity, suggesting potential utility of this bioactive as a nutraceutical.

We further investigated the role of nBalsamin as a potent therapeutic agent for cancer. The anti-proliferative effects of nBalsamin and its associated mechanism on liver
(HepG2 and H4IIE) and breast cancer (MCF-7 and BT549) cells were evaluated. nBalsamin inhibited the viability of liver and breast cancer cells in a dose and time dependent manner. nBalsamin-induced apoptosis involved DNA fragmentation, G/S phase cell cycle arrest, increase in caspase-3 and -8 activities, upregulation of Bax, Bid, Bad and p53 and downregulation of Bcl-2 and Bcl-XL, suggesting that nBalsamin activates intrinsic and extrinsic apoptotic pathways in these cells. Increase in the expression of GRP78 and CHOP in liver cancer cells and not in breast cancer cells, indicated that nBalsamin activates an additional, ER-stress mediated, apoptotic pathway in liver cancer cells. These findings suggest that nBalsamin with anti-tumor activities could be used as a nutraceutical for the treatment of liver and breast cancer.

Recovery and purification of Balsamin from natural sources involves multiple purification steps that compromises protein yield. Heterologous protein expression in E.coli strain offers an alternative strategy with higher yields and shorter fermentation time. Herein, we reported a successful heterologous expression of Balsamin (rBalsamin) in E.coli strain. The protein was expressed in E.coli BL21(DE3) strain and purified by nickel affinity chromatography. A total of 45 mg of protein was obtained from a 1 L of induced culture. rBalsamin was found to be sensitive to change in temperature and pH, suggesting that any change in pH and temperature are likely to affect its structure and function. Further, rBalsamin exhibited RNA N-glycosidase, DNase-like and broad-spectrum antimicrobial activity suggesting that rBalsamin carries a high potential for the design of new antimicrobial drugs against human and plant infections.

Investigation of the cytotoxicity of rBalsamin on cancer cells could be beneficial to explore its potential use in cancer therapeutics. Therefore, we investigated the anti-tumor potential of rBalsamin on liver (HepG2 and H4IIE) and breast cancer (MCF-7 and BT549) cells. rBalsamin significantly inhibited the growth and proliferation of liver and breast cancer cells in a dose and time dependent manner. rBalsamin induced apoptosis in these cells via DNA fragmentation, cell cycle arrest, increasing the activity of caspase-3 and -8, and regulating the expression of various pro-apoptotic (Bax, Bid, Bad, p53) and anti-apoptotic (Bcl-2 and Bcl-XL) genes. Interestingly, rBalsamin upregulated the expression of genes, GRP78 and CHOP, involved in ER-stress mediated apoptosis in liver cancer cells and not in breast cancer cells. These
findings suggest that rBalsamin with such variant characteristics could find potential application in the field of cancer therapeutics.

Flavonoids are known to increase and synergize the effects of various chemotherapeutic drugs. We investigated the apoptotic effects of nBalsamin (nBal) in presence of three flavonoids, namely naringenin (Nar), naringin (Nir) and quercetin (Qu). Three concentrations (0.5 X IC₅₀, 1.0 X IC₅₀ and 2.0 X IC₅₀) of flavonoids were tested in combination with nBal (25µg/ml). Nar Nir and Qu increased the anti-proliferative effects of nBal on HepG2 and MCF-7 cells compared to nBal treatment alone. The effect appeared to be more pronounced with increasing flavonoid concentrations. Interestingly, low concentration (0.5 X IC₅₀) of flavonoids appeared to produce an additive but higher concentrations did not (1.0 X IC₅₀ and 2.0 X IC₅₀). Therefore, low concentration of flavonoids; Nar, Nir and Qu in combination with nBal were selected for further study. Mechanically, Nar, Nir and Qu tended to increase the activation of caspase-3 and -8, upregulate the expression of pro-apoptotic gene (Bax, Bid, Bad, p53) and downregulate the expression of anti-apoptotic genes (Bcl-2 and Bcl-XL), indicating that these flavonoids could possibly enhance nBal-induced apoptosis in HepG2 and MCF-7 cells. These findings suggest that nBalsamin in combination with flavonoids could serve as a novel combinational strategy for the treatment of liver and breast cancer. However, in vivo studies are warranted to establish its potential as an effective therapeutic agent.

**Scope of work**

The present study provides an insight into the anti-tumor properties of nBalsamin and rBalsamin and unveils the molecular mechanism involved in Balsamin-induced apoptosis in liver and breast cancer cells. Combination of nBalsamin with flavonoids enhanced the cytotoxic potential of nBalsamin towards liver and breast cancer cells. This approach could serve as a novel combinational strategy for the treatment of liver and breast cancer. The present study offers far-reaching implications of exploring new balsamin targets and delivery systems for cancer therapy.
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List of abbreviations

α        alpha
β        beta
%        percentage
°C       degree celsius
bp       base pairs
h        hours
KB       kilo base
kDa      kilo dalton
L        litre
m        metre
mg       milligram
mg/ml    milligram per millilitre
ng/ml    nanogram/millilitre
min      minutes
ml       milliliter
μg       microgram
μg/ml    micrograms per milliliter
μl       microlitre
μm       micrometre
U/μl     units/microliter
rpm      revolutions per minute
ds       double stranded
ss       single stranded
Akt      serine/threonine kinase
ANOVA    analysis of variance
ATCC     American Type Culture Collection
CD       circular dichroism
cDNA     complementary deoxyribonucleic acid
CRTAM    class-I restricted T cells associated molecule
DEPC     diethylpyrocarbonate
DNA      deoxyribonucleic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EF2</td>
<td>elongation factor 2</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>ERα</td>
<td>estrogen receptor alpha</td>
</tr>
<tr>
<td>GAP31</td>
<td><em>Gelonium</em> anti-HIV protein 31</td>
</tr>
<tr>
<td>GLUT4</td>
<td>insulin responsive glucose transporter</td>
</tr>
<tr>
<td>HBD</td>
<td>human derived cell penetrating peptide</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency disease</td>
</tr>
<tr>
<td>HLTV-1</td>
<td>human T-cell leukemia virus-1</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>JEV</td>
<td>japanese encephalitis virus</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>KSHV</td>
<td>kaposi’s sarcoma-associated herpesvirus</td>
</tr>
<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LRP1</td>
<td>LDL receptor related protein 1</td>
</tr>
<tr>
<td>MAP30</td>
<td><em>Momordica</em> antiviral protein 30</td>
</tr>
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<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCI</td>
<td><em>Momordica charantia</em> inhibitor</td>
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<tr>
<td>MCP30</td>
<td><em>Momordica charantia</em> protein 30</td>
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<td>MIC</td>
<td>minimum inhibitory concentration</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>NaCl</td>
<td>sodium chloride</td>
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<tr>
<td>Nar</td>
<td>naringenin</td>
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<tr>
<td>nBalsamin</td>
<td>natural balsamin</td>
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<tr>
<td>Nir</td>
<td>naringin</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light chain enhancer of activated B cells</td>
</tr>
<tr>
<td>P13K</td>
<td>phosphoinositide 3-kinase</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAP</td>
<td>pokeweed antiviral protein</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>pNA</td>
<td><em>p</em> nitroaniline</td>
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<tr>
<td>PSMA</td>
<td>prostate-specific membrane antigen</td>
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<td>Full Form</td>
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<tr>
<td>Qu</td>
<td>quercetin</td>
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<tr>
<td>rBalsamin</td>
<td>recombinant balsamin</td>
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<td>RIPs</td>
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<td>ribonucleic acid</td>
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<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>TCS</td>
<td>trichosanthin</td>
</tr>
<tr>
<td>TRIP</td>
<td>tobacco ribosome-inactivating protein</td>
</tr>
<tr>
<td>TSLC-1</td>
<td>tumor suppressor lung cancer 1</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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Chapter 1: General introduction and research objectives
1.1 Introduction

Plants represent the first stage in the evolution of living things. During evolution, there has been association between plants and man. In ancient times, human beings solely depended on plants for food, medicine, animal feed, fuel and clothes. Plants with medicinal values were extensively used by Vedas to develop medicines applied for the cure and relief of various illnesses (Petrovska 2012). Plants have been considered a natural reservoir of organic compounds with great importance. These compounds have been employed in the manufacture of food items, cosmetics, paints and medicines that hold major significance in everyday life. Medicinal remedies from plant products have been encouraged as the formulations obtained are cheap and can significantly contribute towards prevention and treatment of various diseases (Howard 2005).

With the development in the scientific world, higher plants have provided a good source for the development of therapeutic agents. Around one quarter of the modern medicinal products are directly or indirectly obtained from plants (Shu 1998). It has been reported by World Health Organisation (WHO) that around 80% of the human population rely on medicinal plants for their primary health care. During the last 30 years, up to 50% of the approved therapeutic agents are either directly or indirectly obtained from natural products (Veeresham & Chitti 2013).

Plants are known to contain variety of secondary metabolites (such as tannins, terpenoids, alkaloids, flavonoids, phenols, oils, etc) that exhibit therapeutic importance and are employed by herbalists in the treatment of various ailments. Despite the presence of secondary metabolites, plants are rich in variety of constitutive and inducible defense mechanisms, such as constitutive inhibitine and phytoalexin, and certain proteins such as peroxidases, proteinase inhibitors, chitinases that accumulate to high concentration in plants in response to infection by viruses, bacteria and fungi and other stresses (Wittstock & Gershenzon 2002). New approaches, involving the production of therapeutic proteins or peptides, have been recently used for the treatment of various diseases such as arthritis, skin ulcers, metabolic disorders, cancer, HIV, myocardial infarction and so on. However, large scale, safe and economic production of these therapeutic proteins remains a
challenge as various parameters need to be considered while manufacturing (Katsila, Siskos & Tamvakopoulos 2012). The by-products generated during downstream processing of therapeutic proteins need to be removed, which incurs cost. Unlike synthetic therapeutic peptides, purifying naturally occurring therapeutic proteins from plants is safe and involves less cost as plant system utilizes its own machinery to produce the protein (Menkhaus et al. 2004). Instead of large capital investment in the manufacturing unit, simply growing and harvesting additional plants can expand a plant protein production system. Apart from this, a plant based production system also has advantage over animal cells, as plants do not present risk of viral infection. Also, the safety advantage over microbial systems is related to the endotoxins and related impurities that come from microbes. However, these kinds of contaminations are not associated with plant-based systems. A number of therapeutic proteins such as hirudin, enkephalins, lactoferrin, serum albumin and many more have been obtained through plant based systems (Thomas, Van Deynze & Bradford 2002) and one such protein that naturally occurs in the plant kingdom is ribosome inactivating protein (RIP). The section below describes in detail the various aspects of RIPS.

1.2 Ribosome inactivating proteins (RIPS)

RIPs are a group of ribotoxins that inhibit protein synthesis in various prokaryotic and eukaryotic cells. These proteins are categorized as RNA N-glycosidases that inactivate ribosomes in an irreversible manner through depurination of specific adenine residues, A2660 in E.coli 23S rRNA or A4324 in eukaryotic 28S rRNA, found in a highly conserved SR (α-sarcin/ricin) loop on the large ribosomal subunit. The inactivation of ribosomes prevents the binding to elongation factor-2 (eF-2), thus arresting protein synthesis (Endo et al. 1987) (Figure 1.1). These proteins are widely distributed in Angiosperms and highly expressed in the seeds of many plants (Puri et al. 2009; Stirpe & Barbieri 1986). Besides RNA N-glycosidase activity, RIPs also display additional activities including DNA, poly(A) and mRNA polynucleotide depurination (Barbieri et al. 1997), superoxide dismutase (Sharma et al. 2004), phospholipase and antioxidant activity (Bhaskar et al. 2008). The activities of these proteins probably explain their putative role in plant protection against various pathogenic infections. The other activities of RIPS include anti-tumor, antiviral, anti-HIV, antiproliferative, immunosuppressive and abortifacient activities.
These proteins have been employed in therapeutics to develop immunotoxins for cancer (Cimini et al. 2012) and AIDS therapy (Uckun et al. 1998). Therefore, RIPS with various beneficial activities are of considerable interest.

Figure 1.1: The site of action of RIPS on 28S rRNA in eukaryotic ribosomes (Fracasso, Stirpe and Colombatti, 2010).

### 1.3 Classification of RIPS

In 1986, based on their primary structure, RIPS were categorized as type I or type II RIPS (Stirpe & Barbieri 1986). However, later in 1994, these RIPS were classified into three groups (type I, type II and type III RIPS) based on their physical properties (Mundy et al. 1994) (Figure 1.2).

Type I RIPS, also called Halo RIPS, are comprised of a single polypeptide chain, with a molecular weight ranging between 26-35 KDa, either linked to carbohydrate moieties or not. These RIPS are known to possess N-glycosidase activity (Peumans, Hao & van Damme 2001). Type II RIPS, also called Chimero RIPS, are heterodimers that comprise an A (active) chain and a lectin B (binding) chain linked through a disulphide bond. The A chain resembles Type I RIPS and exhibits N-glycosidase activity. The B chain with lectin properties recognizes receptors on cell membranes and promotes endocytosis (Ng et al. 2011). These proteins are highly cytotoxic as
the lectin B-chain has the ability to bind to galactose residues present on the surface of animal cells that mediate retrograde transport of the A-chain to the cytosol where it interferes with the translational machinery and arrests protein synthesis (Lord & Roberts 1996). The molecular weight of type II RIPs ranges between 60-65 KDa with each polypeptide chain of approximately 30 KDa. They are basic proteins and their active site contains highly conserved secondary structure and amino acid residues. However, their sequence homology and post-translational modifications are distinctly different (Hartley & Lord 1993). Type III RIPs are rarely found and consist of single polypeptide chain synthesized as a zymogen (inactive precursors) that undergoes proteolytic cleavage to produce two noncovalently linked active chains identical to Type I RIPs (Mak et al. 2007). These RIPs have been characterized from maize, including sorghum, and barley. In maize, these RIPs are synthesised as acidic proteins that enter proteolytic processing events involving removal of short polypeptides from the N- and C- terminals, and amino acids from the middle region resulting in a protein with two polypeptide subunits (Walsh, Morgan & Hey 1991). b-32 is an example of a type III RIP that is synthesised in maize endosperm in an inactive form and undergoes cleavage of amino acid 1-16 from the N-terminal, 25 amino acids (position 162-186) from middle region of the peptide and 14 amino acids (position 289-301) from the C-terminal to produce two peptides, α and β-RIP, in the active form (Hey, Hartley & Walsh 1995). In barley, jasmonate induced protein, a type III RIP is induced by jasmonic acid and undergoes proteolytic cleavage of the middle and C-terminal domains to become active (Chaudhry et al. 1994). The function of the extra domain in type III RIP is still unknown. However, once this domain is cleaved, the active protein produced displays activity similar to that of Type I RIPs.
1.4 Distribution of RIPS

RIPS are widely distributed in several plants, and certain fungi, bacteria and algae. In addition, RIP activity has been reported in mammalian tissues (Girbes et al. 2004). RIPS are ubiquitous in the plant kingdom, mostly found in Angiopermae including monocotyledons and dicotyledons, and have been reported from over 50 different plant species covering 14 families. These families include Cucurbitaceae, Euphorbiaceae, Iridaceae, Fabaceae, Amaranthaceae, Basellaceae, Lamiaceae, Lauraceae, Nyctaginaceae, Sambucaceae, Poaceae, Caryophyllaceae and
Phytolaccaceae (Schrot, Weng & Melzig 2015; Stirpe 2004). RIP producing plants are distributed in diversified regions such as tropical trees, desert succulents, weedy opportunists, spring annuals and obligate parasites. RIPS are found in plants at various concentrations including plants that are eaten raw (such as spinach, tomato) (Stirpe & Battelli 2006). Synthesis of RIPS in plants can be influenced by various factors such as senescence, stress, viral infection and development. Both type I and type II proteins are located in more than one plant tissue and organ including stems, leaves, bark, fruit, latex, endosperm and seeds. However, the most abundantly found RIPS are type I ribosome inactivating proteins (Hartley, Chaddock & Bonness 1996). Pokeweed antiviral protein (PAP) was the first type I RIP purified from Phytolacca americana and thereafter the number has dramatically increased with type I RIPS isolated from different plant tissues and organs (Stirpe & Barbieri 1986). Some of the examples of type I RIPS include α-, β- momorcharin (MMC) and momordica anti-viral protein 30 (MAP-30) (Momordica charantia); gelonin and gelonium anti-HIV protein 31 (GAP-31) (Gelonium multiflorum); saporin (Saponaria officinalis); trichosanthin (TCS) (Trichosanthes kirilowii); cucurmosin (Cucurbita moschata); luffin (Luffa cylindrica); and velin and flammin (Flammulina velutipes) (Puri et al. 2012). It has been noted that more than one RIP isolated from same source of plant exhibits similar physicochemical properties and structure, and consists of highly conserved amino acid sequence. The examples include RIPs isoforms identified from Momordica charantia, Dianthus carophyllus and Phytolacca americana leaves (Stirpe et al. 1992). RIP isoforms may sometimes be found in more than one plant tissue. Different isoform of RIPS such as PD-L1, PD-L2, PD-L3 and PD-L4 have been isolated from the leaves of Phytolacca dioica L. (Di Maro et al. 2009). Another example includes RIP isoforms, PAP-I, PAP-II and PAP-III obtained from the leaves of Phytolacca americana (Domasevshkiy & Goss 2015). Type I RIPS isolated from different plant species, with various activities, are listed in Table 1.1.

Type II RIPS are less abundant and are more toxic in nature than single chain type I RIPS. Ricin from the seeds of Ricinus communis, and abrin from the seeds of Abrus precatorius were the first type II RIPS discovered at the end of the 19th century (Olsnes, Refsnes & Pihl 1974). To date few type II RIPS have been identified and recent findings are shown in Table 1.1. Some of the examples of Type II RIPS include viscumin (Viscum album L.) (Olsnes et al. 1982), nigrin-b (Sambucus nigra
L.) (Girbes et al. 1993), aralin (*Aralia elata*) (Tomatsu, Ohnishi-Kameyama & Shibamoto 2003), riproximin (*Ximenia americana*) (Voss et al. 2006) and ebulin I (*Sambucus ebulus*) (Ferreras et al. 2011).

Type III RIPs are neither a subset of type I RIPs nor type II RIPs and are less prevalent than other two RIPs. These proteins have been identified in a few plants, such as sorghum and barley, certain algal and fungal species. Examples include Shiga toxin I and II (*Shigella dysenteria*); α-sarcin (*Aspergillus niger*); restrictocin and mitogillin (*Aspergillus restrictus*) and tricholin (*Trichoderma viride*) (Nielsen & Boston 2001).

Off all the natural reservoirs exploited for RIPs, RIPs have been obtained largely from plants with a purpose to understand the role of RIPs in plants.
Table 1.1: Overview of RIPs with various activities.

<table>
<thead>
<tr>
<th>RIP</th>
<th>Source</th>
<th>Activity</th>
<th>References</th>
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<tbody>
<tr>
<td>Type I RIPS</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Saporin-6</td>
<td><em>Saponaria officinalis</em></td>
<td><em>N</em>-glycosidase, protein synthesis inhibition, DNase activity, antitumor and anti-HIV activity</td>
<td>(Au et al. 2000; Bagga S 2003)</td>
</tr>
<tr>
<td>MAP 30</td>
<td><em>Momordica charantia</em></td>
<td><em>N</em>-glycosidase, anti-HIV and antitumor, cell free translation inhibitory, anti-HSV activity, DNA glycosylase activity</td>
<td>(Bourinbaiar &amp; Lee-Huang 1996; Lee-Huang et al. 1995b; Wang et al. 1999)</td>
</tr>
<tr>
<td>β- momorcharin</td>
<td><em>Momordica charantia</em></td>
<td>Abortifacient, antitumor and anti-HIV activity, DNase activity, immunosuppressive</td>
<td>(Puri et al. 2009)</td>
</tr>
<tr>
<td>Cochinin</td>
<td><em>Momordica cochinchinensis</em></td>
<td><em>N</em>-glycosidase, protein synthesis inhibition, antitumor activity</td>
<td>(Chuethong et al. 2007)</td>
</tr>
<tr>
<td>GAP-31</td>
<td><em>Gelonium multiflorum</em></td>
<td><em>N</em>-glycosidase, protein synthesis inhibition, anti-HIV and antitumor activity, anti-HSV activity</td>
<td>(Bourinbaiar &amp; Lee-Huang 1996; Lee-Huang et al. 1999)</td>
</tr>
<tr>
<td>Lyophyllin</td>
<td><em>Lyophyllum shimeji</em></td>
<td>Antimitogenic, HIV-1 reverse transcriptase inhibitory, antifungal activity</td>
<td>(Ng, Wong &amp; Wang 2010)</td>
</tr>
<tr>
<td>RIP</td>
<td>Source</td>
<td>Activity</td>
<td>References</td>
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<tr>
<td><strong>Type I RIPs</strong></td>
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</tr>
<tr>
<td>Hispin</td>
<td><em>Benin hispada</em></td>
<td>tRNA ribonuclease, <em>N</em>-glycosidase, antifungal activity</td>
<td>(Ng &amp; Parkash 2002)</td>
</tr>
<tr>
<td>Heterotpalins</td>
<td><em>Phytolacca heterotepala</em></td>
<td>tRNA ribonuclease, <em>N</em>-glycosidase, antifungal activity</td>
<td>(Di Maro et al. 2007)</td>
</tr>
<tr>
<td>Velin</td>
<td><em>Flammulina velutipes</em></td>
<td><em>N</em>-glycosidase, cell-free translation inhibitory</td>
<td>(Ng &amp; Wang 2004)</td>
</tr>
<tr>
<td>Flammin</td>
<td><em>Flammulina velutipes</em></td>
<td><em>N</em>-glycosidase, cell-free translation inhibitory</td>
<td>(Ng &amp; Wang 2004)</td>
</tr>
<tr>
<td>Amaranthin</td>
<td><em>Amaranthus viridis</em></td>
<td><em>N</em>-glycosidase, <em>in vitro</em> translation inhibitory</td>
<td>(Kwon et al. 2000)</td>
</tr>
<tr>
<td>Bouganin</td>
<td><em>Bougainvillea spectabilis</em></td>
<td>Adenine polynucleotide glycosylase, protein synthesis inhibition</td>
<td>(Fermani et al. 2009)</td>
</tr>
<tr>
<td>Lychnin</td>
<td><em>Lychnis chalcedonica</em></td>
<td>Adenine polynucleotide glycosylase, protein synthesis inhibition</td>
<td>(Fermani et al. 2009)</td>
</tr>
<tr>
<td>PAP isoforms</td>
<td><em>Phytolacca americana</em></td>
<td><em>N</em>-glycosidase, protein synthesis inhibition, anti-HIV and antitumor activity, TMV growth inhibitor, DNA cleavage activity</td>
<td>(Domashesvkiy &amp; Goss 2015; Irvin 1975; Mansouri, Kutky &amp; Hudak 2012; Taylor et al. 1994)</td>
</tr>
<tr>
<td>RIP</td>
<td>Source</td>
<td>Activity</td>
<td>References</td>
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<tr>
<td><strong>Type II RIPS</strong></td>
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<tr>
<td>Ricin</td>
<td><em>Ricinus communis</em></td>
<td><em>N</em>-glycosidase, protein synthesis inhibition, DNase activity, antitumor and anti-HIV activity</td>
<td>(Au et al. 2014; Brigotti et al. 2002; Kumar et al. 2004; Rao et al. 2005)</td>
</tr>
<tr>
<td>Abrin</td>
<td><em>Abrus precatorius</em></td>
<td><em>N</em>-glycosidase, protein synthesis inhibition, antitumor activity</td>
<td>(Bora, Gadadhar &amp; Karande 2010; Narayanan, Surolia &amp; Karande 2004)</td>
</tr>
<tr>
<td>Viscumin</td>
<td><em>Viscum album L.</em></td>
<td><em>N</em>-glycosidase, cell free protein synthesis inhibition, catalytic and hemagglutinating activity, antitumor activity</td>
<td>(Olsnes et al. 1982; Pryme et al. 2006; Stirpe et al. 1982)</td>
</tr>
<tr>
<td>Riproxiimin</td>
<td><em>Ximenia americana.</em></td>
<td><em>N</em>-glycosidase, protein synthesis inhibition, antineoplastic activity, antitumor activity</td>
<td>(Adwan et al. 2014; Voss et al. 2006)</td>
</tr>
<tr>
<td>Aralin</td>
<td><em>Aralia elata</em></td>
<td><em>N</em>-glycosidase, protein synthesis inhibition, antitumor activity</td>
<td>(Tomatsu et al. 2004; Tomatsu, Ohnishi-Kameyama &amp; Shibamoto 2003)</td>
</tr>
<tr>
<td>Nigrin-b</td>
<td><em>Sambucus nigra L.</em></td>
<td>RNA <em>N</em>-glycosidase, protein synthesis inhibition, antitumor activity, hemagglutinating activity</td>
<td>(Girbés et al. 1993; Girbes et al. 2004; Muñoz et al. 2001)</td>
</tr>
<tr>
<td>Lanceolin</td>
<td><em>Adenia lanceolata</em></td>
<td>Polynucleotide glycosylase activity, cell-free translation inhibitory, hemagglutinating activity</td>
<td>(Stirpe et al. 2007)</td>
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<tr>
<td>Stenodactylin</td>
<td><em>Adenia stenodactyla</em></td>
<td>Polynucleotide glycosylase activity, cell-free translation inhibitory, hemagglutinating activity</td>
<td>(Stirpe et al. 2007)</td>
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<td>Foetidissimin II</td>
<td><em>Cucurbita foetissima</em></td>
<td><em>N</em>-glycosidase, anticancer activity, cell-free protein synthesis inhibition</td>
<td>(Zhang &amp; Halaweish 2007)</td>
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<tr>
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<td>Source</td>
<td>Activity</td>
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<td>Type II RIPS</td>
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<tr>
<td>Articulatin D</td>
<td><em>Viscum articulatum</em></td>
<td><em>N</em>-glycosidase, hemagglutinating activity, cell-free translation inhibitory</td>
<td>(Das, Sharma &amp; Mishra 2011)</td>
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<td>Ebulin I</td>
<td><em>Sambucus ebulus</em></td>
<td><em>N</em>-glycosidase, protein synthesis inhibition</td>
<td>(Ferreras et al. 2011)</td>
</tr>
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<td>Cinnamomin</td>
<td><em>Cinnamomum camphora</em></td>
<td><em>N</em>-glycosidase, antitumor activity</td>
<td>(He &amp; Liu 2003)</td>
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</tbody>
</table>
1.5 Role of RIPs in plants

The importance of RIPs in plant activities and metabolism is not well understood. However, evidences gained from both *in vitro* and *in vivo* studies indicate that some RIPs play a role in plant defense. Two type I RIPs from *Mirabilis expansa* roots were found to be active against several soil borne bacterial pathogens. In addition, these RIPs were active against wide variety of both pathogenic and non-pathogenic fungal pathogens including *Trichoderma* and *Fusarium* species (Vivanco, Savary & Flores 1999). Besides bacterial and fungal pathogens, RIPs are also known to be active against certain insect pests. Cinnamomin, a type-II RIP from *Cinnamomum camphora*, was toxic to mosquito larvae and cotton bollworm (Zhou et al. 2000). In other studies, saporin and ricin demonstrated significant toxicity towards larvae of two *Coleopteran* species but had no effect on *Lepidoptera* (Gatehouse et al. 1990). These studies suggested that RIPs have varied degree of susceptibility and resistance to plant insects and that defense is atleast one function of RIPs in plant.

In addition, some studies indicated that RIPs provide seeds with nutritional benefits and protection against pathogenic attacks. Saporin activity was found to be highest in mature seeds (Barbieri, Battelli & Stirpe 1993), suggesting that RIPs might play an important role during seed germination. Besides this, RIPs have been reported to accumulate in abundance in storage organs in response to stress. RIP activity increased in leaves of pokeweed and *Hura crepitans* in response to osmotic stress and leaf senescence (Stirpe et al. 1996) and in *Mesembryanthemum crystallinum* plant in response to salt stress (Rippmann et al. 1997). Stress induction of RIPs in plant is not limited to abiotic factors but also occurs in response to biotic stress in some plants. The activity of trichosanthin, a type-I RIP from *Trichosanthes kirilowii*, increased upto 19 fold in roots of plant co-cultivated with pathogens than in sterile soil (Wong et al. 1995). These evidences suggest that RIPs play an influential role in plant defense and protection during abiotic and biotic stress.
1.6 Mechanism of action of RIPs

The most remarkable and unique feature of RIPs that distinguishes them from other proteins is RNA N-glycosidase activity. It is this activity through which RIPs act on the cellular machinery. Endo and co-workers demonstrated for the first time that RIPs are enzymes (N-glycosidases) that cause hydrolysis of the N-glycosylic bond at A-4324 in 28S rRNA from eukaryotic ribosomes (Endo et al. 1987). This activity was demonstrated by Ricin, a type-II RIP isolated from the seeds of *Ricinus communis*. Ricin consists of two-polypeptide chains joined by a single disulfide bond. This protein removes the adenine residue located at position 4324 in the 28S rRNA of rat liver ribosomes, defining RIPs as ribosome specific N-glycosidases (Endo & Tsurugi 1987). The mechanism of action consists of two steps; the first step involves recognition of a highly conserved (GAGA) sequence in the stem loop region or sarcin/ricin loop in 28S rRNA, and depurination of adenine from this sequence that is known to be important for the interaction with ribosome elongation factor, making the site unstable. The site is located in domain VII, around 400 nucleotides from the 3’ end of the rRNA. The second step involves the β-elimination reaction that results in cleavage of the 3’ end of the rRNA on treatment of RNA with acidic aniline (Figure 1.3) (Endo et al. 1987). Subsequent work revealed that this particular site-specific RNA N-glycosidase activity towards the ribosome is a unique characteristic of all RIPs identified to date.

However, RIPs are highly substrate dependent for this activity. It has been observed that most type I RIPs possess broad substrate specificity while type II RIPs are highly specific towards animal ribosomes. Ricin (*Ricinus communis*) exhibited highest activity toward ribosomes from animals and yeast, but showed low adenine-cleavage activity towards *E.coli* and plant ribosomes (Barbieri, Battelli & Stirpe 1993). Contrary to this, PAP can act upon bacterial, plant, animal and fungal ribosomes (PAP-2015). PAP, a type I RIP, inactivated ribosomes isolated from RIP-free pokeweed and endod suspension culture cells. The sensitivity of pokeweed ribosome to PAP was found to be similar to wheat ribosome (Bonness et al. 1994). Further, α- and
β-MMC from *Momordica charantia* showed high specificity towards eukaryotic ribosomes and cleaved 28S rRNA, but not on 18S, 5.8S and 5S rRNA, releasing around 400 bp long nucleotide fragment, named as Endo’s fragment. The activity was influenced by different physicochemical conditions. The increase in concentration of [NH$_4^+$] from 0.1-0.25 M slightly affected the enzyme activity of β-MMC. The N-glycosidase activity of the MMCs remained uninfluenced by a change in pH from 6.5-9. However, the activity increased with increasing K$^+$ concentration (Fong et al. 1996). Recently, balsamin from *Momordica balsamina* was shown to mediate depurination of 28S rRNA. When depurinated rRNA was treated with aniline, cleavage occurred at the depurinated site and a small ~400 nucleotide fragment (Endo fragment) was released (Kaur et al. 2012).

Further, it has been shown that some RIPs are much more active on mammalian ribosomes. However, they also depurinate rRNA from other organisms such as plants, fungi and bacteria (Puri et al. 2012). ME1 and ME2 (*Mirabilis expansa*) and TRIP (*Nicotiana tabacum*) exhibited N-glycosidase activity. These proteins showed specificity towards yeast ribosomes and depurinated 26S rRNA and released a 367-nucleotide fragment upon treatment with aniline (Sharma et al. 2004; Vivanco, Savary & Flores 1999). In other studies, BBAP1 (*Bougainvillea xbutiana*) released a rRNA fragment when incubated with tobacco ribosome (Choudhary, Kapoor & Lodha 2008).

The rRNA in native ribosomes is the best substrate for RIPs. However, RIPs also have the ability to act on protein free rRNA and synthetic oligoribonucleotides that resemble the sarcin/ricin domain of 28S rRNA (Domashevskiy, Miyoshi & Goss 2012; Tan et al. 2009). In addition, several RIPs are able to depurinate naked rRNA from non-substrate ribosomes. For instance, Ricin could depurinate *E.coli* 23S rRNA, however, didn’t possess any activity against the intact *E.coli* ribosome (Endo, Gluck & Wool 1991). Also, many RIPs have the ability to depurinate guanine residues within their substrate. Ricin A-chain, under acidic conditions, can release not only adenine but also guanine from synthetic oligonucleotides that mimic the sarcin/ricin domain or 5S rRNA (Tang et al. 2001). Similarly, PAP cleaved
both adenyl-guanosine (ApG) and adenyl-cytosine-cytosine (ApCpC) nucleotides leaving only adenine bases clearly visible in the active site pocket of PAP (Kurinov et al. 1999).

Interestingly, some RIPs also have the tendency to distinguish between capped and uncapped transcripts. A novel mechanism of inhibition of translation was put forward suggesting that PAP causes specific depurination of capped mRNA but not of uncapped RNA. PAP treated luciferase transcripts inhibited translation by binding to the capped structure and depurinating mRNA downstream of the cap, while no degradation was observed in PAP treated uncapped luciferase mRNAs (Hudak, Bauman & Tumer 2002).

These evidences suggested that RIPs have the ability to act on diverse form of rRNA’s with broad-spectrum substrate specificity that encouraged researchers to further investigate enzymatic activities of RIPs on different substrates.
1.7 Enzymatic activities of RIPs

1.7.1 Inhibition of protein synthesis

Inhibition of protein synthesis is an essential step involved in cell death. Proteins known to block translation can be employed in various ways to treat a number of diseases. Interestingly, plant RIPs are found to be potent inhibitors of protein synthesis in a cell free system. Ricin from *Ricinus communis* was first demonstrated to inhibit protein synthesis *in vitro*. Ricin inhibited the elongation step by blocking the interaction of elongation factor 2 (EF2) with ribosomes, which in turn, blocks GTP hydrolysis catalysed by EF-2 resulting in arrest of protein synthesis. However, the protein did not
affect ribosome linked phenylalanyl-tRNA binding catalysed by EF-1 (Montanaro, Sperti & Stirpe 1973).

RIPs from *Momordica charantia* have also been found to inhibit translational systems *in vitro. α- and β- MMC exhibited inhibition of protein synthesis at nM concentration in rabbit reticulocyte cell free lysate with IC₅₀ of 0.12 nM and 0.11 nM respectively (Yeung et al. 1988). On the other hand, MAP30 inhibited cell free protein synthesis in a dose dependent manner with IC₅₀ of 3.3 nM, same as observed with rMAP30, but did not affect translation in normal cells (Lee-Huang et al. 1995b; Lee-Huang et al. 1990). Recently, balsamin from *Momordica balsamina* demonstrated inhibition of protein synthesis in a rabbit reticulocyte lysate based cell free translation system. It was found that the protein inhibited protein synthesis at IC₅₀ of 90.6 ng/ml (Kaur et al. 2012). Furthermore, the effect of natural and recombinant PD-L4 from *Phytolacca dioica* on protein biosynthesis was determined by radiolabelled methionine incorporation into translational products. Natural and recombinant PD-L4 displayed cell free translation inhibition at IC₅₀ of 151±8 pM and 142±7 pM, respectively (Blanco et al. 1998).

It has been shown that some RIPs require cofactors such as ATP for inhibition of the translation process. Gelonin (*Gelonium multiflorum*), PAP-S (*Phytolacca americana*), Barley RIP (*Hordeum vulgare*) and Tritin-S (*Triticum aestivum*) require ATP to achieve maximum activity. However, other RIPs such as saporin (*Saponaria officinalis*), momordin (*Momordica charantia*) and TCS (*Trichosanthes kirilowii*) inhibited protein synthesis without the requirement of ATP (Carnicelli et al. 1997). The translational inhibitory activity of some RIPs is also influenced by the presence of ions. Out of the 7 saporin isoforms isolated from *Saponaria officinalis*, the activity of one saporin isoform was found to be dependent on magnesium ion concentration in a rat liver cell free system (Ferreras et al. 1993).

Some RIPs are capable of inhibiting translation in several cell free systems from mammals and plants. For instance, TRIP (*Nicotiana tabaccum*) inhibited translation in a rabbit reticulocyte lysate and a wheat germ system. TRIP demonstrated a higher inhibitory effect in the wheat germ system with
IC$_{50}$ of 30 ng/ml compared to 100 ng/ml in the rabbit reticulocyte system (Sharma et al. 2004). The effect of saporin isoforms isolated from various parts (leaves, roots and seeds) of Saponaria officinalis L. was tested on mammalian, plant and bacterial cell free systems. All isoforms inhibited protein synthesis in the cell free system derived from rabbit reticulocyte lysate, rat liver, wheat species, Triticum aestivum L., and plants including Cucumis sativus L. and Vicia sativa L. However, they were found to be poor inhibitors of the E.coli protein synthesis system. In the mammalian cell free system, a greater difference in protein synthesis inhibition was observed in the cell lines of human origin. All of the saporin isoforms inhibited protein synthesis in HeLa, BeWo and NB100 cells with IC$_{50}$ of 76 nM or less (Ferreras et al. 1993). The response of MU isoforms (MU 1, 2 & 3) from Muscari armeniacum on cell free systems from mammals and plants showed that all the isoforms exhibited strong inhibitory activity in the rabbit reticulocyte system with IC$_{50}$ of 7, 9.5 and 4 ng/ml, respectively. The values were higher in case of the rat liver system with IC$_{50}$ of 79, 95 and 101 ng/ml for MU 1, 2 & 3, respectively. However, the protein did not exhibit any activity towards the plant derived cell free system (Arias et al. 2003).

These evidences suggest that RIPs are capable of inhibiting protein translation at different levels in variety of cell free systems. This characteristic of RIPs could prove beneficial in suppressing the growth of diseased cells and be considered as a possible strategy of disease treatment in plants, animals and humans. Apart from rRNA cleavage and translation inhibiting activity, some RIPs also exhibit nuclease activities towards various other substrates.

1.7.2 DNase activity

RIPs from plants have been known to remove adenine from DNA, thereby converting supercoiled DNA into open circular and linear forms. TCS demonstrated deoxyribonuclease activity towards supercoiled DNA (pBluescript II, SV40, Φ X 174 RF) in vitro. TCS cleaved supercoiled dsDNA into nicked circular and linear DNA. However, no effect was observed on linear dsDNA (Li et al. 1991). α- and β- MMC were shown to
cleave supercoiled dsSV-40 DNA into nicked circular and linear forms with no activity towards linear DNA such as lambda, Ad-2 and T7 (Go, Yeung & Fong 1992). Interestingly, it was stated that RIP preparation contaminated with DNase and RNase was responsible for this observation, which raised a serious concern. Day and co-workers presented evidence of contamination of PAP isoforms with nucleases (Day, Lord & Roberts 1998). Subsequent efforts were made to report the DNase activity of R IPs. Dianthin 30, saporin-S6 and gelonin demonstrated DNase activity towards single stranded DNA (Nicolas et al. 1997; Roncuzzi & Gasperi-Campani 1996). In addition, gelonin, ricin and PAP could damage ssDNA (pUC18) via removal of specific adenine residues followed by cleavage at the resulting abasic site. Further studies reported that high concentrations of PAP could also cleave double stranded supercoiled DNA using the same site that depurinates rRNA and pG-1 DNA (Wang & Tumer 1999).

Saporin-6 exhibited fragmentation activity towards genomic DNA of U937 cells (Bagga, Seth & Batra 2003). Higher concentrations of recombinant BBAP1 were able to depurinate supercoiled pBlueScript SK+ plasmid DNA in a concentration dependent manner (Choudhary, Kapoor & Lodha 2008). Recent studies showed that DNase cleavage activity of α-MMC depended on various factors such as treatment time, reaction temperature and DNase buffer pH (Wang et al. 2013).

These evidences suggest that R IPs have DNA nuclease activity towards variety of different forms of DNA, including supercoiled, covalently closed, circular plasmid DNA and single stranded phage DNA, along with rRNA N-glycosidase and translation inhibitory activity, suggesting its variant biological role within the cell.

1.7.3 Lipase, chitinase and superoxide dismutase activity

Besides nuclease activities, R IPs exhibit number of novel enzymatic activities, including phosphatase activity on lipids, chitinase and superoxide dismutase activity. However, there is limited evidence to support lipase, chitinase and superoxide dismutase activity of R IPs. RCA60 demonstrated a
phospholipase activity towards palmitoyl-oleoyl-glycerophosphatidylcholine. The nature of the hydrolysis products were analysed by thin-layer and gas chromatography which supported the conclusion that RCA₆₀ exhibits phospholipase A₁ and A₂ activities (Helmy, Lombard & Pieroni 1999).

TKC 15, TKC 28-I, and TKC 28-II purified from the *Trichosanthes kirilowii* plant cell cultures possess both chitinase and RIP activity. Highly purified preparations of these proteins exhibited endochitolytic as well as the specific 28S rRNA N-glycosidase activity (Shih et al. 1997).

Camphorin, a type-I RIP isolated from the seeds of *Cinnamomum camphora*, exhibits superoxide dismutase activity along with RNA N-glycosidase and supercoiled dependent endonuclease activities (Li et al. 1997). A BLAST (NCBI) database sequence search studies revealed that a 15 amino acid internal polypeptide sequence of TRIP was identical with the internal sequence of the iron-superoxide dismutase (Fe-SOD) from wild tobacco, *Arabidopsis*, and potato. Also, purified TRIP showed SOD and RIP activity demonstrating it as a dual activity enzyme (Sharma et al. 2004). In contrast, pyrogallol autoxidation and NBT staining analysis revealed that MAP30 and α-MMC, simultaneously isolated from *Momordica charantia L.*, did not exhibit superoxide dismutase activity (Meng et al. 2014).

These evidences suggest that RIPS possess dual biochemical activities and have multiple biological roles, encouraging researchers to exploit RIPS in the field of therapeutics. The section below describes various therapeutic applications of RIPS with a major focus towards the use of RIPS in cancer treatment.

### 1.8 Therapeutic function of RIPS

RIPS are one class of pathogenesis-related proteins that could be used as antimicrobial agents. The section below describes some of the antimicrobial properties exhibited by RIPS.

#### 1.8.1 Antibacterial activity
Antibacterial properties of RIPs are less frequently described compared to antitumor and antiviral activities. A number of RIPs have been shown to inhibit the growth of various human and plant bacterial pathogens. Tobacco RIP (TRIP) from *Nicotiana tabacum* possessed broad-spectrum antibacterial activity towards various bacterial pathogens including *Pseudomonas solancearum*, *Erwinia amylovora*, *Shigella asonei*, *Salmonella typhimurium*, *Rhizobium leguminosarum*, *Enterobacter aerogenes*, *Agrobacterium radiobacter*, *Bacillus subtilis* and *Erwinia herbicola* (Sharma et al. 2004). A RIP purified from *Cucurbita moschata* demonstrated growth inhibitory activity towards several phytopathogenic bacteria such as *Phytophthora infestans*, *E. amylovora* and *P. solancearum* (Barbieri et al. 2006). α-MMC from *Momordica charantia* exhibited antibacterial activity against *E.coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *B. subtilis*. α-MMC was found to be active against *P. aeruginosa*, however, had little effect on *E.coli*, *S. aureus* and *B. subtilis* (Wang et al. 2012). This potential of RIPs has been exploited in the field of crop improvement to develop transgenic plants resistant to bacterial infections.

### 1.8.2 Antiviral activity

Apart from antibacterial properties, RIPs exhibit broad range antiviral properties against different viruses, including RNA and DNA viruses, infecting both plants and humans (Akkouh et al. 2015).

Several RIPs have been reported to inhibit the growth of various plant viruses. PAP, a type-I RIP from *Phytolacca americana* exhibited antiviral activity against wide range of plant DNA and RNA viruses, including potato virus X and Y, tobacco mosaic virus, alfalfa mosaic virus, cucumber mosaic virus, cauliflower mosaic virus, african cassava mosaic virus and brome mosaic virus in a dose and time dependent manner (Chen et al. 1991; Di & Tumer 2015; Hudak, Wang & Tumer 2000; Taylor et al. 1994). α-MMC from *Momordica charantia* inhibited replication and growth of plant viruses such as chilli veinal mottle virus, tobacco mosaic virus and cucumber mosaic virus in the infected plants (Zhu et al. 2013). In the case of human viruses, specially human immunodeficiency virus (HIV), RIPs have been known to
target three main enzymes involved in the progression of HIV replication, namely, (i) Reverse Transcriptase, the enzyme involved in the synthesis of viral DNA from HIV-1 mRNA, (ii) Integrase, the enzyme that integrates viral DNA into host genome, and (iii) Viral proteins essential for virion synthesis and release from the host cell for the spread of HIV infection (Figure 1.4). Table 1.2 describes in detail the enzymes and viral proteins targeted by RIPs in the replication cycle of HIV.

Besides human immunodeficiency virus (HIV-1), RIPs are also known to target various other human viruses (Kaur, Gupta & Puri 2011). PAP from *Phytolacca americana* exhibited antiviral activity against various human viruses including influenza virus, poliovirus, human T-cell leukemia virus-1 (HTLV-1), herpes simplex virus (HSV), japanese encephalitis virus (JEV) and lymphocytic choriomeningitis virus (LCMV) (Domashevskiy & Goss 2015; Ishag et al. 2013; Mansouri et al. 2009; Teltow, Irvin & Aron 1983; Uckun et al. 2005). MAP30 from *Momordica charantia* demonstrated inhibitory activity towards kaposi’s sarcoma-associated herpesvirus (KSHV) and retarded the proliferation of KSHV infected BL-2 cells with an EC$_{50}$ ranging between 0.3 to 0.6 nM. It was found that MAP-30 upregulated the genes involved in apoptosis (*Bax*, *caspase-3*, *CRADD*) and lowered the expression of cellular genes related to KSHV pathogenesis (Sun et al. 2001).

Other RIPs such as gelonin (*Gelonium multiflorum*), *Momordica charantia* inhibitor (MCI) (*Momordica charantia*) and dianthin 32 (*Dianthus caryophyllus*) have shown inhibitory effect against HSV-1 and poliovirus. All proteins reduced viral replication, decreased plaque forming efficiency and inhibited protein synthesis in the virus infected cells (Foa-Tomasi et al. 1982).
Figure 1.4: Schematic representations of targets of various RIPs in the life cycle of HIV-1. The RIP causes inhibition at various stages in the replication cycle and further represses its progress.
Table 1.2 Overview of RIPS targeting various enzymes and viral proteins involved in HIV replication.

<table>
<thead>
<tr>
<th>RIPS</th>
<th>Source</th>
<th>RIPs target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balsamin</td>
<td><em>Momordica balsamina</em> (seeds)</td>
<td>p55, p24, p41</td>
<td>(Kaur et al. 2013)</td>
</tr>
<tr>
<td>Bryodin 1 &amp; 2</td>
<td><em>Bryonia cretica spp. dioica</em> (roots)</td>
<td>Reverse Transcriptase</td>
<td>(Wachinger et al. 1993)</td>
</tr>
<tr>
<td>TCS</td>
<td><em>Trichosanthes kirilowii</em> (root tubers)</td>
<td>Reverse Transcriptase, p24</td>
<td>(Wang et al. 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gag budding sites</td>
<td>(Pan &amp; Levy 1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIV- Integrase</td>
<td>(Zhao et al. 2009)</td>
</tr>
<tr>
<td>Saporin</td>
<td><em>Saponaria officinalis</em> (seeds)</td>
<td>HIV- Integrase</td>
<td>(Au et al. 2000)</td>
</tr>
<tr>
<td>GAP 31</td>
<td><em>Gelonium multiflorum</em> (seeds)</td>
<td>Reverse Transcriptase, p24</td>
<td>(Lee-Huang et al. 1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIV- Integrase</td>
<td>(Lee-Huang et al. 1995a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Viral DNA-LTR</td>
<td>(Li et al. 2010b)</td>
</tr>
<tr>
<td>Luffin</td>
<td><em>Luffa cylindrica</em> (seeds)</td>
<td>HIV- Integrase</td>
<td>(Au et al. 2000)</td>
</tr>
<tr>
<td>DAPs 30 &amp; 32</td>
<td><em>Dianthus caryophyllus</em> (leaves)</td>
<td>Reverse Transcriptase, p24</td>
<td>(Lee-Huang et al. 1991)</td>
</tr>
<tr>
<td>MAP 30</td>
<td><em>Momordica charantia</em> (seeds &amp; leaves)</td>
<td>Reverse Transcriptase, HIV- Integrase</td>
<td>(Lee-Huang et al. 1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Viral DNA-LTR</td>
<td>(Lee-Huang et al. 1995a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Wang et al. 1999)</td>
</tr>
<tr>
<td>PAP-S</td>
<td><em>Phytolacca americana</em> (seeds)</td>
<td>Reverse Transcriptase</td>
<td>(Olson, Ramakrishnan &amp; Anand 1991)</td>
</tr>
<tr>
<td>RIPS</td>
<td>Source</td>
<td>RIPs target</td>
<td>Reference</td>
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<tr>
<td>Gelonin</td>
<td><em>Gelonium multiflorum</em> (seeds)</td>
<td>HIV-Integrase</td>
<td>(Au et al. 2000)</td>
</tr>
<tr>
<td>LRIP-1</td>
<td><em>Luffa aegyptiaca</em> (seeds)</td>
<td>Reverse Transcriptase</td>
<td>(Olson, Ramakrishnan &amp; Anand 1991)</td>
</tr>
<tr>
<td>α-MMC</td>
<td><em>Momordica charantia</em> (seeds)</td>
<td>p24</td>
<td>(Zheng, Ben &amp; Jin 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIV-Integrase</td>
<td>(Au et al. 2000)</td>
</tr>
<tr>
<td>PAP isoforms (PAP-I, II, III)</td>
<td><em>Phytolacca americana</em> (leaves)</td>
<td>HIV-1 mRNA</td>
<td>(Rajamohan et al. 1999)</td>
</tr>
<tr>
<td>β-MMC</td>
<td><em>Momordica charantia</em> (seeds)</td>
<td>HIV-Integrase</td>
<td>(Au et al. 2000)</td>
</tr>
<tr>
<td>Luffin P1</td>
<td><em>Luffa cylindrica</em> (seeds)</td>
<td>p24, RRE</td>
<td>(Ng et al. 2011)</td>
</tr>
<tr>
<td>PAP</td>
<td><em>Phytolacca americana</em> (leaves)</td>
<td>Gag protein, p24, p41, Nef, Env</td>
<td>(Mansouri, Kutky &amp; Hudak 2012)</td>
</tr>
<tr>
<td>Luffaculin I</td>
<td><em>Luffa acutangula</em> (seeds)</td>
<td>p24</td>
<td>(Long et al. 2008)</td>
</tr>
</tbody>
</table>

HIV: human immunodeficiency virus; LTR: long terminal repeats; RRE: rev response element; Nef: negative regulatory factor; Env: envelope protein.
1.8.3 Anti-tumor activity

Type I RIPs from different plant sources have shown to have antitumor activity in both in vivo and in vitro studies (Zeng et al. 2015). Table 1.3 provides a detailed overview of Type I RIPs with antitumor activities including studies on cell lines and tumor models.

1.8.3.1 Effect on breast cancer

Type I RIPs such as TCS, MAP30, GAP31, saporin-6, gelonin, marmorin, α–MMC have been known to inhibit the growth of breast tumors in vitro and in vivo. The first RIP that demonstrated anti-breast activity was saporin-6, a plant RIP isolated from Saponaria officinalis. The protein at a concentration of $10^{-9}$ M was found to be highly toxic to breast cancer cells obtained from 27 patients bearing primary breast cancer (Gasperi-Campani et al. 1991). The conjugation of saporin to epithelial cell adhesion molecule (EpCAM) targeting monoclonal antibody, 3-17I demonstrated enhanced toxicity towards MCF-7 breast cancer cells in a light dependent manner after photochemical internalization of 3-17I (Lund et al. 2014). Several other RIPs have been reported as potent inhibitors of breast cancer. TCS exhibited antiproliferative activity and induced apoptosis in both MCF and MDA-MB-231 cells in a dose dependent manner. The protein arrested cell cycle at G1 phase and stimulated apoptosis through caspase -3, -8 and -9 activation, PAP cleavage and DNA fragmentation. In vivo studies demonstrated that TCS could ablate tumor volume and tumor weight from the 6th day of treatment in nude mice bearing MDA-MB-231 cells (Fang et al. 2012c). Other RIPs such as MAP30, GAP31 and α-luffin have also been found to possess antitumor activity against various breast cancer cell lines in a dose dependent manner (Lee-Huang et al. 1999; Liu et al. 2010; Rybak et al. 1994). MAP30 and GAP31 inhibited MDA-MB-231 cell proliferation by downregulating the expression of human epidermal growth factor receptor-2 (Her-2), an oncogene that plays an important role in the development and progression of cancer. This gene is overexpressed in approximately 30% of all human breast cancer, and Her-2 expressing breast tumor cells are sensitive to chemotherapy, therefore, MAP-30 and GAP31 could represent an alternative therapeutic strategy for Her-2 expressing breast tumors (Rybak et al. 1994).
Table 1.3: Summary of RIPs antitumor activities, including *in vivo* and *in vitro* studies.

<table>
<thead>
<tr>
<th>RIP</th>
<th>Tumor</th>
<th>Cell line (<em>in vitro</em>)</th>
<th>Animal (<em>in vivo</em>)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCS</td>
<td>Breast cancer</td>
<td>MCF-7, MDA-MB-231</td>
<td>Nude mouse (transplanted)</td>
<td>(Fang et al. 2012c)</td>
</tr>
<tr>
<td></td>
<td>Cervical cancer</td>
<td>CaSki, HeLa</td>
<td></td>
<td>(Peng et al. 2011; Wang et al. 2009)</td>
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<td></td>
<td>Choriocarcinoma</td>
<td>JAR, H35, BeWo</td>
<td></td>
<td>(Chan, Huang &amp; Tam 2003; Jiao &amp; Liu 2010)</td>
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<td>Colorectal cancer</td>
<td>LoVo</td>
<td></td>
<td>(Gao et al. 2010)</td>
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<td>Liver cancer</td>
<td>HepG2, Hep A-H, H22</td>
<td>Nude mice SW-1116 cells</td>
<td>(Dou &amp; Li 2004; Li et al. 2010c)</td>
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<td>Prostate cancer</td>
<td>RM-1</td>
<td>Balb/c mice with H22 cells</td>
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<td>Nude mouse with A549 cells</td>
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<td>Larynx cancer</td>
<td>AMC-HN-8, HEp-2</td>
<td></td>
<td>(Li et al. 2010a)</td>
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<td>α-MMC</td>
<td>Breast cancer</td>
<td>MCF-7, MDA-MB-231, EMT-6</td>
<td>Nude mouse (transplanted)</td>
<td>(Cao et al. 2015; Deng et al. 2016)</td>
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<td></td>
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<td>JAR</td>
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<td>(Ng et al. 1994)</td>
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<td></td>
<td>Nasopharyngeal cancer</td>
<td>CNE-2, HONE-1</td>
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<td></td>
<td>Lung cancer</td>
<td>A549, COR-L23</td>
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<td>(Fan et al. 2015; Manoharan, Jaiswal &amp; Singh 2014)</td>
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<td>WeriRb-1</td>
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<td></td>
<td>Glioblastoma</td>
<td>1321N1, Gos-3, U-87MG</td>
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<td>(Manoharan, Jaiswal &amp; Singh 2014)</td>
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<td>MCP30</td>
<td>Prostate cancer</td>
<td>PIN, RWPE-1, PC-3, LNCaP</td>
<td>Male mouse with PC-3 cells</td>
<td>(Xiong et al. 2009)</td>
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<td>RIP</td>
<td>Tumor</td>
<td>Cell lines (in vitro)</td>
<td>Animal (in vivo)</td>
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<td>(Manoharan, Jaiswal &amp; Singh 2014)</td>
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<td>(Meng et al. 2012)</td>
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<td>(Hao et al. 2014)</td>
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<td>(Fan et al. 2008)</td>
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<td>(Fan et al. 2015)</td>
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<td>MDA-MB-231</td>
<td>Mouse with MDA-MB-231 cells</td>
<td>(Lee-Huang et al. 1999)</td>
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<td>HepG2 bearing mice</td>
<td>(Fang et al. 2012b)</td>
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<td>(Lee-Huang et al. 1995b)</td>
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<td>(Xie et al. 2011)</td>
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<td>Bladder</td>
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<td>(Shin et al. 2013)</td>
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<td>MCL</td>
<td>Nasopharyngeal cancer</td>
<td>CNE-1, CNE-2</td>
<td>Nude mouse (transplanted)</td>
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<td>Breast cancer</td>
<td>MCF-7</td>
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<td>(Liu et al. 2010)</td>
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<td>Liver cancer</td>
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<td>Breast cancer</td>
<td>MCF-7</td>
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<td>(Gasperi-Campani et al. 1991)</td>
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<td>(Kuroda et al. 2010)</td>
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<td>(Lund et al. 2014)</td>
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<td>Choriocarcinoma</td>
<td>JAR</td>
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<td>(Mi et al. 2005)</td>
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<td>GAP31</td>
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<td>MDA-MB-231</td>
<td>Mouse with MDA-MB-231 cells</td>
<td>(Lee-Huang et al. 1999)</td>
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<td>Cochinin B</td>
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<td>HeLa</td>
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<td>(Chuethong et al. 2007)</td>
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<td>Lung cancer</td>
<td>NCI-H187</td>
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<td>(Chuethong et al. 2007)</td>
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<td>Diocin-2</td>
<td>Cervical cancer</td>
<td>HeLa</td>
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<td>(Iglesias et al. 2016)</td>
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<td></td>
<td>Choriocarcinoma</td>
<td>CoLo320</td>
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<td>(Iglesias et al. 2016)</td>
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Many cell membrane receptors are expressed at a very low level in normal cells but are overexpressed in the tumor cells. Estrogen receptor α (ERα) is a membrane receptor that plays an important role in breast carcinogenesis as more than 70% of breast cancer tissues express elevated levels of ERα compared to normal breast tissue (Ali & Coombes 2000). Number of drugs has been known that target ERα expressed on breast tumor tissue. Marmorin, type I RIP, from Hypsizigus marmoreus suppressed in vivo and in vitro growth of breast tumors. It induced apoptosis in mice bearing MDA-MB-231 tumor xenografts by suppressing the expression of ERα, inducing endoplasmic reticulum (ER) stress and activating mitochondrial and death receptor-mediated apoptotic pathway. In addition, marmorin also inhibited angiogenesis by reducing the viability of human umbilical vein endothelial cells, suggesting that it has an anti-angiogenic and anti-metastatic potential (Pan et al. 2013). All these studies demonstrated that RIPS exhibit potential toxicity on breast tumor cells; however, none of these studies reported about the toxicity of RIPS on normal cells, which is considered as an important aspect before any therapeutic agent is considered for clinical trials. In one of the recent studies, it was demonstrated that α-MMC, a type I RIP exerts toxic effects on Balb/c mice. These studies revealed that α-MMC inhibited cell viability and induced apoptosis in breast cancer cells through cell cycle arrest and caspase-3 activation. In the xenograft tumor models, α-MMC demonstrated significant decrease in the tumor growth at a dose of 1.2 mg/kg and 0.8 mg/kg. However, further, in vivo toxicity experiments revealed that at a dose above 1.2 mg/kg, balb/c mice began to show signs of toxicity such as loss of body weight and changes in serum biochemical parameters, suggesting that α-MMC has a narrow therapeutic window in vivo, which could limits its use in clinical applications (Cao et al. 2015). Therefore, to reduce the toxic effects and achieve improved therapeutic efficacy, researchers modified α–MMC with polyethylene glycol (PEG) and investigated its anti-tumor efficacy in MDA-MB-231 transplanted tumor mouse models. They demonstrated that PEGylation increased plasma life and improved anti-tumor efficacy of α–MMC and mitigated non-specific toxicity, suggesting an alternative approach to reduce toxicity of RIPS on normal cells and improving the effectiveness of RIPS towards breast cancer treatment (Deng et al. 2016).
1.8.3.2 Effect on liver cancer

RIPs from plants have also expressed in vivo and in vitro growth inhibitory activity towards liver cancer cells. MAP30 exerted both cytostatic and cytotoxic effects on human hepatocellular carcinoma HepG2 cells where the upregulation in the expression level of caspase-8, -9 and -3, and PARP after MAP30 treatment suggested that caspase-8 regulated extrinsic and caspase-9 regulated intrinsic cell death pathways were involved in MAP30 induced HepG2 cell apoptosis. Moreover, treatment of HepG2 cells with MAP30 resulted in activation of a serine/threonine kinase (Akt), p38 mitogen activated protein kinase (p38MAPK) and extracellular signal-regulated kinase (ERK), however, it did not had significant effect on JNK activation, suggesting that MAP30 induced apoptosis involves activation of Akt and MAPK/ERK signaling pathway, but not JNK signaling pathway in HepG2 cells. Further, MAP30 induced apoptosis resulted in reduction of tumor volume in HepG2 bearing mice, which merits clinical trials of MAP30 in the management of liver cancer (Fang et al. 2012b). Similar studies showed that α-luffin could inhibit the growth and induce apoptosis in HepG2 cells in a dose dependent manner with an IC50 value 7.03±1.03 µg/ml (Liu et al. 2010). Further, cucurmosin from Cucurbita moschata induced G0/G1 phase cell cycle arrest and apoptosis by increasing Bax protein expression and decreasing Bcl-2 and pro-caspase 3 expressions in HepG2 cells. In vivo studies demonstrated that cucurmosin significantly reduced tumor weight with an inhibitory rate of 78.4% observed in HepG2 bearing mice treated with 1 mg/kg cucurmosin (Xie et al. 2011).

Dexamethosane is a drug used to treat various cancers. It was reported that dexamethosane could enhance TCS induced apoptosis in HepG2 cells. Dexamethosane may inhibit dissociation of nuclear factor kappa-light chain enhancer of activated B cells (NF-κB) in the cytoplasm and transcription of the IκB-α gene leading to suppression of NF-κB activation and enhanced TCS induced anti-tumor effect (Li et al. 2010c). Flow cytometry and chorioallantoic membrane assay studies showed that TCS could inhibit tumor growth and angiogenesis in BALB/c mice bearing H22 cells (Zhang et al. 2011).

Not much has been reported about the potential cytotoxicity of RIPs on liver cancer cells, but based on these evidences, it could be anticipated that RIPs possibly have a
potential anticancer properties against human hepatoma. However, these studies further warrants detailed investigation of RIPs in the management of liver cancer.

1.8.3.3 Effect on other cancer cells

RIPs from plants have been effective against various other tumors including lung cancer, prostate cancer, choriocarcinoma, bladder cancer, cervical cancer, leukemia, colorectal cancer, pancreatic cancer and nasal pharyngeal cancer.

Some early studies in the 20th century showed that TCS exerts significant inhibitory effects on HeLa cells (Dou & Li 2004). TCS induced apoptosis of HeLa cells through S phase cell cycle arrest and activation of caspase family mediated apoptosis that critically involved caspase-3, -8, and -9 (He & Li 2006). Other studies showed that TCS mediates apoptosis in HeLa cells by interfering with the PKC/MAPK and ER-stress signaling pathway (Huang et al. 2010; Wang et al. 2009). Further investigations revealed that cAMP response element binding (CREB) protein plays an important role in TCS induced apoptosis in HeLa cells. CREB is involved in the regulation of Bcl-2 expression. Usually, phosphorylation of CREB increases the expression of Bcl-2. However, TCS treatment decreased the phosphorylated CREB protein level and Bcl-2 expression resulting in HeLa cell apoptosis (Wang, Xu & Zhang 2010). Interestingly, increased cytosolic calcium was also stimulated in TCS induced apoptosis that inhibited cAMP levels through suppression of adenylyl cyclase activity in HeLa cells (Jiang et al. 2011). In CaSki cervical cells, TCS arrested cell cycle at G1/G2 phase and upregulated Smac, a mitochondrial protein that promotes caspase activation in the caspase-9 apoptotic pathway (Cui et al. 2015; Peng et al. 2011). TCS also exerted antiproliferative activity in colorectal carcinoma LoVo cells in vitro (Gao et al. 2010).

Further, the antitumor and immunomodulatory effect of TCS was studied in a murine lewis lung cancer model. The studies revealed that TCS inhibited cell proliferation, induced apoptosis and retarded tumor growth in the 3LL tumor model. The level of IFN-γ producing CD4+ and CD8+ T cells increased in tumor bearing mice. Moreover, TCS upregulated the expression of tumor suppressor lung cancer 1 (TSLC-1) and class-I restricted T cells associated molecule (CRTAM). It was further elucidated that TCS treatment enhanced antitumor immunity through TSLC-1 and CRTAM interaction, as blocking TSLC-1 expression with siRNA completely eliminated the effect of TCS on effector T cells (Cai et al. 2011). In vivo studies on Swiss-Df strain
nude mice transplanted with colorectal adenocarcinoma SW-1116 cells in subrenal capsules showed that TCS at a dose of 0.05 mg/kg was effective enough to inhibit the growth of tumor by 27.5% in this model (Zhenwu, Yuxin & LingJi 1997). Further, low concentration of TCS induced apoptosis in human epithelial type 2 (HEp-2) and AMC-HN-8 human laryngeal epidermoid carcinoma cells via activation of JNK/MAPK signaling pathway and S phase cell cycle arrest (Zhang et al. 2015).

Interestingly, researchers have also attempted to explore the mechanism by which TCS enters inside the cancer cells. Dai and co-workers conjugated colloidal gold to TCS and found that TCS specifically enters cultured choriocarcinoma JAR cells through receptor mediated endocytosis that enables rapid transport and accumulation of TCS in the JAR cells (Dai et al. 1993). Further in depth studies by other group confirmed that LDL receptor family members, LRP and megalin, mediate the entry of TCS in the cells (Chan et al. 2000; Chan, Huang & Tam 2003). Jiao and co-workers elaborated that LDL receptor related protein 1 (LRP1) is an essential receptor that is responsible for binding and internalization of TCS into JAR cells, which might be the molecular basis of the abortifacient and anti-choriocarcinoma activity of TCS. The results were further verified by silencing the LRP1 gene in the cells which ultimately blocked the binding and endocytosis of TCS (Jiao & Liu 2010). However, Xia and colleagues contradicted the above findings and reported that TCS has specificity for two proteins found on the surface of JAR cell and these proteins are neither LRP and megalin nor CCR5 (Xia et al. 2006). Production of ROS and influx of calcium were also stimulated in TCS induced apoptosis of human choriocarcinoma cells suggesting that generation of ROS is a calcium dependent process and is involved in TCS induced apoptosis in JAR cells (Chun-Yang et al. 2000; Zhang et al. 2001). Similarly, trichomislin from Trichosanthes kirilowii could also bind and enter JAR cells and induce apoptosis by releasing cyt-c from mitochondria and stimulating a cascade of caspases in JAR cells (Mi et al. 2005). Based on these evidences, it could be concluded that TCS has specificity for the receptors expressed on the surface of cancer cells, however, detailed investigation is required to identify precise mechanism by which TCS enters inside the choriocarcinoma cells.

RIPs from Momordica charantia have also demonstrated to exhibit antitumor activity against various cancers. α-MMC and MAP30 from Momordica charantia could
inhibit the proliferation of human lung adenocarcinoma epithelial A549 cells. Cell cycle arrest at S phase and DNA fragmentation were the main apoptosis characteristics observed in α-MMC and MAP30 treated cells. However, MAP30 was found to be more potent in inhibiting tumor growth compared to α-MMC (Chuethong et al. 2007; Fan et al. 2015). In choriocarcinoma cells, α-MMC exhibited antiproliferative activity on JAR (human placental choriocarcinoma) cells (Ng et al. 1994). The analysis of the effect of PEGylated and unPEGylated α-MMC and MAP30 on JAR cells showed that PEGylated α-MMC and MAP30 were 21% and 25% more effective in inhibiting cell proliferation at the highest concentration tested compared to their native forms (Meng et al. 2012). In similar studies, α-MMC supressed the growth of normoxic and hypoxic nasopharyngeal carcinoma (NPC) by blocking the expression of growth factors (e.g. HIF1 α, VEGF and UPR) involved in survival signaling and stimulating apoptotic pathways mediated by mitochondria or death receptors (Pan et al. 2014). Interestingly, α- and β- MMC have also been found to be effective against various other tumors including glioblastoma, retinoblastoma, lung cancer and melanoma (Manoharan, Jaiswal & Singh 2014).

Several other RIPs have also been reported to exhibit anti-tumor activity against various cancer cells. Cochinin B from *Momordica cochinchinensis* exerted strong antiproliferative activity on a human small lung cancer cell line (NCI-H187) with IC_{50} of 574 mM (Chuethong et al. 2007). Li and colleagues demonstrated that TH-R (tianhua) extracted from *Trichosanthes kirilowii* inhibited proliferation of lung cancer cells. The protein inhibited cell growth and induced apoptosis via G0/G1 cell cycle arrest. Telomerase activation is an indication of carcinogenesis. Interestingly, it was found that TH-R could supress the telomerase activity in A549 cells, inhibiting the process of lung tumorgenesis (Li et al. 2010a). Similarly, the effect of cucurmosin, from *Cucurbita moschata*, was examined on chronic myeloid leukemia K562 cells. The cells treated with cucurmosin demonstrated cell shrinkage, cell cycle arrest, DNA fragmentation, activation of the caspase-3 cascade and decreased Bel-2/Bax ratio that induced apoptosis in K562 cells (Xie et al. 2011). Nevertheless, the protein also triggered programmed cell death in human pancreatic cancer BxPC-3 and PANC-1 cells by blocking epidermal growth factor receptor (EGFR) signaling pathway (Wang et al. 2014; Zhang et al. 2012). In addition, curcumosin downregulated the expression of proteins (e.g. PI3K, Akt, p-Akt, mTOR, p-mTOR, P70S6K-a, p-P70S6K-a, 4E-BP1
and p-4E-BP1) involved in P13K/Akt/mTOR signaling pathway resulting in apoptosis of human pancreatic cancer CFPAC-1 cells (Xie et al. 2013).

HDAC (histone deacetylases) are a class of enzymes that catalyse the removal of acetyl groups from N-termini of histones resulting in chromatin condensation and transcriptional repression. HDAC1 is usually overexpressed in human premalignant and malignant lesions. HDAC inhibitors selectively induce apoptosis in neoplastic cells and are therefore recognized as a class of antitumor compounds. Recent studies showed that type I RIPs from plants also acts as HDAC inhibitors and can be employed in the treatment of prostate cancer. *Momordica charantia* protein 30 (MCP30) isolated from bitter melon seeds has been shown to induce apoptosis in PIN and PCa cell lines *in vitro*. MCP30 inhibited HDAC1 activity and promoted histone 3 and 4 protein acetylation. Interestingly, MCP30 also inhibited Akt phosphorylation, an essential step in the Akt signaling pathway that promotes survival and growth of cancer cells. The Akt pathway regulates Wnt signaling. MCP30 inhibited Wnt signaling activity through reduction of nuclear accumulation of β-catenin and decreased levels of c-Myc and cyclin-D1 (Xiong et al. 2009). These finding suggest that RIPs can also induce apoptosis by inhibiting HDAC apart from activating other protein kinases such as Akt, p38MAPK and ERK within the cancer cells, which could enhance its importance as a potent anticancer agent, however, side effect and toxicity of RIPs on normal cells remains a challenge. The section below describes various strategies employed to overcome these problems.

### 1.9 RIPs from bioconjugates to nanoconstructs for cancer therapeutics

RIPs exhibit anti-proliferative activity towards various cancer cells and the mechanism of apoptosis varies with the cancer cell type. Although several mechanisms involved in RIP-induced apoptosis have been elucidated, determination of optimal dose of RIPs and precise mechanism is necessary to establish the scientific basis for the potential application of RIPs in the treatment of cancer. Also, it becomes necessary to develop strategies that can enable selective targeting of RIPs to malignant cells in order to minimize its effects on normal biochemical events in the mammalian system.
Monoclonal antibody targeted therapy is one such approach that has been exploited for the treatment of cancer and involves combining monoclonal antibody to RIP to develop chimera with superior antigen binding and pharmacokinetic properties. Kuroda and his co-workers developed an immunotoxin by conjugating humanized anti-PSMA monoclonal antibody (hj591) to saporin. The treatment of prostate-specific membrane antigen (PSMA) positive cell lines, LNCaP and CWR22Rv1, with immunotoxin inhibited the growth of cells with IC\textsubscript{50} of 0.14 nM and 1.99 nM, respectively. The population of apoptotic cells were found to be 60.29\% and 40.73\% in LNCaP and CWR22Rv1 cell lines, respectively. However, no such effect was observed in the PSMA negative cell line, PC-3. Also, hj591 coupled with streptavidin-saporin (SAZAP), named as hj591-SAZAP immunotoxin, exhibited potent antitumor activity in LNCaP xenograft model (Kuroda et al. 2010), suggesting that conjugation of monoclonal antibody with RIPs could be an improved and effective therapy for cancer. Nevertheless, many problems such as competition between free antibodies and immunoconjugates in the recognition of target sites; decrease of intracellular cytotoxic RIPs and increase of side effects on several organs are associated with this approach.

Protein or peptide conjugated RIPs is another strategy that has been employed to improve therapeutic application of RIPs. This approach involves fusion of RIPs with carriers such as cell binding ligands, hormones, protease inhibitors, etc, to develop a bifunctional cytotoxic agent that binds to specific receptors expressed on the surface of tumor cells. Transferrin receptor is widely distributed in different cell types and usually overexpressed in malignant cells. A transferrin receptor peptide (TfRBP) was fused to curcin, a type I RIP, to develop a conjugate that selectively inhibited the growth of HepG2 cells overexpressing transferrin receptor. Interestingly, this conjugate demonstrated low inhibitory effect on SKBR-3 cells expressing low levels of transferrin receptors (Zheng et al. 2013). MAP30 expressed in an E.coli BL21(DE3) strain demonstrated growth inhibitory effects and induce apoptosis in bladder cancer 5637 cells in a dose and time dependent manner (Hao et al. 2014). However, low efficiency of rMAP30 to penetrate into tumor cells restricted its tumoricidial effects. In recent studies, rMAP30 fused with human derived cell penetrating peptide (HBD) exhibited significant improvement in the cytotoxic activity of rMAP30 in tested cancer cell lines, HeLa, MCF-7, 5637, MGC 803, SMMC-7721 and B16, compared to rMAP without HBD (Lv et al. 2015), suggesting that this could
be an alternative approach to increase the effectiveness of RIPS towards cancer cells, however, stability of peptide is a major issue that needs to addressed before this approach can be employed in the treatment of cancer.

In recent years, considerable efforts have been made to develop nanoparticle based RIP delivery system. Apart from greater stability, greater surface area and ease of modification, this system renders intriguing features such as size, shape and biocompatibility. In a recent study, a type I RIP from *Mirabilis jalapa L*. leaves was incorporated into a nanoparticle conjugated with Anti-EpCAM antibodies. Nanoparticles were obtained by polyelectrolyte complexation of chitosan and alginate and then chemically conjugating with Anti-EpCAM antibody by carbodiimide reaction. The RIP nanoconjugate demonstrated selective cytotoxicity against T47D breast cancer cells with no cytotoxic effect on normal cells (Wicaksono et al. 2016). In other study, curcin, a type I RIP was incorporated into gold nanoparticles (AuNPs) conjugated with folate and anti-transferrin antibody to achieve nanoconjugate that had the ability to selectively target glioma cells with minimal non-specific systemic toxicity during circulation (Mohamed et al. 2014; Pizzo and Di Maro 2016)).

Progressive development towards RIP based nanoconjugate system represents an innovative approach aimed to mitigate toxicity associated with the use of RIPS in cancer therapy.
1.10 Research objectives

Keeping in view the therapeutic and agricultural applications of RIPs, the purification of RIPs from plants carries a great importance. *Momordica balsamina* is a medicinal and nutraceutical plant advocated for health care management, however it has not received major international attention (Puri 2010). This plant comprises tendril bearing high climbing vines. The leaves, seeds, bark and fruits of the plant contain large number of medicinal compounds such as phytochemicals (alkaloids, saponins, flavonoids, glycosides and terpenes) and type I RIP. These compounds contribute towards medicinal and nutritional properties, including anti-plasmodial, anti-inflammatory, anti-diarrheal, anti-HIV, antioxidant, antimicrobial and hepatoprotective properties, of the plant (Thakur et al. 2009). Balsamin, a type-I RIP, isolated from *Momordica balsamina* constitute up to 15% of the total protein content in mature dry seeds (Kaur et al. 2012). Previous studies from our group have demonstrated that balsamin possesses potent anti-HIV activity and inhibits the translation step occurring prior to viral budding and release in the HIV-1 replication cycle (Kaur et al. 2013). In the present study, we extended the therapeutic application of balsamin from HIV to cancer with the following research objectives:

1. Purification and functional analysis of natural balsamin (nBalsamin) from *Momordica balsamina*.
2. Evaluation of cytotoxic effects of nBalsamin on liver and breast cancer cells.
4. Determination of anti-tumor activity of rBalsamin on liver and breast cancer cells.
5. Evaluation of *in vitro* cytotoxicity of nBalsamin-flavonoids combination on liver and breast cancer cells.

The studies carried out in this thesis are outlined in Figure 1.5.
Figure 1.5 Research outline and the corresponding chapters shown in the thesis.

Chapter 1

- Isolation and purification of nBalsamin from *M. balsamina* seeds.
- Biophysical characterization of nBalsamin-ESI-MS, CD and FTIR spectroscopy.
- Functional characterization of nBalsamin-RNA N-glycosidase, DNase, antimicrobial and antioxidant activity.
- Evaluation of cytotoxicity of nBalsamin on liver and breast cancer cells.
  - MTT and DNA fragmentation assay
  - Cell cycle analysis
  - Gene expression studies
  - Combined effect of nBalsamin and flavonoids on liver and breast cancer cells.

Chapter 2

- Molecular cloning of Balsamin gene from *M. balsamina* seeds.
- Optimization of parameters for the expression of rBalsamin.
- Purification of rBalsamin via Ni²⁺ affinity and gel filtration chromatography.
- Biophysical characterization of rBalsamin-CD and fluorescence spectroscopy.

Chapter 3

- Functional characterization of rBalsamin-RNA N-glycosidase, DNase, antimicrobial and antioxidant activity.
- Evaluation of cytotoxicity of rBalsamin on liver and breast cancer cells and comparison with nBalsamin.
  - MTT and DNA fragmentation assay
  - Cell cycle analysis
  - Gene expression studies

Chapter 4

- Biophysical characterization of rBalsamin-CD and fluorescence spectroscopy.

Chapter 5

- Functional characterization of rBalsamin-RNA N-glycosidase, DNase, antimicrobial and antioxidant activity.
- Evaluation of cytotoxicity of rBalsamin on liver and breast cancer cells and comparison with nBalsamin.
  - MTT and DNA fragmentation assay
  - Cell cycle analysis
  - Gene expression studies

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Chapter 2: Purification and functional analysis of natural balsamin (nBalsamin) from *Momordica balsamina*.1

1 This chapter has been published in the form of a research article in the Plant Foods for Human Nutrition journal. I exclusively conducted this work with the guidance of Prof. Munish Puri and Prof. Ken Walder.
Functional Analysis of a Type-I Ribosome Inactivating Protein Balsamin from Momordica balsamina with Anti-Microbial and DNase Activity

Parminder Kaur Ajji, Ken Walder, Munish Puri

Abstract Ribosome inactivating proteins (RIPs) have received considerable attention in biomedical research because of their unique activities towards tumor and virus-infected cells. We extracted balsamin, a type-I RIP, from Momordica balsamina. In the present study, a detailed investigation on DNase activity, antioxidant capacity and antibacterial activity was conducted using purified balsamin. DNAse-like activity of balsamin towards plasmid DNA was pH, incubation time and temperature dependent. Moreover, the presence of Mg²⁺ (10–50 mM) influenced the DNA cleavage activity. Balsamin also demonstrated reducing power and a capacity to scavenge free radicals in a dose dependent manner. Furthermore, the protein exhibited antibacterial activity against Staphylococcus aureus, Salmonella enterica, Staphylococcus epidermidis and Escherichia coli, which suggests potential utility of balsamin as a nutraceutical.

Keywords Antioxidant - Antibacterial - Bioactive - Purification - Therapeutic - Nutraceutical

Introduction

Plants contain a wide variety of bioactive proteins that make them an important functional ingredient in the food system. Ribosome inactivating proteins (RIPs) are a family of proteins that have attracted interest because of their antitumor and antiviral properties, making them a promising tool for the treatment of various diseases. RIPs are widely distributed in plants, certain bacteria and fungi [1]. These proteins are named ribosome inactivating protein because of their ability to inactivate ribosomes in an irreversible manner and remove adenine from the highly conserved loop structure of 28S rRNA, which prevents binding of elongation factor-2, resulting in inhibition of protein synthesis [2]. RIPs are categorized into three main types: Type I RIPs (Helo RIPs) comprise a single polypeptide with N-glycosidase activity and a molecular weight ranging between 20-35 kDa. These monomers can be found attached to a carbohydrate moiety. Type II RIPs (Chimero RIPs) are heterodimers where an A-chain with N-glycosidase activity is bound by a disulfide linkage to a galactose-specific lectin B-chain that enables receptor recognition on the membrane and initiates endocytosis [3]. The molecular weight of the Chimero RIPs ranges between 60-65 kDa. Type III RIPs (66-68 kDa) comprise a single polypeptide chain synthesized as an inactive precursor that undergoes proteolytic cleavage to become active [4]. Almost all of the RIPs possess similar activity (RNA N-glycosidase activity). However, they differ in their biological activities where some of the RIPs have also been reported to exhibit antifungal, antiviral and antibacterial activities [5]. For this reason, RIPs have been employed in agriculture biotechnology to produce crops resistant to insect, fungal and viral infections [6].

RIPs have been used as antitumor agents to treat various carcinomas in vitro and in vivo mouse models. It has been found that RIPs induce apoptosis in lung, liver, and prostate...
cancer cells by interfering with specific cell death or signaling pathways [7]. A number of RIPs have also been utilised for the production of heteroconjugates. RIPs have been linked to carrier molecules (such as antibodies, peptides, cytokines or aptamers) either chemically or via gene fusion to specifically target receptors on diseased cells. This has provided a rationale for the treatment of various diseases such as non-Hodgkin’s lymphoma, sarcoma and AIDS [8–10]. RIPs have been extensively explored for their antiviral activities. A significant level of anti-HIV properties possessed by RIPs provided new hope to HIV infected patients [11–13].

Besides N-glycosidase activity, a number of studies have shown additional activities for RIPs including DNA, poly (A), mRNA and polynucleotide depurination [14]. Some RIPs have also shown superoxide dismutase [15], phospholipase and antioxidant activities [16]. DNase activity has been proposed for few RIPs suggesting that RIPs can cleave supercoiled plasmids and produce a linear form of DNA, with activity depending on pH, temperature, RIP concentration and incubation time [17]. Balsamin, a type-I RIP, has been extracted from M. balsamina. Previous studies have demonstrated that balsamin possesses potent anti-HIV activity and inhibits the translation step occurring prior to viral budding and release in the HIV-1 replication cycle [11]. In the present study, the DNase-like activity of balsamin was investigated. DNA is a hereditary material that makes up the human genome. Within the cellular machinery, genes undergo transcription to produce mRNA that gets translated to produce proteins. However, degradation of DNA prevents protein synthesis leading to apoptosis. This unique characteristic is beneficial for generating a sufficient level of cytotoxicity in diseased cells, being of great impact for diseases such as cancer.

In recent years, research into the relationship between antioxidants and prevention of disease has been increasing. Free radicals generated within the body play a predominant role in processes such as inflammation and ageing, and diseases such as cancer, neurodegenerative and cardiovascular disease. This damage induced to the body can sometimes be prevented or alleviated by supplementing the diet with antioxidants [18, 19]. Various food varieties and plants contain antioxidants as active ingredients and exhibit antimicrobial activity [20]. Therefore, in this study we investigated the antioxidant and antimicrobial activity of balsamin.

**Materials and Methods**

*M. balsamina* seeds were purchased from National Seed Stock, India. Gels and columns for ion exchange and size exclusion chromatography were purchased from Sigma (St. Louis, USA). The chemicals used in the study were of analytical grade obtained from either Sigma or Bio-Rad. Bacterial strains *Salmonella enterica* ATCC 43971, *Enterococcus faecalis* ATCC 10100, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 2548 and *Pseudomonas aeruginosa* ATCC 27853 were procured from American Type Culture Collection.

**DNase-Like Activity of Balsamin** Balsamin was purified according to the methods reported by Kaur et al. [21] and was characterized by SDS-PAGE. To initially determine DNase-like activity of balsamin, pET-30b (+) plasmid was used as a substrate. Various concentrations of balsamin were incubated with 0.25 μg pET-30b (+) plasmid DNA in a final volume of 10 μl of assay buffer (50 mM Tris HCl, pH 7.5, 50 mM KCl, 0.1 mM MgCl₂). The reaction mixture was incubated at 37 °C for 2 h. Digestion of the plasmid with restriction endonuclease EcoRI served as a positive control.

To determine the response of incubation time on enzymatic activity, 0.25 μg of plasmid was treated with 0.5 μg balsamin in a final reaction mixture of 10 μl of assay buffer. The reaction setup was incubated at 37 °C for various time intervals (ranging from 30 to 240 min). The effect of temperature (from 5 to 55 °C, with an increment of 10 °C) was also tested for 2 h incubations. To determine the effect of pH on DNase-like activity of balsamin, assay buffer (50 mM Tris HCl, pH 7.5, 50 mM KCl, 0.1 mM MgCl₂) with final pH values of 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 were prepared. 10 μl of reaction mixture was prepared with 0.25 μg DNA, 0.5 μg balsamin and assay buffer at various defined pH. The reaction was incubated at 37 °C for 2 h. To investigate the effect of magnesium ion concentration on enzymatic activity, assay buffer with different Mg²⁺ levels was prepared. Buffer without Mg²⁺ served as a negative control. 0.25 μg DNA was incubated with 0.5 μg balsamin in a final reaction mixture of 10 μl made with the assay buffer varying in Mg²⁺ concentration (0–50 mM MgCl₂). The incubation was carried out at 37 °C for 2 h. At the end of the reaction, DNA was extracted using Qiagen PCR purification kit and analyzed on a 0.8 % agarose gel. DNA bands were visualized with ethidium bromide staining.

**Antioxidant Activity**

**Free Radical Scavenging Activity** The free radical scavenging activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Boonmee et al. [22]. Absorbance was measured at 517 nm using spectrophotometer. The DPPH free radical scavenging activity was calculated using the formula:

% scavenging activity = \( \frac{(Ab\text{ (control)} - Ab\text{ (sample) })}{Ab\text{ (control)}} \) × 100

**Superoxide Radical Scavenging Activity** To different concentrations of balsamin (0.5–2 mg/ml) or standard solution of ascorbic acid (25–500 μg/ml), 100 μl riboflavin (20 μg),
200 µl EDTA solution (12 mM), 200 µl methanol and 100 µl NBT (0.1 mg) was added. The final volume of each sample was made up to 3 ml with phosphate buffer (50 mM, pH 7.8). The absorbance was measured at 590 nm using phosphate buffer as a blank after 5 min of illumination. The superoxide scavenging activity was calculated as follows for free radical scavenging activity (above).

Reducing Power Ability The reducing power of balsamin was determined as described by Hong et al. [23]. The absorbance was measured at 700 nm using a spectrophotometer. Phosphate buffer and ascorbic acid (10–100 µg/ml) served as a blank and positive control, respectively.

Antibacterial Activity

Disc Diffusion Method Bacterial strains were streaked on Iso-Sensitest agar (ISA; Oxoid) plates and incubated overnight at 37 °C. Isolated colonies of each bacterial strain were inoculated in 5 ml of Iso-Sensitest (IS) broth and grown overnight at 37 °C in a shaker incubator. The bacterial cultures were inoculated into fresh IS broth at a ratio of 1:100 and grown with agitation at 37 °C until an absorbance (OD600nm) of 0.6 was reached. 50 µl of the inoculum was evenly spread on the ISA plate. 4 mm filter paper discs impregnated with 20 µl of each of the different dilutions of balsamin (50 µg/ml, 100 µg/ml and 200 µg/ml) were placed onto the surface of the agar. The plates were incubated overnight at 37 °C. After incubation, the zone of inhibition (ZI) was determined by measuring the diameter using a ruler. In all experiments, phosphate buffer saline (PBS) was used as a negative control. Experiments were carried out in triplicate with data expressed as mean ± SD.

Broth Microdilution Method Isolated colonies of each bacterial strain were inoculated in 5 ml of IS broth and grown overnight at 37 °C in a shaker incubator. The next day, the suspension was adjusted to achieve absorbance (OD625nm) between 0.08–0.13 (equivalent to 0.5 Mc Farland turbidity standard). Susceptibility panels in 96-well microtiter plates were prepared by dispensing 100 µl of 200 µg/ml of balsamin into the first column wells and 50 µl of IS broth into the rest wells. Two-fold serial dilutions of balsamin were made drawing up 50 µl of balsamin from the first well into the second well and so on. 50 µl of each bacterial suspension was added into the well to obtain final volume of 100 µl. Ampicillin (50 µg/ml) and PBS was used as positive and negative controls, respectively. The plates were sealed and incubated at 37 °C for 24 h. After incubation, absorbance were measured at 625 nm. The MICs of balsamin were recorded as the lowest concentration where reduced bacterial numbers were observed in the wells based on absorbance measurements. Experiments were carried out in triplicate with data expressed as mean ± SD.

Statistical Analysis

Data was analyzed using SPSS 22.0 software. All data were shown as mean ± standard deviation. Data was assessed for normality using Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) was used to compare the mean differences among multiple groups and repeated measurements within one group. Independent samples t-test was used to compare means between two groups. Probability values less than 0.05 were considered statistically significant.

Results and Discussion

M. balsamina is a medicinal and nutraceutical plant advocated for health care management, however it has not received major international attention [24]. Balsamin is a RIP that has been isolated from the seeds of M. balsamina. Previous studies have shown that balsamin exhibits potent anti-HIV activity [11]. However, the other functional properties of balsamin have not yet been reported and thus have been investigated in this study.

Optimization of DNase-Like Activity of Balsamin To initially determine DNase-like activity of balsamin, pET-30b (+) plasmid was used as a substrate. The method used in our study has been described and applied, for the first time, to a M. balsamina protein. Treatment of supercoiled pET-30b (+) plasmid with balsamin caused changes in the conformation of the DNA as visualized on agarose gels. Balsamin converted supercoiled DNA into open circular and linear forms. Plasmid treated with EcoR1 served as a positive control as it cleaved the DNA and converted it into linear form (Fig. 1a, Lane PC). Normal plasmid without any treatment with balsamin (negative control) showed the presence of supercoiled and open circular forms (Fig. 1a, Lane NC). However, balsamin treated samples produced an additional linear DNA form. With a low amount of balsamin (0.5 µg), the DNA band gradually appeared and became more diffuse while the intensity of the supercoiled DNA form decreased (Fig. 1a). In addition, the amount of open circular DNA initially increased and then decreased suggesting that the balsamin first cleaved the supercoiled form into open circular conformation, and then to a linear form of DNA.

To optimize DNase activity, freshly prepared pET-30b (+) was treated with balsamin under various reaction conditions to examine the changes in DNA conformation. The experiments showed that the activity of balsamin was time dependent. A small amount of linear DNA was produced in 30 min of incubation. However, as the reaction proceeded, the appearance of the linear form of DNA became prominent, while the
superoiled DNA became inconspicuous and completely disappeared after 120 min. In addition, the amount of open circular DNA initially increased and then decreased as the reaction proceeded (Fig. 1b).

Balsamin remained inactive against DNA at 5 and 15 °C as no change in the amount of supercoiled and open circular form of the plasmid was observed. However, with an increase in temperature to 25 °C, appearance of linear DNA was observed with a decline in the amount of supercoiled DNA. At the optimum temperature range of 35 to 45 °C, complete conversion of the supercoiled form to open circular and linear conformations was observed, while the DNase-like activity of balsamin decreased as the temperature was raised to 55 °C (Fig. 1c). In addition, the activity was found to be pH dependent. At a low pH range of 1.5 to 2.5, DNA seemed unstable as no bands were observed after incubation of pET-30b (+) with balsamin. However, with the increase in pH to 3.5, the DNA seemed stable but balsamin was inactive as no change in the amount of closed circular form was observed when compared to control sample. In the pH range of 4.5 to 8.5, balsamin was active against plasmid DNA with complete cleavage of the supercoiled DNA demonstrated in the pH range of 6.5 to 7.5 (Fig. 1d).

Many enzymes require divalent metal ions for nuclease activity. Our investigation showed that the DNase-like activity of balsamin was highly dependent on Mg²⁺ as the exclusion of
Mg\textsuperscript{2+} from the assay buffer completely inhibited balsamin activity and no effect was observed on the supercoiled plasmid DNA. However, the activity was restored by the addition of Mg\textsuperscript{2+} (Fig. 1e). Interestingly, the ionic strength of the assay buffer also affected the enzymatic activity of balsamin with the complete conversion of supercoiled DNA obtained in the ionic strength range of 10 mM–40 mM Mg\textsuperscript{2+} (Fig. 1e).

### Table 1: Antibacterial activity of balsamin towards pathogenic microorganisms

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Zone of inhibition in mm ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein concentration (µg/ml)</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>3 ± 0.2</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>2 ± 0.1</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>4 ± 0.3</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are mean ± SD based on three set of experiments; ND – not detected.

Analysis of DNase-like activity of balsamin revealed that rRNA is not the only substrate of this RNP, but it can also act on deoxyribonucleotides. The dependence of DNase-like activity on pH, temperature and Mg\textsuperscript{2+} suggested that it might play a role in plant protection and other biological processes under inconsistent environmental conditions. Similar results have been reported with α-momorcharin treated pET-32a (+) plasmids [25, 26]. Trichosanthin could also cleave supercoiled φX174 RF, pBluescript II and SV40 DNA into nicked circular and linear DNA [27]. Many nucleases require divalent cations as cofactors for their activity. The nuclease activity of *Arabidopsis sulfurea* (AtSx), a redox dependent sulfenic acid reductase, was significantly enhanced by the addition of metal ions such as Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, Mn\textsuperscript{2+} and Co\textsuperscript{2+} to the reaction mixture containing pUC 19 as a substrate [28]. Interestingly, the requirement of Mg\textsuperscript{2+} for the nuclease activity of balsamin suggested that divalent metal binding site(s) might be present in the DNA cleavage domain of balsamin. The DNase-like activity of balsamin enhances its importance and potential for disease treatment, as damage to the DNA would suppress transcription and translation within the cells, ultimately leading to cell death. This approach could be used to hamper the growth of cancer cells and spread of disease within the body. However, further studies are required to understand the underlying

### Table 2: Minimum inhibitory concentrations (MICs) of balsamin against different pathogenic microorganisms

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>MIC of balsamin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>6.25</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>25.00</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>12.50</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>1.56</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td>12.50</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>50.00</td>
</tr>
</tbody>
</table>
Chapter 2

mechanism of deoxyribonucleolytic activity of RIPs and their possible involvement in the biological processes.

Antioxidant Activity of Balsamin Some RIPs have shown to possess antioxidant activity. TRIP (tobacco ribosome inactivating protein) isolated from tobacco leaves demonstrated 60 % NBT reduction at 500 ng/ml [15]. Similarly, some other RIPs such as camphorin from Cinnamomum camphora [29] and Cucurbita moschata RIP [30] have exhibited superoxide dismutase (SOD) activity. However, α-monomorcharin and momordica anti-HIV protein (MAP) do not possess superoxide dismutase activity [31]. In our study, the concentration of balsamin significantly affected the antioxidant activity. DPPH was used to determine the free radical scavenging activity of balsamin with ascorbic acid as a positive control (Fig. S1). Scavenging increased as balsamin concentration was increased up to a maximum effect at 1.5 mg/ml, and decreased slightly at a higher concentration (Fig. 2a). The superoxide radical scavenging activity of balsamin was measured by riboflavin/NBT reduction with ascorbic acid as a positive control (Fig. S2). Balsamin was able to scavenge superoxide radical in a dose dependent manner with an increase in activity observed up to 1.5–2.0 mg/ml (Fig. 2b). Furthermore, balsamin exhibited strong reducing power in a concentration dependent manner. The reducing ability increased as the concentration of balsamin increased up to a maximum effect at 1.5 mg/ml, and decreased slightly at higher concentrations (Fig. 2c). Ascorbic acid served as a control (Fig. S3). Therefore, balsamin can be considered as a valuable food ingredient with high antioxidant activity.

Antibacterial Activity of Balsamin RIPs are a class of defense proteins, which could function as antimicrobial agents [32]. α-monomorcharin from M. charantia has been found to impede the growth of several bacterial and fungal pathogens including Fusarium oxysporum, Fusarium solani, E. coli, P. aeruginosa, S. aureus and Bacillus subtilis [33]. RIP from C. moschata was found to inhibit the growth of plant pathogens such as Pseudomonas solanacearum, Phytophthora infestans and Erwinia amylovora [34]. In our study, balsamin exhibited a dose dependent inhibition towards all microorganisms with maximum inhibition observed at the highest dose (200 μg/ml). S. aureus, S. enterica, S. epidermidis and E. coli demonstrated susceptibility towards balsamin. However, P. aeruginosa and E. faecalis were found to be less susceptible to balsamin. The zone of inhibition generated by different dilutions of balsamin on different pathogenic bacteria varied between 2–9 mm (Fig. S4, Table 1). Minimum inhibitory concentrations (MICs) of balsamin were determined by the broth microdilution method. The MICs of balsamin were found to be 6.25 μg/ml for S. aureus, 25 μg/ml for P. aeruginosa, 12.5 μg/ml for E. coli and S. enterica, 1.56 μg/ml for S. epidermidis and 50 μg/ml for E. faecalis (Fig. S5, Table 2). Therefore, balsamin could be considered as a promising candidate for disease protection.

Conclusions

Based on our findings it can be inferred that balsamin, a ribosome inactivating protein, is an important molecule in M. balsamin seeds that contributes towards its DNase-like activity, total antioxidant and broad-spectrum antibacterial activity, and could have utility for disease protection in humans as well as in plants. Therefore, it can be considered as a potent functional food or value added ingredient that could find significant importance in preventing and slowing the progress of ageing, neurodegenerative and cardiovascular diseases, cancer and bacterial infections.

Acknowledgments The authors are thankful to Professor Colin J. Barrow, Director, Centre for Chemistry and Biotechnology, Deakin University for providing the necessary facility to carry out the research work. PKA thanks Dr. Inderdeep Kaur for advising on methodology optimization.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights This article does not contain any studies with human or animal subjects.

References


Supplementary information

Supplementary Figure 1: DPPH scavenging activity of ascorbic acid. All values are mean ± SD. SD, standard deviation; n = 3; *P = 0.002, Kruskal-Wallis test.

Supplementary Figure 2: Superoxide radical scavenging activity of ascorbic acid. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.001, Kruskal-Wallis test.
Supplementary Figure 3: Reducing power ability of ascorbic acid. All values are mean ± SD. SD, standard deviation; n = 3; *P = 0.001, Kruskal-Wallis test.
Supplementary Figure 4: Antibacterial activity of different dilutions of balsamin against *Staphylococcus aureus* ATCC 25923 (A), *Pseudomonas aeruginosa* ATCC 27853 (B), *Escherichia coli* ATCC 2348 (C), *Staphylococcus epidermidis* ATCC 12228 (D), *Salmonella enterica* ATCC 43971 (E), *Enteroccus faecalis* ATCC 10100 (F). Disc 1, 2, 3 contains 50 μg/ml, 100 μg/ml, and 200 μg/ml of balsamin respectively; disc 4 contains control PBS in each plate.
Supplementary Figure 5: Dose dependent inhibition of *Staphylococcus aureus* (A), *Pseudomonas aeruginosa* (B), *Escherichia coli* (C), *Staphylococcus epidermidis* (D), *Salmonella enterica* (E) and *Enterococcus faecalis* (F) with balsamin. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.05.
Additional information for Chapter 2:

**Methodology:**

**Purification of natural balsamin (nBalsamin) from *Momordica balsamina:***

Purification was performed according to Kaur et al. (2012) as shown in Supplementary Figure 6. *Momordica balsamina* seeds (10 g) were grounded into fine powder using a motor and pestle and homogenized in 50 ml of 150 mM NaCl. The slurry obtained was centrifuged at 12,000 X g for 20 min at 4°C. Following centrifugation, the proteins were precipitated from the supernatant with the addition of ammonium sulphate. The precipitate obtained after centrifugation was dissolved in 10 mM phosphate buffer (PB), pH 6.5 and dialysed against the same buffer. Following equilibration of a CM-Sepharose column with PB, pH 6.5, the dialysed sample was loaded onto the column and elution of unbound protein was carried out with PB, pH 6.5. The bound proteins were eluted using linear gradient of NaCl (0.1-0.4 M) in PB, pH 6.5. The peak fractions containing the protein of interest (analysed by SDS-PAGE) were pooled, concentrated and loaded onto a Hi Load™ 26/60 Superdex™ 75 prepacked column equilibrated with PB, pH 6.5. The eluted fractions with the desired protein were pooled, concentrated and further used to determine various enzymatic and biological activities.

Protein concentrations were determined using bovine serum albumin as standard. The molecular weight of the purified protein was determined by SDS-PAGE using a 12% separating gel. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 (CBB).

**Assay for RNA N-glycosidase activity**

The assay was performed as per Bagga et al. (2003). nBalsamin was incubated with rabbit reticulocyte lysate at 30°C for 30 min. 10% SDS (w/v) was added to terminate the reaction and total rRNA was extracted using an Ambion RNAqueous® Kit. The RNA obtained was divided into two parts where one part was treated with aniline acetate (pH 4.5) and the other part was left untreated. The samples were incubated at 60°C for 3 min. The treated and untreated samples were analyzed on a 2% agarose gel and visualized using a Gel Doc™ XRS+ system (Bio-Rad, Hercules, CA, USA).
Supplementary Figure 6: Diagrammatic representations of purification strategy.
Biophysical characterization of nBalsamin

Electrospray ionization- Time of flight (ESI-TOF) mass spectrometry

ESI-TOF mass spectrometric analysis was carried out to determine the molecular weight of nBalsamin. All analyses were done on an Agilent 6220 ESI-TOF LC/MS mass spectrometer coupled to an Agilent 1200 LC system (Agilent, Palo Alto, CA) with the following experimental conditions:

- Ionisation mode: Electrospray Ionisation
- Drying gas flow: 7 L/min;
- Nebuliser: 35 psi;
- Drying gas temperature: 325°C;
- Capillary Voltage (Vcap): 4000 V;
- Fragmentor: 250 V;
- Skimmer: 65 V;
- OCT RFV: 250 V;
- Scan range acquired: 100–3200 m/z
- Internal Reference ions: Positive Ion Mode = m/z = 121.050873 & 922.009798
- Chromatographic separation was performed using an Phenomenex Aeris Widepore-C4 2.1 x 150 mm, 3.6μm column (Phenomenex, NSW, Australia) using an acetonitrile gradient (5% (v/v) to 75% (v/v)) over 15 min at 0.25 mL/min.
  - Solvent A = aqueous 0.1% (v/v) formic Acid
  - Solvent B = acetonitrile/0.1% (v/v) formic Acid

All data were acquired and reference mass corrected via a dual-spray electrospray ionisation (ESI) source. Mass spectra were created by averaging the scans across each peak and background subtracted against the first 10 seconds of the TIC. Acquisition was performed using the Agilent Mass Hunter Acquisition software version B.02.01 (B2116.30) and analysis was performed using Mass Hunter version B.03.01.
Circular dichroism (CD) spectroscopy

CD analysis of nBalsamin (450 μg/ml in 10 mM phosphate buffer, pH 6.5) was carried out on Jasco J-815 CD spectrometer (Jasco Inc., Easton, MD, US). Spectra in the far UV range (190-260 nm) were recorded at a scanning speed of 100 nm/min in 1 mm path length cells. The analysis of CD spectrum was performed using Spectra manager™ software (Jasco).

Fourier transform infrared (FTIR) spectroscopy

The FTIR analysis of nBalsamin was performed to determine the functional groups present in the protein. FTIR measurements were performed using an Alpha FTIR spectrometer (Bruker Optik GmbH, Ettlingen, Germany). The FTIR spectra were acquired at 4 cm⁻¹ spectral resolutions with 24 scans. Blackman-Harris 3-Term apodization, PowerSpectrum phase correction, and zero-filling factor of 2 were set as default acquisition parameters using the OPUS 7.0 software suite. Background spectra of a clean attenuated total reflection (ATR) surface and PB (pH 6.5) were acquired prior to sample measurement using the same acquisition parameters.

Results and discussion

Purification of nBalsamin

nBalsamin was extracted from the seeds of *Momordica balsamina* as described by Kaur et al. 2012. Most of the protein precipitated at 60% ammonium sulphate saturation. After dialysis, the sample was subjected to CM-Sepharose column equilibrated with phosphate buffer (10 mM PB, pH 6.5). Most of the protein retained on CM-Sepharose column indicating that proteins collected is mainly basic in nature. Two peaks (BI and BII) were obtained using NaCl linear gradient (0.1-0.4 M) (Supplementary Figure 7a). SDS-PAGE analysis revealed that peak BII contained nBalsamin (Supplementary Figure 7b). Peak BI fractions were pooled, concentrated and further purified using a Superdex 75 gel-filtration column. Two peaks (PI and PII) were obtained (Supplementary Figure 8a) and analysed by SDS-PAGE (Supplementary Figure 8b). Peak PI fractions containing nBalsamin were pooled, concentrated and stored at -20°C until further analysis. The purified
nBalsamin showed a single band with a molecular weight corresponding to ~28 kDa by 12% SDS-PAGE (Supplementary Figure 8c). Multiple forms of RIPs from *Momordica charantia*, namely, α- MMC, β- MMC, γ- MMC, δ-MMC and ε-MMC and MAP30 have been reported (Kaur et al. 2012). Most of the RIPs possess molecular mass in the range of 24–30 kDa (Puri et al. 2009), indicating that the molecular weight of nBalsamin (28 kDa) is in agreement with the published data.

Supplementary Figure 7: a) Profile of ion exchange chromatography of nBalsamin. Fractions corresponding to the peaks were pooled separately and denoted as BI and BII. b) SDS-PAGE of BI and BII fractions. Lane 1: marker, Lane 2: flow through, Lane 3-5: fractions from peak BI and Lane 6-9: fractions from peak BII. The arrow points to the nBalsamin.
Supplementary Figure 8: a) Profile of Superdex-75 gel filtration chromatography of nBalsamin. Fractions corresponding to the peaks were pooled separately and denoted as PI and PII. b) SDS-PAGE of PI and PII fractions. Lane 1 & 2: marker, Lane 3-6: fractions from peak PI and Lane 7-10: fractions from peak PII. c) SDS-PAGE of concentrated nBalsamin. Lane 1: marker, Lane 2: concentrated nBalsamin. The arrow points to the nBalsamin.

ESI-TOF mass spectrometric analysis

ESI-TOF mass spectrometric analysis was carried out to determine the molecular weight of nBalsamin. All analyses were done on an Agilent 6220 ESI-TOF LC/MS mass spectrometer coupled to an Agilent 1200 LC system (Agilent, Palo Alto, CA). The results revealed that the molecular weight of nBalsamin is 28.26 kDa, in agreement with the predicted molecular mass of nBalsamin by SDS-PAGE (Supplementary Figure 9) and the published data (Kaur et al. 2012).

SDS-PAGE (Supplementary Figure 8c) and ESI-TOF mass spectrometric analysis
Supplementary Figure 9 (Supplementary Figure 9) indicates that nBalsamin with greater than 95% purity was obtained through purification process.

Supplementary Figure 9: ESI-TOF mass spectrometric analysis of nBalsamin.

**CD spectroscopic analysis**

CD spectroscopic analysis was carried out to determine the secondary structure of nBalsamin. CD spectra of nBalsamin indicated the presence of predominantly α-helices and random coils. nBalsamin exhibited 35.2% α-helix, 19.6% β-sheet, 12.5% turn and 32.2% random coils at 25°C (Supplementary Figure 10), which was in agreement with the published data (Kaur et al. 2012).
Supplementary Figure 10: CD spectroscopic analysis of nBalsamin.

**FTIR spectroscopic analysis**

The functional groups present in the nBalsamin were identified by FTIR spectroscopy. Peaks recorded between 3000 and 3500 cm\(^{-1}\) were expected to be due to vibration modes of O-H and –NH groups present in the protein. Peak around 2971 cm\(^{-1}\) depicted N-CH\(_3\) stretching. Peaks recorded around 1650 cm\(^{-1}\) arises mainly from -C=O stretching vibration with minor modification from the out-of-phase C-N stretching vibration, the C-C-N deformation and the NH in-phase bend, and depicts amide I of the protein. Peaks recorded around 1542 cm\(^{-1}\) arises from out of phase combination of the -NH in plane bend and the C-N stretching vibration with minor contribution from the -C=O in plane bend and the C-C and N-C stretching vibrations, and depicts amide II of the protein (Supplementary Figure 11).
Supplementary Figure 11: FTIR spectroscopic analysis of nBalsamin.

RNA N-glycosidase activity

RNA N-glycosidase activity is a unique characteristic exhibited by RIPS. The activity was performed as described by Kaur et al. 2012. nBalsamin produced a specific RNA fragment of about 400 nucleotides, named Endo-fragment. The treatment of rRNA with nBalsamin resulted in removal of adenine thereby weakening the phosphodiester bond. Therefore no fragment appeared at this stage on a 2% agarose gel. Only after the treatment of rRNA with aniline was the bond cleaved resulting in generation of an Endo fragment (Supplementary Figure 12). These results were in agreement with previous reports on RIPS from different plant species (Choudhary, Yadav & Lodha 2008; Sharma et al. 2004) confirming that nBalsamin extracted from *Momordica balsamina* belong to a family of ribosome inactivating proteins.
Supplementary Figure 12: RNA N-glycosidase activity of nBalsamin. rRNA was treated (+) with or without aniline (-) and analysed on a 2% agarose gel. The Endo-fragment generated after nBalsamin treatment is denoted with an arrow.

Besides RNA N-glycosidase activity, nBalsamin demonstrated DNase-like activity in a concentration dependent manner. The DNase-like activity of nBalsamin was found to be temperature dependent with the complete conversion of the supercoiled form of plasmid to open circular and linear conformation observed in the temperature range of 35-45°C. However, below 35°C and above 45°C, partial conversion in the supercoiled form of plasmid was observed. It could be anticipated that increasing and decreasing the temperature outside the tolerable range might cause conformational changes and affect the chemical bonds in the active site of nBalsamin decreasing their specificity towards DNA. Further, the DNase-like activity of nBalsamin was found to be pH dependent with the pH range of 6.5-7.5 observed to be optimum. The possible reason could be that the isoelectric focusing point (pI) of nBalsamin is 9.43. At pH below pI, nBalsamin establishes a net positive charge thereby increasing its specificity towards negatively charged DNA. However, lowering the pH outside the optimal range causes nBalsamin to denature resulting in loss of DNase-like activity.
Many enzymes require divalent metal ions for nuclease activity. It was found that the DNase-like activity of nBalsamin was dependent on Mg$^{2+}$ as the exclusion of Mg$^{2+}$ from the assay buffer completely inhibited balsamin activity. However, the activity was restored by the addition of Mg$^{2+}$, suggesting that divalent metal binding site(s) could be present in the DNA cleavage domain of nBalsamin. RIPs are known to depurinate adenine from highly conserved (GAGA) sequence in the stem loop region in 28S rRNA, resulting in inhibition of protein synthesis (Endo et al. 1987). Similarly, it is assumed that the DNase-like activity of nBalsamin possibly involves recognition and binding of DNA cleavage domain of nBalsamin to specific sequence in the DNA, and depurination of adenine from this sequence results in DNA damage. Nevertheless, further studies are required to understand the precise mechanism of DNase-like of nBalsamin and its possible involvement in biological processes.

Besides enzymatic activities, nBalsamin exhibited antibacterial activity against various pathogens including *S. aureus*, *S. enterica*, *S. epidermidis* and *E. coli*. Further, the protein demonstrated free radical and superoxide radical scavenging activity in a dose dependent manner. Based on these findings, it can be inferred that nBalsamin with multifunctional properties could be considered as a nutraceutical or functional food ingredient that could find significant importance in preventing and slowing down the progress of various human diseases and bacterial infections. However, further investigation of the cytotoxicity of nBalsamin on diseased cells would be beneficial to explore its potential use in the field of therapeutics.
Chapter 3: Cytotoxic effects of nBalsamin from *Momordica balsamina* on liver and breast cancer cells.
3.1 Introduction

RIPs are RNA N-glycosidases that have gained attention as potential therapeutic agents. A number of RIPs have been reported to inhibit tumor cell proliferation via inducing apoptosis in a variety of cancers, such as hepatoma, breast carcinoma and leukemia (Zeng et al. 2015). Some studies have also unveiled the mechanisms and pathways involved in RIP-induced apoptosis in tumor cells. MAP30 induced apoptosis in liver cancer cells via activation of both caspase-8 regulated extrinsic and caspase-9 regulated intrinsic apoptotic cascades (Fang et al. 2012b). α-MMC induced apoptosis in human breast cancer cells in vivo and in vitro by activating caspase-3 and arresting cell cycle at G0/G1 phase or G2/M phase (Cao et al. 2015). MCP30 evoked apoptosis in prostate cancer cells by inhibiting histone deacetylase and Wnt signaling activity (Xiong et al. 2009). Pan et al. (2013) demonstrated that marmorin activated the extrinsic apoptotic pathway and ER-stress induced apoptosis in both MCF-7 and MDA-MB-231 breast cancer cells (Pan et al. 2013). Cucurmosin downregulated the expression of PDGFR-β at the protein level and activated the PI3K/Akt/mTOR signaling pathway in cystic fibrosis pancreatic adenocarcinoma cells leading to apoptosis (Xie et al. 2013). TCS generated reactive oxygen species (ROS) and induced nitric oxide mediated apoptosis in human choriocarcinoma cells (Zhang et al. 2001). Thus, it is suggested that RIPs do not target a specific signaling pathway to induce apoptosis; rather the pathway varies with the type of RIP and tumor cell type.

Balsamin, a type-I RIP, has been extracted from *Momordica balsamina* (Kaur et al. 2012) (mentioned in Chapter 2, named nBalsamin). Studies have demonstrated that nBalsamin exhibited potent anti-HIV activity and inhibited the translation step occurring prior to viral budding and release in the HIV-1 replication cycle (Kaur et al. 2013). In our previous study, we reported that nBalsamin exhibits antioxidant, DNase-like and broad-spectrum antimicrobial activity (Ajji, Walder & Puri 2016). To further investigate its role as a potential therapeutic agent, we evaluated the cytotoxicity of nBalsamin on liver (HepG2 and H4IIE) and breast cancer (MCF-7 and BT549) cells.

Liver cancer is the sixth most common cancer and the third leading cause of cancer related deaths worldwide. Hepatocellular carcinoma (HCC) is one of the most
common forms of liver cancer. Around 6% of all types of cancer in men and 3% in women are HCC (Venook et al. 2010). Risk factors include cirrhosis, hepatitis B and C infections, aflatoxin, gender, hormones and obesity (Curry & Beattie 1996). Most cases of HCC (around 80%) are associated with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections. Approximately 5% of the world’s population is chronically infected with HBV and 2% with HCV (El-Serag 2012). The only therapy for HCC remains surgery that includes liver transplantation and partial liver resection. Although liver transplantation is considered the most efficient treatment in patients with small HCC (<3cm), less than 30% of the patients are eligible for this treatment due to donor shortage, the expense of the procedure, tumor size and localization. For large HCC, liver resection is the preferred treatment, however, the treatment involves a high risk of deterioration in liver function after the operation (Belghiti & Kianmanesh 2005). Non-surgical therapies such as percutaneous ethanol injection and radiofrequency ablations are alternatives, but are not suitable for large peripheral lesions (>3cm in diameter) and moreover data on efficacy are limited. In addition, HCC has high resistance to conventional chemotherapy (Ryder 2003). All of these therapies have limitations associated with their use. Therefore, in this study, we investigated the efficacy of nBalsamin on HepG2 and H4IIE liver cancer cells.

Breast cancer is also one of the most prevalent and severe health problems in women worldwide with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers). Since 2008, the worldwide incidence of breast cancer has increased by more than 20% with the death rate increased by 14% (www.bcrfcure.org/breast-cancer-statistics). Despite novel drugs being developed as first-line therapies for breast cancer, drawbacks such as metastasis and relapse make breast cancer a major challenge (Kretowski et al. 2016; Qu et al. 2014).

Keeping in view the limitations involved in the treatment of cancer, identification and development of a single natural plant product that can be used to treat various cancers is highly desirable. Therefore, in this study, we investigate the antitumor potential of nBalsamin on two different cancer cell types, liver (HepG2 and H4IIE) and breast (MCF-7 and BT549) cancer cells.
3.2 Materials and methods

3.2.1 Chemicals, reagents and kits

RPMI, DMEM media and FBS were purchased from Life Technologies Corporation (California, USA). Trypsin, In vitro toxicology assay kit, Caspase-3 assay kit and Caspase-8 assay kit were purchased from Sigma-Aldrich (USA). DNasey and total RNA isolation kit were purchased from Qiagen (Hilden, Germany). Propidium iodide (PI) flow cytometry kit was procured from BD Biosciences (San Jose, CA). Reagents for electrophoresis were procured from Bio-Rad Laboratories (California, USA). All other reagents were purchased from either Sigma-Aldrich (USA) or Bio-Rad Laboratories (California, USA) and were of analytical grade.

3.2.2 Cell lines and cell culture

Liver (HepG2 and H4IIE) and breast (MCF-7 and BT549) cancer cells used were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). HepG2 and H4IIE cells were maintained in RPMI medium supplemented with 10% FBS, in an atmosphere of 5% CO2 at 37°C. The cells were grown in T-25 flasks until 80% confluence and split 1:10 in culture dishes before assays were performed. MCF-7 and BT549 cells were maintained in DMEM supplemented with 10% FBS, in an atmosphere of 5% CO2 at 37°C. The cells were grown in T-25 flasks until 80% confluence and split 1:4 in culture dishes before assays were performed.

3.2.3 Cell viability/proliferation assay

Liver (HepG2 and H4IIE) and breast (MCF-7 and BT549) cancer cells were seeded in a 6-well plate at a density of 1 X 10^5 cells per well and incubated overnight at 37°C in 5% CO2. After incubation, the media was aspirated and fresh media was added to each well. The cells were treated with increasing concentrations (3.125-200 µg/ml) of nBalsamin and PBS (control) for 24 h, 48 h and 72 h. After incubation, the media was discarded and cell viability was determined using an In vitro toxicology (MTT based) assay kit. For this, 45 µl media and 5 µl of MTT reagent was added in each well. The plates were incubated at 37°C for 2 h. After incubation, the resulting formazan crystals were dissolved in 50 µl of MTT solubilisation reagent.
Absorbance was measured at 570 nm and 690 nm. The background absorbance of each well at 690 nm was subtracted from the 570 nm measurements. The % inhibition of cell viability was calculated with the formula:

\[
\% \text{ inhibition of cell viability} = \left(1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \right) \times 100
\]

Results were expressed as mean ± standard deviation. Each experiment was performed in triplicate.

For the cell proliferation assay, counting of total cell number was performed using a haemocytometer after treatment of cells with nBalsamin (3.125-200 µg/ml) and PBS (control). Results were expressed as mean ± standard deviation. Each experiment was performed in triplicate.

3.2.4 DNA fragmentation assay

Liver (HepG2 and H4IIE) and breast (MCF-7 and BT549) cancer cells were seeded in a 6-well plate at a density of 1 X 10^5 cells per well and incubated overnight at 37°C in 5% CO₂. The cells were then treated with nBalsamin at a final concentration of 6.25 µg/ml, 25 µg/ml or 100 µg/ml for 48 h, and harvested for genomic DNA extraction using DNeasy kits (Qiagen). Briefly, the cells were suspended in 200 µl of 1X PBS and 0.6 U/µl proteinase K. To this, 200 µl of Buffer AL was added. The suspension was vortexed and incubated at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the suspension. The suspension was then applied to the spin column and centrifuged at 10,000 X g for 1 min. The flow through was discarded and the column was washed with 500 µl of Buffer AW1 and 500 µl of Buffer AW2. Finally, 30 µl of nuclease free water was added to the column and DNA was eluted by centrifugation at 10,000 X g for 1 min. DNA was analysed on 1.5% agarose gel and the image of the gel was captured in a Gel Doc™ XRS+ system (Bio-Rad, Hercules, CA, USA).

3.2.5 Cell cycle analysis

Cell cycle analysis was performed according to the manufacturer’s instructions (BD Biosciences, San Jose, CA). Briefly, liver (HepG2 and H4IIE) and breast (MCF-7 and BT549) cancer cells were seeded in 6-well plates at a density of 2 X 10^5 cells per
well and incubated overnight at 37°C in 5% CO₂. The cells were then treated with 6.25 µg/ml, 25 µg/ml or 100 µg/ml of nBalsamin and PBS (control) for 48 h. After 48 h, the cells were harvested by centrifugation at 500 X g for 5 min and fixed in 1 ml of 66% ice-cold ethanol for 2 h. After incubation, the cells were centrifuged at 500 X g for 5 min to remove the ethanol and washed twice with PBS. The cells were then stained with propidium iodide (PI) medium (50 µg/ml PI in PBS, containing 10 µg/ml RNase A) and incubated at 37°C in the dark for 20-30 min before analysis. Finally, the cells were analyzed on a FACSCanto™ II (BD Biosciences, US) and PI fluorescence was collected. The data was analyzed using FCS Express 4 software.

3.2.6 Caspase-3 and -8 activity assay

Caspase-3 and -8 activity was measured according to the manufacturer’s instructions (Sigma-Aldrich, USA). Liver (HepG2 and H4IIE) and breast (MCF-7 and BT549) cancer cells were seeded in a 24-well plate at a density of 6 X 10⁴ cells per well. The plates were placed overnight at 37°C in 5% CO₂. After incubation, the cells were treated with 6.25 µg/ml, 25 µg/ml or 100 µg/ml of nBalsamin and PBS (control) for 48 h. Media was aspirated from each well and 100 µl of 1X lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS and 5 mM DTT) was added. The cells were incubated on ice for 20 min and scraped from the surface to ensure proper lysis. The suspension was then transferred in an eppendorf tube and centrifuged at 16,000 X g for 15 min at 4°C. The supernatant was transferred into fresh tubes and stored at -80°C for further analysis.

The caspase-3 activity assay was carried out in 96 well plates. To 5 µl of cell lysate from each treatment or caspase-3 positive control, 85 µl of 1X assay buffer (20 mM HEPES, pH 7.4, 0.1% CHAPS, 5 mM DTT and 2 mM EDTA) was added. The reaction was initiated by adding 10 µl of 2 mM caspase-3 substrate (acetyl-Asp-Glu-Val-Asp p-nitroanilide) to each well. The plates were covered and incubated at 37°C for 2 h. After incubation, absorbance was measured at 405 nm. The caspase-3 activity was calculated as µmol of p-nitroaniline (pNA) released (obtained from pNA calibration curve) per min per ml of cell lysate or positive control based on the formula:

Caspase-3 activity, µmol pNA/min/ml = µmol pNA x d / t x v

where:
The caspase-8 activity assay was performed in 96 well plates. To 10 µl of cell lysate from each treatment or caspase-8 positive control, 80 µl of 1X assay buffer (20 mM HEPES, pH 7.4, 0.1% CHAPS, 5 mM DTT, 2 mM EDTA and 5% sucrose) was added. The reaction was initiated by adding 10 µl of 2 mM caspase-8 substrate (Acetyl-Ile-Glu-Thr-Asp p-nitroaniline) to each well. Absorbance was measured at 405 nm (t = 0). The plates were then placed at 37°C for 30 min. After incubation, absorbance was measured at 405 nm (t = 35 min). The caspase-8 activity was calculated as nmoles of pNA released (obtained from pNA calibration curve) per minute per ml of cell lysate or positive control based on the formula:

\[
\text{Caspase-8 activity (nmole/min/ml)} = \frac{(A_t - A_0) \times d}{(A_{1 \text{ nmole}}) \times t \times v}
\]

where:

\(v\) = volume of sample in ml
\(d\) = dilution factor
\(t\) = reaction time in minutes
\(A_{1 \text{ nmol}}\) = absorbance of 1 nmole of pNA in the well
\(A_t\) = absorbance at time t minutes
\(A_0\) = absorbance at zero time

### 3.2.7 RNA extraction and RT-PCR

Liver (HepG2 and H4IIE) and breast (MCF-7 and BT549) cancer cells were seeded in 24 well-plates at a seeding density of 6 X 10^4 cells per well and incubated overnight at 37°C in 5% CO₂. After incubation, the cells were treated with 6.25, 25 or 100 µg/ml of nBalsamin and PBS (negative control) for 48 h. After treatment, the media was removed and the cells were washed with PBS. The total RNA from each sample was extracted using a Total RNA isolation kit based on the manufacturer’s protocol. For this, 400 µl of RLT buffer was added to each well. The lysate was collected in the centrifuge tube and centrifuged at 16,000 X g for 3 min to remove debris. An equal volume of 64% ethanol was then added to the supernatant. The suspension was applied to the mini-isolation column and centrifuged at 16,000 X g for 30 sec. The flow through was discarded and the column was washed with 700 µl
of RW1 buffer and twice with 500 µl RPE buffer. Finally, the spin column was dried by centrifugation at 16,000 X g for 30 sec. The spin column was then transferred to a fresh collection tube for RNA elution. 30 µl of nuclease free water was added to the column, incubated for 1 min and then centrifuged at 16,000 X g for 1 min.

RNA of each sample was subjected to first strand complementary DNA (cDNA) synthesis using a Maxima H minus first strand cDNA synthesis kit in a total volume of 20 µl. Reverse transcription reaction was performed at 50°C for 30 min, followed by 85°C for 5 min and 4°C for 5 min. PCR amplification of the newly synthesised cDNA was performed using the primers listed in Table 3.1. PCR, in a total volume of 10 µl, was carried out at 95°C for 7 min, 40 cycles at 95°C for 30 s and 60°C for 1 min, 60°C for 60 s, 55°C-95°C (hold time: 1 s, temperature increment after hold: 0.2°C) and 20°C for 10 s. Results were expressed as mean ± standard deviation with each sample tested in replicates of six.

Table 3.1: Primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
</tr>
</thead>
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<td>GATCAGCTCGGGCACTTTAG</td>
</tr>
<tr>
<td></td>
<td>human</td>
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<td>TGCCACTCGGAAAAAGACCT</td>
</tr>
<tr>
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<td>TGTCGTTCCTCATGTCCTTA</td>
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<tr>
<td></td>
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<td>GCTATCTCCAGCCTGCTTTCT</td>
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<tr>
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<td>TCAAGCTAGGATGAGGAC</td>
</tr>
<tr>
<td></td>
<td>human</td>
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<td>AAAATTCGGATCCCCAGG</td>
</tr>
<tr>
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<tr>
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<tr>
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<tr>
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<td></td>
<td>human</td>
<td>TGCTTCTGGCTTTGCTGAC</td>
<td>CCAAGGGAAACCCAGGAAACCG</td>
</tr>
</tbody>
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3.2.8 Statistical analysis

Data was analysed using SPSS Statistics software version 22.0. Data was assessed for normality using Kolmogorov-Smirnov test. Two-way analysis of variance was used to compare the mean difference between groups that have been separated on two independent factors. One-way analysis of variance (ANOVA) was used to compare the mean difference among multiple groups and repeated measurements within one group. Probability values less than 0.05 were considered statistically significant.

3.3 Results and discussion

3.3.1 nBalsamin inhibits liver and breast cancer cell proliferation and viability

Recent statistics revealed that cancer is one of the leading causes of deaths worldwide. In Australia, 130,466 new cancer cases and 46,880 cancer deaths are expected to occur in Australia in the current year (http://www.aihw.gov.au/). The most common treatment available is chemotherapy that involves the use of anti-cancer drugs to weaken and destroy cancer cells. However, drawbacks such as toxic side effects and development of resistance to chemical agents limit its effectiveness (Chabner & Roberts 2005). Thus, the development of naturally occurring therapeutic agents is highly desirable. In recent years, RIPs have gained increasing attention as they target conserved host protein synthesis machinery, which makes it difficult to develop drug resistance, a common problem observed during chemotherapy. Moreover, it has been reported that RIPs exhibit low or no detectable side effect on normal cells as they recognize specific receptors expressed on the surface of tumor cells, which makes them a promising tool for cancer therapeutics (Fang et al. 2012b). RIPs from many plants have shown antitumor activity (Akkouh et al. 2015; Fan et al. 2008; Lee-Huang et al. 1999). TCS, a type-I RIP exhibits anti-tumor activity against various tumor cell lines including cervical carcinoma, breast carcinoma, hepatoma, colon carcinoma, choriocarcinoma, melanoma, prostate carcinoma, lung carcinoma and stomach adenocarcinoma (Sha et al. 2013). α-MMC possesses anti-tumor activity against breast cancer, lung carcinoma, nasopharyngeal cancer, retinoblastoma and glioblastoma (Akkouh et al. 2015; Cao et al. 2015; Manoharan, Jaiswal & Singh 2014). However, this is the first study carried out to
investigate the anti-tumor effects of nBalsamin on liver (HepG2 and H4IIE) and breast (MCF-7 and BT549) cancer cells.

To determine the potential cytotoxicity of nBalsamin on these cancer cell lines, the growth inhibitory effects of nBalsamin were evaluated. The results showed that nBalsamin inhibited the growth of HepG2, H4IIE, MCF-7 and BT549 cells in a dose-dependent manner with a maximum effect observed on MCF-7 cells (Figure 3.1). Based on this, we further evaluated the cytotoxicity of nBalsamin on these cell lines using an In vitro toxicology assay kit.

![Graphs showing growth inhibition rate vs. nBalsamin concentration](image)

**Figure 3.1: Effect of different concentrations of nBalsamin on the growth of HepG2 (a), H4IIE (b), MCF-7 (c) and BT549 (d) cells after 48 h treatment. All values are mean ± SD. SD, standard deviation; n = 3.**

The results showed that nBalsamin exhibited the potential to inhibit cell viability in all the four cell lines in a time and dose dependent manner (Figure 3.2). After 24 h treatment with nBalsamin, the IC₅₀s for the HepG2, H4IIE, MCF-7 and BT549 cancer cell lines were 183.09 µg/ml, 372.41 µg/ml, 179.47 µg/ml and 399.41 µg/ml respectively. After treatment with nBalsamin for 48 h, the IC₅₀s for the HepG2, H4IIE, MCF-7 and BT549 cancer cells were 60.95 µg/ml, 72.97 µg/ml, 49.40 µg/ml
and 103.54 µg/ml respectively. To further investigate the effect of treatment time on cell viability, cell viability was determined after 72 h of treatment. The IC50s observed were 33.12 µg/ml, 28.50 µg/ml, 24.53 µg/ml and 32.79 µg/ml for HepG2, H4IIE, MCF-7 and BT549 cells, respectively (Figure 3.2), suggesting that nBalsamin exhibits significant antiproliferative effects on HepG2, H4IIE, MCF-7 and BT549 cells, however, the degree of cytotoxicity varies with cancer cell type. Based on this, we further attempted to explore the main cause of death in these cells, which could be apoptosis, necrosis or autophagy. Since RIPs are known to cause cell death through apoptosis, we decided to further align our studies towards investigating the key hallmarks of apoptosis in nBalsamin treated liver and breast cancer cells.

Figure 3.2: Effect of different concentrations of nBalsamin on the viability of HepG2 (a), H4IIE (b), MCF-7 (c) and BT549 (d) cells after 24 h, 48 h and 72 h treatment. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.001, main effect of protein concentration; main effect of time; interaction between nBalsamin and time, Two-way ANOVA.
3.3.2 nBalsamin induces changes in apoptotic morphology and causes DNA fragmentation in liver and breast cancer cells.

Apoptosis is characterized by a series of morphological changes in the cell (Saraste & Pulkki 2000). Therefore, we examined the morphology of HepG2, H4IIE, MCF-7 and BT549 cells after nBalsamin treatment. Brightfield microscopy showed that the cells in the negative control (PBS) grew well, and were in dense and uniform arrangement. However, after treatment with 6.25, 25 or 100 µg/ml of nBalsamin, the number of cells decreased significantly with increasing nBalsamin concentration. Cells treated with nBalsamin-exhibited characteristics of apoptosis, including cell shrinkage, cavitation and a decrease in cell connections. More dead cells and less adherent cells were observed in treated cells compared to untreated cells (Figure 3.3).

![Morphology of HepG2, H4IIE, MCF-7 and BT549 cells treated with different concentrations of nBalsamin (6.25, 25 and 100 µg/ml) for 48 h.](image)

Figure 3.3: Morphology of HepG2, H4IIE, MCF-7 and BT549 cells treated with different concentrations of nBalsamin (6.25, 25 and 100 µg/ml) for 48 h.
In addition, DNA fragmentation into different size fragments is one of the biochemical hallmarks of apoptosis (Zhang & Ming 2000). Therefore, the genomic DNA of HepG2, H4IIE, MCF-7 and BT549 cells, treated with 6.25, 25 or 100 µg/ml of nBalsamin and PBS (control) was extracted and analysed on a 1.5% agarose gel. As shown in Figure 3.4, cells treated with different concentrations (6.25, 25 or 100 µg/ml) of nBalsamin for 48 h resulted in degradation of DNA into small oligonucleosomal fragments. Treated cells showed characteristic DNA laddering with a significant amount of DNA fragments appearing around the 180-200 bp interval on an agarose gel compared to untreated cells with intact DNA (Figure 3.4, Lane C), suggesting that DNA damage could be one reason for apoptosis in nBalsamin treated liver and breast cancer cells.

![Figure 3.4: DNA fragmentation in HepG2 (a), H4IIE (b), MCF-7 (c) and BT549 (d) cells treated with different concentrations of nBalsamin for 48 h. M and C denotes marker (1 KB DNA ladder) and control, respectively.](image-url)
3.3.3 nBalsamin induces cell cycle arrest in liver and breast cancer cells

Cell cycle is a key regulator of processes crucial to cell survival, including the detection and repair of DNA damage, cell proliferation and cell migration. The molecular events that regulate the cell cycle are ordered and directional. Any dysregulation of cell cycle machinery can lead to the development of cancer (Boehm & Nabel 2001). The induction of cell cycle arrest leading to apoptosis is a common mechanism through which a number of anticancer drugs inhibit tumor growth (Gerl & Vaux 2005). It has been reported that a variety of natural occurring plant proteins can arrest cell cycle in G0/G1, S or G2/M phase and then induce apoptosis of cancer cells (Zeng et al. 2015). Therefore, we determined the proportion of nBalsamin-treated HepG2, H4IIE, MCF-7 and BT549 cells in different phases of the cell cycle. Cell cycle analysis revealed that nBalsamin arrested HepG2 cells in G phase as a significant dose dependent increase in the cell number was observed in G phase, after 48 h of treatment. Arrest of G phase was accompanied by a dose dependent decrease in the number of HepG2 cells in S phase, with no significant changes observed in M phase (Figure 3.5a). Compared to HepG2 cells, H4IIE, MCF-7 and BT549 cells had increased cell numbers in S phase in a dose dependent manner and decreased in G phase, which indicated that nBalsamin induced S phase cell cycle arrest in these cells after 48 h of nBalsamin treatment (Figure 3.5b, 3.6a and b).

Furthermore, an increase in the proportion of apoptotic cells was observed in nBalsamin treated HepG2, H4IIE, MCF-7 and BT549 cells compared to untreated cells, suggesting that nBalsamin induces apoptosis in these cells, however, the extent of apoptosis and the phase of cell cycle arrest varies with different cell types. Similar evidence has been reported with other RIs. α-MMC from Momordica charantia blocks cell cycle at G phase in MDA-MB-231 cells and M phase in MDA-MB-453 and MCF-7 breast cancer cells (Cao et al. 2015). While in lung (A549) cancer cells, α-MMC induces cell cycle arrest at S phase (Fan et al. 2015), suggesting that the mode of action of RIs could vary in different cancer cell types.
Figure 3.5: Cell cycle analysis of HepG2 (a) and H4IIE (b) cells treated with different concentrations of nBalsamin for 48 h. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.007; **P < 0.05, One-way ANOVA.
Figure 3.6: Cell cycle analysis of MCF-7 (a) and BT549 (b) cells treated with different concentrations of nBalsamin for 48 h. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.001; **P = 0.001, One-way ANOVA.
3.3.4 nBalsamin induced apoptosis involves activation of caspases in liver and breast cancer cells.

Apoptosis, also known as programmed cell death, is one of the most fundamental biological processes that occurs in response to external stimuli. Apoptosis can occur via caspase dependent and caspase independent mechanisms. Caspase-3, a key effector that is activated by several initiator caspases, such as caspase-8, is activated in apoptotic cells by the extrinsic (death ligand) pathway, and caspase-9 is activated in apoptotic cells by the intrinsic (mitochondrial) pathway, resulting in apoptosis (McIlwain, Berger & Mak 2013; Porter & Jänicke 1999). Since RIPs have been reported to induce caspase-mediated apoptosis in cancer cells, we determined the activity of caspase-3 and -8 in nBalsamin treated liver and breast cancer cells.

nBalsamin significantly increased the activity of caspase-3 (P < 0.001) in HepG2, H4IIE, MCF-7 and BT549 cells in a dose dependent manner. The effect appeared to be more pronounced in H4IIE cells compared to HepG2, MCF-7 and BT549 cells (Figure 3.7a). Furthermore, nBalsamin significantly increased the activity of caspase-8 (P < 0.001) in HepG2, H4IIE, MCF-7 and BT549 cells in a dose dependent manner. The effect was similar and appeared to be more pronounced in liver (HepG2 and H4IIE) cancer cells compared to breast (MCF-7 and BT549) cancer cells (Figure 3.7b). An increase in the activity of caspase-8 coupled with an up-regulation of caspase-3 suggested that nBalsamin induced the death receptor (extrinsic) apoptotic pathway in liver and breast cancer cells. However, activation of caspase-3 can also be due to activation of a cascade of caspases or regulation of genes involved in the mitochondrial (intrinsic) cell death pathway. Therefore, to discover whether nBalsamin activates the mitochondrial apoptotic pathway in liver and breast cancer cells, we investigated the expression of pro-apoptotic and anti-apoptotic genes involved in the mitochondrial cell death pathway.
Figure 3.7: Effect of different concentrations of nBalsamin on caspase-3 (a) and caspase-8 (b) activity in HepG2, H4IIE, MCF-7 and BT549 cells after 48 h treatment. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.001, One-way ANOVA.

3.3.5 nBalsamin regulates the expression of various apoptotic genes involved in the mitochondrial cell death pathway

The tumor suppressor gene p53 plays a significant role in the regulation of apoptosis. It has been reported that DNA damage can lead to activation of p53. p53 normally exists in inactive form that does not have the ability to bind to DNA and activate transcription. In response to DNA damage, the ability of p53 to bind to DNA increases leading to activation of genes that initiate apoptosis (Lakin & Jackson 1999). Our studies showed that nBalsamin damages DNA, therefore, we
further investigated if this damage could activate p53 expression in nBalsamin treated liver and breast cancer cells. The qRT-PCR results showed the expression of p53 significantly increased in HepG2 (P < 0.007), H4IIE (P < 0.05), MCF-7 (P < 0.007) and BT549 (P < 0.007) cells in a dose dependent manner with the effect appearing to be more pronounced in liver cancer cells compared to breast cancer cells (Figure 3.8a).

Caspase-8 is a major activator of Bid, cleaving Bid to an active form called tBid, that initiates the transcription of genes involved in mitochondrial cell death pathway (Kantari & Walczak 2011; Wei et al. 2000). In addition, it has been reported that p53-mediated apoptosis can also lead to an increase in the expression of Bid (Sax et al. 2002). Therefore, we further determined the expression of Bid in nBalsamin treated liver and breast cancer cells. The qRT-PCR results showed that nBalsamin significantly increased the expression of Bid in HepG2 (P < 0.007), H4IIE (P < 0.05), MCF-7 (P < 0.007) and BT549 (P < 0.007) cells in a dose dependent manner. The effect appeared to be more pronounced in HepG2 cells compared to other cell lines (Figure 3.8b).

Apart from p53 and Bid, Bcl-2 family genes also regulate the process of apoptosis. The genes associated with the Bcl-2 family can be either pro-apoptotic (Bax, Bak and Bok) or anti-apoptotic (including Bcl-2, Bcl-XL, and Bcl-W) and govern mitochondrial outer membrane permeabilization, which is a key indicator of the mitochondrial cell death pathway (Czabotar et al. 2014; Hong, Park & Lyu 2014). Therefore, we determined the expression of pro-apoptotic and anti-apoptotic markers in nBalsamin treated liver and breast cancer cells. The results showed that nBalsamin significantly increased the expression of Bax in HepG2 (P < 0.007), H4IIE (P < 0.007), MCF-7 (P < 0.007) and BT549 (P < 0.007) cells in a dose dependent manner. The effect appeared to be more pronounced in HepG2, H4IIE and BT549 compared to MCF-7 cells (Figure 3.9a). Further, the expression of Bad was also observed to increase significantly in HepG2 (P < 0.007), MCF-7 (P < 0.007) and BT549 (P < 0.007) cells in a dose dependent manner. The expression tended to increase in H4IIE cells, however, the results were not significant demonstrating that nBalsamin does not activate Bad in H4IIE cells (Figure 3.9b).
Figure 3.8: Expression of p53 (a) and Bid (b) genes in HepG2, H4IIE, MCF-7 and BT549 cells treated with different concentrations of nBalsamin for 48 h. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.007; **P < 0.05, One-way ANOVA.

In addition, we also evaluated the expression of anti-apoptotic genes (Bcl-2 and Bcl-XL) involved in the mitochondrial cell death pathway. The qRT-PCR results showed that nBalsamin significantly decreased the expression of Bcl-2 and Bcl-XL in MCF-7 (P < 0.007) and BT549 (P < 0.007) cells in a dose dependent manner with the similar effects observed in both cell lines (Figure 3.10a and b).

However, in the case of liver cancer cells, the level of Bcl-2 expression tended to decrease in nBalsamin treated HepG2 and H4IIE cells compared to control (Figure 3.10a). A trend for an increase in the expression of Bcl-XL was observed in
nBalsamin treated H4IIE cells, however, it appeared opposite in HepG2 cells (Figure 3.10b). However, none of these results were statistically significant. Based on these findings, it can be concluded that nBalsamin regulates the expression of anti-apoptotic genes in breast (MCF-7 and BT549) cancer cells but not in liver (HepG2 and H4IIE) cancer cells.

Figure 3.9: Expression of Bax (a) and Bad (b) genes in HepG2, H4IIE, MCF-7 and BT549 cells treated with different concentrations of nBalsamin for 48 h. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.007; **P < 0.05, One-way ANOVA.
Figure 3.10: Expression of anti-apoptotic genes, Bcl-2 (a) and Bcl-XL (b), in HepG2, H4IIE, MCF-7 and BT549 cells treated with different concentrations of nBalsamin for 48 h. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.007, One-way ANOVA.

3.3.6 nBalsamin induces ER-stress mediated apoptosis in liver cancer cells but not in breast cancer cells.

The endoplasmic reticulum (ER) is a cell organelle that plays a significant role in cell function, including the synthesis, post-translational modification and proper folding of proteins, and transport of newly synthesised proteins to the Golgi apparatus (Shiraishi et al. 2006). Several ER chaperone proteins, such as Hsp70 family members GRP78, protein disulphide isomerase (PDI) and the peptidylpropyl
isomerase family are involved in the correct folding of newly synthesised proteins. However, perturbations in ER function, such as viral infection, redox dysregulation, nutrient deprivation and calcium dysregulation or over-expression of proteins, can lead to ER stress (Kober, Zehe & Bode 2012). When ER function is severely affected, the organelle evokes apoptotic signals. One of the most important mediators in ER-stress mediated apoptosis is C/EBP homologous protein (CHOP), also known as DNA damage-inducible gene 153 (GADD153). CHOP forms a heterodimer with various other C/EBP family members and acts as a dominant negative inhibitor of transcription. CHOP is usually expressed at undetectable levels, however, its expression is induced by cellular stress (Bento, Andersson & Aman 2009). The 78-kDa glucose regulated protein (GRP78), also known as BiP, is a major regulator of ER function, as it forms complex with heterologous proteins that are processed through the ER. It is involved in protein folding and assembly, targeting misfolded proteins for degradation and regulating the activation of ER stress signals. In response to ER stress, GRP78 expression is upregulated to assist proper protein folding and it is considered to be an important component involved in ER-stress induced apoptosis (Li & Lee 2006).

In order to assess whether nBalsamin induces ER-stress mediated apoptosis in liver and breast cancer cells, we determined the expression of CHOP and GRP78 in nBalsamin treated cells. The qRT-PCR results confirmed that nBalsamin significantly increased the expression of both GRP78 and CHOP in a dose dependent manner in liver cancer, HepG2 (P < 0.001) and H4IIE (P < 0.001), cells. However, nBalsamin neither affected the expression of GRP78 nor CHOP in breast cancer, MCF-7 and BT549, cells (Figure 3.11 a and b). These results suggest that, in addition to activation of intrinsic and extrinsic apoptotic pathways, nBalsamin-induced apoptosis also involves activation of an ER stress-mediated pathway in liver cancer cells but not in breast cancer cells. Overexpression of CHOP has been reported to lead to a decrease in the levels of Bcl-2. Moreover, increased levels of CHOP leads to translocation of Bax protein from the cytoplasm to the mitochondria. Thus, CHOP mediated apoptotic signals are finally transmitted to the mitochondria, which act as an amplifier and integrator of apoptosis (Oyadomari & Mori 2004; Shiraishi et al. 2006). Hence, it could be anticipated that overexpression of CHOP is
linked to activation of the mitochondrial cell death pathway in nBalsamin treated liver cancer, HepG2 and H4IIE, cells.

Figure 3.11: Expression of genes involved in ER-stress mediated apoptosis, *GRP78* (a) and *CHOP* (b), in HepG2, H4IIE, MCF-7 and BT549 cells treated with different concentrations of nBalsamin for 48 h. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.001, One-way ANOVA.

Although several RIPs are known to exhibit anti-proliferative activity and induce apoptosis in cancer cells by activating various apoptotic pathways, however, toxicity and side effect of RIPs to normal cells remains a challenge (Zeng et al. 2015). This could also be one possible problem associated with the use of nBalsamin in cancer
therapy. Various strategies have been employed to tune the selectivity of RIPS to cancer cells. Antibody targeted therapy is one such approach that has been used to develop RIP immunotoxin and involves conjugation of RIP to monoclonal antibody that specifically binds to the antigen expressed on the surface of cancer cells. Humanized anti-PSMA monoclonal antibody (hj591) was fused with saporin, a type I RIP, to develop immuno-toxin that had specificity towards PSMA positive LNCaP and CWR22Rv1 cell lines and not towards PSMA negative cell line, PC-3 (Kuroda et al. 2010). Nevertheless, problems such as generation of immune response, decrease of intracellular cytotoxic RIPS and increase of side effects on several organs are associated with this approach.

Protein or peptide conjugated RIPS is another approach that has been used to improve therapeutic applications of RIPS. This strategy involves fusion of RIPS with carrier such as cell binding ligands, hormones and protease inhibitors to develop a bifunctional cytotoxic agent that binds to specific receptors expressed on the surface of tumor cells. A transferrin receptor peptide (TfRBP) was fused to curcin, a type I RIP, to develop a conjugate with improved selectivity towards HepG2 cells overexpressing transferrin receptors. This conjugate demonstrated low inhibitory effect on SKBR-3 cells expressing low levels of transferrin receptors (Zheng et al. 2013). Nonetheless, stability of peptide is a major issue that restricts the use of this approach.

Similar strategies could be employed to tune the selectivity of nBalsamin to cancer cells. However, drawbacks associated with these approaches might limit the use of nBalsamin in cancer therapeutics. In recent years, efforts have been made to develop nanoparticle based RIP delivery system. Apart from greater stability, greater surface area and ease of modification, this system renders intriguing features such as size, shape and biocompatibility. Keeping in view the versatile and durable nature of nanoconstructs, the development of nanoparticle based nBalsamin delivery system carries a great importance. Thus, in the near future, our research will be focused in this area.
3.4 Conclusions

The present study identifies the role of nBalsamin in exerting anti-tumor effects on hepatoma (HepG2 and H4IIE) and breast cancer (MCF-7 and BT549) cells, and its associated mechanism of action. The results indicated that nBalsamin could inhibit the growth and viability of liver and breast cancer cells in a dose and time dependent manner. nBalsamin could induce cell cycle arrest at G phase in HepG2 cells and S phase in H4IIE, MCF-7 and BT549 cells. Based on our findings, it may be proposed that nBalsamin induces apoptosis through three mechanisms: (i) by activating the extrinsic cell death pathway as indicated by increased level of caspase-8 leading to activation of the downstream effector caspase-3, (ii) by inducing the intrinsic apoptotic pathway as manifested by upregulation of Bax, Bid, Bad and p53 and downregulation of Bcl-2 and Bcl-XL and (iii) by triggering ER-stress mediated apoptosis in liver cancer cells and not in breast cancer cells, as indicated by overexpression of CHOP and GRP78. The signaling pathways involved in nBalsamin-induced apoptosis in liver and breast cancer cells are summarized in Figure 3.12a and b. This characteristic of nBalsamin might prove beneficial in the treatment of liver and breast cancer, as the cells would be less likely to mutate and develop resistance compared to other chemotherapeutic agents that activate a specific apoptotic pathway. Moreover, compared to synthetic drugs, natural products such as nBalsamin can be added as a valuable dietary supplement in the diet of cancer patients for the treatment of cancer. Further analysis of nBalsamin-induced apoptosis and in vivo studies are needed to develop this biomolecule as an effective therapeutic and/or functional food.
Figure 3.12: Signaling pathways involved in nBalsamin-induced apoptosis in liver (a) and breast (b) cancer cells.
Chapter 4: Molecular cloning, expression and functional analysis of recombinant Balsamin (rBalsamin) from *Momordica balsamina*.
4.1 Introduction

Balsamin isolated from *Momordica balsamina* is a type-I RIP that can inhibit HIV-1 replication at the translation step occurring prior to viral budding and release (Kaur et al. 2013). Our recent studies have shown that natural balsamin (nBalsamin) also possesses antibacterial and DNase-like activity (Ajji, Walder & Puri 2016), however, the amount of nBalsamin in *Momordica balsamina* seeds is limited. Moreover, recovery and purification of balsamin from natural sources involves multiple purification steps that compromises protein yield. Heterologous protein expression in a suitable recombinant strain offers an attractive alternative with higher yields, shorter fermentation time and cheaper production costs. Therefore, in this study the balsamin gene was cloned and expressed, and the biological functions of recombinant balsamin (rBalsamin) were investigated.

Several RIP genes from *Momordica* species have been cloned and expressed in *E. coli*. α-MMC was the first gene to be cloned using a λgt11 cDNA library constructed from *Momordica charantia* seeds, followed by its mass production in a heterologous system, the *E. coli* Rosetta (DE3) pLysS strain (Ho et al. 1991; Wang et al. 2012). The MAP30 gene was cloned in a pET-28 vector and expressed in *E. coli* BL21(DE3) cells (Han et al. 2011). In other studies, MAP30 was fused with the human-derived cell penetrating peptide (HBD) to overcome the low uptake efficiency by tumor cells and improve the cytotoxicity of MAP30 (Lv et al. 2015). In our previous studies, we investigated the anti-tumor effect of nBalsamin on liver and breast cancer cells. However, to explore animal studies and clinical trials a large quantity of pure protein is required. Thus, in this study we attempted heterologous expression of balsamin.

Immobilized metal affinity chromatography (IMAC) is a versatile technology used for industrial scale purification of proteins fused to short affinity tags consisting of polyhistidine residues and provides high specificity and ease of purification (Chaga 2001). A polyhistidine tag consists of at least 6 histidine residues, often fused at the N- or C-terminus of the protein. The ability of histidine residues to bind to an immobilized metal ion matrix with high affinity in the presence of denaturing agents and mild elution conditions has made the His-tag a powerful tool for protein purification (Bornhorst & Falke 2000). The immobilization of a His-tag on an IMAC
matrix is a rapid and inexpensive method used to study protein-protein interactions and to purify recombinant proteins with up to 95% purity (Porath et al. 1975).

In this study, we describe an IMAC based method of obtaining a biologically active rBalsamin from an E.coli expression system. In addition, physicochemical parameters were optimized to obtain maximum expression of balsamin. Further, biophysical characterization of rBalsamin and its other functional properties were investigated. Of all expression systems, E.coli was used for this study because of its advantage over other host cells, namely fast growth kinetics, ease of transformation with exogenous DNA and low cost of rich complex growth medium.

Here we report the successful heterologous expression of balsamin in E.coli. It is noteworthy that the cloning and expression of His-tagged rBalsamin has not been attempted before. This study carries great importance as it not only enables large-scale production of balsamin, but also a versatile system for genetic manipulation in the rational development of this potential therapeutic agent.

4.2 Materials and methods

4.2.1 Enzymes, chemicals and purification kits

All chemicals used in this study were of analytical grade and were obtained from either Sigma-Aldrich (St. Louis, MO, USA) or Bio-Rad Laboratories (Hercules, CA, USA). DNA modifying enzymes and DNA markers were from New England Biolabs Inc (Ipswich, MA, USA). Precision Plus™ dual colour protein marker was from Bio-Rad Laboratories (Hercules, CA, USA). Restriction enzymes (BamHI and XhoI) and pET- 30a(+) were procured from Novagen Inc. (Wisconsin, USA). RNAqueous® Total RNA Isolation Kit was obtained from Life Technologies Corporation (California, USA). Plasmid DNA purification kit, QIAquick PCR purification kit and QIAquick gel extraction kit were from Qiagen Pty. Ltd. (Hilden, Germany). MasterPure™ DNA Purification Kit was from Epicentre (Wisconsin, USA). Immobilized metal affinity gel (Ni²⁺ Sepharose 6 fast flow) and Hi Load™ 26/60 Superdex™ 75 preparation grade column were procured from GE Healthcare (Little Chalfont, UK).
4.2.2 Bacterial strains

*E.coli* DH10β and BL21(DE3) were obtained from New England Biolabs Inc. (Ipswich, MA, USA). Bacterial strains *Salmonella enterica* ATCC 43971, *Enterococcus faecalis* ATCC 10100, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 2348 and *Pseudomonas aeruginosa* ATCC 27853 were procured from American Type Culture Collection (Rockville, MD).

4.2.3 Molecular cloning of the gene encoding balsamin

4.2.3.1 Genomic DNA extraction

Genomic DNA was extracted from *Momordica balsamina* seeds using MasterPure™ DNA Purification Kit. Seeds were ground to powder form under liquid nitrogen. 500 μl of lysis buffer and 2 μl of RNase were added to 50 mg of seed powder and incubated at 65°C for 30 min. After incubation, the sample was placed on ice for 10 min and 200 μl of protein precipitation reagent was added. The suspension was vortexed for 10 sec and centrifuged at 10,000 X g for 15 min. To the supernatant, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added followed by centrifugation at 10,000 X g for 15-20 min. The aqueous layer obtained after centrifugation was transferred into a fresh eppendorff tube and mixed with an equal volume of 100% ethanol. The sample was placed at -20°C for 30-45 min and then centrifuged at 10,000 X g for 15-20 min at 4°C to obtain a DNA pellet. The pellet was washed with 70% ethanol, air dried under laminar airflow and resuspended in 35 μl of Milli Q water. 5 μl of the above sample was visualized on a 0.8% agarose gel.

4.2.3.2 Agarose gel electrophoresis

Integrity and quality of isolated genomic DNA was checked by agarose gel electrophoresis. A 0.8% agarose gel (50 ml) was prepared in 1X TAE buffer (40 mM Tris, pH 7.6, 20 mM acetic acid and 1 mM EDTA) in an Erlenmeyer flask using a microwave oven. The gel was allowed to cool and 2.5 μl of 10 mg/ml of ethidium bromide was added with proper mixing. The gel was poured into a horizontal gel
electrophoresis tray with a comb inserted in place and allowed to stand at room temperature until solidified (covered with aluminium foil). After solidification, the comb was removed and the gel was placed in the electrophoresis chamber. The chamber was filled with 1X TAE buffer until the wells were fully submerged. 1 μg of genomic DNA sample was mixed with 2 μl of 6X DNA loading dye (40% sucrose and 0.04% bromophenol blue) and loaded into the respective well with 1KB DNA ladder. Electrophoresis was carried out at 100 V for 50 min. After electrophoresis, the DNA was visualized under UV light using a Gel Doc™ XRS+ system (Bio-Rad, Hercules, CA, USA).

4.2.3.3 PCR gene amplification

Seven different degenerate PCR primers were designed for balsamin (Bal) gene amplification based on published data (Barthelemy et al. 1993; Han et al. 2011; Liu et al. 2010; Nolan, Garrison & Better 1993; Poyet & Hoeveler 1997; Poyet, Radom & Hoeveler 1994; Wang et al. 2012). The list of forward and reverse primers used is given in Table 4.1. The PCR reaction was performed in 50 μl volume containing; 0.5 μg of genomic DNA as template, 0.5 μM each of forward and reverse primer, 1 μl of 10mM dNTP, 1.5 μl of DMSO, 0.02 U/μl of Phusion DNA polymerase, 10 μl of 5X Phusion HF buffer and 30 μl of Milli Q water. The PCR procedure included: 98°C for 2 min as initial denaturation, 35 cycles at 98°C for 15 sec (denaturation), 65°C for 30 sec (annealing), 72°C for 60 sec (extension) and then 72°C for 5 min as a final extension step. Finally, the PCR products were analyzed by electrophoresis on a 0.8% agarose gel.
### Table 4.1 Degenerate primers used for balsamin gene amplification.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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| MAP  | Forward primer: 5’-CATGCGGGATCCATGTTGGAATGGCTTAC-3’  
       | Reverse primer: 5’-CGAGCCTCGAGTCAGTCAACAACAG-3’ |
| Gel  | Forward primer: 5’-CATGCGGGATCCACTGRTCNACNGTNA CRTARTAYTTYTT-3’  
       | Reverse primer: 5’-CGAGCCTCGAGTTTTAARGAYGCNCCN GAYGNGCTAYGARGG-3’ |
| Sap  | Forward primer: 5’-CATGCGGGATCCACTGCAGAATTCGCATGGATCTGGAAT-3’  
       | Reverse primer: 5’-CGAGCCTCGAGCTGCAGAATTCGCCTCGTTTGACTACTTTG-3’ |
| Luf  | Forward primer: 5’-CATGCGGGATCCATGRTTAAATCTGIGCIGCTT -3’  
       | Reverse primer: 5’-CGAGCCTCGAGTGATTGAAATACGATCACCTTTGATC-3’ |
| PAP  | Forward primer: 5’-CATGCGGGATCCGATGTTAGCTTTCGTTTTCGAGT-3’  
       | Reverse primer: 5’-CGAGCCTCGAGCGGATCCGAATCCTTCAAATAGATCAC-3’ |
| Mom  | Forward primer: 5’-CATGCGGGATCCATGRTTAAATCTGIGCIGCTT -3’  
       | Reverse primer: 5’-CGAGCCTCGAGTGATTGAAATACGATCACCTTTGATC-3’ |
| PAPII| Forward primer: 5’-CATGCGGGATCCATGRTTAAATCTGIGCIGCTT -3’  
       | Reverse primer: 5’-CGAGCCTCGAGTGATTGAAATACGATCACCTTTGATC-3’ |

MAP: *Momordica* antiviral protein; gel: gelonin; sap: saporin; Luf: α-luffin; PAP: pokeweed antiviral protein; Mom: α-momorcharin and PAPII: pokeweed antiviral protein II.
4.2.3.4 Extraction and purification of DNA fragments from an agarose gel

The gene fragments obtained after PCR were purified from the gel using a QIAquick gel extraction kit. The DNA fragment of interest was excised from the agarose gel using a clean razor blade and placed in 2 ml eppendorff tubes and 3 volumes of buffer QG was added to 1 volume of gel. To help dissolve the gel, the sample was incubated at 50°C with regular mixing every 2-3 min until completely dissolved. The sample was then applied to a QIAquick spin column and centrifuged for 1 min at 10,000 X g for proper binding of DNA to the column. After centrifugation, the flow through was discarded and the column was washed with 750 μl of Buffer PE by centrifugation at 10,000 X g for 60 sec. The above step was repeated for proper column washing. The spin column was then dried by centrifugation at 10,000 X g for 1 min and transferred to a fresh collection tube for DNA elution. 30 μl of milliQ water was added to the centre of the column. After 2 min, the column was centrifuged as before. The DNA fragment was stored at -20°C.

4.2.3.5 DNA sequencing

The samples (prepared by mixing 0.5 μg DNA template; 0.5 μM forward primer; 2.5 μl Milli Q water) were sent to Macrogen Inc. (Seoul, Korea) for sequencing.

4.2.3.6 Primer design and DNA amplification

Specific sense and antisense primers were designed to obtain balsamin mature peptide: forward primer: 5‘-CGGGATCCATGTTTATCAAAGC-3’; and reverse primer: 5’-CAGCTCGAGTTAGGAGCTGAAGCCT-3’. The underlined bases in the primers were designated as BamHI and XhoI restriction sites, respectively. The PCR reaction mixture was performed in 50 μl volume containing; 0.5 μg of DNA fragment (sequenced), 0.5 μM each of specific forward and reverse primer, 1 μl of 10mM dNTP, 1.5 μl of DMSO, 0.02 U/μl of Phusion DNA polymerase, 10 μl of 5X Phusion HF buffer and 30 μl of Milli Q water. The PCR procedure included: 98°C for 1 min (initial denaturation), 30 cycles at 98°C for 10 sec, 64°C for 20 sec, 72°C for 45 sec and then 72°C for 5 min as a final extension step. The PCR product was analyzed on a 0.8% agarose gel and purified from the gel using a QIAquick gel extraction kit as described in section 4.2.3.4.
4.2.3.7 Restriction digestion and ligation of amplified gene and pET-30a(+)

Digestion of DNA and pGEM-T with restriction enzymes was performed using type II restriction endonucleases. The sample volume and the amount of enzyme were adjusted according to the amount of DNA. The restriction mixture consisted of 1 μg of DNA/pGEM-T, 1X digestion buffer (enzyme specific), 10 U/μl BamHI and XhoI restriction enzymes, Milli Q water to make the final volume to 50 μl. The restriction digestions were performed for 4 h at 37°C. The DNA fragments obtained after restriction digestion of DNA and pGEM-T were directly analyzed by agarose gel electrophoresis and purified from the gel using a QIAquick gel extraction kit as described in section 4.2.3.4.

The ligation reaction was performed by using T4 ligase, which catalyzes the formation of a phosphodiester bond between 3'-OH and free 5'-phosphate of double-stranded DNA fragments. The ligation reaction mixture consisted of 50 ng vector DNA, 150 ng insert, 1.5 μl of 10X T4 DNA ligase buffer, 10 U/μl T4 DNA ligase, nuclease-free water to make the final volume to 20 μl. The components were mixed and incubated overnight at 16°C for ligation. The ligated product (pGEM-T-Bal) was then transformation into E.coli DH10β. Besides colony PCR with vector-specific primers, the correct insertion of Bal gene was confirmed by DNA sequencing (Macrogen, Inc., South Korea). The plasmid was double digested and directionally subcloned between the BamHI and XhoI sites of the pET-30a(+) vector, as described above. The recombinant vector thus generated was designated pET-Bal.

4.2.3.8 Preparation of competent cells

Competent cells were prepared from the BL21(DE3) strain of E.coli bacteria using the calcium chloride method. 50 μl of E.coli BL21(DE3) culture was inoculated in 5 ml of Luria-Bertani (LB) broth and incubated overnight at 37°C at 250 rpm in a shaker incubator. The next day, 0.5 ml of these cells were cultured in 50 ml of LB broth and incubated at 37°C at 250 rpm until the O.D_{600nm} reached between 0.4-0.6. After incubation, the culture was transferred to a 50 ml falcon tube (pre-chilled) and placed on ice for 30 min. The culture was then centrifuged at 4000 X g and 4°C for 15 min. The supernatant was discarded and 5 ml of 0.1 M calcium chloride solution (cold) was added to the pellet and mixed gently. The suspension was further placed
on ice for 30 min and then centrifuged at 4000 X g at 4°C for 15 minutes. The supernatant was discarded and 2 ml of 0.1 M calcium chloride containing 20% glycerol (cold) was added to the pellet and mixed gently. From this, 50 μl aliquots were prepared in 1.5 ml pre-chilled eppendorff tubes and stored at -80°C until further use.

4.2.3.9 Transformation of competent cells

Transformation of ligated products into E.coli BL21(DE3) was carried out using the heat shock method. Around 100 ng of the ligated product was mixed with competent E.coli BL21(DE3) (thawed on ice for 10 min) and incubated on ice for 30 min. The cells were given heat shock for 45 sec at 42°C followed by incubation on ice for 5 min. 950 μl LB broth was added to the cells and incubated at 37°C and 250 rpm for 45 min. After incubation, 100 μl of the bacterial suspension was spread onto LB agar plates containing kanamycin. The plates were incubated overnight at 37°C.

4.2.4 Expression of rBalsamin and optimization of parameters

For bacterial expression of the gene encoding Balsamin, pET-Bal was transformed into freshly prepared E.coli BL21(DE3) competent cells. A single colony harbouring the pET-Bal recombinant plasmid was inoculated into 20 ml of LB broth containing 50 μg/ml of kanamycin and grown overnight at 37°C and 250 rpm. The next day, the overnight culture was inoculated into 500 ml of LB broth containing 50 μg/ml of kanamycin at a ratio 1:100 and grown at 37°C and 250 rpm until an OD$_{600}$nm of 0.4-0.6 was reached. Protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). Culture conditions, such as growth temperature (20°C, 25°C, 30°C and 37°C), IPTG concentration (0.2, 0.4, 0.6, 0.8 and 1.0 mM) and culture time (4, 8, 12, 16 and 20 h) were optimized for the production of rBalsamin. After induction, the cells were harvested by centrifugation at 12,000 X g for 20 min at 4°C. The cell pellet was dissolved in lysis buffer (50 mM Tris-HCl buffer, pH 7.5, 300 mM NaCl and 20 mM imidazole) and the cells were disrupted using an ultra-sonicator (Vibra-Cell VCX 500, Sonics, USA). Sonication was performed for 15 min at 30% amplitude using a 3mm tapered micro tip probe. After sonication, the cells were centrifuged at 12,000 X g for 15 min at 4°C. Samples of
the soluble (supernatant) and insoluble (cell pellet) fractions were analysed by SDS-PAGE.

The insoluble fraction obtained after centrifugation was dissolved in buffer A (50 mM Tris-HCl buffer, pH 7.5, 300 mM NaCl, 20 mM imidazole and 8M urea) and stirred at room temperature for 30 min for cell disruption. The suspension was then centrifuged at 12,000 X g for 20 min at 4°C and then passed through a 0.22 µm filter.

4.2.5 Purification of rBalsamin

4.2.5.1 Ni^{2+} affinity chromatography

Immobilized metal affinity chromatography (IMAC) is a common technique used to purify proteins fused to a His-tag. Since the rBalsamin included an N-terminal His-tag, therefore Ni^{2+} Sepharose 6 fast flow was used for purification. A suspension of Ni^{2+} Sepharose 6 fast flow was poured gently in the column of dimensions 10 X 1.5 cm through the sides avoiding bubble formation and plugging the column at the bottom with a stopper. The Ni^{2+} Sepharose 6 fast flow was allowed to settle to make a bed and the column was then flushed with 10-column volumes of Milli Q water. Once the resin in the column settled, the column was equilibrated with 30-40 ml buffer A (50 mM Tris-HCl buffer, pH 7.5, 300 mM NaCl, 20 mM imidazole and 8M urea). The protein solution was loaded on the top of Ni^{2+} Sepharose column and incubated for 1 h for effective binding of the proteins to the matrix. To remove the unbound protein, the column was washed with 5-column volumes of buffer A. To refold the bound protein on the column, the column was washed with 5-column volumes of linear gradient of urea (8-0 M) in buffer A. The bound proteins were then eluted from the column using 15 ml of Buffer B (50 mM Tris-HCl buffer, pH 7.5, 300 mM NaCl, and 250 mM imidazole). The protein content in each fraction was measured using the Bradford method (1976) and analysed on SDS-PAGE. Finally, the fractions were concentrated using an ultrafiltration membrane (Amicon Ultra-15, 10 kDa, US) and stored at -20°C until further use.
4.2.5.2 Gel filtration chromatography

Gel filtration is a simplest and mildest chromatographic technique used to separate molecules based on their size and molecular weight. In our study, a Hi Load™ 26/60 Superdex™ 75 prepacked column was used for further purification of rBalsamin. Prior to its use, the tubing connected to the column and the pump valves (A1, A2, B1, and B2) was flushed with distilled water to remove any salt residues left over from previous runs. Using a syringe and a needle, the sample loop was flushed with ethanol and then with water. The column was washed with 2 column volumes of 20% ethanol (degassed) and 2 column volumes of distilled water (degassed). Once the prewashing cycles were complete, the column was equilibrated with 2 column volumes of 50 mM Tris-HCl, pH 7.5 (degassed). Using a syringe and needle, 2 ml of concentrated fraction was loaded onto the column. The protein was eluted with 50 mM Tris-HCl, pH 7.5 (degassed) at a flow rate of 0.5 ml min⁻¹ and 6 ml of fraction were collected. The absorbance (A₂₈₀) of each fraction was measured automatically with a Quad Tech 280 spectrophotometer attached to the system. The peak fractions were pooled and analysed on SDS-PAGE. The fractions containing the target protein were concentrated by ultrafiltration, and stored in aliquots at -80°C.

4.2.6 rRNA N-glycosidase activity

RNA N-glycosidase activity is a unique characteristic exhibited by RIPs that causes hydrolysis of the N-glycosyl bond at A-4324 in 28S rRNA resulting in the release of a 400 bp fragment (the Endo-fragment) on aniline treatment. The assay was conducted as described by (Bagga S 2003) with minor modification. Rabbit reticulocyte lysate was used as a source of ribosome. 70 μl of rabbit reticulocyte was incubated with 50 μg/ml of nBalsamin and rBalsamin at 30°C for 30 min. 10 μl of 10% SDS (w/v) and 170 μl of diethylpyrocarbonate (DEPC) water was added to terminate the reaction. Following termination, total rRNA from the reaction mixture was extracted using RNAqueous® Total RNA Isolation Kit. For this, equal volume of lysis buffer was added to the sample. The sample was vortexed and centrifuged for 2-3 min at top speed to remove debris. An equal volume of 64% ethanol was then added to the supernatant. The sample was vortexed and the suspension was applied onto the spin column. The column was centrifuged at 12,000 X g for 1 min
to ensure that the RNA was completely bound to the column. After centrifugation, the flow through was discarded and the column was washed with 700 μl of wash solution-1, to remove any unbound biomolecules, followed by centrifugation at 12,000 X g for 1 min. The flow through collected in the column was removed and a second column washing was given with 500 μl wash solution-2/3. The column was centrifuged at 12,000 X g for 1 min and flow through was discarded. The above step was repeated for proper column washing. The spin column was dried by centrifuging at 12,000 X g for 30 sec and then transferred to a fresh collection tube for RNA elution. 25 μl of prewarmed Milli Q water was added to the spin column. The column was incubated for 2 min and then centrifuged at 12,000 X g for 30 sec at room temperature. The above step was repeated to elute any remaining RNA from the column.

50 μl of RNA was divided into two parts. One part was treated with 25 μl of aniline acetate (10% aniline and 12% glacial acetic acid in DEPC water, pH 4.5), whereas the other part was left untreated. The samples were incubated at 60°C for 3 min, aniline was evaporated under vacuum and the RNA pellet was dissolved in 25 μl of DEPC water. The samples were analysed on a 2% agarose gel. For this, a loading buffer containing 32% formamide, 4mM EDTA, 0.04% xylene cyanol and 0.04% bromophenol blue was added to the aniline treated and untreated samples. The samples were heated at 65°C for 5 mins and loaded on the agarose gel. The gel was stained with ethidium bromide and the RNA was visualized using a Gel Doc™ XRS+ system (Bio-Rad, Hercules, CA, USA).

4.2.7 Biophysical characterization of rBalsamin

4.2.7.1 Circular dichroism (CD) spectroscopy

The secondary structure of rBalsamin (450 μg/ml in 50 mM Tris-HCl buffer, pH 7.8) was determined using a Jasco J-815 CD spectrometer (Jasco Inc., Easton, MD, US) with a temperature controlled cuvette chamber. Spectra in the far UV range (190-260 nm) were recorded at a scanning speed of 100 nm/min in 1 mm path length cells.

To study the effect of temperature on the secondary structure of rBalsamin (450
μg/ml in 50 mM Tris-HCl buffer, pH 7.8), the spectra were recorded over a temperature range from 20 to 90°C with a heating rate of 1°C/min. In addition, to study the effect of pH, spectra of rBalsamin dissolved in buffer with pH ranging from 4-11, were measured. The analysis of the CD spectra was then performed using Spectra manager™ software.

4.2.7.2 Fluorescence spectroscopy

The fluorescence of the rBalsamin was recorded using a spectrofluorometer. The intrinsic fluorescence of rBalsamin (100 μg/ml in 50 mM Tris-HCl buffer, pH 7.8) was measured over a temperature range of 20 to 90°C with a heating rate of 1°C/min. Fluorescence spectra were recorded by excitation at 280 nm (slit width 5 nm) and emission in the range of 300-500 nm (slit width 5 nm). To study the effect of pH on the tertiary structure, the spectra of rBalsamin dissolved in buffer with pH ranging from 4-11 were recorded.

4.2.8 Bioinformatics analysis

4.2.8.1 Protein sequence identification and homology

EXPASY translate is a tool that allows the translation of nucleotide (DNA/RNA) sequence to a protein sequence. The Bal gene sequence obtained after sequencing was submitted to the EXPASY translate tool to obtain a protein sequence. The similarity of the predicted protein sequence with other RIP sequences was determined using the BLASTP tool. The BLASTP program searches for similarity between query protein sequence and other protein sequences available in the NCBI database. The protein sequence obtained from the EXPASY translate tool was submitted to BLASTP and the % sequence identity of the protein with other proteins in the database was reported.

4.2.8.2 Prediction of biochemical, biophysical and physicochemical parameters

The PROTPARAM program of the expasy protein analysis tool computes various physico-chemical parameters (such as molecular weight, theoretical pI, amino acid composition, extinction coefficient, estimated half-life, instability index and aliphatic index) from a protein sequence. The protein sequence obtained from the
EXPASY translate tool was submitted to PROTPARAM. The values of various predicted physico-chemical parameters were reported.

4.2.9 DNase-like activity of rBalsamin

4.2.9.1 Optimization of rBalsamin concentration for DNase-like activity

DNase-like activity of rBalsamin was evaluated as described in Chapter 2. pUC19 plasmid (0.25 μg) was incubated with different amounts of rBalsamin (0.5 μg, 1 μg, 1.5 μg or 2 μg) for 2 h at 37°C in a final volume of 10 μl made with DNase buffer (50 mM Tris HCl, pH 7.5, 50 mM KCl, 0.1 mM MgCl₂). Plasmid DNA (without any protein) and treated with the restriction endonuclease EcoRI (10 U/μl) served as negative and positive controls, respectively.

4.2.9.2 DNase-like activity of nBalsamin and rBalsamin

To compare the DNase-like activity of nBalsamin and rBalsamin with other restriction endonucleases, 0.25 μg of pUC 19 plasmid was incubated with 1.5 μg of nBalsamin and rBalsamin, and 10 U/μl restriction enzymes (BamHI, HindIII and NdeI) for 2 h at 37°C in a final volume of 10 μl made with DNase buffer (50 mM Tris HCl, pH 7.5, 50 mM KCl, 0.1 mM MgCl₂). Plasmid DNA (without any protein) served as a negative control. At the end of the reaction, DNA was extracted and analysed on a 0.8% agarose gel.

4.2.9.3 DNase-like activity for the evaluation of stability of rBalsamin

To determine the stability of rBalsamin, 0.25 μg of pUC 19 plasmid was incubated with 1.5 μg of five months old rBalsamin sample (stored at -80°C), and 10 U/μl restriction enzyme (BamHI) for 2 h at 37°C in a final volume of 10 μl made with DNase buffer (50 mM Tris HCl, pH 7.5, 50 mM KCl, 0.1 mM MgCl₂). Plasmid DNA (without any protein) served as a negative control. At the end of the reaction, DNA was extracted and analysed on a 0.8% agarose gel.
4.2.10 Antioxidant activity

4.2.10.1 Determination of DPPH radical scavenging activity

The activity was determined using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) as described by (Boonmee et al. 2011). For the analysis, 40 μl of various concentrations of rBalsamin (0.1, 0.5, 1, 1.5, 2, 2.5 mg/ml) and standard solution of ascorbic acid (1.56-200 μg/ml) were incubated with 160 μl of 0.2 mM DPPH solution at room temperature for 30 min in the dark. After incubation, the absorbance of the samples was measured at 517 nm. The DPPH free radical scavenging activity was calculated using the formula:

\[
\% \text{ scavenging activity} = \left[ \frac{Ab \text{ (control)} - Ab \text{ (sample)}}{Ab \text{ (control)}} \right] \times 100
\]

4.2.10.2 Determination of superoxide radical scavenging activity

Superoxide radical scavenging activity is based on the capacity of the protein to inhibit photochemical reduction of nitroblue tetrazolium (NBT). For this, 100 μl of various concentrations of rBalsamin (0.1, 0.5, 1, 1.5, 2, 2.5 mg/ml) and standard solution of ascorbic acid (25-500 μg/ml) were mixed with 100 μl riboflavin (20 μg), 200 μl EDTA solution (12 mM), 200 μl methanol and 100 μl NBT (0.1 mg). The final volume of each sample was made up to 3.0 ml with 50 mM phosphate buffer (pH 7.8). The samples were kept in front of fluorescent light for 5 min and absorbance was then measured at 590 nm using phosphate buffer as a blank. The superoxide scavenging activity was calculated with the formula:

\[
\% \text{ scavenging activity} = \left[ \frac{Ab \text{ (control)} - Ab \text{ (sample)}}{Ab \text{ (control)}} \right] \times 100
\]

4.2.10.3 Evaluation of reducing power ability

The reducing power of the proteins was determined according to (Hong et al. 2014) with minor modifications. For this, 0.2 ml of rBalsamin was mixed with 0.5 ml of 0.2 M sodium phosphate buffer (pH 7.4) and 0.5 ml of 1% (w/v) potassium ferricyanide solution. The reaction mixture was incubated at 50°C for 20 min. At the end of incubation, 0.5 ml of 10% (w/v) trichloroacetic acid was added to terminate the reaction. The mixture was then centrifuged at 2500 X g at 25°C for 10 min. 0.5
ml of the supernatant was mixed with 0.5 ml distilled water and 0.1 ml of 0.1% (w/v) ferric chloride. The absorbance was then measured at 700 nm. Phosphate buffer and ascorbic acid served as blank and positive controls, respectively.

### 4.2.11 Antimicrobial activity of rBalsamin

The antimicrobial activity of rBalsamin was determined by disc diffusion and broth microdilution methods, as described in Chapter 2.

### 4.3 Results and discussion

#### 4.3.1 PCR amplification of Bal gene

RIP genes have been reported to be intronless, therefore obtaining an open reading frame of the Bal gene from genomic DNA by PCR was possible. Genomic DNA from the seeds of Momordica balsamina was extracted (Figure 4.1) and used as a template for the amplification of the gene encoding balsamin. Among the 7 degenerate primers used to amplify the Bal gene fragment, one primer resulted in the amplification of an open reading frame of the mature peptide (Figure 4.2). The amplified gene (Figure 4.3) was purified and sent for sequencing. The sequencing results were confirmed by comparing the sequence with the nucleotide database using basic local alignment search tool (BLAST software, NCBI). A BLAST search of the sequenced gene showed 89% homology to α-MMC, a type-I RIP obtained from Momordica charantia (Figure 4.4). To obtain the maximum efficiency of translation, the sequence was sent to GenScript (NJ, USA) for rare codon optimization. The analysis report showed that the codon adaptation index (CAI) of the balsamin open reading frame was adjusted to 0.87 (a CAI of 0.1 was considered to be ideal) with average GC content of 52.51% (Annexure 8.1).
Figure 4.1 Genomic DNA isolated from *Momordica balsamina* seeds. Lane 1: 1KB DNA ladder; Lane 2 and 3: genomic DNA.

Figure 4.2 PCR amplification of genomic DNA with 7 degenerate primers. Lane 1: 1 KB DNA ladder; Lane 2-8: amplification with MAP, gel, sap, luf, PAP, mom and PAP-II primers, respectively. Arrow points to an amplified product.
Figure 4.3: Gene amplification with mom primers. Lane 1: 1 KB DNA ladder; Lane 2: amplification with mom primer.

4.3.2 Cloning of the Bal gene in pGEM-T cloning vector

Based on the sequence, specific primers with restriction sites (BamHI and XhoI) were designed. The sequenced gene (Bal gene) was amplified using these primers and purified from the gel. The Bal gene with restriction sites and pGEM-T cloning vector were double digested with BamHI and XhoI and ligated. The resultant recombinant plasmid (pGEM-Bal) was then transformed into E.coli DH10β. The cloning of the Bal open reading frame into the pGEM-T cloning vector was confirmed by purifying the plasmid from ampicillin-resistant colonies and performing colony PCR analysis (Figure 4.5 a) and digesting it with the endonucleases BamHI and XhoI (Figure 4.5 b).
Figure 4.4: Homology of Bal gene sequence with $\alpha$-MMC, a type-I RIP obtained from *Momordica charantia*.
Figure 4.5: Recombinant plasmid screening. (a) Colony PCR of recombinant plasmid. Lane 1: 1 KB DNA ladder; Lane 2: pGEM-T vector; Lane 3: \textit{Bal} gene; Lane 4-7: colony PCR of recombinant plasmids. (b) Restriction digestion of recombinant plasmid. Lane 1: 1 KB DNA ladder; Lane 2: pGEM-T vector; Lane 3: \textit{Bal} gene; Lane 4-7: recombinant plasmids digested with \textit{BamHI} and \textit{XhoI} restriction enzymes.
4.3.3 Cloning of *Bal* gene in pET-30a(+) expression vector

The *Bal* gene was excised from pGEM-T vector by digestion with *BamHI* and *XhoI* and ligated into pET-30a(+) vector digested with the same endonucleases, *BamHI* and *XhoI*. The resultant recombinant plasmid pET-Bal was then transformed into *E.coli* BL21(DE3) competent cells (Figure 4.6).

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**Figure 4.6** Schematic representations of steps involved in the cloning of *Bal* gene in pET-30a(+) vector.
4.3.4 Optimization of rBalsamin expression

A single colony harbouring the Bal gene was grown in LB broth containing 50 μg/ml kanamycin overnight at 37°C. 1% of the overnight culture was inoculated in 1 L of LB broth containing kanamycin, and incubated at 37°C, 250 rpm until an OD600 reached approximately 0.6. The expression of rBalsamin was then induced by IPTG. The recombinant bacterial cells were collected by centrifugation and disrupted by sonication. The expression and molecular weight of rBalsamin was assessed in the supernatant and cell pellet by 12% SDS-PAGE. A band at the expected size (~ 29 kDa) was observed in the cell pellet of the induced cells (Figure 4.7, Lane 3, arrow). No band was visible at this position in the supernatant (Figure 4.7, Lane 2) indicating that rBalsamin was expressed in the insoluble fraction and possibly got accumulated in inclusion bodies rather than the soluble fraction. Also, the absence of a protein band corresponding to the rBalamin in the uninduced cells (Figure 4.7, Lane 1) indicated tight control of induction of expression.

![Figure 4.7: SDS-PAGE analysis of expression of rBalsamin in E.coli. Lane M: protein marker, Lane 1: cell lysate of uninduced E.coli BL21(DE3) cells, Lane 2: supernatant (soluble fraction) of the induced E.coli BL21(DE3) cells, Lane 3: cell pellet (insoluble fraction) of the induced E.coli BL21(DE3) cells.](image)

To obtain a maximum yield of the rBalsamin, parameters such as temperature (20-37°C), IPTG concentration (0.2-1.0 mM) and induction time (4-20 h) were
optimized. It was observed that the optimum expression of insoluble rBalsamin was obtained at 30 °C (Figure 4.8a) after 4 h (Figure 4.8b) of induction with 0.4 mM IPTG (Figure 4.8c).

![Figure 4.8: Optimization of expression of rBalsamin in E.coli. Cultures were induced with IPTG at an OD$_{600\text{nm}}$ of 0.6. Equal volumes (10 μl) of culture supernatants from different conditions were analysed by 12% SDS-PAGE. a) Temperature optimization. SDS-PAGE analysis of supernatants of the uninduced (UI) and induced cultures grown at different temperatures (20-37 °C). b) Time kinetics of balsamin expression in E.coli BL21(DE3). SDS-PAGE analysis of the supernatants of the induced cultures harvested at different time periods (4-20 h). c) Optimisation of IPTG concentration. SDS-PAGE analysis of the supernatants of the UI and cultures induced with different IPTG concentrations (0.2-1.0 mM). M in all the panels denotes protein marker and the arrow points to the rBalsamin.](image-url)
4.3.5 Purification of rBalsamin

The His-tagged rBalsamin was purified by a two-step purification strategy. rBalsamin was expressed in the insoluble fraction, therefore, the purification of rBalsamin was carried out in a Ni Sepharose 6 fast flow column under denaturing conditions (buffer containing 8M urea). The rBalsamin in the cell suspension was adsorbed to the Ni\textsuperscript{2+} Sepharose column. The majority of the unadsorbed proteins were removed by washing the column with 15-column volumes of buffer A (wash buffer). Refolding of the bound protein was performed by the use of a linear gradient of urea (8-0 M) in the wash buffer. Finally, the rBalsamin was eluted from the column by increasing the concentration of imidazole to 250 mM in the elution buffer (with no urea). 10 μl of sample from each of the collected fractions was loaded on a 12% SDS-PAGE gel to assess the purity of the protein. The fractions were pooled and concentrated for gel filtration chromatography. The affinity tag has previously been used to facilitate purification of many recombinant proteins expressed in E.coli BL21(DE3) by employing Ni\textsuperscript{2+} Sepharose affinity chromatography (Han et al. 2011).

Gel filtration (GF) chromatography was carried out immediately after the first step of IMAC purification using a Superdex 75 gel-filtration column. The column was washed with 50 mM Tris-HCl, pH 7.5 (degassed) at a flow rate of 0.5 ml/min until a baseline was achieved. Two peaks were obtained (Figure 4.9) and analyzed using 12% SDS-PAGE. The purified fraction PII was subsequently found to contain most of the protein of approximately 29 kDa, corresponding to the predicted molecular mass of rBalsamin. The purity of the eluted rBalsamin was confirmed by the presence of single band on the gel. Figure 4.10 summarizes the purification of rBalsamin. Approximately 45 mg rBalsamin was purified from 1 L of culture medium, by this two-step purification procedure. A similar strategy has previously been employed for purification of other RIPs in E.coli strains. α-luffin, a type-I RIP, was expressed in E. coli BL21(DE3)pLysS strain and purified by Ni- nitrilotriacetic acid (NTA) agarose affinity chromatography. However, the final yield of soluble protein was reported to be 0.45 mg/l bacterial cell culture (Liu et al. 2010), 100 times lower compared to the protein yield in our study. α-MMC expressed in E.coli Rosetta (DE3)pLysS strain and purified by Ni- NTA agarose affinity
chromatography produced 85 mg/l of soluble α-MMC (Wang et al. 2012), around double the yield reported in our study. This difference might be due to the fact that rBalsamin was expressed in the insoluble fraction, which required additional purification and protein-refolding steps leading to reduced protein yield.

Figure 4.9: Profile of superdex-75 gel filtration chromatography. Fractions corresponding the peaks were pooled separately and designated as PI and PII.
Figure 4.10: SDS-PAGE analysis of rBalsamin expressed in *E. coli*. Lane M: protein marker, Lane 1: cell lysate of uninduced *E. coli* BL21(DE3) cells, Lane 2: cell lysate of IPTG induced *E. coli* BL21 (DE3) cells, Lane 3: supernatant of the cell lysate loaded onto Ni$^{2+}$ sepharose column, Lane 4: flow through, Lane 5 & 6: Ni$^{2+}$ fractions, Lane 7: concentrated Ni$^{2+}$ fraction, Lane 8: GF PII fraction and Lane 9: concentrated PII fraction (purified rBalsamin).

4.3.6 rRNA N-glycosidase activity

Reports have shown that RIP-mediated cleavage of the 28S rRNA leads to the release of a 300-400 nucleotide product, named the Endo-fragment (Choudhary, Yadav & Lodha 2008; Girbes et al. 2004). To confirm whether the purified rBalsamin was properly refolded during purification and is biologically active, an rRNA N-glycosidase assay was performed. The activity was determined by analyzing rRNA treated with the purified rBalsamin. It was observed that the 28S rRNA generated a typical Endo fragment after treatment with rBalsamin. The rRNAs seemed stable towards rBalsamin without the treatment of aniline as no fragment appeared at this stage on a 0.8% agarose gel. Only after the treatment with aniline, the rRNA was cleaved resulting in generation of the Endo-fragment (Figure 4.11). nBalsamin isolated from *Momordica balsamina* served as a positive control. These results were in agreement with previous reports on RIPs from different plant species (Choudhary, Yadav & Lodha 2008; Sharma et al. 2004).
Figure 4.11: RNA N-glycosidase activity of rBalsamin when incubated with rabbit reticulocyte lysate. The rRNA was extracted and treated (+) with or without aniline (-) and analysed on a 2% agarose gel. The Endo-fragment generated after balsamin treatment is denoted with an arrow.

4.3.7 Biophysical characterization of rBalsamin

4.3.7.1 Circular dichroism (CD) spectroscopy

CD spectra of rBalsamin indicated the presence of predominantly α-helices and random coils. rBalsamin exhibited 34.8% α-helix, 19.3% β-sheets, 12.5% turns and 32.5% random coils at 25°C, which is comparable to the CD profile reported for nBalsamin (Kaur et al. 2012). Some similar structures have been reported with other RIPS. CD spectral analysis of pulchellin isoforms from Abrus pulchellus showed the presence of α-helix (10-16%), β-sheets (30-32%), turns (20-24%) and random coils (33-36%) (Castilho et al. 2008). Trichosanthin (TCS), a type-I RIP purified from Trichosanthes kirilowii maxim, comprises of α-helices, β-sheets, random coils and turns and the percentage of secondary structure confirmation of TCS varies with change in pH (Xia & Sui 2000).
In our study, the secondary structure of rBalsamin was found to be stable in the temperature range of 20-60°C as little or no change in the CD spectrum was observed within this temperature range. However, above 60°C, a slight distortion was observed in the secondary structure indicating that rBalsamin is unstable at high temperatures (Figure 4.12a).

The effect of pH on the secondary structure of rBalsamin was studied to determine the stability of the protein. rBalsamin seemed stable as no change in the secondary structure was noted between pH 6-11. However, below pH 6, a slight disruption in the secondary structure was observed indicating that rBalsamin is unstable under acidic conditions (Figure 4.12b).

4.3.7.2 Fluorescence spectroscopy

The effect of temperature and pH on the native structure of rBalsamin was studied using fluorescence spectroscopy. rBalsamin was found to be stable in the temperature range of 20-60°C as no shift in the emission maximum was observed within this temperature range. However, above 60°C, a decrease in the fluorescence intensity with a shift in emission maximum was observed, indicating that rBalsamin is unstable at higher temperatures (Figure 4.13a).

An increase in the relative fluorescence intensity with a slight red shift was observed with an increase in pH from 4 to 5. rBalsamin seemed stable between pH 6 to 9 as no change in the emission maximum was observed within this pH range. A further increase in pH from 9 to 11 resulted in a decrease in relative fluorescence and slight shift in the emission maximum, suggesting that rBalsamin is unstable at pH below 6 and above 9 (Figure 4.13b).
Figure 4.12: CD spectra of rBalsamin at different temperature (a) and pH (b).
Figure 4.13: Fluorescence spectra of rBalsamin at different temperature (a) and pH (b).
4.3.8 Bioinformatics analysis

4.3.8.1 Protein sequence identification and homology

The Bal gene sequence was translated using the EXPASY translate tool. The conceptually translated rBalsamin sequence was used to determine the homology of the protein with other type-I RIPS in GenBank. A BLAST search of the predicted amino acid sequence of rBalsamin was performed to determine its similarity with other RIP sequences (Table 4.2). The analysis showed that the rBalsamin has highest similarity (91%) with α-MMC from Momordica charantia followed by 89% with momordin from Momordica charantia. The protein has less similarity, ranging between 64-72%, with luffaculin 1, luffin-B, trichomisin, trichosanthin, trichobakin, bryodin-I and karasurin-C (Table 4.2).

4.3.8.2 Prediction of biochemical, biophysical and physicochemical parameters

The biochemical, biophysical and physicochemical parameters of rBalsamin were deduced using the PROTPARAM program of the EXPASY protein analysis tool. The analysis showed that rBalsamin consisted of 247 amino acids with a predicted molecular weight of 27.23 kDa and isoelectric point of 9.19. The protein consisted of 25 positively and 20 negatively charged amino acids. The instability index was estimated to be 33.40 indicating that rBalsamin is a stable protein (Table 4.3). Through EXPASY analysis, the predicted molecular weight of the protein was 27.23 kDa. However, the calculated molecular mass of rBalsamin obtained from SDS-PAGE was around 29 kDa. This difference was due to the presence of His-tag at the N-terminal of the purified rBalsamin.

In contrast, nBalsamin consisted of 262 amino acids with a molecular weight of 28.90 kDa and isoelectric point of 9.43. The protein consisted of 27 positively and 20 negatively charged amino acids with estimated index stability of 28.45 (Kaur et al. 2012). This difference in the physicochemical parameters of rBalsamin and nBalsamin might be due to the fact that open reading frame of Bal gene was selected for expression instead of the whole gene.
Table 4.2 BLASTP analysis of rBalsamin with the protein sequences in the NCBI database.

<table>
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<tr>
<th>Species</th>
<th>Name of protein</th>
<th>Accession no.</th>
<th>Identity (%)</th>
<th>E-value</th>
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<tr>
<td><em>Momordica charantia</em></td>
<td>α-MMC</td>
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<td>P84530.2</td>
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<td>1e-109</td>
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<tr>
<td><em>Luffa aegyptiaca</em></td>
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<td>P22851.1</td>
<td>70</td>
<td>1e-109</td>
</tr>
<tr>
<td><em>Trichosanthes kirilowii</em></td>
<td>Trichomislin</td>
<td>AA592579.1</td>
<td>66</td>
<td>1e-104</td>
</tr>
<tr>
<td><em>Trichosanthes kirilowii</em></td>
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<td>P09989.2</td>
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<td>6e-108</td>
</tr>
<tr>
<td><em>Trichosanthes kirilowii</em></td>
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<tr>
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<td>P33185.3</td>
<td>65</td>
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<tr>
<td><em>Trichosanthes kirilowii</em></td>
<td>Karasurin-C</td>
<td>P24478.2</td>
<td>64</td>
<td>4e-109</td>
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</tbody>
</table>

E-value (Expect value) describes the likelihood that a sequence with a similar score will occur by chance when searching a database of particular size. The lower the E-value, the more significant a match to a database sequence is.
### Table 4.3 Biophysical and physicochemical parameters of rBalsamin predicted using the PROTPARAM tool of the EXPASY protein analysis server.

<table>
<thead>
<tr>
<th>Biochemical/biophysical/physicochemical parameters</th>
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</tr>
<tr>
<td>Molecular weight</td>
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</tr>
<tr>
<td>Theoretical $pI$</td>
<td>9.19</td>
</tr>
<tr>
<td>Total number of negatively charged residues (Asp + Glu)</td>
<td>20</td>
</tr>
<tr>
<td>Total number of positively charged residues (Arg + Lys)</td>
<td>25</td>
</tr>
<tr>
<td>Total number of atoms</td>
<td>3877</td>
</tr>
<tr>
<td>Extinction coefficient</td>
<td>24870</td>
</tr>
<tr>
<td>Estimated half-life</td>
<td>30 h (mammalian reticulocytes, <em>in vitro</em>)</td>
</tr>
<tr>
<td></td>
<td>&gt;20 h (yeast, <em>in vivo</em>)</td>
</tr>
<tr>
<td></td>
<td>&gt;10 h (<em>E. coli, in vivo</em>)</td>
</tr>
<tr>
<td>Instability index</td>
<td>33.40</td>
</tr>
<tr>
<td>Aliphatic index</td>
<td>103.12</td>
</tr>
<tr>
<td>Grand average of hydrophathicity (GRAVY)</td>
<td>-0.011</td>
</tr>
</tbody>
</table>
4.3.9 DNase-like activity

4.3.9.1 Optimization of rBalsamin concentration for DNase-like activity

To optimize rBalsamin concentration for DNase-like activity, pUC19 plasmid was used as a substrate. Treatment of supercoiled pUC19 with various concentrations of rBalsamin (0.5-2 μg) resulted in conversion of supercoiled plasmid into the open circular and linear forms (Figure 4.14). 0.5 and 1 μg of rBalsamin treatment partially converted the supercoiled form into the open circular form. However, when the concentration of rBalsamin was increased to 1.5 μg, almost complete disappearance of supercoiled plasmid was observed with the appearance of the linear form of the DNA. At a concentration of 2 μg, rBalsamin completely converted supercoiled and open circular forms of DNA to the linear form, suggesting that 2 μg of rBalsamin is the optimum concentration for DNase-like activity (Figure 4.14).

![Figure 4.14: Effect of rBalsamin concentration on DNase-like activity. “NC”, untreated plasmid DNA (negative control); “PC”, plasmid treated with EcoRI (positive control); remaining lanes represent plasmid treated with different concentrations of balsamin (0.5-2 μg). “OC”, “L” and “SC” denotes open circular, linear and supercoiled plasmid respectively.](image)

4.3.9.2 DNase-like activity of nBalsamin and rBalsamin

To compare DNase-like activity, pUC19 plasmid was treated with 2.0 μg of nBalsamin, rBalsamin and restriction enzymes (HindIII, BamHI and NdeI). The experiments showed that rBalsamin completely converted supercoiled form of plasmid into the linear form, equivalent to the results observed with restriction enzymes. However, nBalsamin could only partially convert the supercoiled form of
plasmid as a mixture of both open circular and linear confirmations were observed after the treatment (Figure 4.15). These results suggest that rBalsamin is more effective in terms of DNase-like activity at a concentration of 2.0 μg compared to nBalsamin.

Figure 4.15: DNase-like activity of rBalsamin, nBalsamin and various restriction enzymes. “NC”, untreated plasmid DNA (negative control); remaining lanes represent plasmid treated with rBalsamin, nBalsamin and restriction enzymes (positive controls). “OC”, “L” and “SC” denotes open circular, linear and supercoiled plasmid respectively.

4.3.9.3 DNase-like activity for the evaluation of stability of rBalsamin

To evaluate the stability and efficacy of rBalsamin, pUC19 plasmid was treated with 2.0 μg of five months old rBalsamin (stored at -80°C) sample and restriction enzyme, BamHI. The experiments showed that rBalsamin completely converted supercoiled form of DNA into the linear form (Lane 1-3, Figure 4.16) equivalent to the results observed with restriction enzyme (Lane PC, Figure 4.16). These results suggest that rBalsamin is stable when stored at -80°C for several months, confirming the efficacy and stability of rBalsamin.
Figure 4.16: DNase-like activity of five months old rBalsamin sample. “NC”, untreated plasmid DNA (negative control); “PC”, plasmid treated with *BamHI*; “Lane 1-3”, plasmid treated with rBalsamin taken from three different aliquots stored at -80°C for 5 months. “OC”, “L” and “SC” denotes open circular, linear and supercoiled plasmid respectively.

4.3.10 Antioxidant activity of rBalsamin

DPPH is a free radical compound and has been widely used to test free radical-scavenging ability (Priyanka et al. 2013; Yan, Cao & Yang 2014; Yang, Li & Chuang 2012). This stable free radical with a characteristic absorption at 517 nm was used to determine the radical scavenging activity of rBalsamin with ascorbic acid as a standard. The results showed that rBalsamin concentration did not affect free radical scavenging of DPPH as no DPPH reduction was observed (Annexure 8.2a).

The superoxide radical scavenging assay is based on the capacity of the protein to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Harman 1992). However, in our study, it was observed that rBalsamin did not exhibit this activity as no NBT reduction was observed (Annexure 8.2b). Some RIPs such camphorin from *C. camphora* (Li et al. 1997) and *C. moschata* (Park et al. 2004) have been reported to exhibit superoxide dismutase activity. However, reports on similar RIPs such as α-MMC and MAP30 from *Momordica charantia* have shown that these RIPs do not possess superoxide dismutase activity (Meng et al. 2014).

Reducing power measures the total antioxidant ability of a compound and the efficiency of an antioxidant to donate electrons. The reducing ability of the compound ferricyanide complex (Fe\(^{3+}\)) to ferrous serves as an important indicator of
Chapter 4

antioxidant activity (Benzie & Strain 1999). The assay showed that the reducing power ability tended to increase as the concentration of rBalsamin increased. However, none of these results were statistically significant demonstrating that the protein does not possess reducing ability (Annexure 8.2c).

It is interesting to note that Balsamin extracted from a natural source (nBalsamin) exhibits free radical scavenging and reducing power ability, however, rBalsamin does not. Further investigation is required to identify the main cause of this difference.

4.3.11 Antimicrobial activity of rBalsamin

RIPs have been classified as defense proteins, which could function as antimicrobial agents (Girbes et al. 2004). α-MMC from Momordica charantia inhibited the growth of several bacterial and fungal pathogens such as Fusarium oxysporum, Fusarium solani, E. coli, P. aeruginosa, S. aureus and Bacillus subtilis (Wang et al. 2012). ME1 and ME2, two type I RIPs, from Mirabilis expansa roots exhibited strong inhibitory activity against an array of fungal pathogens including Fusarium and Trichoderma species (Vivanco, Savary & Flores 1999). Therefore, we investigated the antimicrobial potential of rBalsamin. The disc diffusion method was performed to determine the zone of inhibition of different dilutions of rBalsamin on various pathogenic microorganisms. The assay demonstrated that rBalsamin inhibited the growth of pathogens in a dose dependent manner with a maximum inhibition observed at the highest dose. S. epidermidis, S. aureus, S. enterica and E. coli demonstrated higher susceptibility to rBalsamin while P. aeruginosa and E. faecalis demonstrated comparatively less susceptibility to rBalsamin (Figure 4.17). The zone of inhibition caused by different dilutions of rBalsamin on the different pathogens ranged between 2.2-9.0 mm (Table 4.4).
Figure 4.17: Antibacterial activity of different dilutions of rBalsamin against Staphylococcus aureus ATCC 25923 (A), Pseudomonas aeruginosa ATCC 27853 (B), Escherichia coli ATCC 2348 (C), Staphylococcus epidermidis ATCC 12228 (D), Salmonella enterica ATCC 43971 (E), Enterococcus faecalis ATCC 10100 (F). Disc 1 contains PBS (control); Disc 2, 3 and 4 contains 50 μg/ml, 100 μg/ml, and 200 μg/ml of rBalsamin respectively, in each plate.
Table 4.4: Antibacterial activity of rBalsamin towards different pathogenic microorganisms.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Zone of inhibition in mm ± SD</th>
<th>rBalsamin concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>3.1 ± 0.2</td>
<td>6.2 ± 0.5</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>ND</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>2.2 ± 0.1</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>4.1 ± 0.3</td>
<td>6.2 ± 0.4</td>
</tr>
<tr>
<td>Salmonella enterica</td>
<td>2.7 ± 0.2</td>
<td>5.5 ± 0.6</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are mean ± SD based on three sets of experiments; ND denotes not detected.

The minimum inhibitory concentrations (MIC’s) of rBalsamin were determined by the broth microdilution method. The MIC’s of rBalsamin were found to be in the following order: \( S.\ epidermidis < S.\ aureus < S.\ enterica = E.\ coli < P.\ aeruginosa = E.\ faecalis \) (Annexure 8.3, Table 4.5). A number of RIPs have been reported to function as antimicrobial agents to defend plants from various fungal, viral and bacterial attacks (Girbes et al. 2004). \( \alpha\)-MMC from \textit{Momordica charantia} inhibited the replication of chilli veinal mottle virus, tobacco mosaic virus and cucumber mosaic virus in plants treated with various concentration of \( \alpha\)-MMC (Zhu et al. 2013). \( \alpha\)-MMC has also been found to inhibit the growth of several pathogens including \textit{Fusarium oxysporum, Fusarium solani, E. coli, P. aeruginosa, S. aureus and Bacillus subtilis} (Wang et al. 2012). Our studies demonstrated that rBalsamin exhibited growth inhibitory activity towards various pathogens that could prove beneficial in plant protection.
Table 4.5: Minimum inhibitory concentrations (MICs) of rBalsamin against different pathogenic microorganisms.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>MICs of rBalsamin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>6.25</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>25.00</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>12.50</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>1.56</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td>12.50</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>25.00</td>
</tr>
</tbody>
</table>
4.4 Conclusions

The present study discusses the development of a two-step purification procedure for the production of functionally bioactive balsamin from *Momordica balsamina*. The open reading frame of the *Bal* gene was successfully obtained from *Momordica balsamina* genomic DNA. After optimizing the expression parameters such as induction time, IPTG concentration and temperature, maximum protein yield was obtained at 0.4 mM IPTG induction at 30°C for 4 h in the *E.coli* BL21(DE3) expression system. The incorporation of a His-tag on the N-terminus of the protein allowed purification of rBalsamin by Ni²⁺ Sepharose affinity chromatography. A total of 45 mg of protein was obtained from a 1 L culture of induced *E.coli* BL21(DE3) cells. Functional analysis indicated that rBalsamin exhibited RNA N-glycosidase activity, releasing the Endo-fragment from rabbit reticulocyte rRNA, and DNase-like activity, converting the supercoiled form of a plasmid DNA into the linear form in a concentration-dependent manner. The structure of rBalsamin was found to be sensitive to change in temperature and pH, suggesting that any changes in pH and temperature are likely to affect its structure and function. Antimicrobial assay revealed that rBalsamin could inhibit the growth of various pathogens with MICs ranging between 1.56-25 µg/ml, which may enable the development of transgenic crops resistant to microbial infections. However, further investigation of the cytotoxicity of rBalsamin on cancer cells would be beneficial to explore its potential use in the field of cancer therapeutics.
Chapter 5: Antitumor activity of rBalsamin from *Momordica balsamina* on liver and breast cancer cells.
5.1 Introduction

In our previous study, we developed a molecular based strategy to produce rBalsamin in an *E.coli* strain. We reported that besides RNA N-glycosidase activity, rBalsamin exhibits DNase-like activity towards the pUC-19 plasmid and broad-spectrum antimicrobial activity towards various pathogens. Further in depth investigation is required to ascertain its function as a potent therapeutic agent for cancer. Therefore, in this study, we extended the potential biological application of rBalsamin from nuclease and bactericide to cancer.

RIPs are considered by some as a potential future treatment against various tumors as they have the potential to inhibit tumor growth and induce apoptosis *in vitro* and *in vivo* through different molecular mechanisms. rMAP30 from *Momordica charantia* has been reported to inhibit the viability of bladder (5637), colorectal (LoVo), breast (MCF-7), cervical (HeLa), liver (SMMC-7221), gastric (BGC823 and MGC803) and skin (B16) cancer cells in a dose and time dependent manner (Fan et al. 2008; Fan et al. 2015; Hao et al. 2014). The apoptotic events in rMAP30 treated LoVo cells included increased *Bax* and decreased *Bcl-2* expression (Fan et al. 2008), and in BGC823 cells, included decreased mitochondrial membrane potential and increased caspase-3, caspase-9, cyt C content and Apaf-1 mRNA expression (Han et al. 2011). rTCS from *Trichosanthes kirilowii* demonstrated cytotoxicity towards various cancer cells, including lung (95D), cervical (HeLa), lung (A549), hepatocellular (SMMC-7221), breast (MCF-7), colorectal (LoVo) and cervical (Caski) cancer cells (Gao et al. 2010; Lu et al. 2016; Peng et al. 2011). rTCS-induced apoptosis in Caski cells involved activation of caspase-9, decreased *Bcl-2/Bax* ratio and cleavage of PARP (Lu et al. 2016). Although a number of studies have shown that recombinant RIPs could inhibit proliferation and induce apoptosis in a variety of cancer cells, little is known about their cytotoxicity and the underlying mechanism(s) of apoptosis in liver and breast cancer cells. Therefore, in this study, we investigated the anti-proliferative activity of rBalsamin towards liver (HepG2 and H4IIE) and breast (MCF-7 and BT549) cancer cells and investigated the possible molecular mechanism(s) of action.

Breast cancer is the second most common cancer worldwide. Estrogen receptor α (ERα) plays an important role in breast carcinogenesis as more than 70% of breast
cancer tissues express elevated levels of ERα compared to normal breast tissue (Ali & Coombes 2000). The most common treatment available for ERα-positive breast cancer is endocrine therapy that involves the use of drugs that are either endocrine receptor modulators (such as tamoxifen) or endocrine receptor downregulators (such as fluvestrant). However, the majority of ERα-positive breast cancer does not respond to endocrine therapy and eventually become resistant (Osborne & Schiff 2011; Pan et al. 2013). Therefore, identification of therapeutic agents that can target both ERα-positive and ERα-negative tumors without developing resistance would prove beneficial for breast cancer therapy. Therefore, in this study, we investigated the antitumor potential of rBalsamin on MCF-7 (ERα-positive) and BT549 (ERα-negative) breast cancer cells.

In addition, hepatocellular carcinoma (HCC) is the most common type of liver cancer (Venook et al. 2010). Surgical treatments (such as liver transplantation and partial liver resection) and non-surgical treatments (such as percutaneous ethanol injection and radiofrequency ablations) are available, however they have limitations associated with their use (Peng et al. 2015; Shi et al. 2013). Thus, in this study we also explored the efficacy of rBalsamin on liver (HepG2 and H4IIE) cancer cells as developing a single therapeutic bioactive that can target a variety of cancer cell types would be a promising tool in the field of cancer therapeutics.

5.2 Materials and methods

5.2.1 Chemicals, reagents and kits

RPMI and DMEM media, and FBS were purchased from Life Technologies Corporation (California, USA). Trypsin, In vitro toxicology assay kit, Caspase-3 assay kit and Caspase-8 assay kit were purchased from Sigma-Aldrich (USA). Total RNA isolation kit was purchased from Qiagen (Hilden, Germany). Propidium iodide (PI) flow cytometry kit was procured from BD Biosciences (San Jose, CA). Pierce high-capacity endotoxin removal spin columns were purchased from Thermo Fisher Scientific (Victoria, Australia). Reagents for electrophoresis were procured from Bio-Rad Laboratories (California, USA). All other reagents were purchased from either Sigma-Aldrich (USA) or Bio-Rad Laboratories (California, USA) and were of analytical grade.
5.2.2 Cell lines and cell culture

The two liver cancer cell lines, HepG2 (human hepatoma) and H4IIE (rat hepatoma), and two breast cancer cell lines, MCF-7 (ERα-positive) and BT549 (ERα-negative) were used for the present study. These cell lines were obtained from the ATCC (Rockville, MD). HepG2 and H4IIE cells were maintained in RPMI medium supplemented with 10% FBS, in an atmosphere of 5% CO₂ at 37°C. The cells were grown in T-25 flasks until 80% confluence and split 1:10 in culture dishes before assays were performed. MCF-7 and BT549 cells were maintained in DMEM supplemented with 10% FBS, in an atmosphere of 5% CO₂ at 37°C. The cells were grown in T-25 flasks until 80% confluence and split 1:4 in culture dishes before assays were performed.

5.2.3 Endotoxin removal from rBalsamin

Since rBalsamin was expressed and purified from E.coli (bacterial) strain, there were chances that the purified product (rBalsamin) could contain endotoxin, which could contaminate the mammalian cell cultures used in the study. Therefore, prior to use, rBalsamin was made endotoxin free by the use of Pierce high-capacity endotoxin removal spin columns as per manufacturer’s protocol. Briefly, the spin column was placed in a collection tube and centrifuged at 500 X g for 1 min to remove the storage buffer. The flow through was discarded. The column was then washed with 0.2 N NaOH in 95% ethanol and 2 M NaCl. After discarding the flow through, the column was finally washed 4 times with endotoxin-free buffer. The column was then placed in a fresh collection tube. rBalsamin was loaded onto the column and incubated at 4°C for 1 h. The column was then centrifuged at 500 X g for 1 min to collect the sample (endotoxin free rBalsamin), which was stored at -20°C until further use.

5.2.4 rBalsamin treatment groups

For cell viability assays, rBalsamin at doses of 3.125-200 µg/ml was used. PBS was used as a negative (vehicle) control. For all other assays, liver (HepG2 and H4IIE) and breast (MCF-7 and BT549) cancer cells were categorized into four groups:
control (PBS); low (6.25 µg/ml), medium (25 µg/ml) and high (100 µg/ml) dose rBalsamin groups, unless otherwise stated.

5.2.5 Cell viability assay

The cell viability assay was performed as described in section 3.2.3.

5.2.6 DNA fragmentation assay

The DNA fragmentation assay was performed as described in section 3.2.4.

5.2.7 Cell cycle analysis

The cell cycle distribution was analysed by FACSCanto™ II (BD Biosciences, US) using propidium iodide (PI), as described in section 3.2.5.

5.2.8 Caspase-3/-8 activity assay

The caspase-3 and -8 activity was determined using caspase-3 and caspase-8 colorimetric assay kits (Sigma, USA), as described in section 3.2.6.

5.2.9 RNA extraction and RT-PCR for gene expression studies

The gene expression studies were carried out as described in section 3.2.7.

5.2.10 Statistical analysis

Data was analyzed using SPSS Statistics software version 22.0. Data was presented as the mean ± SD for at least three independently performed experiments unless otherwise stated. Data was assessed for normality using Kolmogorov-Smirnov test. One-way ANOVA was used to compare the difference among multiple groups and repeated measurements within one group. Two-way ANOVA was used to compare the mean difference between groups that have been separated based on two independent factors. Probability values with p < 0.05 were considered to be statistically significant.
5.3 Results and discussion

5.3.1 Growth inhibitory effect of rBalsamin on liver and breast cancer cells

RIPs occurring naturally in plants have gained attention as potential anticancer therapeutic agents because of their ability to inhibit tumor growth with nominal or no side effects (Fang et al. 2012b; Schreiber et al. 1999). Recombinant RIPs from *Momordica charantia* have been shown to produce different degrees of inhibition on various tumor cells, including bladder (5637), colorectal (LoVo), breast (MCF-7), cervical (HeLa), liver (SMMC-7221), gastric (BGC823 and MGC803) and skin (B16) cancer (Zeng et al. 2015). However, little has been reported about the efficacy of recombinant RIPs on breast and liver cancer cells and associated mechanisms. Therefore, in this study, we evaluated the cytotoxic effect of rBalsamin on liver (HepG2 and H4IIE) and breast (MCF-7 and BT549) cancer cells and explored the mechanism(s) by which it induces apoptosis in these cell lines.

Firstly, the effect of rBalsamin treatment on cell viability was evaluated. Liver and breast cancer cells were treated with different concentrations of rBalsamin (3.125-200 µg/ml) to investigate the dose- and time-dependent response of rBalsamin. The results showed that rBalsamin suppressed cell proliferation in a dose and time-dependent manner with IC$_{50}$ values ranging from 25.53 to 190.57 µg/ml in HepG2 cells (Figure 5.1a), 43.82 to > 200 µg/ml in H4IIE cells (Figure 5.1b), 18.92 to 112.17 µg/ml in MCF-7 cells (Figure 5.1c), and 38.09 to > 200 µg/ml in BT549 cells (Figure 5.1d). rBalsamin exhibited maximum cytotoxicity towards MCF-7 followed by HepG2, BT549 and H4IIE cells. From this, it can be concluded that rBalsamin possesses different degrees of cytotoxicity in these cell lines.

In contrast to nBalsamin, rBalsamin was found to be more effective towards HepG2 and MCF-7 cells and less effective towards H4IIE and BT549 cells after 72 hr of treatment. It is interesting to note that the level of cytotoxicity of rBalsamin and nBalsamin varies within the same cells. Further investigation is required to identify the cause of this variability of nBalsamin and rBalsamin.

Cell death occurs as a result of apoptosis (Type I programmed cell death), autophagy (Type II programmed cell death), or necrosis (non-physiological process that occurs...
as a result of infection or injury to the cell) (Kroemer et al. 2009). Since RIPs have been reported to induce apoptosis in cancer cells by regulating the activation of various apoptotic pathways, we decided to focus towards studying the key hallmarks of apoptosis (including DNA fragmentation, cell cycle arrest and expression of various apoptotic genes) in rBalsamin treated liver and breast cancer cells.

Figure 5.1: Effect of different concentrations of rBalsamin on the viability of HepG2 (a), H4IIE (b), MCF-7 (c) and BT549 (d) cells after 24 h, 48 h and 72 h treatment. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.001, main effect of protein concentration; main effect of time; interaction between rBalsamin and time, Two-way ANOVA.
5.3.2 rBalsamin induces changes in apoptotic morphology and DNA fragmentation in liver and breast cancer cells

Apoptosis is characterized by specific morphological changes in the cell. These morphological changes include cell shrinkage, condensation of nuclear chromatin, nuclear membrane twisting, and loss of cell connections (Ziegler & Groscurth 2004). Therefore, we examined the morphology of HepG2, H4IIE, MCF-7 and BT549 cells after rBalsamin treatment. Brightfield microscopy showed that cancer cells treated with PBS (negative control) grew efficiently in a uniform pattern with prominent cellular outlines. However, after rBalsamin treatment, the growth of HepG2, H4IIE, MCF-7 and BT549 cells was inhibited. The inhibition increased with increased rBalsamin concentration. Cells treated with rBalsamin exhibited characteristics of apoptosis including cell shrinkage, decrease or disappearance of cell connections, increased cytoplasmic density and increased cell detachment from the surface. A significant increase in the proportion of dead cells was observed in rBalsamin treated groups compared to untreated groups (Figure 5.2), similar to the result observed with nBalsamin (Figure 3.3).

Apart from the morphological changes of apoptosis observed in most of the cell types, one of the main causes of cell death is DNA fragmentation (Roos & Kaina 2006). Therefore, we analysed the DNA of H4IIE, HepG2, MCF-7 and BT549 cells treated with different concentrations of rBalsamin (6.25, 25 and 100 µg/ml) on 1.5% agarose gels. As shown in Figure 5.3, rBalsamin treatment resulted in fragmentation of DNA into small oligonucleotides appearing at an interval of 180-200 bp in all four cell lines, however, no DNA fragmentation was observed in the cells treated with PBS (negative control). DNA laddering appeared to be more conspicuous with increasing rBalsamin concentration. These results were similar to the result observed with nBalsamin (Figure 3.4).
Figure 5.2: Morphology of HepG2, H4IIE, MCF-7 and BT549 cells treated with different concentration of rBalsamin for 48 h.
Figure 5.3: DNA fragmentation in HepG2 (a), H4IIE (b), MCF-7 (c) and BT549 (d) cells treated with different concentrations of rBalsamin for 48 h. M and C denotes marker (1 KB DNA ladder) and control, respectively.
5.3.3 rbalsamin induces cell cycle arrest in liver and breast cancer cells

Regulation of cell cycle is coupled to cell death and has major significance in cell turnover and tumorigenesis (Alenzi 2004). It has been reported that extensive DNA damage can result in activation of cell cycle checkpoints and lead to cell cycle arrest and apoptosis (Pietenpol & Stewart 2002). Therefore, in our study, we determined the distribution of rbalsamin-treated HepG2, H4IIE, MCF-7 and BT549 cells in different phases of the cell cycle. Cell proliferation is regulated by the cell cycle; therefore sub G1 phase of the cell cycle can also give an estimate of apoptosis. As shown in Figure 5.4a and b, treatment with different concentrations of rbalsamin for 48 h led to a dose-dependent increase in the proportion of HepG2 (p < 0.001) and H4IIE (p < 0.001) cells in the apoptotic phase compared to the untreated cells. This increase in the apoptotic population in HepG2 cells was accompanied by a dose-dependent increase in the proportion of cells in G phase (p < 0.001) and decrease in S phase (Figure 5.4a), suggesting that rbalsamin induces apoptosis in HepG2 cells by blocking the cell cycle at the G phase, similar to the result observed with nbalsamin (Figure 3.5a). Comparatively, an opposite trend was observed in the case of H4IIE cells (Figure 5.4b), indicating that rbalsamin induces apoptosis by arresting the cell cycle at the S phase in H4IIE cells, similar to the result observed with nbalsamin (Figure 3.5b).

In the case of breast cancer cells, treatment with different concentrations of rbalsamin for 48 h led to a dose-dependent increase in the distribution of cells in the apoptotic phase in MCF-7 (p < 0.001) and BT549 cells (p < 0.05). This increase was accompanied by an increase in the proportion of cells in G phase at rbalsamin concentration of 6.25 µg/ml in MCF-7 and BT549 cells. However, with further increases in the rbalsamin dose (25 µg/ml and 100 µg/ml), the proportion of cells decreased in G phase in MCF-7 and BT549 cells. Comparatively, the proportion of cells decreased in a dose-dependent manner in S phase in both cell lines (Figure 5.5a and b). These results indicated that rbalsamin tends to arrest the cell cycle at the G phase at low rbalsamin dose (6.25 µg/ml) in both MCF-7 and BT549 cells. However, when the dose is increased (25 µg/ml and 100 µg/ml), rbalsamin drives the cells more towards apoptosis from G phase of the cell cycle. Unlike rbalsamin, nbalsamin isolated from Momordica balsamina induced S-phase cell cycle in MCF-
7 and BT549 cells which was accompanied by decrease in the proportion of cells in M and G phase (Figure 3.6 a and b).

It is noteworthy that rBalsamin induced cell cycle arrest varies with cancer cell type. Similar evidence has been reported with other RIPs. α-MMC from *Momordica charantia* blocks cell cycle at S phase in lung cancer (A549) cells (Fan et al., 2015). While in breast cancer cells, α-MMC induces cell cycle arrest at G phase in MDA-MB-231 cells and M phase in MDA-MB-453 and MCF-7 cells (Cao et al., 2015), suggesting that RIPs could act differently in similar cancer cell types as well.

**Figure 5.4:** Cell cycle analysis of HepG2 (a) and H4IIE (b) cells treated with different concentrations of rBalsamin for 48 h. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.001, One-way ANOVA.
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5.3.4 rBalsamin induced apoptosis involves activation of caspases in liver and breast cancer cells

Caspases are a family of cysteine proteases that are considered as the key regulators of apoptosis. Caspase-3, a key effector is activated by several initiator caspases, including caspase-2, -8, -9, -10, -11 and -12, that in turn executes apoptosis by regulating the expression of various apoptotic genes (Shi 2004). Therefore, in our study, we determined the activity of caspase-3 and -8 in rBalsamin treated liver and breast cancer cells. It was observed that rBalsamin significantly increased the activity of caspase -3 (P < 0.001) and -8 (P < 0.001) in H4IIE, HepG2, MCF-7 and BT549 cells in a dose dependent manner. The increase in caspase-3 and -8 activity appeared to be more pronounced in liver cancer (H4IIE and HepG2) cells compared to breast cancer (MCF-7 and BT549) cells (Figure 5.6 a and b), similar to the effect

Figure 5.5: Cell cycle analysis of MCF-7 (a) and BT549 (b) cells treated with different concentrations of rBalsamin for 48 h. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.001; **P < 0.05, One-way ANOVA.
observed with nBalsamin (Figure 3.7 a and b). The results indicated that apoptosis in rBalsamin treated liver and breast cancer cells involves the activation of caspase-dependent pathways, most likely the death receptor (extrinsic) apoptotic pathway as both caspase-3 and -8 are associated with this pathway. However, caspase-8 is also involved in activation of the mitochondrial (intrinsic) cell death pathway. Therefore, we investigated the expression of pro-apoptotic and anti-apoptotic genes involved in the mitochondrial cell death pathway.

Figure 5.6: Effect of different concentrations of rBalsamin on caspase-3 (a) and caspase-8 (b) activity in HepG2, H4IIE, MCF-7 and BT549 cells after 48 h treatment. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.001, One-way ANOVA.
5.3.5 rBalsamin-induced apoptosis involves regulation of various pro-apoptotic and anti-apoptotic genes in liver and breast cancer cells

During mitochondrial apoptosis, caspase-8 cleaves Bid to release a truncated form of the protein named tBid that rapidly accumulates in the mitochondria and activates molecular interactions between different members of the Bcl-2 family (Kantari & Walczak 2011; Wei et al. 2000). Therefore, we evaluated the expression of Bid in rBalsamin treated liver and breast cancer cells. The qRT-PCR confirmed that the level of Bid was significantly upregulated in rBalsamin treated H4IIE, HepG2, MCF-7 and BT549 cells compared to the untreated cells. The effect appeared to be more conspicuous in HepG2 cells compared to the other cell lines (Figure 5.7a), similar to the effect observed with nBalsamin (Figure 3.8b). It has been reported that Bid is transcriptionally upregulated in response to stabilized p53 (Sax et al. 2002). The tumor suppressor protein, p53, is a key regulator of apoptosis. It integrates multiple stress signals into a diverse series of antiproliferative responses to execute apoptosis (Fridman & Lowe 2003). Consequently, we determined the expression of p53 in rBalsamin treated cancer cells. The qRT-PCR results showed that the expression of p53 significantly increased in a dose dependent manner in rBalsamin treated H4IIE, HepG2 and MCF-7 and BT549 cells compared to the untreated cells. The effect was similar in HepG2, H4IIE and MCF-7 cells, while it appeared to be less prominent in BT549 cells (Figure 5.7b). Unlike rBalsamin, the effect appeared to be similar in HepG2 and H4IIE cells while it appeared to be less prominent in MCF-7 and BT549 cells treated with nBalsamin (Figure 3.8a).

The cleavage of Bid and activation of p53 can increase the expression of pro-apoptotic (Bax, Bad, Bak and Bok) genes and reduce the expression of anti-apoptotic (Bcl-2 and Bcl-XL) genes to promote mitochondrial apoptosis (Lee & Chang 2010; Pawlowski & Kraft 2000; Westphal et al. 2011). Therefore, to confirm the involvement of the mitochondrial apoptotic pathway in rBalsamin treated liver and breast cancer cells, we further evaluated the expression of pro-apoptotic (Bax and Bad) and anti-apoptotic (Bcl-2 and Bcl-XL) genes. The qRT-PCR results showed that the expression of Bax significantly increased in rBalsamin treated liver and breast cancer cells with a maximum effect observed in HepG2 and BT549 cells (Figure 5.8a), similar to the effect observed with nBalsamin (Figure 3.9a). The expression of
Bad increased with rBalsamin treatment in all the four cell lines. However, the effect appeared to be more pronounced in HepG2 cells while it was similar in all the other cell lines (Figure 5.8b). These observations were similar to those observed with nBalsamin (Figure 3.9b).

Figure 5.7: Expression of Bid (a) and p53 (b) genes in HepG2, H4IIE, MCF-7 and BT549 cells treated with different concentrations of rBalsamin for 48 h. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.001, One-way ANOVA.
In addition, we also evaluated the expression of anti-apoptotic genes (Bcl-2 and Bcl-XL) in rBalsamin treated liver and breast cancer cell lines. The qRT-PCR results showed that the expression of Bcl-2 and Bcl-XL decreased in a dose dependent manner when HepG2, H4IIE, MCF-7 and BT549 cells were treated with different concentrations of rBalsamin (Figure 5.9 a and b). In contrast, nBalsamin decreased
the expression of Bcl-2 and Bcl-XL in MCF-7 and BT549 in a dose dependent manner with no effect observed in HepG2 and H4IIE cells (Figure 3.10 a and b), indicating that the function of nBalsamin and rBalsamin varies within the same cell type.

Figure 5.9: Expression of anti-apoptotic genes, Bcl-2 (a) and Bcl-XL (b), in HepG2, H4IIE, MCF-7 and BT549 cells treated with different concentrations of rBalsamin for 48 h. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.001; **P = 0.004, One-way ANOVA.
Results of cell cycle arrest, the activation of caspases, upregulation of pro-apoptotic genes (Bax, Bid, Bad and p53) and downregulation of anti-apoptotic genes (Bcl-2 and Bcl-XL) confirm the involvement of the mitochondrial apoptotic pathway in rBalsamin induced apoptosis in HepG2, H4IIE, MCF-7 and BT549 cells. A number of RIPs naturally occurring in plants exhibit anti-liver and anti-breast cancer activity. α-MMC from *Momordica charantia*, and TCS from *Trichosanthes kirilowii* induced apoptosis in MCF-7, MDA-MB-231 and MDA-MB-453 via the mitochondrial cell death pathway and cell cycle arrest (Cao et al. 2015; Fang et al. 2012c). MAP30 from *Momordica charantia* induced apoptosis in HepG2 cells via cell cycle arrest, extrinsic and intrinsic (mitochondrial) apoptotic pathways (Fang et al. 2012b). However, little has been reported on the anti-liver and anti-breast cancer potential of recombinant RIPs. rMAP30 and rMAP30 fused with human derived cell penetrating peptide (HBD) have been shown to induce apoptosis in HeLa, MCF-7, 5637, SMMC-7721, MGC803 and B16 cells (Lv et al. 2015). However, the actual mechanism by which it promotes apoptosis in MCF-7 cells has not been described.

5.3.6 rBalsamin induces ER-stress mediated apoptosis in liver and breast cancer cells

In addition to the mechanisms of death receptor and mitochondrial mediated apoptosis, ER-stress can also lead to programmed cell death (Zhang & Kaufman 2004). C/EBP homologous protein (CHOP), also known as DNA damage-inducible gene 153 (GADD153), is one of the key mediators of ER stress-mediated apoptosis. It is usually expressed at undetectable levels, however, its expression is induced by cellular stress (Bento, Andersson & Aman 2009). The 78 kDa glucose regulated protein (GRP78), also known as BiP, is a major regulator of ER function and is involved in protein folding and assembly, targeting misfolded proteins for degradation and regulating the activation of ER stress signals. In response to ER stress, GRP78 expression is upregulated to assist proper protein folding and it is considered to be an important component involved in ER stress-induced apoptosis (Li & Lee 2006). RIPs have also been reported to induce ER stress-mediated apoptosis in cancer cells (Pan et al. 2013). TCS, a type I RIP induced apoptosis in cervical cancer (HeLa) cells and leukemia (HL-60) cells via ER stress signaling pathway (Huang et al. 2010; Li et al. 2007a). Therefore, we studied the expression of
genes (*CHOP* and *GRP78*) involved in this pathway. The qRT-PCR results showed that the expression of *GRP78* and *CHOP* was significantly upregulated in rBalsamin treated liver cancer (H4IIE and HepG2) cells, however, the expression of neither *GRP78* nor *CHOP* were affected in rBalsamin treated breast cancer (MCF-7 and BT549) cells, compared to the untreated cells (Figure 5.10 a and b). Interestingly, our results indicate that the apoptotic effect of rBalsamin on liver cancer (H4IIE and HepG2) cells but not on breast cancer (MCF-7 and BT549) cells may be partially attributed to ER stress. These observations were similar to those observed with nBalsamin (Figure 3.11 a and b).


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Figure 5.10: Expression of genes involved in ER-stress mediated apoptosis, GRP78 (a) and CHOP (b), in HepG2, H4IIE, MCF-7 and BT549 cells treated with different concentrations of rBalsamin (6.25, 25 and 100 µg/ml) for 48 h. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.001, One-way ANOVA.
5.4 Conclusions

The present study demonstrates for the first time the potential cytotoxicity of rBalsamin on liver (HepG2 and H4IIE) and breast (MCF-7 and BT549) cancer cells. It was found that rBalsamin could inhibit the proliferation of liver and breast cancer cells in a dose and time dependent manner with $IC_{50}$ ranging between 18.92 to > 200 µg/ml. rBalsamin induced apoptosis in these cells via DNA fragmentation, cell cycle arrest and regulating the expression of various pro-apoptotic and anti-apoptotic markers. Cell cycle analysis revealed that rBalsamin could arrest cell cycle at G and S phases of the cell cycle suggesting that rBalsamin interferes with the cell cycle progression and induces apoptosis in cancer cells. In addition, caspase-3 and -8 activity significantly increased in a dose dependent manner in these cell lines after rBalsamin treatment. RT-PCR results demonstrated that rBalsamin significantly upregulated the expression of pro-apoptotic genes, Bax, Bid, Bad and p53, and downregulated the expression of anti-apoptotic genes, Bcl-2 and Bcl-XL, in liver and breast cancer cells, indicating that the intrinsic and extrinsic cell death pathways are involved in rBalsamin-induced apoptosis in liver and breast cancer cells. Interesting, the expression of genes, GRP78 and CHOP, involved in ER-stress mediated apoptosis significantly increased in liver cancer cells but not in breast cancer cells, suggesting that rBalsamin might activate an additional pathway of apoptosis (ER-stress mediated pathway) in liver cancer cells. This multifunctional biomolecule with variable characteristics could be a new hope for cancer patients. However, further in vivo studies would be beneficial to develop this biomolecule as an effective therapeutic agent.
Chapter 6: Combination of nBalsamin and flavonoids enhances *in vitro* cytotoxicity in HepG2 and MCF-7 cells.
6.1 Introduction

Flavonoids are group of polyphenolic compounds found in fruits, vegetables, herbs, cereals and dairy products (Cook & Samman 1996). The basic structure of flavonoids consists of 15 carbon atoms and two benzene rings (A and B, Figure 6.1), connected via a heterocyclic pyran ring (C, Figure 6.1). Based on their chemical structure, flavonoids are categorized into 12 major subclasses, six of which, namely anthocyanidins, flavonols, flavanones, flavan-3-ols, flavones and isoflavones are of dietary significance (Kumar & Pandey 2013). Flavonoids as a complementary medicine have attracted the attention of researchers due to their diverse pharmacological properties, including antioxidation, antibacterial, antiviral, antitumor, anti-atherosclerosis, anti-diabetic, anti-inflammatory, antithrombogenic, hypolipidemic, and neuroprotective effects (Huang et al. 2016). Such biological diversity of flavonoids appears to be associated with their ability to regulate a number of cell signaling cascades, and could play a pivotal role in human health (Williams, Spencer & Rice-Evans 2004).

![Figure 6.1: Basic chemical structure of flavonoids (Kumar & Pandey 2013).](image)

Of all the polyphenols known, naringenin (Nar) and its derivatives are known to possess strong antioxidant potential along with other biologically beneficial effects (Sumathi, Tamizharasi & Sivakumar 2015). Nar is a flavone, a type of flavonoid that is abundantly found in citrus fruits. It is derived from the hydrolysis of the glycone form of flavanone, such as naringin (Figure 6.2a) (Erlund 2004; Puri et al. 2012). Naringin (Nir) consists of two sugar units (glucose and rhamnose) attached to its aglycon portion, naringenin, at the 7-carbon atom (Figure 6.2a) (Alam et al. 2014). Studies have shown that both Nar and Nir exert a variety of common
pharmacological effects including antioxidant, anti-inflammatory, anticarcinogenic and hepatoprotective effects (Bharti et al. 2014). Quercetin (Qu) is a flavonol, another type of flavonoid that is abundantly found in edible fruits and vegetables, and has also gained attention in human health. It consists of two aromatic rings connected via oxygen containing heterocyclic ring (Figure 6.2b) (Gibellini et al. 2011). Qu has reported health benefits similar to Nar and Nir, and it has also been used therapeutically in allergic conditions (such as asthma, hayfever, eczema and hives), arthritis, metabolic syndrome and mood disorders (Kelly 2011).

Figure 6.2: Structure of naringin and its hydrolysed product (a), and quercetin (b).

Several studies have demonstrated that these flavonoids exhibit anticancer properties towards various cancers, including liver, breast, lung, colon, and bladder cancer (Park & Pezzuto 2012). Nar inhibited the proliferation of breast cancer (MCF-7) cells by blocking insulin stimulated glucose uptake in GLUT4 (insulin responsive glucose transporter) expressing, insulin responsive MCF-7 cells. Nar blocked glucose uptake by impairing the activation of phosphoinositide3-kinase (P13K), a key regulator of insulin induced GLUT4 translocation, and inhibiting the phosphorylation of p44/p42 mitogen-activated protein kinase (MAPK), a step
essential for the insulin signaling pathway (Harmon & Patel 2004). Nir induced apoptosis in breast cancer cells (MDA-MB-231 and BT549) by increasing the expression of p21 and decreasing the expression of survivin (inhibitor of apoptosis protein, IAP) and active β-catenine (Li et al. 2013). Similarly, Qu suppressed the expression of survivin and induced G0/G1 phase cell cycle arrest in breast cancer (MCF-7) cells (Deng et al. 2013). In the case of liver cancer (HepG2) cells, Qu induced apoptosis via inhibition of FASN (fatty acid synthase), a metabolic enzyme that is usually upregulated during early stages of tumorigenesis (Zhao et al. 2014). Nar and Nir induced apoptosis in HepG2 cells via mitochondrial mediated activation of caspase-9 and caspase-8 mediated proteolysis of Bid (Arul & Subramanian 2013; Banjerdpongchai, Wudtiwai & Khaw-on 2016). This evidence suggests that the anticancer effects of these flavonoids involve various mechanisms, a desirable trait in cancer therapeutics.

Balsamin, a type I RIP from *Momordica balsamina*, exhibits potent anti-HIV activity. It blocks the replication of HIV by inhibiting the translation step, occurring prior to viral budding and release (Kaur et al. 2013). Our recent studies have shown that nBalsamin (nBal) exhibits DNase-like activity, and broad spectrum antimicrobial and antioxidant activity (Ajji, Walder & Puri 2016). Further, in subsequent studies on the anti-proliferative effects of nBal on liver (HepG2 and H4IIE) and breast cancer (MCF-7 and BT549) cells, we observed that nBal induced apoptosis in liver cancer cells via increasing the expression of pro-apoptotic markers involved in mitochondrial mediated (caspase-3, Bax, Bid, Bad and p53), death receptor mediated (caspase -3 and -8) and ER-stress mediated (GRP78 and CHOP) apoptotic pathways, with no profound effect observed in the expression of anti-apoptotic genes (*Bcl*2 and *Bcl*-XL). However, in breast cancer cells, nBal increased the expression of pro-apoptotic markers and also simultaneously decreased the expression of anti-apoptotic genes, triggering mitochondrial mediated and death receptor mediated apoptosis. Interestingly, nBal did not activate the ER stress-mediated pathway in breast cancer cells, suggesting diverse mechanisms of apoptosis induction in different cancer cells.

nBal and flavonoids (Nar, Nir and Qu) appear to exert antitumor effects and induce apoptosis through similar mechanisms in liver (HepG2) and breast (MCF-7) cancer
cells, therefore, we hypothesized that nBal-flavonoid (Nar, Nir and Qu) combinations might have additive apoptotic effects on these cells. Thus, in this study, we examined the effect of co-treatment of nBal and three flavonoids, namely Nar, Nir and Qu, on HepG2 and MCF-7 cells.

6.2 Materials and methods

6.2.1 Chemicals, reagents and kits

Nar, Nir and Qu, trypsin, In vitro toxicology assay kit, caspase-3 assay kit and caspase-8 assay kit were procured from Sigma-Aldrich (USA). RPMI and DMEM media, and FBS were purchased from Life Technologies Corporation (California, USA). Total RNA isolation kit was purchased from Qiagen (Hilden, Germany). Propidium iodide (PI) flow cytometry kit was procured from BD Biosciences (San Jose, CA). All other reagents were either purchased from Sigma-Aldrich (USA) or Bio-Rad Laboratories (California, USA), and were of analytical grade.

6.2.2 Cell lines and cell culture

For the present study, liver (HepG2) and breast (MCF-7) cancer cells lines were investigated. These cell lines were procured from the ATCC (Rockville, MD). HepG2 and MCF-7 cells were maintained in RPMI and DMEM media supplemented with 10% FBS, respectively, as described in section 3.2.2.

6.2.3 Treatment groups

For cell viability assay, HepG2 and MCF-7 cells were treated for 24 h with a treatment regime given in Figure 6.3. Based on data, treatments highlighted in Figure 6.3 were selected for all the other assays.
Figure 6.3: Treatment regime used for the study.
6.2.4 Cell viability assay

The cell viability was determined using In vitro toxicology assay kit, as described in section 3.2.3.

6.2.5 DNA fragmentation assay

The DNA fragmentation assay was performed as described in section 3.2.4.

6.2.6 Caspase-3/8 activity assay

The caspase-3 and -8 activity was determined using caspase-3 and caspase-8 colorimetric assay kits, as described in section 3.2.6.

6.2.7 RNA extraction and RT-PCR for gene expression studies

The gene expression studies were carried out as described in section 3.2.7.

6.2.8 Statistical analysis

Data was analysed using SPSS Statistics software version 22.0. Data was presented as the mean ± SD for at least three independently performed experiments unless otherwise stated. The non-parametric test, Kruskal-Wallis H test, also known as one-way ANOVA on ranks, was used to compare the difference between two or more groups. Mann-Whitney U test was used to compare the difference between two groups. Probability values with p < 0.05 were considered to be statistically significant.
6.3 Results and discussion

6.3.1 Growth inhibitory effects of nBal combined with flavonoids on HepG2 and MCF-7 cells.

The relationship between diet and cancer has been implicated in number of epidemiological studies (Ross 2010). Vegetables, fruits and cereals contain flavonoids that have antioxidant, anti-inflammatory and anticancer properties. Nar, Nir and Qu are three such flavonoids that have been explored for their anti-proliferative effects towards various cancer cells. Nar, a natural flavone exhibits anti-tumor activity towards various tumor cell types including hepatoma (HepG2), breast cancer (MCF-7, HTB26 and HTB132), leukemia (K562), human epidermoid carcinoma (A431), colorectal cancer (SW1116 and SW837) and glioma cells (C6) (Abaza et al. 2015; Ahamad et al. 2014; Arul & Subramanian 2013; Harmon & Patel 2004; Li et al. 2015; Sabarinathan, Mahalakshmi & Vanisree 2011). Nir exerts antiproliferative effects on cancer cells such as breast cancer (MDA-MB-231 and BT549), liver cancer (HepG2), bladder cancer (5637), melanoma (A375) and glioblastoma (U87MG) (Guo et al. 2016; Kim et al. 2008; Li et al. 2016). Qu possesses antitumor activity against liver cancer (HepG2), breast cancer (MCF-7), leukemia (HL-60) and prostate cancer (DU-145 and PC-3) cells (Deng et al. 2013; Nair et al. 2004; Niu et al. 2011; Zhao et al. 2014).

Several studies have reported that flavonoids with antioxidant properties synergize the effect of chemotherapeutic agents (Lewandowska et al. 2014). Qu increased cisplatin-induced apoptosis in human laryngeal cancer (Hep-2) cells (Kuhar, Imran & Singh 2007). 4T1 breast tumor mice treated with Qu and doxorubicin demonstrated inhibition of tumor growth and reduction of doxorubicin side effects, leading to prolonged survival of the mice (Du et al. 2010). Qu enhanced the antitumor effect of trichostatin-A, a novel anticancer agent, in human lung cancer cells through upregulation of p53 expression (Chan et al. 2013). Our previous studies demonstrated that nBal, a type-I RIP isolated from Momordica balsamina, exhibits inhibitory effects towards liver and breast cancer cells, highlighting its potential as a therapeutic agent. However, nothing is known about the combined effects of RIPS and flavonoids on cancer cells. Therefore, in this study, we
investigated the apoptotic effects of nBal in the presence of three flavonoids, namely Nar, Nir and Qu, on liver (HepG2) and breast (MCF-7) cancer cells.

To test this, we first examined the effect of co-treatment of nBal (25 µg/ml) and flavonoids (Nar, Nir or Qu) on the viability of HepG2 and MCF-7 cells using an In vitro toxicology assay kit. As shown in Figure 6.4a and b, the combination of nBal with low dose (0.5 X IC_{50}) of Nar appeared to inhibit the viability of HepG2 and MCF-7 cells compared to the cells treated with nBal alone. When the concentration of Nar (1.0 X IC_{50} and 2.0 X IC_{50}) was increased in combination with nBal, the inhibitory effect tended to increase compared with nBal treatment, however, did not increase much compared to Nar treatment. Interestingly, the effects appeared to be additive at low dose of Nar with nBal, but not at higher doses of Nar (Figure 6.4a and b).

Further, the trend appeared to be similar when HepG2 and MCF-7 were treated with nBal-Qu combinations (Figure 6.5a and b). However, when HepG2 and MCF-7 cell were co-treated with low dose (0.5 X IC_{50}) of Nir and nBal, the cell viability tended to decrease slightly compared to singly treated groups, however, the effect was not as prominent as observed with low dose (0.5 X IC_{50}) of Nar or Qu with nBal. Further, when the concentration of Nir (1.0 X IC_{50} and 2.0 X IC_{50}) was increased in combination with nBal, the inhibitory effect tended to increase compared with nBal treatment alone, however, did not increase much compared to Nir treatment alone. (Figure 6.6a and b). These results suggest that these flavonoids could increase the anti-proliferative effects of nBal on HepG2 and MCF-7 cells, with low dose (0.5 X IC_{50}) of flavonoids (Nar, Nir and Qu) tending to have an additive effect in combination with nBal. Therefore, we selected this flavonoid dose in combination with nBal for further study.
Figure 6.4: Effect of nBal and Nar on the viability of HepG2 (a) and MCF-7 (b) cells compared with nBal. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.05, Kruskal-Wallis H test.
Figure 6.5: Effect of nBal and Qu on the viability of HepG2 (a) and MCF-7 (b) cells compared with nBal. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.05, Kruskal-Wallis H test.
Figure 6.6: Effect of nBal and Nir on the viability of HepG2 (a) and MCF-7 (b) cells compared with nBal. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.05, Kruskal-Wallis H test.
6.3.2 Effect of nBal-flavonoid combinations on the morphology of HepG2 and MCF-7 cells

Flavonoids, such as Nar, Nir and Qu are known to induce apoptosis in various cancer cells by regulating the expression of apoptotic markers (Arul & Subramanian 2013; Banjerdpongchai, Wudtiwai & Khaw-on 2016). In addition, our studies have also shown that nBal induces apoptosis via a similar pathway to flavonoids. Therefore, we next investigated whether flavonoids (Nar, Nir or Qu) could increase nBal induced apoptotic effects in HepG2 and MCF7 cells. For this, we examined the morphology of HepG2 and MCF-7 cells treated with combinations of nBal and Nar, Nir or Qu, and compounds alone under brightfield microscopy. The results showed that apoptotic morphological changes, such as loss in cell connections, cell shrinkage, cell surface detachment, increased cytoplasmic density and more dead cells appeared to be more prominent in cells treated with nBal-flavonoid (Nar, Nir or Qu) combinations compared to single treatments (Figure 6.7).
Figure 6.7: Morphology of HepG2 and MCF-7 cells treated with nBal-Nar, nBal-Qu and nBal-Nir compared with nBal, Nar, Qu and Nir alone.
6.3.3 Caspase-3 and -8 activity of nBal combined with Nar, Nir or Qu in HepG2 and MCF-7 cells

Apoptosis is a programmed and often energy dependent process that involves activation of caspases, a group of cysteine proteases, and a complex cascade of signals (Elmore 2007). Flavonoids such as Nar, Nir and Qu have been reported to activate caspase dependent apoptosis in cancer cells (Ramos 2007). Nar and Nir induced apoptosis in HepG2 cells via activating caspase-8 and caspase-9 mediated cell death pathways (Arul & Subramanian 2013; Banjerdpongchai, Wudtiwai & Khaw-on 2016). Qu induced cell death in glioblastoma U373MG cells through proteolytic activation of caspase-3 and caspase-7, a decrease in mitochondrial membrane potential and increases in caspase-3 and caspase-9 activities (Kim et al. 2013). Our studies have demonstrated that nBal also induces apoptosis by increasing the activity of caspase-3 and -8 in liver and breast cancer cells. Therefore, we further decided to evaluate whether flavonoids (Nar, Nir and Qu) increase caspase-mediated apoptotic effects in nBal treated HepG2 and MCF-7 cells. As shown in Figure 6.8a, nBal-Nar treatment tended to increase and appeared to have an additive effect on caspase-3 and -8 activity in HepG2 and MCF-7 cells compared to nBal and Nar alone. However, no significant differences were observed between co-treated, nBal and Nar treated groups. Further, the trend appeared to be similar in HepG2 and MCF-7 cells treated with the nBal-Qu combination (Figure 6.8b). However, when HepG2 and MCF-7 cells were treated with the nBal-Nir combination, the caspase-3 and -8 activity tended to increase as compared to Nir and nBal treatment alone, however, did not appear to have an additive effect (Figure 6.8c).

These results indicate that out of all the nBal-flavonoid combinations tested, nBal-Nar and nBal-Qu appeared to be a more effective combination compared to nBal-Nir in HepG2 and MCF-7 cells.
Figure 6.8: Effect of nBal-Nar (a), nBal-Qu (b) and nBal-Nir (c) on caspase-3 and -8 activity compared with nBal in HepG2 and MCF-7 cells. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.05, Kruskal-Wallis H test.
6.3.4 Effect of nBal-flavonoid combinations on the expression of various apoptotic genes involved in the mitochondrial cell death pathway.

Apoptosis is an essential physiological process that is regulated by the Bcl-2 family of pro-apoptotic and anti-apoptotic genes. Flavonoids, such as Nar, Nir and Qu, have been reported to trigger apoptosis via regulating the expression of pro-apoptotic and anti-apoptotic genes involved in the mitochondrial cell death pathway (Ramos 2008). Nar isolated from Thymus vulgaris upregulated pro-apoptotic markers, p18, p19, p21, Bax and Bak, and downregulated anti-apoptotic markers, Cdk4, Cdk6, Cdk7 and Bcl-2 in human colorectal and breast cancer cells leading to apoptosis (Abaza et al. 2015). Qu induced apoptosis in HepG2 hepatoma cells by decreasing the Bcl-XL:Bcl-XS ratio, activating caspase-3 and -9 and increasing the translocation of Bax to the mitochondrial membrane, leading to mitochondrial mediated apoptosis (Granado-Serrano et al. 2006). Our previous studies demonstrated that nBal induces the mitochondrial cell death pathway in HepG2 and MCF-7 cells by increasing the expression of pro-apoptotic genes, Bax, Bid, Bad and p53, and decreasing the expression of anti-apoptotic genes, Bcl-2 and Bcl-XL. Therefore, we studied whether these flavonoids could increase nBal-induced mitochondrial apoptotic effects in HepG2 and MCF-7 cells. The qRT-PCR results showed that nBal-Nar treatment tended to increase and appeared to have an additive effect on the expression of Bax, Bid, Bad and p53 compared to nBal treatment alone in HepG2 and MCF-7 cells. However, no significant difference was observed between the groups (Figure 6.9a). Further, the trend appeared to be similar in HepG2 and MCF-7 cells treated with the nBal-Qu combination (Figure 6.9b), suggesting that Nar and Qu increase nBal-induced apoptotic effect in HepG2 and MCF-7 cells.

In addition, the nBal-Nir combination appeared to increase the expression of Bax, Bid, Bad and p53 as compared nBal treatment alone in HepG2 and MCF-7 cells. However, the effect did not appear to be pronounced and additive compared to nBal-Nar and nBal-Qu treatment (Figure 6.9c), suggesting that the nBal-Nar and nBal-Qu combinations could be considered more effective than the nBal-Nir combination.
Figure 6.9: Effect of nBal-Nar (a), nBal-Qu (b) and nBal-Nir (c) on the expression of pro-apoptotic genes, Bax, Bid, Bad and p53, compared with nBal in HepG2 and MCF-7 cells. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.05, Kruskal-Wallis H test.
Further, we also evaluated the expression of anti-apoptotic genes (Bcl-2 and Bcl-XL) in HepG2 and MCF-7 cells treated with nBal-flavonoid (Nar, Nir or Qu) combinations. The qRT-PCR results showed that nBal-Nar treatment tended to decrease the expression of Bcl-2 and Bcl-XL compared to nBal and Nar treatment alone in HepG2 and MCF-7 cells, indicating a possible additive effect. However, no significant difference was observed between the groups (Figure 6.10a). Likewise, the trend appeared to similar, but less pronounced in HepG2 and MCF-7 cells treated with nBal-Qu and nBal-Nir combinations (Figure 6.10b and c).

Increase in activation of caspase-3 and -8, upregulation of pro-apoptotic genes (Bax, Bid, Bad, p53) and downregulation of anti-apoptotic genes (Bcl-2 and Bcl-XL) with nBal-Nar, nBal-Nir and nBal-Qu treatment compared to nBal and flavonoid (Nar, Nir or Qu) treatment alone in HepG2 and MCF-7 cells, suggests that these flavonoids increase nBal-induced mitochondrial mediated apoptosis with a possible additive effect in HepG2 and MCF-7 cells, which could be considered as a promising strategy to sensitize cells to nBal treatment.
Figure 6.10: Effect of nBal-Nar (a), nBal-Qu (b) and nBal-Nir (c) on the expression of anti-apoptotic genes, *Bcl-2* and *Bcl-XL*, compared with nBal in HepG2 and MCF-7 cells. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.05, Kruskal-Wallis H test.
6.3.5 Effect of nBal-flavonoid combinations on the expression of genes involved in ER-stress mediated apoptosis

The ER-stress response has been reported to initiate apoptosis in various cancer cells, through three different pathways; (i) activation of CHOP, also known as DNA damage-inducible gene 153 (GADD153), (ii) activation of ER associated caspases and (iii) activation of c-Jun N- terminal kinase (JNK)-mediated cell death (G Johnson, C White & Grimaldi 2011). Qu induced ER-stress mediated apoptosis by increasing the expression of ATF, GRP78 and CHOP in prostate cancer PC-3 cells (Liu et al. 2014). It has been reported that Qu pre-treatment elicits ER-stress to enhance cisplatin cytotoxicity in ovarian cancer cells, indicating that Qu has a potential to enhance the efficacy of cisplatin induced apoptosis in ovarian cancer cells (Yang et al. 2015). Our studies have shown that nBal induces ER-stress mediated apoptosis in liver cancer (H4IIE and HepG2) cells by increasing the expression of CHOP and GRP78, however, it does not activate this pathway in breast cancer (MCF-7 and BT549) cells. Therefore, to investigate whether nBal-flavonoid treatment could increase nBal-induced ER stress in HepG2 cells and activate this pathway in breast cancer (MCF-7) cells, we evaluated the expression of CHOP and GRP78 in nBal-flavonoid (Nar, Nir and Qu) treated HepG2 and MCF-7 cells.

The qRT-PCR results showed that the expression of GRP78 and CHOP tended to increase in HepG2 cells treated with nBal-Nar (Figure 6.11a), nBal-Qu (Figure 6.11b) and nBal-Nir (Figure 6.11c) combinations compared to nBal and flavonoids (Nar, Nir and Qu) treatment alone, indicating a possible additive effect of co-treatment in HepG2 and MCF-7 cells. In the case of MCF-7 cells, nBal treatment alone did not increase the expression of GRP78 and CHOP. However, when nBal was combined with the flavonoids (Nar, Qu and Nir), the expression tended to increase with respect to nBal treatment, but did not increase significantly with respect to flavonoid (Nar, Qu and Nir) treatment alone, suggesting that the effect was entirely due to the presence of the flavonoid (Nar, Qu or Nir) in the nBal-flavonoid combinations (Figure 6.11a, b and c). It is interesting to note that these flavonoids activate ER-stress mediated apoptosis, an additional apoptotic pathway, in breast cancer MCF-7 cells that is not activated by nBal treatment alone.
Figure 6.11: Effect of nBal-Nar (a), nBal-Qu (b) and nBal-Nir (c) on the expression of genes, *GRP78* and *CHOP*, involved in ER-stress mediated apoptosis, compared with nBal in HepG2 and MCF-7 cells. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.05, Kruskal-Wallis H test.
6.4 Conclusions

The present study demonstrates the efficacy of flavonoids (Nar, Qu and Nir) to enhance nBal-induced apoptosis in HepG2 and MCF-7 cells. Three doses (0.5 X IC\textsubscript{50}, 1.0 X IC\textsubscript{50} and 2.0 X IC\textsubscript{50}) of flavonoids (Nar, Qu and Nir) were tested in combination with nBal (25 µg/ml). It was observed that low dose (0.5 X IC\textsubscript{50}) of flavonoid in combination with nBal appeared to inhibit the viability of HepG2 and MCF-7 cells compared to the cells treated with nBal. When the concentration of flavonoid (1.0 X IC\textsubscript{50} and 2.0 X IC\textsubscript{50}) was increased in combination with nBal, the inhibitory effect tended to increase compared with nBal treatment, however, did not increase much compared to flavonoid (Nar, Nir and Qu) treatment. Interestingly, low dose of flavonoid with nBal appeared to produce an additive effect but higher doses did not. Based on this, low dose of flavonoids in combination with nBal was selected for further study. Our studies demonstrated that combination of nBal and low dose (0.5 X IC\textsubscript{50}) of flavonoids (Nar, Qu and Nir) appears to exhibit a more apparent anti-tumor activity \textit{in vitro} compared to each of them alone. Mechanically, these flavonoids (Nar, Qu and Nir) tended to increase the activation of caspase-3 and -8, upregulate pro-apoptotic genes (Bax, Bid, Bad and p53) and downregulate anti-apoptotic genes (Bcl-2 and Bcl-XL), suggesting that these flavonoids could possibly enhance nBal-induced mitochondrial mediated apoptosis in HepG2 and MCF-7 cells. Out of the three flavonoids (Nar, Qu and Nir) tested, the nBal-Nir combination appeared to be least effective compared with the nBal-Nar and nBal-Qu combinations. In addition, it was observed that these flavonoids could also activate ER-stress mediated apoptosis in breast cancer (MCF-7) cells, which was not activated by nBal treatment alone. These results suggest that nBal, in a combined treatment with flavonoids (Nar, Qu and Nir), could be a promising therapeutic strategy to reduce HepG2 and MCF-7 proliferation and to sensitize cells to nBal treatment. However, \textit{in vivo} studies are required to further evaluate its effectiveness against liver and breast cancer.
Chapter 7: Summary and future directions
Cancer is one of the leading causes of deaths worldwide. Although scientific advancements have been made to treat cancer, limitations and room for improvement still remains. Chemotherapy is the most common treatment available for cancer patients. However, drawbacks such as serious side effects and development of resistance to drugs limit its effectiveness. Nowadays, great deal of research efforts are being made to discover natural therapeutics such as plant derived products with minimal side effects.

*Momordica balsamina* is a medicinal and nutraceutical plant advocated for health care management, however it has not received major international attention (Puri 2010). This plant comprises tendril bearing high climbing vines. The leaves, seeds, bark and fruits of the plant contain large number of medicinal compounds such as phytochemicals (alkaloids, saponins, flavonoids, glycosides and terpenes) and type I RIP (Thakur et al. 2009). RIPs are naturally occurring plant proteins that have received considerable attention in biomedical research due to their activities towards tumor and virus-infected cells. Balsamin, a type-I RIP, has been extracted from *Momordica balsamina*, denoted as nBalsamin in this study. Previous studies from our group have reported that nBalsamin exhibits potent anti-HIV activity. In this study, we investigated the other biological activities including antimicrobial, antioxidant and DNase-like activity of nBalsamin with a major focus towards exploring the therapeutic potential of nBalsamin against liver and breast cancer cells. In addition, we attempted the heterologous expression of balsamin in *E.coli* strain, denoted as rBalsamin, and investigated its various biological and anti-tumor activities. We further determined the efficacy of flavonoids in enhancing nBalsamin-induced apoptosis in liver and breast cancer cells.

In Chapter 2, the functional properties of nBalsamin purified from *Momordica balsamina* were investigated. nBalsamin exhibited DNase-like activity, converting the supercoiled form of a plasmid into the linear form in a concentration dependent manner. DNase-like activity of nBalsamin was found ideal at pH range 6.5-7.5, temperature range 35°C-45°C and incubation time of 2 h. Moreover, the presence of Mg²⁺ (10-50 mM) influenced the DNA cleavage activity. nBalsamin also possessed antibacterial activity against various pathogens including *S. aureus, S.enterica,*
Further, the protein demonstrated free radical and superoxide radical scavenging activity in a dose dependent manner.

In Chapter 3, the role of nBalamin in exerting anti-tumor effects on liver (HepG2 and H4IIE) and breast (MCF-7 and BT549) cancer cells, and its associated mechanism was identified. The studies demonstrated that nBalsamin significantly inhibited the proliferation of liver and breast cancer cells in a dose and time dependent manner. nBalsamin induced apoptosis in these cells via DNA fragmentation, cell cycle arrest, activation of caspase-3 and -8, upregulating the expression of pro-apoptotic gene, Bax, Bid, Bad and p53, and downregulating the expression of anti-apoptotic genes Bcl-2 and Bcl-XL. Based on these finding, it was proposed that nBalsamin probably induces apoptosis through death-receptor mediated and mitochondria mediated cell death pathway. Interestingly, nBalsamin upregulated the expression of GRP78 and CHOP, genes involved in ER-stress mediated apoptosis, in liver cancer (HepG2 and H4IIE) cells but not in breast cancer (MCF-7 and BT549) cells, suggesting that nBalsamin potentially activates an additional pathway (ER-stress mediated pathway) in liver cancer cells and not in breast cancer cells. These findings suggested that nBalsamin could serve as a novel biomolecule for the treatment of liver and breast cancer. However, further in vivo studies are warranted to develop this biomolecule as an effective therapeutic agent.

To explore animal studies and clinical trials a large quantity of pure and bioactive protein is required, therefore, in Chapter 4, we cloned and expressed balsamin in E.coli and developed a two-step purification strategy for the production of functionally bioactive balsamin. The open reading frame of the Bal gene was successfully obtained from Momordica balsamina genomic DNA. The Bal gene was expressed in E.coli BL21(DE3) cells and various expression parameters such as IPTG concentration, induction time and temperature were optimized. Optimum expression of balsamin was obtained at 30°C after 4 h of induction with 0.4 mM IPTG. The incorporation of His-tag on the N-terminus of the protein enabled purification of rBalsamin by Ni²⁺ Sepharose affinity chromatography. Approximately, 45 mg of rBalsamin was obtained from a 1 L culture of induced E.coli cells. The structure of rBalsamin was found to be stable in the temperature range of 20°-60°C and pH range of 6-11. Further, functional analysis revealed that rBalsamin exhibited RNA N-
glycosidase and DNase-like activity. Also, rBalsamin inhibited the growth of various pathogens including *S. aureus*, *S. enterica*, *S. epidermidis* and *E. coli* with MICs ranging between 1.56-25 µg/ml.

In Chapter 5, we further investigated the potential cytotoxicity of rBalsamin on liver (HepG2 and H4IIE) and breast (MCF-7 and BT549) cancer cells. The studies revealed that rBalsamin could inhibit the proliferation of liver and breast cancer cells in a dose and time dependent manner with IC₅₀ ranging between 18.92-200 µg/ml. Further, rBalsamin induced apoptosis in liver and breast cancer cells through DNA fragmentation and by increasing caspase-3/8 activities, arresting cell cycle at G and S phase, increasing *Bax*, *Bid*, *Bad* and *p53* gene expression and decreasing *Bcl-2* and *Bcl-XL* gene expression, indicating that death-receptor and mitochondrial cell death pathways are involved in rBalsamin-induced apoptosis in liver and breast cancer cells. Interestingly, rBalsamin increased the expression of genes (*GRP78* and *CHOP*) involved in ER-stress mediated apoptosis in liver cancer cells but not in breast cancer cells, indicating that rBalsamin activates an additional, ER-stress mediated apoptotic pathway in liver cancer cells. *In vivo* studies would further enable us to highlight the potential of rBalsamin as an effective therapeutic agent.

In Chapter 6, the potential of three flavonoids (Nar, Nir and Qu) in enhancing nBalsamin (nBal)-induced apoptosis in HepG2 and MCF-7 cells was investigated. Three doses (0.5 X IC₅₀, 1.0 X IC₅₀ and 2.0 X IC₅₀) of flavonoids were tested in combination with nBal (25µg/ml). MTT assay results indicated that flavonoids, Nar, Nir and Qu, increased the inhibitory effects of nBal on HepG2 and MCF-7 cells compared to nBal treatment alone. The effect appeared to be more pronounced with increasing flavonoids concentration. Interestingly, low concentration (0.5 X IC₅₀) of flavonoids appeared to produce an additive effect but higher concentrations (1.0 X IC₅₀ and 2.0 X IC₅₀) did not. Based on these findings, lower concentration of flavonoids, Nar, Nir and Qu in combination with nBal were selected for further study. Combination of low dose of flavonoids with nBal appeared to exhibit enhanced anti-tumor activity compared to nBal treatment alone. Mechanically, Nar, Nir and Qu tended to increase the activation of caspase-3 and -8, upregulate pro-apoptotic genes (*Bax*, *Bid*, *Bad* and *p53*) and downregulate anti-apoptotic genes (*Bcl-2* and *Bcl-XL*), suggesting that these flavonoids could possibly enhance nBal-induced mitochondrial
mediated apoptosis in HepG2 and MCF-7 cells. Interestingly, out of the three combinations (nBal-Nar, nBal-Nir and nBal-Qu) tested, nBal-Nir combination was found to be least effective compared to other two combinations. These flavonoids appeared to activate genes, GRP78 and CHOP, involved in ER-stress mediated apoptosis, which were not activated with nBal treatment alone in MCF-7 cells, suggesting that nBal-flavonoid combination activates an additional, ER-stress mediated, apoptotic pathway in MCF-7 cells. These findings suggested that combinational therapy could be considered as a promising strategy to increase nBal-induced apoptosis in HepG2 and MCF-7 cells. However, in vivo studies are further required to explore the potential of nBal-flavonoid combinations in liver or breast cancer therapy.

To the best of our knowledge, this is the first study that explored the anti-tumor potential of Balsamin, a type-I RIP, on liver and breast cancer cells. This biomolecule could be considered as a potential therapeutic agent for liver and breast cancer. However, further in vivo studies are warranted to ascertain its effectiveness. Moreover, possible side effects and toxicity of this novel biomolecule on normal cells could limit its use in cancer therapeutics. Therefore, an efficient delivery system needs to be explored to overcome this problem. Nowadays, nanoparticle based drug delivery system has received majority of attention due to their stability, greater surface area and ease of modification. Several different natural materials have been explored for nanoparticle-based delivery. They may be of biological origin such as chitosan, phospholipids, dextran, lipids, or chemical origin such as polymers, carbon, silica and gold. Nanoparticles obtained from these materials can be tailored-made to achieve controlled drug release or drug-specific localization in cells. Thus, in the near future, an attempt would be made to encapsulate balsamin in a nanoparticle conjugated with ligand that specifically binds to the receptor expressed on the surface of breast cancer cells. It is expected that the fusion of ligand with the receptor will facilitate selective targeting of balsamin into the cytosol of cancer cells where it will interfere with the cellular machinery and lead to the activation of apoptotic markers resulting in cell death. Developing this efficient delivery system aimed to mitigate toxicity of balsamin to normal cells could prove significant and remarkable discovery in the field of cancer therapeutics.
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8. Annexures
Annexure 8.1: Rare codon optimization report.

OptimumGene™ Codon Optimization Analysis

Optimization Parameters:
OptimumGene™ algorithm optimizes a variety of parameters that are critical to the efficiency of gene expression, including but not limited to:
- Codon usage bias
- GC content
- CpG dinucleotides content
- mRNA secondary structure
- Cryptic splicing sites
- Premature PolyA sites
- Internal ribosome entry sites
- Negative Cpg islands
- RNA instability motif (ARE)
- Repeat sequences (direct repeat, reverse repeat, and dyad repeat)
- Restriction sites that may interfere with coding

Additional sequences we propose to improve translational performance:
(1) To increase the efficiency of translational initiation
   - Kozak sequence
   - Shine-Dalgarno Sequence
(2) To increase the efficiency of translational termination
   - Stop codon (TAA)
Results *E. coli*

1. Codon usage bias adjustment

**Codon Adaptation Index (CAI)**

![CAI Chart](image)

Figure 1a. The distribution of codon usage frequency along the length of the gene sequence. A CAI of 1.0 is considered to be perfect in the desired expression organism, and a CAI of > 0.8 is regarded as good, in terms of high gene expression level.

**Frequency of Optimal Codons (FOP)**

![FOP Chart](image)

Figure 1b. The percentage distribution of codons in computed codon quality groups. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism.

2. GC Content Adjustment

**GC Content Adjustment**

![GC Content Chart](image)

Figure 2. The ideal percentage range of GC content is between 30-70 %. Peaks of %GC content in a 60 bp window have been removed.
# 3. Restriction Enzymes and CIS-Acting Elements

<table>
<thead>
<tr>
<th>Restriction Enzymes</th>
<th>Optimized</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI (GGATCC)</td>
<td>1(1)</td>
</tr>
<tr>
<td>XhoI (CTCGAG)</td>
<td>1(751)</td>
</tr>
<tr>
<td>Polymerase slippage site 1</td>
<td>0</td>
</tr>
<tr>
<td>Polymerase slippage site 2</td>
<td>0</td>
</tr>
<tr>
<td>Frameshift element</td>
<td>0</td>
</tr>
<tr>
<td>Ribosome binding site</td>
<td>0</td>
</tr>
</tbody>
</table>

* Green: filtered sites; Blue: checked sites (not filtered); Red: kept sites.

<table>
<thead>
<tr>
<th>CIS-Acting Elements</th>
<th>Optimized</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli_RBS (AGGAGG)</td>
<td>0</td>
</tr>
<tr>
<td>PolyT (TTTTTT)</td>
<td>0</td>
</tr>
<tr>
<td>PolyA (AAAAAA)</td>
<td>0</td>
</tr>
<tr>
<td>Chi_sites (GCTGGTG)</td>
<td>0</td>
</tr>
<tr>
<td>T7Css (ATCGTT)</td>
<td>0</td>
</tr>
<tr>
<td>SD_like (GGRGGT)</td>
<td>0</td>
</tr>
</tbody>
</table>

# 4. Remove Repeat Sequences

**After Optimization**

- Max Direct Repeat: Size 9 Distance 909 Frequency 2
- Max Inverted Repeat: None
- Max Dyad Repeat: None
5. Optimized Sequence (Optimized Sequence Length: 756, GC%: 52.51)

CGATCC
ATCTTTATGAAAAGCTCATCATCCACCCCTCTGCTGGCAAAAGTCTAATATCTTCCTGCTGCTGGCTCC
GTCCTGGGCGCCGGCCGTGATGCTACACCTGACCAATCTTTATCAGGTGAACTTACCATCGCATCAAGC
CTCAAGAACGTCTATATATTGAGCCACATCATCAGGTGGATGTATTCTTACATCAGGTGAGCTGAG
GCATCCAAATACGGTTTTGGCAAGCTGCTGCTGCTGCAAAATCAACCCCTCCCTGATTAGCTAACGCAACCGCTCGAG
ATTCCACGCTGCAAGCTGGGCGCCGGCCGAAAAATCCCGGCTGGCTGCTGCTGGCCCTGGGAACTGGGCGCTCTCG
CAGACTCATAGCACCACGCACGGCGAAGTGCCTGCTGTTCCTCAGCCAAGGCCACCGCGAGCTTCTCCTCCAAC
TACATCAAACAGCAAAATCGCAACCCCTACAAACTGAACTGCCCTCACACTCGCTGCAGATTGGGAAAAC
AATGCTGTCTGCCTGCCTAACAACATTCAGTCTACGCGCCAGGCCAAATGCTAATCTTCCTGACCCGGAATTCTCTTG
ATCGCAACGCTGCTAACTTGCTGTACACCAATGTCTACGAGCAGAAATGCTTAGCTGCTAACATCCAAACCTCCTG
CTGAAATACAAAACATAGGGGCGCGGCAATAAGGGCTGTGCTGACCCGGAACGGGAGCTCTCTAT
TAACTCCAC
Conclusion

A wide variety of factors regulate and influence gene expression levels, and our OptimumGene™ algorithm takes into consideration as many of them as possible, producing the single gene that can reach the highest possible level of expression.

In this case, the native gene employs tandem rare codons that can reduce the efficiency of translation or even disengage the translational machinery. We increased the codon usage bias in E. coli by upgrading the CAI to 0.87. GC content and unfavorable peaks have been optimized to prolong the half-life of the mRNA. The Stem-Loop structures, which impact ribosomal binding and stability of mRNA, were broken. In addition, our optimization process has screened and successfully modified those negative cis-acting sites as listed in the introduction.

We are honored to deliver the analysis that you requested. We hope that you are pleased with your GenScript OptimumGene™ results.
Annexure 8.2: Antioxidant activity of rBalsamin. a) Effect of concentration on DPPH scavenging activity of rBalsamin. b) Effect of concentration on superoxide radical scavenging activity of rBalsamin. c) Effect of concentration on reducing power ability of rBalsamin. All values are mean ± SD. SD, standard deviation; n = 3.
Annexure 8.3: Dose dependent inhibition of *Staphylococcus aureus* (A), *Pseudomonas aeruginosa* (B), *Escherichia coli* (C), *Staphylococcus epidermidis* (D), *Salmonella enterica* (E) and *Enterococcus faecalis* (F) with rBalsamin. All values are mean ± SD. SD, standard deviation; n = 3; *P < 0.05.*