CELLULAR EVALUATION FOR ANTIOXIDATIVE AND IMMUNOMODULATORY POTENTIAL OF BUFFALO CASEIN PEPTIDE(S)

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

ANIMAL BIOCHEMISTRY

BY

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(M.Sc. Biochemistry)

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KARNAL-132001 (HARYANA), INDIA

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This is to certify that the thesis entitled, **"CELLULAR EVALUATION FOR ANTIOXIDATIVE AND IMMUNOMODULATORY POTENTIAL OF BUFFALO CASEIN PEPTIDE(S)"** submitted by **KANDUKURI SOWMYA** in partial fulfilment of the requirement for award of the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL BIOCHEMISTRY** of the ICAR-National Dairy Research Institute (Deemed University), Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

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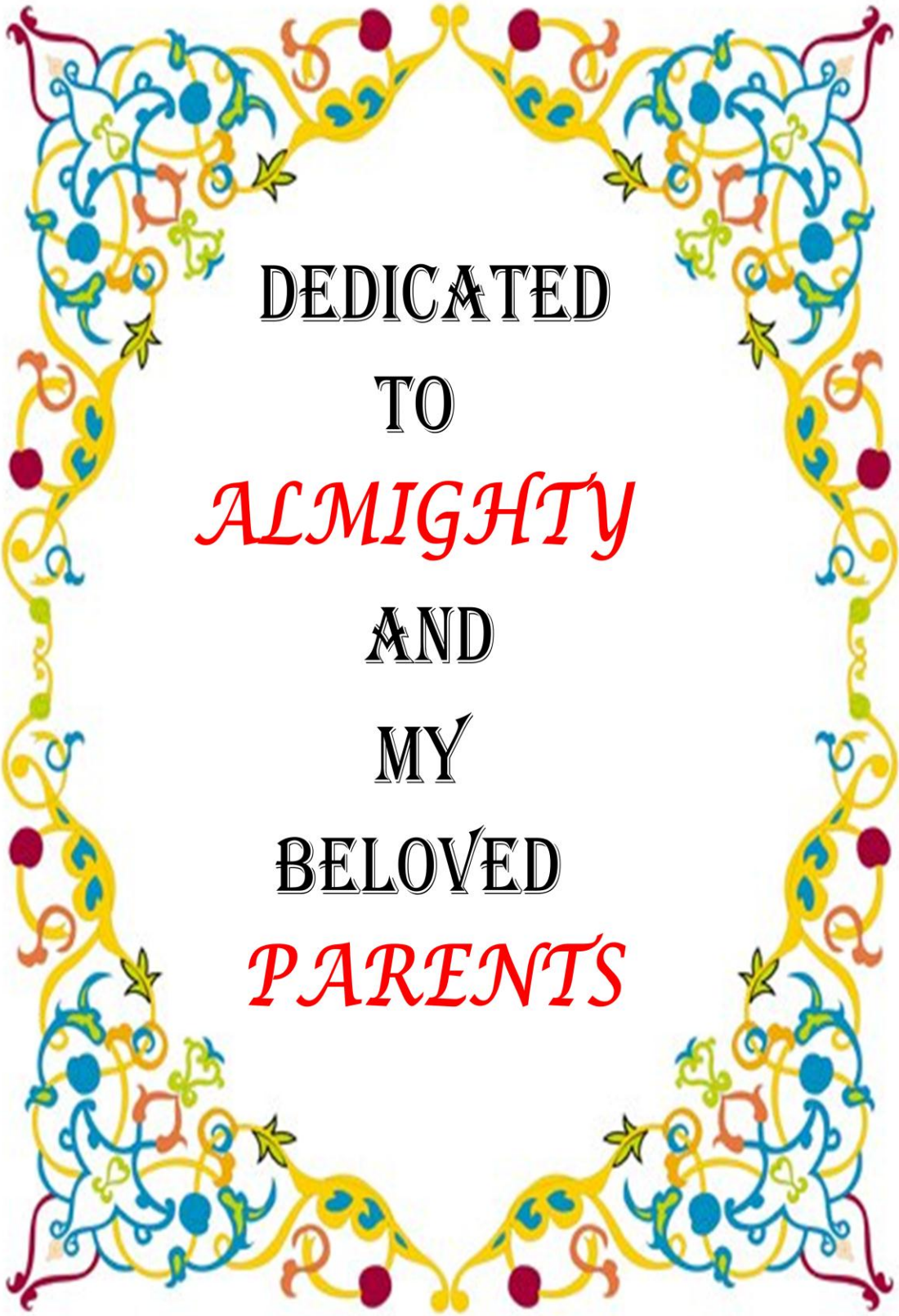
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TO
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AND
MY
BELOVED
PARENTS

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ABSTRACT

Among the natural sources, milk derived peptides have attracted increasing attention due to their wide varieties of bioactivities such as antioxidative, antimicrobial, antihypertensive and immunomodulatory etc. Bioactive peptides with overlapping antioxidative and immunomodulatory properties may have the potential to reduce the risk of lifestyle associated diseases such as cancer, heart attack, diabetes and hypertension as oxidative stress associated with inflammatory reactions is primary cause of them. To date, very limited numbers of studies are available which would reveal such overlapping properties of milk casein derived peptides at cellular level. Therefore, in the present study buffalo casein derived peptides were examined for their antioxidative and immunomodulatory potential with focus on overlapping attributes under cellular milieu. Ten peptides (A to J) from literature and ongoing work in laboratory were selected from bovine milk casein and modified wherever required according to buffalo sequence. The selected peptides were screened for their free radical scavenging and immunomodulatory properties by using chemical and *ex vivo* murine tissues respectively. Out of ten peptides, four peptides (B, C, F and G) showed maximum ($p<0.01$) free radical scavenging capacity at 100 µg/ml and 1 mg/ml dose by both ABTS and ORAC assays. Under *ex vivo* conditions, peptides-B,C and D at 10 ng to 1 µg/ml and peptides F, G, H and I at 10 ng to 1 mg/ml of dose were found to suppress ($p<0.01$) the proliferation of murine splenocytes. On the other hand, addition of peptides B,F,G,H and I significantly ($p<0.01$) stimulated the phagocytosis of murine peritoneal macrophages by adding varying amounts of respective peptides but peptide C showed this response only at much higher concentration (1 mg/ml). On the basis of the above screening, four peptides (B, C, F and G) having overlapping free radical scavenging and immunomodulatory properties were selected for further evaluation under cellular conditions using human intestinal epithelial Caco-2 cells or mouse splenocytes. Treatment of Caco-2 cells with the four selected peptides (B, C, F and G) for 24 hr at different concentrations (10 ng to 1 mg/ml) did not effect the cell viability and membrane integrity. In addition, the increased ROS generation in response to H_2O_2 induced oxidative stress was also inhibited by these peptides. Moreover, pre-treatment with peptides C and G (100 µg to 1 mg/ml) protected ($P<0.05$) the cell viability against H_2O_2 induced oxidative cell death. Further, treatment with peptides B, C, F and G regardless of their concentration significantly ($P<0.01$) reduced the formation of oxidative products such as MDA. The assessment of antioxidative enzymes (catalase: CAT, superoxide dismutase: SOD and glutathione peroxidase: GPx) activities additionally supplemented the anti-oxidative potential of peptides where significant ($P<0.05$) increase in catalase activity was recorded with the addition of selected peptides. But GPx have higher activities ($P<0.01$) with peptide C and F. These results were also confirmed by studying the mRNA expression and western blotting analysis of Nrf-2. Under evaluation of immunomodulatory potential of peptides, all the four selected peptides (B, C, F and G), on one hand reduced the secretion of IFN-γ while on the other increased the levels of IL-10 in the supernatant of cultured spleen cells regardless of their amount used. Moreover, peptide-C at 1 µg to 1 mg/ml also increased the release of IL-4 where other peptides did not show such response. Additionally peptides F and G showed increase ($P<0.05$) secretion of TGF-β at various concentrations used (10 ng to 1 mg/ml). Bioavailability and transport route studies conducted on two peptides (C and G) showed their sufficient transport of 0.95% and 1.72% across the epithelial membrane in their intact form through transcytosis and PepT1 mediated transport mechanisms respectively. Thus present study establishes the overlapping antioxidative and immunomodulatory potential of four peptides (B,C,F and G) to varying extent using different approaches with sufficient bioavailability of the two of the peptides (C and G) studied across the human intestinal epithelial cells.

कोशकीय-स्तर पर भैंस के कैसइन व्युत्पन्न पेप्टाइड्स की ऑक्सीकरण रोधी और प्रतिरक्षण निश्चायक क्षमता का अध्ययन

सारांश

प्राकृतिक स्रोतों के मध्य, दूध से व्युत्पन्न पेप्टाइड्स, अपने वस्तृत गुणों जैसे ऑक्सीकरण रोधी, रोगाणुरोधी, उच्च रक्त दाब रोधी और प्रतिरक्षण निश्चायक आदि जैव गति व धयों के कारण आकर्षण का *केन्द्र बने* है। जैव स क्रय पेप्टाइड्स के ऑक्सीकरण रोधी और प्रतिरक्षण निश्चायक अ धव्याप्त गुणों *से* रोग कैंसर, हृदयाघात, मधुमेह और उच्चरक्तचाप के खतरे को कम करने की क्षमता हो सकती है, जिनका प्राथ मककारणऑक्सीकरण द्वारा सूजन *संबं धत* प्रति क्रयाएं हैं । अभी तक को शकीय स्तर पर दूध व्युत्पन्न कै सइन पेप्टाइड्स के ऐसे अति व्यापीगुण *से संबं धत अध्ययनों* की सी मत संख्या उपलब्ध हैं I इस लए, वर्तमान अध्ययन में भैंस के दूध की कै सइन व्युत्पन्न पेप्टाइड्स को उनके ऑक्सीकरण रोधी और प्रतिरक्षण निश्चायक क्षमता पर स्परव्याप्त गुणों को को शकीय वातावरण के तहत जाँचा गया है। साहित्य और प्रयोगशाला में चल रहे शौध द्वारा मवे शयों के केसीन*से दस* पेप्टाइड (A-J) चयनित कया गये और उन्हें भैंस अनुक्रम के अनुसार जहां कहीं आवश्यक लगा संशो धत कया गयाI चयनित पेप्टाइड्स की जांच मुफ्त मूलक और प्रतिरक्षण निश्चायक गुणों के लए क्रमशः रासायनिक और एक्स-वीवो मूषक के ऊतकों पर *कया गया* I दस पेप्टाइड्स में *से*, चार पेप्टाइड (B, C, F और G) *ने* (पी<0.01)मुफ्त मूलक की सफाई क्षमता को 100 माइक्रो ग्राम/ म० ल० और 1 म०ग्रा०/ म० ल० की खुराक पर ABTS और ORAC दोनों तरीकों से अ धकतम असर दिखाया । एक्स-वीवोशर्तों, पेप्टाइड्स B, C और D *ने* खुराक 10 नैनोग्राम से 1 माइक्रोग्राम/ म० ल० और पेप्टाइड F, G, H, और I *ने* 10 नैनोग्राम से 1 म०ग्रा०/ म० ल० खुराक पर मूषक के तिल्ले के प्रसार को अवरोध कर दिया। दूसरी ओर,पेप्टाइड्स B, F, G, H, और I काफी अलग-अलग मात्रा पर (पी<0.01) मूषक पेरिटोनियल मैक्रोफेज द्वारा निगलने की प्रति क्रया को दिखाया, ले कन पेप्टाइड C *ने* अ धकतम (1 म०ग्रा०/ म० ल०) मात्रा पर समान असर दिखाया I उपरोक्त जांच के आधार पर चार (B, C, F और G) पेप्टाइड्स को मानव आंत्र उपकला Caco-2 को शकाओं या मूषक तिल्ले की *को शकाओं* के अंतर्गत तहत आगे मूल्यांकन के लए चुना गया। चार चयनित पेप्टाइड (B, C, F और G) की व भन्न सांद्रता (10 नैनो ग्राम से 1 म०ग्रा० / म० ल०) *ने* 24 घंटे तक Caco-2 को शकाओं के व्यावहारिकता और झल्ली अखंडता पर कोई प्रभाव नहीं दिखाया। इस के अलावा, H_2O_2 के प्रभाव में बढ़ी हुई ROS की मात्रा *से* प्रेरित ऑक्सीडेटिव तनाव को भी इन पेप्टाइड द्वारा अवरोध कया गया। इसके अलावा, पेप्टाइड-C और G (100 माइक्रो ग्राम से 1 म०ग्रा०/ म० ल०) के पूर्व-उपचार ने (पी< 0.05) H_2O_2 के खलाफ उत्पन ऑक्सीडेटिव तनाव से को शका व्यवहार्यता को सुर क्षत कया। आगे, पेप्टाइड्स B, C, F और G के साथ उन की मात्रा की परवाह कए बिना उपचार कया गया तो, ऑक्सीडेटिव उत्पादों (पी< 0.01) के गठन में कमी हो गई। ऑक्सीकरण रोधी एंजाइमों (catalase, superoxide dismutase और glutathione peroxidase*)* के आकलन से पेप्टाइड्स ऑक्सीकरण रोधी क्षमता के मूल्यांकन के तहत, catalase की कार्य क्षमता सभी पेप्टाइड्स में काफी मात्रा में बढ़ गई, ले कन GPx ने C और F के साथ ज्यादा क्षमता दिखाई। इन सब परिणामो की पुष्टि Nrf-2 के एम.आर.एन.ए. अ भव्यक्ति और वेस्टर्न ब्लॉटिंग द्वारा की गई Iसभी चार चयनित पेप्टाइड्स (B, C, F और G) ने, सुसंस्कृत तिल्ली को शकाओं के सूपर्नेटन्ट में IFN-γ का स्राव कम और IL-10 के स्राव में वृ द्ध की। इसके अलावा, पेप्टाइड-C 1माइक्रो ग्राम -1 म०ग्रा०/ म० ल० में IL-4 स्तर में वृ द्ध की, जहाँ अन्य पेप्टाइड्स ऐसी प्रति क्रया नहीं दिखा सकेI साथ ही पेप्टाइड-F और G ने व भन्न (10 नैनो ग्राम-1 म०ग्रा०/ म० ल०) मात्रा में TGF-β के स्राव में वृ द्ध कीI पेप्टाइड्स C और G पर जैव-उपलब्धता और परिवहन मार्ग का अध्ययन कया गया I पेप्टाइड्स C और G पर आयोजित इस अध्ययन में एपथीलीअम झल्लीसे 0.95% और 1.72% ट्रांसपोर्ट क्रमशः ट्रान्ससैटो सस और पेप टी 1 ट्रांसपोर्टर के माध्यम से देखा गया I इस प्रकार चार पेप्टाइड (B, C, F और G) ऑक्सीकरण रोधी और प्रतिरक्षण निश्चायक अ धव्याप्त गुणों को अलग-अलग तरीकों से सद्ध कया गया और पेप्टाइड्स C और G एपथीलीअम झल्ली में सर्वोत्तम जैव-उपलब्धता दिखाई I

CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

Abbreviations

AAPH	-	2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS	-	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACE	-	Angiotensin converting enzyme
ANOVA	-	Analysis of variance
BCM	-	β-casomorphin
BCM-5	-	β-casomorphin-5
BCM-7	-	β-casomorphin-7
BSA	-	Bovine serum albumin
CAT	-	Catalase
cDNA	-	Complementary deoxyribonucleic acid
ConA	-	Concanavalin A
CPPs	-	Caseinophosphopeptides
DCF-DA	-	2′,7′-Dichlorofluorescein diacetate
DEPC	-	Diethylpyrocarbonate
DMEM	-	Dulbecco's minimal essential medium
DMSO	-	Dimethyl Sulfoxide
dNTP	-	Deoxyribonucleotide triphosphate
DPP IV	-	Dipeptidyl peptidase IV
EDTA	-	Ethylenediaminetetraacetic acid
ELISA	-	Enzyme linked immunosorbant assay
FBS	-	Fetal Bovine Serum
GPx	-	Glutathione peroxidase
H2O2	-	Hydrogen peroxide
HBSS	-	Hanks balanced salt solution
HEPES	-	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	-	Horse radish peroxidase
IFN-γ	-	Interferon-γ
IL-10	-	Interleukin-10
IL-4	-	Interleukin-4
LDH	-	Lactate dehydrogenase
MDA	-	Malondialdehyde

mRNA	-	Messenger ribonucleic acid
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	-	Nuclear factor κB
Nrf-2	-	Nuclear factor (erythroid-derived 2)-like 2
ORAC	-	Oxygen radical absorption capacity
PBS	-	Phosphate buffer saline
PCR	-	Polymerase chain reaction
PVDF	-	Polyvinylidene difluoride
ROS	-	Reactive oxygen species
RP-HPLC	-	Reverse phase-high pressure liquid chromatography
RPMI	-	Roswell park memorial institute
RT	-	Retention time
SDS-PAGE	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	-	Standard error mean
SOD	-	Superoxide Dismutase
TEAC	-	Trolox equivalent antioxidant capacity
TGF –β	-	Transformed growth factor-β
TJ	-	Tight junctions
TRI reagent	-	Trade name for solutions having guanidium thiocyanate and phenol, used for isolation for DNA, RNA and proteins

Chapter 1

Introduction

Introduction

Oxidative stress is a complex process which occurs due to persistent imbalance between antioxidants and pro-oxidants (reactive oxygen species) in favour of the later, resulting in irreversible cellular damages. Its impact on the organism depends on the type of oxidant, on the site and intensity of its production, on the composition and activities of various antioxidants, and on the ability of repair systems (Durackova, 2007). Free radicals or reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) are primarily produced from electron transport chain of mitochondria during oxygen metabolism are highly reactive due to the unpaired electrons in their outermost shell. They participate in a large number of subsequent reactions in which other very reactive metabolites are formed thereby play an important role in the generation of oxidative stress (Breitenbach and Eckl, 2015). Oxidative stress and inflammation are closely related pathophysiological processes, one of which can be easily induced by another. Thus, both processes are simultaneously found in many pathological conditions. There is no doubt that the chronic low-grade inflammatory process plays a central role in the pathogenesis of many chronic diseases. Epidemiological and experimental studies strongly suggest a contribution of oxidative stress in many human diseases including cancer, neurological disorders, atherosclerosis, hypertension, ischemia/perfusion, diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, and asthma (Zhang *et al.,1999*; Chaitanya *et al.,* 2010). If oxidative stress appears as the primary abnormality in an organ, inflammation will eventually develop and will further accentuate oxidative stress. Conversely, if inflammation is the primary event, oxidative stress will develop as a consequence which will further exaggerate inflammation.

The basic mechanism involved in cellular damage by oxidative stress and initiating inflammation (Shi *et al.,* 2014) involves susceptibility of major bio molecules including lipids, proteins and DNA to free radicals (Shafaq, 2012). ROS production induce potentially toxic responses in the cells by modulating gene expression through activation of vital signal transduction pathways involving inflammation associated master regulator (eg.NF-*kB*). This results in the release of pro-

inflammatory cytokines (Hsieh *et al.,* 2013; Newton and Dixit, 2012) which is a highly coordinated process involving margination, rolling, and adhesion of leukocytes to the vascular endothelium, transmigration across the endothelium, and migration toward a chemo-tactic stimulus with the participation of a number of adhesion molecules, including selectins, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), and their respective leukocyte receptors etc. leading to inflammation. Therefore, oxidative stress linked inflammation can cause a variety of life style associated diseases (Reuter *et al.,* 2010).

The free radical induced inflammatory processes might be neutralized by the antioxidants and radical scavengers which can attenuate the adverse effects of oxidative stress as well as inflammation (Delaporte *et al.,* 2002; Geronikaki and Gavalas, 2006). Although, humans have evolved with various endogenous (produced in the body) and exogenous (obtained from diet) antioxidative and anti-inflammatory systems to protect against damage caused by free radical induced inflammation. If the endogenous sources fail to overcome the reactive metabolites production during excessive stress and inflammatory conditions, then exogenous compounds would be necessary to protect the organism. A vast number of researchers all over the world have investigated whether use of only antioxidants are capable of preventing diseases like cardiovascular diseases, cancer, diabetic complications, Alzheimer's disease, and so forth (Bjelakovic *et al.,* 2007). However, results of these antioxidant trials are largely frustrating in human patients; although some of the trials show beneficial health effects, others show either no effect or even harmful effects (Sesso *et al.,* 2008). Thus, the findings of antioxidant trials have raised a great deal of uncertainty about the role of oxidative stress in the pathogenesis of human diseases. Although a number of explanations have been proposed to clarify this discrepancy between findings of clinical trials and those of epidemiological/experimental studies, it has appeared as a new puzzle in medical science and is known as antioxidant paradox (Halliwell, 2000; Halliwell, 2013).

Among the exogenous sources, various herbal drugs such as Curcumin (diferuloylmethane), *Magnolia officinalis*, AS-IV (Saponin astragaloside-IV) and other food poly-phenolic rich food ingredients (Gu and Lambert, 2013) used for the treatment of such lifestyle associated diseases were found to have overlapping antioxidative and anti-inflammatory properties. These drugs showed antioxidative

property by inhibiting lipid peroxidation and by enhancing antioxidative enzymes such as GPx and SOD under *in vitro* conditions along with anti-inflammatory effects by abolishing COX-2, LPS and TNF-α mediated NF*k*B activation (Huang *et al.,* 1991; Jobin *et al.,* 1999; Wang *et al.,* 2002; Luo *et al.,* 2004; Zhang *et al.,* 2003). However, the extraction of these drugs is laborious and their prolong usage found to pose adverse effects (Moron *et al.,* 2010; Zang *et al.,* 2003). Further, their poor absorption and rapid metabolism severely curtails bioavailability of these drugs. These limitations have compelled researchers to explore new areas involving food derived bioactive compounds which are gaining importance because they are toxicologically safe, have a wide spectra of therapeutic action, exhibit less side effects as compared to synthetic drugs and are better absorbed in the intestinal tract (Erdman *et al.,* 2008; Agyei and Danquah 2011). Moreover, the failure of antioxidant trials might result from failure to select appropriate agents that specifically target both inflammation and oxidative stress or failure to use both antioxidants and anti-inflammatory agents simultaneously or use of nonselective agents that block some of the oxidative and/or inflammatory pathways but exaggerate the others might be responsible for the failures of the antioxidant.

Current studies have shown that among the food sources, milk proteins produce various biologically active peptides after digestion with wide varieties of activities, which play an important role in the nervous, cardiovascular, digestive and immune systems (Lopez-Exposito *et al.,* 2007; Kitts, 2005). Initially most of the researchers explored various peptides hidden in the casein system with single activity such as antioxidative, immunomodulatory, ACE inhibitory, antimicrobial and metal chelating properties (De Gobba *et al.,* 2014; Del Mar Contreras *et al.,* 2009; Silva and Malcata, 2005). But recently researchers appeared to have growing interest in identifying peptides with multifunctional or overlapping bioactivities (Sistla, 2013) over single-activity peptides because they can simultaneously trigger, modulate, or inhibit multiple physiological pathways (Aguilar-Toala *et al.,* 2016). Such overlapping peptides may play a great role in reversing the adverse effects of both oxidative stress and inflammatory conditions in the organism which appeared to be a failure with antioxidative therapies earlier. Most of the studies reported the free radical scavenging capacity of casein derived peptides and hydrolysates using different chemical methods (including ABTS, ORAC, DPPH and FRAP assays) which

determine the hydrogen atom donating capacity of bioactive compounds (Suetsuna *et al.,* 2000; Bezerra *et al.,* 2013) but only scanty information is available on antioxidative potential of peptides under cellular conditions which is quite necessary before the onset of biological trials. Similarly isolated studies on casein derived bio active peptides were reported on immunomodulation without delineating antioxidative and immunomodulatory functions in conjunction (Minkiewicz *et al.,* 2000; Hata *et al.,* 1999; Parker *et al.,* 1984). Further, studies on bioavailability of casein derived peptides are insufficient which establish the peptides with potential activity will cross the gastrointestinal barrier, and reach the circulation and target sites in an active form (Vermeirssen *et al.,* 2004; Regazzo *et al.,* 2010) to exert the biological activity. Keeping in view the foregoing discussion the present study has been designed with following objectives.

1) To screen buffalo casein peptides for antioxidative and immunomodulatory activity.
2) To investigate the cellular response of potential casein peptide(s) *in vitro.*
3) To evaluate bioavailability and transepithelial transport of potential anti-oxidative and immunomodulatory peptide(s)

Chapter 2

Review of literature

REVIEW OF LITERATURE

2.1 Oxidative stress and Reactive oxygen species

The term "oxidative stress" was first introduced in the eighties by Helmut Sies (1985), defining it as a disturbance in the pro oxidant-oxidant balance in favor of the first. There is a close association between oxidative stress and lifestyle-related diseases cancer, cardiovascular diseases, atherosclerosis, hypertension, arthritis, alcoholism, neurological disorders, diabetes, obesity, metabolic syndrome, skin and tumor diseases (Pechan *et al.,* 2003; Ballinger, 2005; Cherubini *et al*., 2005; Aruoma *et al*., 2007; Klaunig and Kamendulis 2004). Oxidative stress is defined as a "state in which oxidation exceeds the antioxidant systems in the body due to the loss of balance between them" (Betteridge, 2002). Production of Reactive oxygen species (ROS) or free radicals including superoxide anion (O_2^{-}), hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\cdot}$), alkoxyl radical ($RO^{\cdot}$) and singlet oxygen ($O^{\cdot}$) is mainly associated with the generation of oxidative stress (Murrant and Reid, 2001). Usually, an atom is composed of a central nucleus with pairs of electrons orbiting around it. However, some atoms and molecules have unpaired electrons and these are called free radicals. These are unstable and highly reactive molecules capable of engaging in rapid chain reactions that destabilize other molecules and generate many more free radicals. They can interact with various bio molecules such as lipids, proteins and DNA of the living cells and damage cell structure and function (Valko *et al.,* 2005). ROS can be generated through various mechanisms including (i) irradiation by UV light, by X-rays and by gamma rays (ii) as products of metal-catalyzed reactions (iii) are present as pollutants in the atmosphere (iv) are produced by neutrophils and macrophages during inflammation (v) are by-products of mitochondria-catalyzed electron transport reactions and other mechanisms (Cadenas,1989). Mitochondrial electron transport chain is mainly responsible for the production of ROS. In mitochondria, during electron transport chain superoxide anions (O_2^{-}) are produced by the reduction of molecular oxygen. These superoxides are converted to H_2O_2 by the action of superoxide dismutase. However, when this hydrogen peroxide interacts with ions of transition metals such as iron and copper, the most reactive free radicals are formed by Fenton's reaction (Kowaltowski *et al.,* 2001).

Moreover, there are other cellular sources are also responsible for the production superoxide radicals in the cell, for example xanthine oxidase (XO), an important source for the formation of oxygen-free radicals. It is a highly versatile enzyme that is widely distributed among species from bacteria to man and within the various tissues of mammals. It is a member of a group of enzymes known as molybdenum-sulphur flavin hydroxylases and catalyzes the hydroxylation of purines (Li and Jackson, 2002). XO catalyzes the reaction of hypoxanthine to xanthine and xanthine to uric acid. In both steps, molecular oxygen is reduced, forming the superoxide anion in the first step and hydrogen peroxide in the second step (Valko *et al.,* 2004).

In addition, immune cells such as macrophages, neutrophils and eisinophils are also acts as source of ROS production in the body (Schreck and Baeuerle,1991). Although, these immune cells will help defend the body against invading micro organisms. In this case, however, ROS production is beneficial and even essential to the organism because it plays a central role in destroying foreign pathogens (Rosen *et al.,* 1995). Macrophages and neutrophils contain a group of enzymes called NADPH oxidase complex, which, when activated, they initiate an increase in the uptake of oxygen and leads to the generation of various free radicals such as superoxide radicals, nitric oxide and hydrogen peroxide (Conner and Grisham, 1996). Figure 2.1 showing the sources and cellular responses to reactive oxygen species. Higher production of ROS are toxic to the cells because they can interact with most of cellular macromolecules, including DNA, proteins and lipids and also activates several stress-induced transcription factors, and accelerates the production of pro-inflammatory and anti-inflammatory cytokines (Dalton *et al.,* 1999; Scandalios, 2004). Modifications in DNA due to ROS can occur in several ways which involves single or double stranded DNA breaks, degradation of bases, purine, pyramidine or sugar-bound modifications, deletions, mutations or translocations and cross linking with proteins (Cooke *et al.,* 2003). Most of these DNA modifications are highly relevant to carcinogenesis, aging, neurodegenerative, cardiovascular, and autoimmune diseases. Formation of 8-hydroxyl guanine (8-OH-G) is the best known marker of DNA damage occurring via oxidative stress and is a potential biomarker for carcinogenesis (Ghosh and Mitchell, 1999). Proteins, important macromolecules of the cell are also susceptible to the modification by ROS. They can cause oxidation of specific amino acids of the proteins, cross linking of the proteins, fragmentation of the peptide chain and therefore leads to increased susceptibility to degradation by

specific proteases. Particularly cysteine and methionine residues in proteins are more susceptible to oxidation and can cause conformational changes, unfolding, and degradation of proteins (Lyras *et al.,* 1997). ROS can also induce lipid peroxidation and disrupt the membrane lipid bilayer arrangement that may inactivate membrane-bound receptors and enzymes and increase tissue permeability. Products of lipid peroxidation, such as MDA and unsaturated aldehydes, are capable of inactivating many cellular proteins by forming protein cross-linkages (Davi *et al.,* 2005). Thiobarbituric acid reactive substances such as MDA has been used as a biomarker of lipid peroxidation (Montuschi *et al.,* 2000).

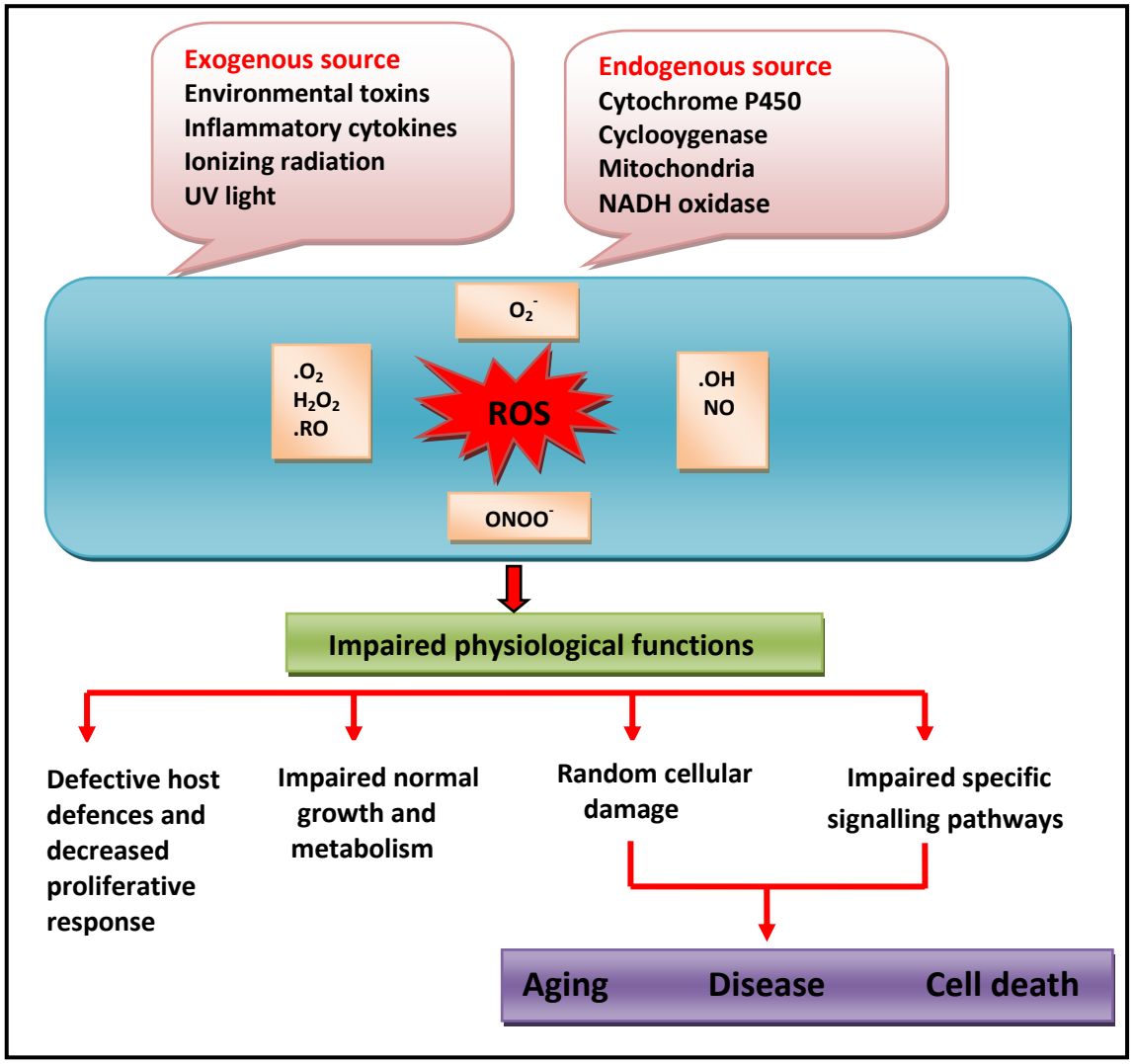


Figure 2.1. The sources and cellular responses to reactive oxygen species (ROS)

2.2 Antioxidants

Antioxidants are molecules, prevent oxidation reactions through neutralising free radicals by donating an electron to them (Percival, 1998). In the human body, a

highly sophisticated and complex antioxidant systems has evolved to protect the cells and organ systems against reactive oxygen species (Aruoma, 1998). Antioxidants are divided in to natural and synthetic antioxidants.

2.2.1 Natural antioxidants

Which involves a variety of components, both enzymatic and non enzymatic origin, that function interactively and synergistically to neutralize free radicals.

2.2.1.1 Enzymatic antioxidants

This includes three major enzymes i) Superoxide dismutase

ii) Catalase

iii) Glutathione peroxidase

i) Superoxide dismutase (SOD)

SODs are a group of metallo enzymes, present almost in all aerobic cells and extra cellular fluids (Johnson and Giulivi, 2005). In humans there are three different types of SODs have been identified which vary in their location and metal ion composition as cofactor. For example, copper/zinc containing SOD is present in the cytosol, while manganese containing SOD is present in the mitochondria and third form of SOD present in extracellular fluids, which also contains copper and zinc as its cofactor (Grayck *et al.,* 2005). SOD catalyses the conversion of superoxide anion to hydrogen peroxide and di oxygen. This reaction acts as a source of cellular H_2O_2 (Hohmann,1997).

ii) Catalase

H_2O_2 formed by SOD, from metabolic reactions is scavenged by a ubiquitous heme protein catalase using either iron or manganese as cofactor (Chelikani *et al.,* 2004; Zamocky and Koller,1999). This enzyme is localized in peroxisomes of almost all eukaryotic cells and catalyzes the breakdown of hydrogen peroxide into water and molecular oxygen (Santoro and Thiele, 1997).

iii) Glutathione peroxidase

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidase and glutathione transferase and this system is found in animals, plants and microorganisms (Meister and Anderson, 1983; Creissen *et al.,* 1996). Glutathione peroxidase contains four selenium cofactors that catalyzes the breakdown of hydrogen peroxide and organic hydroperoxides (Arteel and Sies 2001).

2.2.1.2 Non enzymatic antioxidants

Non enzymatic antioxidants include low-molecular-weight compounds, such as vitamins (vitamins C and E), glutathione (GSH) and β-carotene.

i) Vitamin C (Ascorbic Acid)

Water-soluble vitamin C provides intracellular and extracellular aqueous-phase antioxidant capacity primarily by scavenging oxygen free radicals. It is a reducing agent and can reduce and there by neutralize reactive oxygen species such as hydrogen peroxide (Ortega, 2006). It converts vitamin E free radicals back to vitamin E. Its plasma levels have been shown to decrease with age (Bunker, 1992; Mezzetti *et al.,*1996).

ii) Vitamin E (α-Tocopherol)

Lipid-soluble vitamin E is concentrated in the hydrophobic interior site of cell membrane and is the principal defense against oxidant-induced membrane injury (Herrera and Barbas, 2001). α-Tocopherol is the most active form of vitamin E and the major membrane-bound antioxidant in the cell. It donates electron to peroxyl radical, which is produced during lipid peroxidation. Vitamin E triggers apoptosis of cancer cells and inhibits free radicals formation (White *et al.,*1997).

iii) Glutathione (GSH)

GSH, a tri peptide (glutamyl-cysteinyl-glycine) that comprise a thiol (sulfhydryl) group, is highly abundant in all cell compartments and is the major soluble antioxidant. GSH shows its antioxidant effects in several ways (Curello *et al.,* 1985). It is involved in the detoxification of hydrogen peroxide and lipid peroxides. GSH donates its electron to H_2O_2 to reduce it into H_2O and O_2. It also donates protons to membrane lipids and protects them from oxidant attacks (Masella *et al.,* 2005).

iv) Carotenoids (β-Carotene)

Carotenoids are pigments found in plants. Primarily, β-carotene has been found to react with peroxyl, hydroxyl and superoxide radicals (Miller and Rice-Evans, 1997). Carotenoids show their antioxidant effects in low oxygen partial pressure but may have pro-oxidant effects at higher oxygen concentrations (EI-Agamey *et al.,* 2004).

v) Lipoic acid

Lipoic acid an important sulfur-containing antioxidant involved in the reaction that catalyzes the oxidative decarboxylation of alpha-keto acids, such as pyruvate and α-ketoglutarate in the Krebs cycle. Lipoic acid and its reduced form dihydrolipoic acid (DHLA) are capable of quenching free radicals during lipid peroxidation (Packer *et*

al., 1995). Lipoic acid may also exert its antioxidant effect by chelating pro-oxidant metals. *In vivo* studies have demonstrated that supplementation of lipoic acid gave protection against the symptoms of vitamin E or vitamin C deficiency.

2.2.2 Synthetic antioxidants

These days, most of the food and pharmaceutical products contain synthetic antioxidants. These compounds are added to food in order to prolong product shelf life, mainly by preventing the oxidation of unsaturated double bonds of fatty acids. In pharmaceutical products, for reducing the susceptibility of food compounds to chemical degradation by oxidation various synthetic antioxidants are added. Nowadays, Butylated hydroxyanisole (BHA) and Butylated hydroxyl toluene (BHT) are the two most commonly used synthetic antioxidants. First introduced in 1947, BHA is primarily used as a food preservative. The molecular structure of the BHA compound allows it to scavenge free radicals and prevent other free radical reactions from occurring. BHA is also incorporated in statins, a class of drug used to treat high cholesterol and prevent cardiovascular disease. It also began to appear in food packaging, animal feed, rubber and petroleum products. Similar to BHA, BHT is a synthetic variant of vitamin E. It is incorporated into food to prevent free radical damage, elicit a change in taste and produce putrid odors. In addition to being added to fats in food, BHT is also found in cosmetics, pharmaceuticals, jet fuels, rubber, petroleum products and embalming fluid. Despite the controversy surrounding the safety and carcinogenic potential of BHT, it is conventionally used as a replacement for BHA (Ito *et al.,* 1985). In addition, propyl gallate and tert butyl hydro quinone (TBHQ) are other widely used synthetic antioxidants in the food industry. TBHQ stabilizes and preserves the freshness, nutritive value, flavour and colour of animal food products (Anton *et al.,* 2004).

However, synthetic antioxidants found to pose adverse effects in *in vivo* studies. A study carried out by the National Library of Medicine, USA demonstrated the link between BHT and lung tumour development. BHT promotes the development of tumours in previously initiated cells (Thompson *et al.,* 1991). An another study done in the 1980s tested the behavioural and developmental effects of BHT on the body weight of neonatal rats. The results suggested that a daily dose of BHT had a significantly adverse effect on body weight (Olsen *et al.,* 1986). Studies suggest that BHA can induce the formation of stomach tumours. From the research, it is clear that there is an associated link between BHA and BHT and the instigation and promotion

of tumour formation. Therefore, due to the potential risks of synthetic antioxidants, these days antioxidants from natural sources have been preferred (Ito *et al.,*1985).

2.3 Bioactive peptides

The role of proteins as physiologically active components in the diet is being increasingly acknowledged. In recent years it has been recognized that dietary proteins provide a rich source of biologically active peptides. From a nutritional point of view, peptides represent a more bio available form of essential amino acids than proteins, even compared to free amino acids (Adibi, 1997). Bioactive peptides have been defined as specific protein fragments that have a positive impact on human body functions and conditions and may ultimately influence health (Kitts and Weiler, 2003). Such peptides are inactive within the sequence of the parent protein and display their specific activities after releasing from parent protein through enzymatic hydrolysis. Various bioactive peptides are found in animal as well as in different plant protein sources (Vercruysse *et al.,* 2005; Hernandez-Ledesma *et al.,* 2005; Motoi and Kodama, 2003; Takahashi *et al.,* 1994). Table 2.1 showing the list of bioactive peptides derived from plant and animal sources.

Table 2.1. Bioactive peptides from plant and animal sources and their corresponding biological activity

Origin	Encrypting protein	Sequence of peptide	Biological activity	Reference
Soy	Soy protein	NWGPLV	ACE inhibitory/ hypotensive	Kodera and Nio, 2006
Wheat	Wheat gliadin	IAP	ACE inhibitory/ hypotensive	Motoi and Kodama, 2003
Rice	Rice albumin	GYPMYPLR	Immunomodulatory	Takahashi *et al.,* 1994
Fish	Fish muscle protein	LKP IKP LRP	ACE inhibitory/ hypotensive	Nagai *et al.,* 2006
Meat	Meat muscle protein	IKW, LKP	ACE inhibitory/ hypotensive	Vercruysse *et al.,* 2005
Egg	Ovotransferrin	KVREGTTY	ACE inhibitory/ hypotensive	Lee *et al.,* 2006
Milk	α-LA, β-LG	MHIRL YVEEL WYSLAMAASDI	Antioxidant	Hernandez-Ledesma *et al.,* 2005

2.3.1. Bioactive peptides of milk origin

Today, milk proteins are considered the most important source of bioactive peptides with important physiological functions and prove that their role is not only to feed the neonate but also to regulate the complete growth of the body (Zabielski, 2007). Due to the increasing awareness of the effect of food ingredients upon promotion of health, several studies have arisen that cover recent advances on milk peptide research (Clare *et al.,* 2003; Florisa *et al.,* 2003; Janecka *et al.,* 2004; Kilara and Panyam, 2003). List of bioactive peptides derived from milk proteins and their biological activities are given in table 2.2.

Table 2.2. Bioactive peptides from milk

Fragment	Peptide sequence	Activity	Reference
Bovine αS1-CN f (23-24)	FF	Antihypertensive	Maruyama and Susuki (1982); Maruyama *et al.* (1987a); Meisel and Schlimme (1994)
Bovine αS1-CN f (102-109)	KKYKVPQ	Antihypertensive	Gomez-Ruiz *et al.* (2002)
Bovine β-CN f (74-760)	IPP	Antihypertensive	Nakamura *et al.* (1995)
Human β-CN f (41–44)	YPSFQ	Opioid (agonistic)	Fiat *et al.* (1993)
Human β-CN f (54-59)	VEPIPY	Immunomodulatory	Parker *et al.* (1984)
Bovine αS2-CN f (164-179)	LKKISQRYQKFAL-PQY	Antimicrobial	Recio and Visser (1999)
Bovine k-CN f (103-111)	LSFMAIPPK	Antithrombic	Fiat *et al.* (1993)
Goat αS1-CN f (143-146)	AYFY	ACE- inhibitory	Lee *et al.,* 2005

2.3.2. Production of bioactive peptides

Most of the bioactive peptides are encrypted within the primary sequence of the native protein and are released through the following ways:

2.3.2.1. Microbial fermentation

Several lactic acid bacteria (LAB) (e.g. *Lactococcus lactis*, *Lactobacillus helveticus*) have been reported to release bioactive peptides by the process of fermentation.

This system consists of a number of distinct intracellular peptidases including endo peptidases, amino peptidases, di peptidases, and tri peptidases (Christensen *et al.,* 1999). Recent studies have reviewed the production of various bioactive peptides including antimicrobial, immunomodulatory, antioxidative and ACE-inhibitory peptides through microbial proteolysis (Gobbetti *et al.,* 2004; Korhonen and Pihlanto, 2003). Several studies have demonstrated that *Lactobacillus helveticus* strains, in particular, are capable of releasing antihypertensive peptides, the best known of which are ACE-inhibitory tri peptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP). The antihypertensive capacity of these peptides has been demonstrated in several rat model and human studies (Hata *et al.,* 1996; Sipola *et al.,* 2002; Seppo *et al.,* 2003; Mizushima *et al.,* 2004; Aihara *et al.,* 2005; Jauhiainen *et al.,* 2005; Hirota *et al.,* 2007). In a recent study, Virtanen *et al.* (2006) demonstrated that fermentation of milk with single industrial dairy cultures generated antioxidant activity in the whey fraction. The activity correlated positively with the degree of proteolysis suggesting that peptides were responsible for the antioxidative property. Table 2.3 representing the examples of the identified bioactive peptides in fermented milk and their corresponding physiological activity (Vasiljevic and Shah, 2008).

2.3.2.2. Enzymatic hydrolysis

The most common way to produce bioactive peptides from milk is through enzymatic hydrolysis of the whole protein molecules. Digestive enzymes and combinations of different proteases including chymotrypsin, pepsin and trypsin have been shown to release a number of antihypertensive peptides, calcium-binding phosphopeptides (CPPs), antibacterial, antioxidative, immunomodulatory and opioid peptides from different casein (α-, β- and *k*-casein) and whey proteins (α-lactalbumin, β-lactoglobulin). The biological activity of the released peptides depends on the enzyme used for their production. For example, ACE-inhibitory peptides and calcium-binding phosphopeptides are most commonly produced by trypsin (FitzGerald *et al.,* 2004; Meisel and FitzGerald, 2003; Yamamoto *et al.,* 2003). Moreover, ACE-inhibitory peptides have recently been identified in the tryptic hydrolysates of bovine αS-2 and *k*-casein (Tauzin *et al.,* 2002) and in ovine and caprine *k*-casein macro peptides (Manso and Lopez-Fandino, 2003).

Table 2.3. Bioactive peptides released from food proteins by various microorganisms and microbial enzymes

Substrate	Micro organism Used	Precursor protein	Peptide sequence	Bioactivity	Reference
Milk	*Lactobacillus helveticus*, *Saccharomyces cerevisiae*	β-cn, k-cn	VLP IPP	ACE-inhibitory, Antihypertensive	Takano,2002 Nakamura *et al.,*1995
	Lactobacillus GG enzymes + pepsin and trypsin	β-cn as1-cn	YPFP TTMPLW	Opioid, Immunostimulatory	Rokka *et al.,*1997
	Lactobacillus delbrueckii subsp. *bulgaricus* SS1 *Lactococcus lactis* subsp. *cremoris* FT4	β-cn, k-cn	Many fragments	ACE-inhibitory	Gobbetti *et al.,* 2000
	Lactobacillus delbrueckii subsp. *bulgaricus* IFO13953	k-casein	ARHPHP HLSFM	Antioxidative	Kudoh *et al.,* 2001
Whey	*Kluyveromyces marxianus* var. *Marxianus*	β-lg	YLLF	ACE-inhibitory	Belem *et al.,* 1999
	Tritirachium album derived proteinase K	β-lg	IPA	Antihypertensive	Abubakar *et al.,*1998
Casein	*Lactobacillus helveticus* CP90 proteinase	β-cn	KVLPVPE	ACE-inhibitory	Maeno *et al.,*1996
Soybean	*Bacillus natto* or *Bacillus subtilis*		contain Ala, Phe and His residues	ACE-inhibitory	Cho *et al.,* 2000

Other digestive enzymes and different enzyme combinations of proteases including alcalase, chymotrypsin, pepsin and thermolysin as well as enzymes from bacterial and fungal sources have also been utilized to generate bioactive peptides from various proteins (Kilara and Panyam, 2003; Korhonen and Pihlanto, 2003). Biologically active peptides derived from milk proteins particularly casein offer a promising approach for the promotion of health by means of a tailored diet and provide interesting opportunities to the food industry for expansion of its field of

operation (Nagpal *et al.,* 2011). Various physiologically important bioactive peptides are produced from the digestion of casein proteins, which play an important role in the nervous, cardiovascular, digestive and immune systems (Kitts, 2005; Silva and Malcata, 2005) through their bioactivities such as antioxidative, antimicrobial, ACE inhibitory, immunomodulatory and metal chelating properties (Gobba *et al.,* 2014; Contreras *et al.,* 2009; Rival *et al.,* 2001; Malinowski *et al.,* 2014).

2.4. Different experimental models to determine the antioxidative efficacy of bioactive peptides

2.4.1. Chemical methods (cell free systems)

For assessing the antioxidant capacity of bioactive peptides several chemical assays such as ABTS, FRAP, DPPH and ORAC methods have been frequently used due to their simple, rapid, sensitive and reproducible procedures. Each method relates to the generation of a different radical, acting through a variety of mechanisms and the measurement of a range of end points at a fixed time point or over a range. Thus, the chemical assays in the cell-free systems for antioxidant studies are often considered by researchers before further studies by cell lines and/or animal models.

2.4.1.1. ABTS assay

ABTS method was first reported by Miller and Rice-Evans (1997), which is based on the scavenging ability of antioxidants to the long-life $ABTS^{\cdot+}$ radical anion. This method is frequently used by the food industry and agricultural researchers to measure the antioxidant capacities of foods (Huang *et al.,* 2005). In this assay, ABTS is converted to its radical cation ($ABTS^{\cdot+}$) by the addition of potassium persulfate. This radical cation is a dark green in color and absorbs light at 734 nm (Roberta *et al.,* 1999).The ABTS radical cation is reactive towards most antioxidants including phenolics, thiols, Vitamin C and E (Richard and Jace 2009). During this reaction, the green ABTS radical cation is converted back to its colourless neutral form and the reaction will be monitored spectrophotometrically. This assay is often referred to as the Trolox equivalent antioxidant capacity (TEAC) assay. The reactivity of the various antioxidants tested are compared to that of Trolox, an water-soluble analog of vitamin E.

2.4.1.2. ORAC assay

The ORAC assay is based upon the early work of Ghiselli *et al.* (1995) and Glazer (1990), as developed further by Cao *et al.* (1993). ORAC measures antioxidant inhibition of peroxyl radical induced oxidations and thus reflects classical radical

chain breaking antioxidant activity by H atom transfer (Ou *et al.,* 2001). The ORAC assay is based upon the inhibition of the peroxyl radical-induced oxidation of fluorescent probe such as fluorescein initiated by thermal decomposition of azo compounds such as AAPH (2,2'-azobis(2-amidino-propane) dihydrochloride). Antioxidants supress this reaction by a hydrogen atom transfer mechanism, there by inhibiting the oxidative degradation of the fluorescein signals. The fluorescence signal is measured over 80 minutes by excitation at 485 nm and emission at 538 nm. The antioxidative capacity of the test sample is proportional to the fluorescence intensity through the course of the assay and values are expressed as trolox equivalent antioxidative capacity (TEAC).

2.4.1.3. FRAP assay

The ferric reducing antioxidant power (FRAP) method (Benzie and Strain, 1996) is based on the reduction of a ferroin analog, the Fe^{3+} complex of tripyridyltriazine Fe(TPTZ)3+, to the intensely blue coloured Fe^{2+} complex $Fe(TPTZ)^{2}$+ by antioxidants in acidic medium. Results are obtained as absorbance increases at 593 nm and can be expressed as micromolar Fe^{2}+ equivalents or relative to an antioxidant standard. The authors claim the method to be simple and rapid and both manual and automated procedures have been described (Benzie and Strain, 1999). However, the measured reducing capacity in FRAP assay does not necessarily reflect antioxidant activity. It provides instead a very useful 'total' antioxidant concentration, without measurement and summation of the concentration of all antioxidants involved. The method was originally applied to plasma but has been extended to other biological fluids, foods, plant extracts, juices (Gardner *et al.,* 2000).

2.4.1.4. DPPH assay

The $DPPH^{\cdot+}$ radical is one of the few stable organic nitrogen radicals, which bears a deep purple colour. It is commercially available and does not have to be generated before assay like $ABTS^{\cdot+}$ (Ohnishi *et al.,* 1994). This assay is based on the measurement of the reducing ability of antioxidants toward $DPPH^{\cdot+}$ and the ability of antioxidant can be evaluated by measuring the decrease of its absorbance spectrophotometrically at 515nm. The test is simple and rapid which probably explains its widespread use in antioxidant screening.

An antioxidant is not just a good free radical scavenger but it should be a molecule that can exert its antioxidant capacity by activating various antioxidative systems in the cell thus, ameliorating oxidative stress (Jones, 2006). However, the chemical

methods do not provide the information about the antioxidative potential of the peptide under cellular conditions. In addition, the chemical structure of free radicals produced in the chemical methods having poor correlation with the free radicals produced in biological systems. Therefore, it is very important to evaluate the *in vitro* antioxidative potential of the peptides after screening their activity with chemical methods (Lopez-Alarcon and Denicola, 2013). This does not mean that chemical methods should be considered ineffective when compared to cell-based assays. It is possible that some antioxidative peptides can work chemically, while they may not work cellularly and physiologically (Wolfe and Liu 2007). Therefore, to evaluate a peptide as potential antioxidant, it must be able to change the redox cellular state because participation of different components of the cell are vital to finally develop an antioxidant response.

2.4.2. Cellular systems

2.4.2.1. *In vitro* model

Cell-based or *in vitro* assays as intermediate methods have been used increasingly recently to evaluate the protective effects of antioxidants against oxidative stressors and to elucidate mechanism of action of peptides within cells. These methods can be used to elucidate the mechanism of action of antioxidant peptides within live cells. Moreover, cell culture assays are helpful for the elucidation of peptide dosage to exert beneficial antioxidant effects without cytotoxicity for *in vivo* experiments. Therefore, cell lines are well suited as high capacity screening models and different cell types have been used for assessing the antioxidative capacity of bioactive peptides. List of commonly used cell lines and their characteristic features are given in table.2.4. Among different cell lines, nowadays, Caco-2 cells have been successfully used to examine the antioxidative effects of different food compounds including milk and plant derived compounds, carotenoids and flavonoids against oxidative stress (Mahler *et al.,* 2009; During *et al.,* 2005; Gonzales *et al.,* 2015). Although Caco-2 cells derived from colon (large intestine) carcinoma, when cultured under specific conditions the cells become differentiated and polarized such that their phenotype, morphologically and functionally, resembles the enterocytes lining the small intestine. These cells express tight junctions, microvilli and a number of enzymes such as peptidases, esterases and transporters for the uptake of amino acids, bile and carboxylic acids etc (Shimizu *et al.,* 1997).

Table 2.4. List of commonly used cell lines (Balimane and Chong, 2005)

Cell line	Species of origin	Tissue of origin	Morphology	Ploidy	Characteristics
IMR-90	Human	Lung	Fibroblast	Diploid	Susceptible to human viral infections
3T3-A31	Mouse	Connective tissue	Fibroblast	Aneuploid	Contact inhibited, readily transformed
BHK21-C13	Hamster (Syrian)	Kidney	Fibroblast	Aneuploid	Readily transformable
CHO-K1	Chinese hamster	Ovary	Fibroblast	Dipolid	Simple karyotype
NRK49F	Rat	Kidney	Fibroblast	Aneuploid	Induction of suspension growth by TGF-α, β
BRL 3A	Rat	Liver	Epithelial	Aneuploid	Produces IGF-2
Vero	Monkey	Kidney	Fibroblast	Aneuploid	Viral substrate and assay
HeLa-S_3	Human	Cervical carcinoma	Epithelial	Aneuploid	Rapid growth, high plating efficiency
Sk/HEP-1	Human	Hepatoma	Epithelial	Aneuploid	Factor VIII
Caco-2	Human	Colorectal carcinoma	Epithelial	Aneuploid with polarised	Forms tight monolayer support
MCF	Human	Breast tumor (effusion)	Epithelial	Aneuploid	Estrogen receptor positive

The Caco-2 cell line is widely used across the pharmaceutical industry as an *in vitro* model of the human small intestine because of their cost effectiveness, less time consuming to grow, quick and easy *in vitro* model to generate reliable and reproducible data. Thus, Caco-2 cells are widely used as *in vitro* models in several scientific fields such as nutrition, physiopathology, pharmacology and toxicology.

Although, the use of *in vitro* model systems provide no information about the organ specific effects of the bioactive compounds in the organism. Therefore *ex vivo* and *in vivo* models are preferred to determine the efficacy of the compounds at specific organ level.

2.4.2.2. *Ex-vivo* model

Ex-vivo (Latin: "out of the living") means that which takes place outside an organism. *Ex-vivo* conditions allow experimentation on cells or tissue from an organism in an external environment with minimal alteration of natural conditions. For the purpose of *ex-vivo* experiments, tissues can be removed in many ways, including in part, as whole organs, or as larger organ systems. For example, collection of spleen tissue from the mice and measurement of the proliferative response of splenocytes is the most commonly used technique for evaluating cell-mediated immune response. Similarly, collection of peritoneal fluid from the mice under ex-vivo conditions for determining the phagocytosis rate of murine peritoneal macrophages provides the information about innate immune response. A primary advantage of using *ex-vivo* studies is the ability to perform tests or measurements that would otherwise not be possible or ethical in living subjects under *in vivo* conditions.

2.4.2.3. *In vivo* model

Ex vivo models are not able to fully test how the alterations would affect the rest of the organisms body. Therefore, *in vivo* studies such as animal experiments or human trials should be conducted after identification of the bioactivity of compounds through chemical and *in vitro* assays to demonstrate their activity in an intact organism. The most frequently used *in vivo* methods include inhalation (IH) (whole body and nose-only), intratracheal (IT) instillation, intrapleural injection-implantation and intraperitoneal (IP) injection (McConnell, 1995). *In vivo* testing is often employed over *in vitro* because it is better suited for observing the overall effects of an experiment on a living subject. *In vivo* methods facilitate to reflect organ specific effects and some mammalian models reflect the situation in humans adequately and also provide information concerning absorption and metabolism of the biological compounds in humans (Knasmulle *et al.,* 2008). *In vivo* experimental research became widespread with the use of animal models in genetic manipulation experiments as well as to study drug toxicity in pharmacology.

2.5. Antioxidative attributes of milk derived peptides

Casein is the main proteinaceous ingredient of milk, where it accounts for 80% of the total protein. In recent years, nutrition and food sciences are focusing more on milk casein derived bioactive peptides with growing commercial interest in the context of health-promoting functional foods (Korhonen and Pihlanto, 2006). Current studies have shown that antioxidative peptides can be released from caseins by enzymatic

digestion and by fermentation of milk with proteolytic lactic acid bacterial strains (Korhonen and Pihlanto, 2003). Casein derived antioxidative peptides are composed of 5–11 amino acids majorly consisting hydrophobic amino acids such as proline, histidine, tyrosine or tryptophan in their sequence. Although many food-originated antioxidant peptides have been identified, their structure-bioactivity relationships have not fully been understood. In general, there are three factors that mainly affect the antioxidant activities of peptides. Firstly, the molecular weight of peptides can impact the antioxidant activity. Most of the peptides purified from enzymatic hydrolysates are in the size of 2 to 20 amino acids and peptides less than 6000 Da are most possibly to show antioxidant activity (Sarmadi and Ismail, 2010). Secondly, the amino acid composition has much contribution to the activity of the antioxidant peptides. For example, Trp, Tyr, and Phe, which contain aromatic residues, can donate protons to electron deficient radicals (Rajapakse *et al.,* 2005b) and the imidazole group containing amino acid His has good lipid peroxyl radical trapping capability (Chen *et al.,* 1998).Thirdly, the structurally unique linkage type of peptide chain has been demonstrated to influence antioxidant capacity. For example, tripeptides containing Trp/(Tyr) residues on the C-terminus, or presence of Val/(Leu) at N-terminus, have shown strong radical scavenging activity (Elias *et al.,* 2008).

It was hypothesised that antioxidative peptides can act at different levels in an oxidation sequence. The mechanisms of antioxidative action of proteins, peptides and individual amino acids may include free radical scavenging or product reduction, adduct formation, substrate shielding, metal chelating, oxidative enzyme inhibition and antioxidative enzyme induction. However, the exact mechanism is usually not fully established.

2.5.1. Free radical scavengers

Most of the casein derived peptides and hydrolysates acts as free radical scavangers by scavenging ABTS and DPPH free radicals. Suetsuna *et al.* (2000) studied the free radical scavenging property of a synthetic hexa peptide with the sequence Tyr-Phe-Tyr-Pro-Gln-Leu (YFYPQL) derived from bovine αS1-casein and found that hexa peptide has a potent capability to scavenge hydroxyl radicals and DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals. In another study, three peptides were obtained from Moxoto goat milk casein hydrolysates with papain and their antioxidative activity was determined by using ABTS method (Bezerra *et al.,* 2013). In this study, all the three peptides (NENLL, NPWDQVK and LLYQEPVLGPV) were

noticed to have highest ABTS free radical scavenging property. Ledesma *et al.,* (2005) also investigated the antioxidant activity of β-Lactoglobulin (β-Lg) derived hydrolyzates by using commercial proteases (pepsin, trypsin, chymotrypsin, thermolysin and corolase PP) and found that Corolase PP was the most appropriate enzyme to produce β-Lg hydrolyzates with high oxygen radical scavenging activity.

In another study, Kumar *et al.* (2013) isolated yak milk casein hydrolysates by using three commercially available proteases (Trypsin, Pepsin and Chymotrypsin) at different hydrolysis time and their radical scavenging capacity was assessed by using DPPH method. They observed that tryptic hydrolysates showed an highest scavenging capacity followed by chymotryptic and peptic hydrolysates and further reported increased scavenging activity of casein hydrolysates with increasing incubation time. López-Expósito *et al.* (2007) also reported significant oxygen radical scavenging property in ORAC assay with two hydrolysates prepared from ovine αS2-casein having FALPQYLK and PYVRYL peptides than the other hydrolysates. Similarly, peptides with the sequence YFYPEL and ARHPHPHLSFM were identified to possess potent radical scavenging ability which were derived from α- and k-casein respectively (Suetsuna *et al.,* 2000; Kudoh *et al.,* 2001). List of bovine milk derived peptides with free radical scavenging activity has been shown in table 2.5.

2.5.2. Metal chelators

Metal ions such as iron is the major element responsible for the oxidation reactions. One of the mechanism of antioxidative activity of milk casein derived peptides is metal chelation. The metal ion chelating actions of casein derived peptides and inhibition of lipid peroxidation have been reported in different model systems. For example, the tryptic peptide from β-CN (f1-25) was found to protect polyunsaturated fatty acids (PUFA) rich phospholipid liposomes against long-term iron induced oxidation, mainly due to iron chelation (Kansci *et al.,* 2004). Kim *et al. (*2007) compared chelating ability of casein phosphopeptides (CPPs) and casein hydrolysates in a cell membrane model system and found that iron-induced lipid oxidation was inhibited by both casein hydrolysates and CPPs, though casein hydrolysates being less effective than CPPs and it was identified that they have been shown to bind pro-oxidant metals, such as iron and to inhibit lipid oxidation as metal chelators.

Table 2.5. Antioxidative peptides derived from bovine milk

Protein source	Treatment	Peptide	Antioxidative Activity	Reference
Casein (cn)	Pepsin	Y-F-Y-P-Q-L αs1-cn f(144-149)	Scavenging of Superoxide anion Hydroxy radical DPPH radical	Suetsuna *et al.,* (2000)
	Trypsin	V-K-E-A-M-A-P-K β-cn f(98-105) A-V-P-Y-P-Q-R β-cn f(177-183) K-V-L-P-V-P-E-K β-cn f(169-176) V-L-P-V-P-E-K β-cn f(170-176)	Inhibition of enzymatic and non enzymatic lipid oxidation DPPH radical scavenging activity	Rival *et., al* (2001b)
Milk	Fermentation With Lactobacill-us delbrueckii subsp. bulgaricus	A-R-H-P-H-P-H-L-S-F-M κ-cn f(96-106)	DPPH radical scavenging activity	Kudoh *et al.,* (2001)
β-Lactoglobulin (β-lg)	Corolase-PP	W-Y-S-L-A-M-A-A-S-D-I β-1g f(19-29) M-H-I-R-L β-1g f(145-149) Y-V-E-E-L β-1g f(42-46)	Radical scavenging activity (ORAC)	Ledesma *et al.,* (2005a)

2.5.3. Inhibition of oxidative enzymes:

LOXs are present in animals and plants where they catalyze the oxidation of polyunsaturated fatty acids (PUFAs) and play a key role in the production of signalling compounds (Farmer, 2001; Funk, 2001) and in the degradation of cellular and intracellular membranes during development (van Leyen, 1998). Hydrolyzates of whole casein and β-casein from bovine origin were found to inhibit soybean LOX-1 (Rival *et al.,* 2001b). The tryptic β-casein digests VKEAMAPK, AVPYPQR, KVLPVPQK, and VLPVPQK were reported to acts as potent inhibitors of LOX activity. It was proposed that caseins and casein-derived peptides interact with free radical intermediates in the LOX reaction and hence cause an inhibitory effect

against both enzymatic and nonenzymatic lipid peroxidation (Rival, 2001a). These antioxidative casein-derived peptides behave like chain-breaking antioxidants reacting with carbon and oxygen centered radicals formed in the reaction.

2.5.4. Stimulation of antioxidative enzymes system

Oxygen is the major component of the respiratory chain and is the major source for the formation of highly reactive free radical such as superoxide anion (O_2^-) by accepting electrons. In the biological system, antioxidant defence mechanisms are evolved to cope up with these endogenously produced free radicals and neutralizing them to water (H_2O) in a two step mechanism (Johansen *et al.,* 2005).The first step involves the abstraction of electron from O_2^- producing hydrogen peroxide (H_2O_2) catalysed by superoxide dismutase. In the second step, H_2O_2 is reduced to water (H_2O) with the involvement of either catalase or glutathione peroxidase. Therefore, the above these three antioxidative enzymes are crucial for the efficient detoxification of free radicals and protects the biomolecules of the cell such as lipids, proteins and nucleic acids. Thus the imbalance between the above mechanism leads to the formation even more reactive hydroxyl radicals (OH) through the fenton reaction which damages the cell. It was studied that peptides derived from casein were shown to increase antioxidative enzyme levels. Caseinophosphopeptides (CPPs) showed cytoprotective effect against H_2O_2-induced oxidative stress in Caco-2 cells by preserving cell viability and were found to induce catalase enzyme activity and also elevate GSH content in the cells (Garcia-Nebot *et al.,* 2011). Laparra *et al.* (2008) stated that CPPs exerting a potential antioxidant capacity by increasing reduced GSH content and GSH reductase levels in Caco-2 cells. Phelan *et al.* (2009) identified that antioxidant peptides from casein significantly inducing catalase activity and glutathione (GSH) content in Jurkat cells.

2.6. Over view of the immune system

The immune system is a host defence system comprising many biological structures and processes within an organism that protects against disease. To function properly, an immune system must detect a wide variety of agents, known as pathogens and distinguish them from the organism's own healthy tissue. Immune system is divided in to innate and adaptive branches in the human body (figure.2.2). The innate immune system recognizes and responds to pathogens in a generic way, not conferring long-lasting or protective immunity to the host (Grasso *et al.,* 2002). The cells of innate immune system such as monocytes, neutrophils, basophils,

eoisinophils and natural killer cells (NK cells) are different in nature with particular ro*le (*Janeway *et al.,* 2002). Monocytes circulate in the bloodstream for about one to three days and then typically move into tissues, where they differentiate into tissue resident macrophages or dendritic cells, which are responsible for phagocytosis of pathogens. Neutrophils are mainly involved in the clearance of bacterial pathogens (Nathan, 2006). Basophils are granulocytic cells that release granules containing histamine and they play a role in both parasitic infections and allergies (Schroeder, 2009*)*. Mast cells are very similar in morphology and function to basophils but they resident cells of several types of tissues (Heneberg *et al.,* 2011*)*. Natural killer (NK) cells are a type of cytotoxic lymphocytes and are mainly involved the tumour rejection. They kill the virus infected cells by releasing the proteins called granzyme and perforin that causes the apoptosis of the target cell (Middleton *et al.,* 2002).

Cooperating with the innate immune system to eliminate pathogens, the other part of the immune system is the adaptive immune system that is composed of highly specialized, systemic cells and processes that recognize and remember specific pathogens. In this way the response to the pathogen is more selective and efficient each time the pathogen is encountered. The most important cells intervening in this system are lymphocytes. These cells are divided into two types including B and T-lymphocytes. The B cells turn into plasma cells which are majorly responsible for the production of specific antibodies (Janeway *et al.,* 2001)*.* Antibodies are large soluble proteins used to recognize, identify and neutralize specific antigens. There are different types of antibodies, differing in biological properties, each has evolved to handle different kinds of antigens. T-lymphocytes are of different types such as T_{H}-cells, T_{C}-cells, memory T-cells and T_{reg}-cells and coordinate the entire immune response to eliminate the pathogen in infected cells. T-helper cells (both T_{H1} and T_{H2} cells) promote the maturation of B cells and also activate macrophages and cytotoxic T cells. Cytotoxic T cells (T_{C} cells) destroy virally infected cells and tumor cells, and are also implicated in transplant rejection. Memory T cells quickly expand to large numbers of effector T- cells upon re-exposure to their cognate antigen, thus providing the immune system with memory against past infections. Finally regulatory T cells also called suppressor T- cells are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T-cells that escaped the process of negative selection in the thymus.

The cells of the immune system release chemical mediators called cytokines, which include interleukins, growth factors and interferons and communicate different parts of the immune system by binding to the specific receptors present on the target cells. Lymphocytes, including both T cells and B cells, secrete cytokines called lymphokines, while the cytokines of monocytes and macrophages are called monokines. Many of these cytokines are also known as interleukins because they serve as a messenger between leukocytes. In particular, two important cytokines involved in lymphocytes proliferation and activation are IL-2 and IFN-γ. IL-2 is a T cell growth factor produced by T helper 1 (T_{H1}) and NK cells. IL-2 stimulates the growth of NK cells and enhances the cytolytic function of these cells, producing lymphokine activated killer cells. IL-2 can also induce IFN-γ secretion by NK cells. IFN-γ is an important macrophage activating lymphokine. Because of its role in mediating macrophages and NK cell activation, IFN-γ is important in host defense against intracellular pathogens, viruses and tumours thus influencing downstream. immunological responses. The T_{H2} type cytokines include IL- 4, 5 and 13 which are associated with the promotion of IgE and eosinophilic responses in atopy, and also interleukin-10, which has more of an anti-inflammatory response.

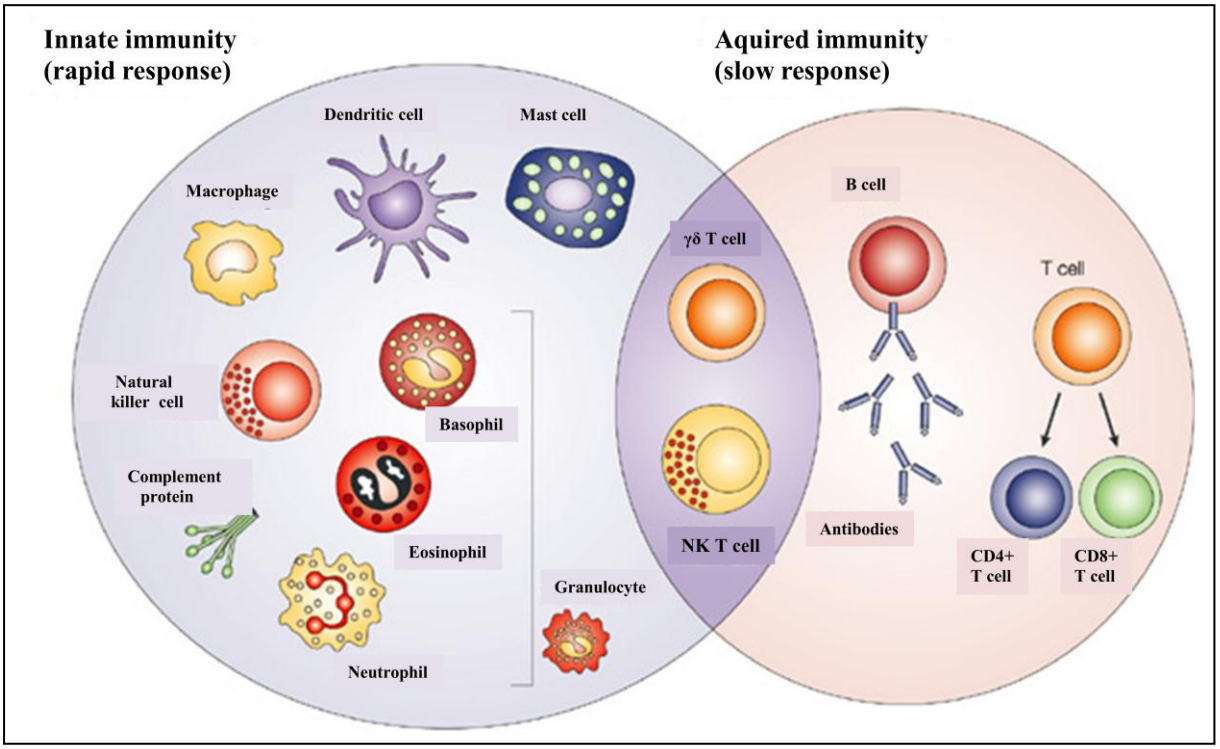


Figure: 2.2 Overview of the immune system

T_{H1} response inhibits T_{H2} path way by inhibiting the cytokine formation from T_{H2} cells. Whereas, T_{H2} pathway inhibits the secretion of cytokines responsible for T_{H1} response. This reciprocal inhibition between T_{H1} and T_{H2} pathways is mainly responsible for the regulation of immune system in the body. The homeostasis between T_{H1} and T_{H2} pathways is mediated by transforming growth factor beta (TGF-β), a multifunctional cytokine belonging to the transforming growth factor super family of cytokines. It is secreted by most of the immune cells (or leukocytes) and plays an important role in controlling the immune system and shows different activities on different types of cell or cells at different developmental stages.

There has been increased interest in the study of the relationship between nutrition and immunity due to the hypothesis that consumption of certain foods may reduce susceptibility for the establishment and/or progression of immunological diseases (Sandre *et al.,* 2001).

Numerous reports demonstrate that milk derived bioactive peptides can interact with immune system at different levels. They may contribute to the overall immune response and may improve the immune system function. It was suggested that casein derived peptides are involved in the stimulation of the newborn's immune system and probably even have a direct effect on the resistance to bacterial and viral infection of adult humans (Migliore-Samour *et al.,* 1989). Immunomodulatory peptides derived from casein enhance the immune system by regulating cytokine expression, antibody production, phagocytosis, lymphocyte proliferation and ROS-induced immune functions (Migliore-Samour *et al.,* 1989; Sandre *et al.,* 2001; Jing and Kitts, 2004; Bennett *et al.,* 2005; Phelan *et al.,* 2009).

2.6.1. Efficacy of milk derived immunomodulatory peptides

2.6.1.1. Splenocytes proliferation index (SPI)

In the following years, a number of potential immunomodulatory peptides were identified from bovine milk proteins (Otani and Hata, 1995). It was demonstrated that peptides derived from αS1-, β- and *k*-casein have been shown to both stimulate and suppress lymphocyte proliferation (Migliore-Samour and Jollés, 1988; Elitsur and Luk, 1991; Otani and Hata, 1995; Sutas *et al.,* 1996). Maruyama *et al.* (1987) and later Kayser and Meisel (1996) reported that, Casomorphin-7 and Casokinin-10 were immune-enhancing at high concentrations (10^{-6} to 10^{-4} M) and immune-suppressing at low concentrations (10^{-12} to 10^{-8} M) based upon splenocytes proliferation.

Sizemore *et al.* (1991) also studied the effects of cow milk-derived immunomodulatory peptide YGG (Tyr-Gly-Gly) on Con A induced T-cell proliferation and observed a biphasic effect as YGG stimulated proliferation at low concentrations (10^{-13}-10^{-14} M) and inhibited proliferation at higher concentrations (10^{-5} to 10^{-4} M). Peptides derived from bovine casein (f1-28) noticed to modulate the proliferative responses and immunoglobulin production in mouse spleen cell cultures (Otani *et al.,*2001). Glycomacropeptide, a *k*-casein derivative, inhibits the proliferation of splenocytes (Otani and Hata, 1995) and enhances IgA production in mice (Yun *et al.,* 1996). Also peptides obtained from the fermentation of milk with *L. helveticus* showed the ability to stimulate proliferation of lymphocytes (Laffineur *et al.,* 1996). In addition, whey protein derived peptides particularly from β-Lactoglobulin f(15-20), f(55-60), f(84-91), f(92-105), f(139-148), and f(142-148) were reported to have stimulatory effect on murine splenocytes proliferation through the modulation of cytokine secretion (Jacquot *et al.,* 2010).

2.6.2.2. Phagocytosis

In addition to effecting splenocytes proliferation, milk derived peptides were found accelerate the rate of phagocytosis of peritoneal macrophages. For example, casein derived peptides (residues 54-59 of human β-casein and residues 194-199 of αS1-casein) were found to stimulate the phagocytosis of sheep red blood cells by murine peritoneal macrophages (Jolles *et al.,*1993) and exert a protective effect against *Klebsiella pneumonia* (Migliore-Samour *et al.,*1989). Similarly, Miyauchi *et al.* (1998) identified the stimulatory effect of lactoferricin B derived from the hydrolysis of lactoferricin by trypsin on the phagocytosis of human neutrophils.

2.6.2.3. Cytokine release

Casein hydrolysates (CHs) were also found to have the significant effects on cytokine expression. Phelan *et al.* (2009) demonstrated that CHs generated a T_{H1} response by enhancing ConA induced IL-2 production while having no effect on the release of IL-10. Peptides derived from bovine β-lactoglobulin and α-lactalbumin also found to stimulating T_{H1} immune response through the induced production of IL-2 and IFN-γ in murine splenocyte culture (Jacquot *et al.,* 2010). Similarly, Mao *et al.* (2007) studied the effect of yak milk casein hydrolysate on the secretion of cytokines in murine spleen culture supernatant and found that casein hydrolysate stimulates T_{H1} mediated immune response through the elevation of IL-2 and IFN-γ. The table

2.6 shows the peptides having immunomodulatory activity derived from bovine milk (Gill *et al*., 2000).

Table: 2.6. Immunomodulatory peptides derived from milk proteins

Peptide fragment	Activity
αs1-CN (1-23)	Stimulation of phagocytosis and immune responses against bacterial infections
αs1-CN (23-34)	Stimulation of phagocytosis and immune responses against bacterial infections
αs1-CN (90-95)	Stimulation effect on lymphocytes proliferation, NK activity, neutrophil locomotion
αs1-CN (194-199)	Stimulation of phagocytosis and immune response against bacterial infections
αs2-CN (1-32)	Stimulatory effect on spleen cells
β-CN (191-193)	Stimulatory effect on spleen cells
β-CN (191-209)	Stimulation of phagocytosis of sheep red blood cells by murine peritoneal macrophages
β-CN (193-202)	Modulation of lymphocyte proliferation
β-CN (193-209)	Induction of proliferative response in rat lymphocytes; Modulation of cytokine production by murine macrophages
B- Lactoferricin (17-41)	Suppressed interleukin-6 production in human monocytic cell line
Glycophosphopeptide	Stimulatory effect on spleen cells

Very less number of studies describe the overlapping or multifunctional properties of milk derived bioactive peptides. Studies of Aguilar-Toala *et al.* (2016) demonstrated the multifunctional activities of peptides derived from fermented milk with specific *Lactobacillus plantarum* strains. Two peptides (QKALNEINQF, TKKTKLTEEEKNRL) obtained from bovine casein were also identified to show overlapping properties (Sistla, 2013). In addition, peptide fractions derived from fermented skim milk with *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus helveticus* strains also displayed such multifunctional activities (Qian *et al.,* 2011; Elfahri *et al.,* 2014)

2.7. Mechanism of action of antioxidative and immunomodulatory peptides

Increased generation of ROS leads to tissue injury and dysfunction by attacking, denaturing, and modifying structural and functional molecules of cells and there by activating redox-sensitive transcription factors and signal transduction pathways (Kim and Vaziri, 2009). The mechanisms by which the milk-derived bio active peptides exert antioxidative effects on the cell are not yet fully elucidated. A recently

elucidated pathway to induce antioxidant enzymes in response to oxidative stress involves the activation of Nrf2 (Nuclear factor-erythroid-2-related factor 2) pathway in the cell (Itoh *et al .,* 2004; Gong *et al.,* 2002). Nrf2 plays a critical part in basal activity and coordinated induction of genes encoding numerous antioxidant and phase II detoxifying enzymes and related proteins such as catalase, superoxide dismutase (SOD), UDP-glucuronosyl transferase, NAD(P)H:quinone oxidoreductase- 1, heme oxygenase-1 (HO-1), glutamate cysteine ligase, glutathione *S*-transferase, glutathione peroxidase and thioredoxin, among others (Li *et al.,* 2008). Under normal conditions, Nrf2 is held in the cytoplasm as an inactive complex by bound to a repressor molecule known as Keap1 (Kelch-like ECH-associated protein 1), which facilitates its ubiquitination (figure 2.3). Keap1 contains several reactive cysteine residues that serve as sensors of intracellular redox state. During stress conditions oxidative or covalent modification of thiols in some of these cysteine residues results in the dissociation of Nrf2 from Keap1 and its translocation to the nucleus. In the nucleus, Nrf2 binds to the regulatory sequences, termed antioxidant response elements or electrophile response elements, located in the promoter region of genes encoding antioxidative and phase 2 detoxifying enzymes. This process is mediated by heterodimerization of Nrf2 with other transcription factors, such as small Maf, within the nucleus (Ji, 2007). It is of note that nuclear translocation of Nrf2 may also occur via phosphorylation of some of its threonine or serine residues by upstream kinases such as protein kinase C, mitogen-activated protein kinases, phosphatidylinositol- 3-kinase/Akt, and casein kinase-2 (Surh *et al.,* 2008).The Nrf2-mediated regulation of cellular antioxidant and anti-inflammatory machinery plays an important role in defense against oxidative stress (Li et al., 2008). In fact, disruption of Nrf2 in mice diminishes or abrogates the induction of these antioxidant genes, indicating their Nrf2-dependent regulation. Moreover, Nrf2 gene ablation has been shown to cause a lupus-like autoimmune nephritis and exacerbate diabetes-induced inflammation, oxidative stress, and renal injury in the experimental animals (Yoh *et al.,* 2001). In earlier studies, various antioxidants were reported to stimulate Nrf-2 mediated antioxidative enzyme expression. Penta peptide derived from chick pea protein hydrolysates stimulated the expression of Phase II detoxification and antioxidant enzymes including NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1) and c-glutamylcysteine synthetase (c-GCS) through the increased expression of Nrf2 in Caco-2 cells. Na *et al.* (2008) demonstrated that (-)-

Epigallocatechin gallate (EGCG), a catechin polyphenol from green tea has induced Nrf-2 mediated expression of Mn SOD and heme oxygenase-1 (HO-1) in human breast epithelial (MCF10A) cells. Similarly, curcumin (diferuloymethane), a yellow colouring agent present in the rhizome of Curcuma longa Linn (Zingiberaceae), has been reported to possess antioxidative property. Administration of curcumin to the rats (200 mg/kg dose) for four consecutive days induced the expression of HO-1 enzyme through the activation and translocation of Nrf-2 (Farombi *et al.,* 2007) in liver. Similarly, Lee, (2011) studied the antioxidative efficacy of Sulforaphane (SFN) in human bronchial epithelial BEAS-2B cells. Sulforaphane is a naturally occurring iso thiocyanate with promising chemopreventive activity. They observed an increased nuclear accumulation of Nrf-2 and up-regulation of *HO-1* expression in BEAS-2B cells after exposure to SFN.

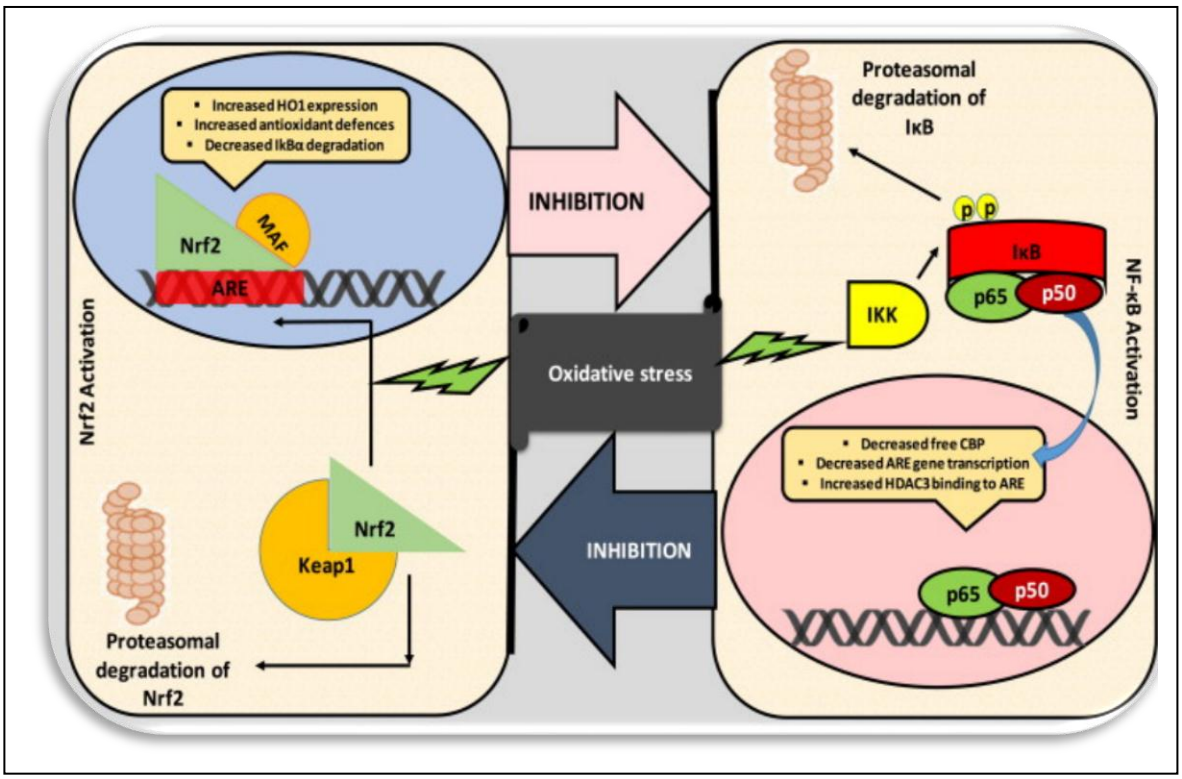


Figure 2.3. Role of Nrf2 and Nf-kβ in oxidative stress

In case of immunomodulatory effect by bioactive peptides, there are less number of intra cellular signalling mechanisms were reported. Nuclear factor-*k*B (NF-*k*B) pathway has long been considered as a proinflammatory signalling mechanism plays a major role in the expression of inflammatory cytokines and chemokines (Federico

et al., 2007*)*. It was identified that Bovine glycomacropeptide (BGMP) induces cytokine production in human monocytes through the stimulation of the MAPK and the NF-kB signal transduction pathways in human THP-1 cells an *in vitro* monocyte model (Requena *et al.,* 2009). In addition, an alternative hypothesis involves a possible immunomodulatory action via ACE-inhibitory mechanism. It was identified that immunomodulatory peptides derived from casein has been found to inhibit ACE, there by increases the levels of bradykinin, a mediator of the acute inflammatory process and is thus able to stimulate macrophages, enhance lymphocyte migration and also able induce the secretion of cytokines (Jolles *et al.,*1993).

Nrf2 and NF-κB signalling pathways are reciprocal in their function and are the key pathways regulating the fine balance of cellular redox status and responses to stress and inflammation (Li *et al.,* 2008). Both of the pathways are regulated by redox sensitive factors and the absence of Nrf2 is associated with increased oxidative stress, leading to the amplification of cytokine production, as NF-κB is more readily activated in oxidative environments (Yerra *et al.,* 2013). The interplay between these pathways occurs through a range of complex molecular interactions and can often depend on the cell type and tissue context. These interactions operate through both transcriptional and post-transcriptional mechanisms, allowing fine-tuning of dynamic responses to ever-changing environmental cues (Wardyn *et al.,* 2015).

2.8. Bioavailability and transport of bioactive peptides across intestinal membrane

Bioavailability refers to the proportion of an ingested nutrient from foods or meals that is absorbed and utilised for normal physiological function and/or storage (Jackson,1997). To exert physiological effects after oral ingestion, it is of crucial importance that milk derived bioactive peptides remain active during gastrointestinal digestion and absorption and reach the circulation (Regazzo *et al.,* 2010). Protein digestion starts in the stomach by the action of enzymes such as pepsin, trypsin, chymotrypsin, elastase, and carboxypeptidase A and B. In the small intestine these enzymes hydrolyse the polypeptides in to mostly oligo peptides and some free amino acids and are absorbed through various transporters and carries in the small intestine (Arhewoh *et al.,* 2005). Oligopeptides again hydrolysed to di and tri peptides and free amino acids through the action of aminopeptidases, endopeptidases, dipeptidases and carboxy peptidases of pancreatic fluid. After passing through the small intestine, undigested and/or unabsorbed peptides enter

the large intestine or colon, where they are metabolized by intestinal microbiota (Vermeirssen *et al.,* 2004). Numerous bacteria, especially colon bacilli, are present normally in the absorbing colon. Therefore, after entering of peptides in to the colon they are either degraded by these bacteria or absorbed intact (Wilson and Basit, 2005).

Peptides that resist the digestive process and arrive intact in the intestine can have a local function or may be able to cross the epithelium, enter the blood stream, and have a systemic effect. Most of the peptides with more than three amino acids are hydrolysed by brush border enzymes of the intestinal epithelium. In contrast, di and tri peptides can be absorbed intact and hydrolysed later (Humphrey and Ringrose, 1986). The bioavailability of peptides depends on a variety of structural and chemical properties, i.e. resistance to proteases, charge, molecular weight, hydrophobicity and the presence of specific amino acid residues (Pauletti *et al.,* 1996).

The route of absorption/transepithelial transport also varies among different peptide. Different pathways were identified for the absorption of compounds through the intestine. This occurs in several ways including a) Transcellular pathway b) Para cellular pathway and c) Transcytosis. Transport of solute through transcellular pathway depends on their size, charge and lipophilicity and might be facilitated by a carrier system and has been observed for most smaller inorganic and organic solutes. In transcellular route the transport of solute occurs across two morphologically and functionally different cell membranes (e.g. the apical and the basolateral membrane), it can be either passive or active process (Brandsch *et al.,* 2008). Peptide transporter 1 (Pep T1) is the most important active system for the transcellular transport of peptides across the intestinal membrane. In human, PepT1 consisting of 729 residues and is a transmembrane protein complex with 12 transmembrane domains. One of the most commonly used and best known reference ways to study the peptide transport mediated by of PepT1 is the substrate glycylsarcosine (Gly-Sar). It is relatively stable against intra and extracellular enzymatic hydrolysis and it acts as competitive substrate of PepT1 (Brodin *et al.,* 2002). Large proteins and peptides which cannot be transported by PepT1 are translocated by transcytosis mechanism, which involves membrane invagination and vesicle internalization of the compound. Cellular internalization via vesicles could be divided in fluid-phase endocytosis, that does not require any interaction between the peptide and the apical membrane (Heyman *et al.,* 1990) and receptor mediated

absorptive endocytosis (Sai *et al.,*1998) that involves a binding with the plasma membrane before being incorporated into endocytotic vesicles. Once internalized inside the vesicles, the proteins or peptides are recycled back to the plasma membrane or processed in the course of a multistep transport sequence through various intracellular organelles, such as endosomes, prelysosomes and lysosomes (Shen *et al.,*1992). If the fusion with lysosomes does not completely disrupt the endocytosed molecules, they could also be transported to the opposite cell surface completing the transcytotic process (Mostov and Simister, 1985). Peptide transport mediated by transcytosis pathway is studied by using inhibitors of that pathway such as wortmannin. It was discovered in 1957 in the broth of fungi *Pencillium wortmannin* Klocker (Brian *et al.,*1957). Which is involved in the inhibition of receptor mediated endocytosis (Kjeken *et al.,* 2001).

In addition to these pathways, an another mechanism called paracellular pathway mediated by tight junctions (TJ) for the transport of organic and inorganic compounds from the gut lumen towards blood circulation (Suzuki and Hara 2004). TJ membrane proteins regulate the functions of TJ by interacting with scaffold proteins to connect them with several transcriptional factors and signalling pathways (Fanning *et al.,* 1998). Para cellular passive diffusion process is mainly responsible for the transport of low molecular weight compounds including peptides (Satake *et al.,* 2002; Adson *et al.,* 1994; Pappenheimer *et al.,*1994). Cytochalasin D, a potent mycotoxin used to disturb TJ. It acts as inhibitor of actin polymerisation and disrupts actin microfilaments. Cytochalasin D might be used to study the mechanism of absorption of the food derived compounds, whose transport is mediated through TJ, as small peptides (Miguel *et al.,* 2008; Shimizu *et al.,* 1997).

2.8.1. Caco-2 intestinal transport model

Nutrient absorption at the intestinal level is studied using an experimental model involving culture of human intestinal epithelial cells, Caco-2 is known to express a variety of small intestinal cell functions, and has been used as a model of the small intestinal epithelium (Hidalgo *et al.,*1989). These cells express various intestinal enzymes and transporters such as PepT1 (Saito *et al.,*1993; Thwaites *et al.,*1993). They are also found to express transcytosis and tight junction mediated transport mechanisms (Heymann *et al.,*1990; Hidalgo *et al.,*1989). Due to the similarity of Caco-2 cells with mature human enterocytes, peptide uptake research can be done using Caco-2 cell culture. In this model, cultured Caco-2 cells grown by attached to a

substrate, forming a monolayer. When they attain confluence, they spontaneously differentiate and acquire the morphofunctional traits of mature enterocytes (Herold *et al.,* 1994). During different growth stages, Caco-2 cells express about eight membrane peptidases with different activities in their apical surface. Among these peptidases DPP-IV (Dipeptidyl peptidase-IV) is found to have highest activity, particularly when these cells are completely differentiated (Howel *et al.,* 1992). DPP-IV is highly expressed on differentiated epithelial cells, endothelial cells and lymphocytes and belongs to prolyl oligopeptidase family of serine proteases. It cleaves N-terminal dipeptides from proteins containing proline or alanine in the penultimate position (Aertgeerts *et al.,* 2004). The activity of DPP-IV can be specifically inhibited by diprotin A, which rapidly binds to the catalytic site of the enzyme and blocks the degradation of substrates (Wiedeman and Trevillyan, 2003). Because of the benficial effects, Caco-2 cells are being used for the determination of absorption behaviour of peptides in the intestinal epithelium. Satake *et al.* (2002) carried out experiments for ACE inhibitory tri peptide VPP using Caco-2 cell monolayer and concluded that the peptide is transported across cell layer via paracellular and transcellular route in intact form, although a significant amount of the peptide is hydrolysed to amino acids by intra cellular peptidases. An another study carried out for the antihypertensive peptide RVPSL derived from egg white (ovotransferrin 328− 332). RVPSL was found to transported from apical to basal side by paracellular transport system through tight junctions (Long *et al.,* 2015). However, during the process of transport, 36 % of the initial RVPSL added to the apical side was degraded, but this degradation decreased to 23% when the Caco-2 cell monolayers were preincubated with diprotin A, suggesting that RVPSL had a low resistance to various brush border membrane peptidases. Sienkiewicz *et al.* (2009) also conducted bioavialbility studies for bovine β-casomorphin-5 and -7 (derived from β-casein) using Caco-2 cells in the presence of DPP-IV inhibitor. During sixty min of incubation, without the presence of inhibitor there was an rapid degradation of BCMs by membrane peptidases has been observed, suggesting that the BCMs are having very low resistance capacity against to the peptidases of the intestinal membrane. Whereas, after the treatment of the Caco-2 cell monolayer with the DPPIV inhibitor, the transport rate was increased about 2.5 fold and 8 fold in the case of bovine BCM-5 and BCM-7 respectively. Although, the resistance capacity of the peptides to brush border enzymatic hydrolysis mainly depends on the amino acid composition of

peptides. Proline and hydroxyproline containing peptides are relatively resistant to degradation by digestive enzymes (Savoie *et al.,* 2005). For example, casein and gelatin derived di and tri peptides were found to shown resistance to the peptidases because of the presence of C-terminal proline residues in their sequence. Bioavailability studies were conducted on antihypertensive peptides LHLPLP using Caco-2 cell monolayer (Quiros *et al.,* 2008). It was identified that LHLPLP was partly hydrolysed by brush border peptidases to HLPLP, which undergoes rapid transport to the basolateral chamber in intact form through paracellular transport route. The presence of proline residue in the penta peptide can have the protective effect against peptidases to prevent its further degradation. Similar results were obtained by Vij *et al.* (2016) they carried out transport studies on β-casein derived antioxidative and ACE inhibitory peptide, VLPVPQK (named as peptide C) using Caco-2 cell monolayer. They found that after the addition of peptide-C to the apical side, a new oligopeptide VLPVPQ was detected to reach the basal side rapidly indicating that the peptide C was in part hydrolyzed by cellular peptidases prior to efflux within five minutes. The resulted breakdown fragment was found to shown maximum transport rate than the intact peptide. However, enzymatic hydrolysis of peptides have shown an increase in their biological function in some peptides while decreasing in others. For example, an antihypertensive hexa peptide with the sequence KVLPVPQ showed low ACE inhibitory activity under *in vitro* conditions. While, the pancreatic digestion of this hexa peptide due to the action of carboxypeptidase A resulted a penta peptide with increased ACE-inhibitory activity. Whereas, an αS1-casein derived peptide, YKAVPQL with strong ACE inhibitory activity, failed to exert an antihypertensive effect due to the above same treatment (Maeno *et al.,* 1996).

2.9. Conclusion

The overlapping antioxidative and immunomodulatory properties of milk derived bioactive peptides have been target of investigation these days due to their potential against oxidative stress and inflammation thus can be used as nutraceuticals in the food and pharmaceutical industries. So far there are very less number of studies carried on such overlapping bioactivities of buffalo milk derived peptides. Apart from the biological activities, in future studies more emphasis should also be given to the bioavailability of milk peptides across the cell membrane before using them as functional ingredients in food industry.

Chapter 3

Materials and methods

MATERIALS AND METHODS

3.1. Chemicals/Reagents

All chemicals required for experiments were of analytical/molecular biology grade and purchased from Sigma-Aldrich Inc., USA; Thermo Scientific, Lithuania; Sisco Research Laboratories Pvt. Ltd., Mumbai; Genetix Asia Pvt. Ltd., New Delhi; S. D. Fine Chem. Ltd, Mumbai; HiMedia Laboratories Pvt. Ltd., Mumbai; Biochem, Life Sciences, New Delhi; Merck Specialities Pvt. Ltd., Mumbai; eBioscience, San Diego, CA, USA;

3.2. Plastic ware

The plastic ware used in cell culture work viz, Tissue culture flask (25 cm^2 and 75 cm^2), Autofill assembly, 6-welled and 96-welled tissue culture plates, 6-welled Transwell polyester plates, falcon tubes (15 ml and 50 ml), Elisa microtiter 96 well plates, eppendorf tubes, micotips etc. The plasticware were purchased from Tarsons products Pvt. Ltd., Kolkata; HiMedia Laboratories Pvt. Ltd., Mumbai; Millipore Pvt. Ltd., UK; Corning Pvt. Ltd., USA; Genetix Asia Pvt. Ltd., New Delhi; Thermo Fisher Scientific Pvt. Ltd; USA.

3.3. Synthesis of peptides

Ten peptides (A to J) used in the present study were custom synthesised from Sigma-Aldrich Inc., USA.

3.4. Cell culture

The human colon adenocarcinoma cell line, Caco-2, was obtained from National Centre for Cell Science, Pune (India). Cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% of antibiotic solution containing amphotericin (0.03%), penicillin (1%) and streptomycin (1%) in 25 cm^2 flask under humidified atmosphere at 37^0C incubator perfused with 5% CO_2.

3.5. Experimental animals

Male Swiss albino mice were used in the experiments were procured from the Small Animal House, National Dairy Research Institute, Karnal, Haryana, India. The animals were about 3 months old and were approximately of similar body weight (25-30 g). They were housed in polypropylene cages in an air conditioned room at 24 ± 1^0 C.

3.6. Evaluation of antioxidative activity by chemical methods

The antioxidant activity of buffalo casein derived peptides was assessed by using three chemical methods. Two of the methods (ORAC: Oxygen radical absorption capacity assay and ABTS method were used based on free radical scavenging property while third depended upon inhibition of lipid peroxidation.

3.6.1. Oxygen radical absorbance capacity (ORAC) assay

ORAC assay was conducted using fluorescein as fluorescent probe, according to the methods of Ou *et al.* (2001) following a slight modification as described by Davalos *et al.* (2004).

Reagents

Phosphate buffer saline (PBS; 10 mM, pH 7.4): Prepared by dissolving NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (1.44 g) and KH_2PO_4 (0.24 g) in 900 ml distilled water. The pH was adjusted to 7.4 and volume was made up to 1000 ml.

Phosphate buffer (75 mM, pH 7.4): Prepared by dissolving Na_2HPO_4 (11.5 g) and NaH_2PO_4 (1.61 g) in 900 ml of distilled water. The pH was adjusted to 7.4 and volume made up to 1000 ml.

Fluorescein disodium salt stock solution (1.17 mM): Dissolved 11.006 mg of fluorescein disodium salt in 25 ml of phosphate buffer (75 mM, pH 7.4).

Fluorescein disodium salt working solution (117 µM): Diluted 10 µl of stock solution to 100 ml with phosphate buffer (75 mM, pH 7.4).

AAPH (2,2'-Azobis(2-aminopropane) dihydrochloride, 40 mM): Prepared freshly by dissolving 109 mg of AAPH in 10 ml of phosphate buffer (75 mM, pH 7.4).

Trolox- stock solution (5 mM): Dissolved 12.5 mg of Trolox in 10 ml of PBS (10 mM, pH 7.4).

Trolox- working stock solution (500 µM): Diluted 1 ml of stock solution with 9 ml of distilled water.

Trolox -working solution: Prepared by diluting 20,40, 60, 80, 100, 120, 140, 160, 180 and 200 µl of working stock Trolox solution to 1.0 ml with distilled water to get 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM solution respectively.

Procedure

The reaction was carried out at 37^0C in 75 mM phosphate buffer (pH 7.4). Mixed 20 µl of various concentrations (10 ng, 100 ng, 1000 ng, 10 µg,100 µg, 1000 µg/ml respectively) of individual peptides solution with 120 µl of fluorescein (117 µM) and incubated at 37^0C for 15 min. Then 60 µl of AAPH (40 mM) was applied to the

mixture. The plate was immediately placed in a micro plate reader (Model: Infinite F200 Pro, Tecan, Austria) and the decay of fluorescence was recorded every 1 min with excitation wavelength 495 nm and 520 nm emission wavelength. The plate was automatically shaken before the first reading and the fluorescence was recorded every 1 min. The whole assay lasted for 80 min. Phosphate buffer was used for blank determination. The area under the fluorescence decay curve (AUC) was calculated as

$$AUC = 1 + \sum_{i=1}^{i=80} f_i / f_0$$

Where, f_0 is the fluorescence reading at zero min and f_i is the fluorescence reading at time i. The net AUC corresponding to a sample was calculated as follows:

$$\text{Net AUC} = \text{AUC}_{\text{antioxidant}} - \text{AUC}_{\text{blank}}$$

Based on net AUC of the sample, Trolox equivalent antioxidant capacity (TEAC) was calculated from the standard curve of Trolox (range from 10 µM to 100 µM).

3.6.2. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay

The assay was conducted according to the method described by Re *et al.* (1999).

Reagents

Phosphate buffer saline (PBS; 10 mM, pH 7.4)

Potassium persulfate (140 mM): Dissolved 0.38 g of potassium per sulfate in 5 ml of distilled water and the volume was made to 10 ml in volumetric flask.

ABTS (7 mM) stock solution: Dissolved 19.2 mg of ABTS in 5 ml of distilled water.

ABTS working solution: ABTS radical cations ($ABTS^{\bullet+}$) were produced by reacting 5 ml of ABTS stock solution with 88 µl of 140 mM potassium persulfate (final concentration of 2.45 mM) and incubated in the dark at room temperature for 12 to 16 hr in amber colour bottle. $ABTS^{\bullet+}$ solution was diluted with PBS (10 mM, pH 7.4) to an absorbance of 0.70 ($\pm$ 0.02) at 750 nm.

Trolox- stock solution (5 mM): Dissolved 12.5 mg of Trolox in 10 ml of PBS (10 mM, pH 7.4).

Trolox-working stock solution (500 µM): Diluted 1 ml of working stock solution with 9 ml of distilled water.

Trolox-working solution: Prepared by diluting 100, 200, 300, 400, 500, 600, 700, 800, 900 µl of working stock Trolox solution to 1.0 ml with distilled water to get 50, 100, 150, 200, 250, 300, 350, 400, 450 µM solution respectively.

Procedure

Before use, ABTS stock solution was diluted with PBS (10 mM, pH 7.4) till it gave an absorbance of 0.70 (± 0.02) at 750 nm. Added 180 µl of ABTS working solution and 20 µl of PBS (10 mM, pH 7.4) to a well of 96 well micro plate and initial absorbance was recorded at 750 nm using microplate reader (Model: Infinite F200 Pro, Tecan, Austria). Added 20 µl of different concentrations of individual peptides solution (10 ng, 100 ng, 1000 ng, 10 µg, 100 µg and 1000 µg/ml respectively) to 180 µl of ABTS working solution. The contents were mixed for 5 sec and change in absorbance at 750 nm was recorded after 10 min. Appropriate solvent blank was run in each assay. All measurements were performed in triplicate. Percent inhibition was calculated by using the following equation

$$\% \text{ Inhibition} = (A_{734} \text{ control} - A_{734} \text{ sample}) / A_{734} \text{ control} \times 100$$

Based on the percent inhibition of absorbance of sample, Trolox equivalent antioxidant capacity (TEAC) was determined from standard curve of Trolox (range from 50 µM to 450 µM).

3.6.3. Inhibition of linoleic acid auto-oxidation

The lipid peroxidation inhibition capacity of the peptides was measured in a linoleic acid model system (Osawa and Namiki, 1985).

Reagents

Phosphate buffer (50 mM, pH 7.0):

Solution A: Dissolved 4.45 g of Na_2HPO_4 in 500 ml of distilled water

Solution B: Dissolved 3.40 g of KH_2PO_4 in 500 ml of distilled water

Mixed solution A and B and adjusted the pH to 7.0

Linoleic acid (Sigma, USA)

Ammonium thiocyanate (30%): Dissolved 30 g of ammonium thiocyanate in 100 ml of distilled water

Hydrochloric acid (HCL, 3.5%)

Ferrous chloride stock solution (2 mM): Dissolved 12.6 mg of ferrous chloride in 50 ml of 3.5% HCL.

Ferrous chloride working solution (0.02 mM): Working solution was prepared by mixing 1 ml of stock solution in 99 ml of 3.5% HCL.

75% ethanol

Procedure

Briefly, peptide sample (100 µg/ml) was dissolved in 10 ml of phosphate buffer (pH 7.0) and added to a solution containing 0.13 ml of linoleic acid and 10 ml of 99.5% ethanol. Then the total volume was adjusted to 25 ml with distilled water. The mixture was incubated in a conical flask with a screw cap at 60 ± 1^0C in dark. The degree of oxidation was evaluated by measuring ferric thiocyanate values (Mitsuda *et al.,* 1996). The reaction solution (100 µl) incubated in the linoleic acid model system described above was mixed with 4.7 ml of 75% ethanol, 0.1 ml of ammonium thiocyanate (30%) and 0.1 ml of ferrous chloride solution in 3.5% HCl. After 3 min, the thiocyanate value was measured by reading the absorbance at 500 nm at different intervals during the incubation period at 60 ± 1^0C. The inhibition activity was calculated as follows:

$$\text{Inhibition activity (\%)} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

3.7. Evaluation of immunomodulatory potential

3.7.1. Splenocytes proliferation index (SPI)

Reagents

RPMI-1640 medium: Dissolved RPMI -1640 (16.4 g), sodium bicarbonate (2.10 g), sodium pyruvate (110.1 mg), HEPES (5.96 g), penicillin (61 mg) and streptomycin (100 mg) in 1 liter of autoclaved distilled water. The pH of the resulting solution was adjusted to 7.2 with 1N NaOH and filter sterilized through 0.22 µm Millex-GV filters (Millipore). The prepared media was distributed in small aliquots and stored at 4^0C and protected from light.

Erythrocyte lysis buffer: Mixed 10 ml of 0.17 M Tris and 90 ml of 0.6 M NH_4Cl and pH was adjusted to 7.2 using HCL and stored at 4°C.

MTT solution (5 mg/ml): Dissolved 5 mg of MTT in filter sterilised RPMI-1640.

Dimethyl suphoxide (DMSO)

Peptides (5 mg/ml): Four peptides (B, C, F and G) were dissolved in RPMI-1640 medium.

Procedure

Isolation of splenocytes

Splenocytes were isolated as described by Jain *et al.,* (2009). Incised the abdominal cavity of mice and spleen was carefully removed and transferred to 1 ml RPMI-1640 medium. Spleen was gently teased in RPMI-1640 medium in 35 mm petri dishes to release cells by using sterile forceps and needles. The cell suspension was kept undisturbed for 5 min to allow sedimentation of clumps following which the upper portion of the medium (containing splenocytes) was carefully removed and transferred to 15 ml sterile centrifuge tube. Centrifuged the cell suspension at 1800 g for 5 min. Supernatant was aspired and the cell pellets were washed twice with RPMI-1640 medium. Subsequently, 1 ml of erythrocyte lysis buffer was added to give hypotonic shock to RBCs. After 30 sec, the suspension was centrifuged again at 1800 g for 5 min and supernatants along with lysed RBC ghosts were carefully removed. The remaining lymphocyte cell population was washed twice with RPMI-1640 medium to remove any traces of lysis buffer. The cells were finally suspended in 1 ml of RPMI-1640 medium containing 10% FBS and 50 nM 2- mercaptoethanol.

Cell counting and viability check

After isolation, splenocytes were counted using Neubauer haemocytometer under high power objective (40X) of a compound microscope. The counting chamber was charged with 10 µl of cell suspension in RPMI-1640 medium. Counting was successively carried out in four large squares of Neubauer haemocytometer present in each corner. Each of these squares further has 16 sub-squares which were counted for cells. Those cells which were half in and half out of the upper and left hand line were included in the counting while those cells which were present half in and half out of the lower and right hand lines were excluded. With the cover slip in place, each corner square represents a total volume of 0.1 mm^2 or 10^{-4} cm^3. As 1 cm_3 is equivalent to approximately 1 ml, the cell concentration per ml was obtained as follows:

$$\text{Total cells per ml} = \text{Average count} \times \text{dilution factor} \times 10^4$$

Trypan blue exclusion method was used to determine the proportion of viable cells using a Neubauer chamber and an optical microscope (40X). This method is based on the principle that dead cells take up the dye and appear blue, whereas live cells appear colourless since their intact cell membrane resist the dye. For estimating the

number of live cells in cell suspension, a 50 µl aliquot of cell suspension was mixed with an equal amount of 0.4% Trypan blue solution (w/v). The counting chambers were charged with 10 µl of the above mixture. The number of colourless viable cells, which had not taken up the dye, and blue dead cells were counted in the four corner squares. The cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{Total viable cells (appearing colourless)} \times 100}{\text{Total number of cells}}$$

Splenocytes proliferation index

The isolated splenocytes were adjusted to 1×10^6 viable cells/ml in RPMI-1640 culture medium containing 10% FBS and 50nM 2-mercaptoethanol and subsequently dispensed (100 µl per well) in 96 well flat bottomed tissue culture plates. Cells were stimulated with 10 µl of mitogen (ConA, 5 µg/ml final concentration) in positive control well. Cells were incubated with the peptides dissolved in the RPMI-1640 medium at different concentrations (10 ng, 100 ng, 1 µg, 10 µg, 100 µg and 1000 µg/ml respectively) for 24 hr. The plates were incubated for 48 hr at 37^0C in a humidified CO_2 incubator perfused with 5% CO_2. After incubation, 10 µl of MTT solution (5 mg/ml in filter sterilized RPMI-1640) was added to each well and plates were incubated for 4 hr in CO_2 incubator. During this period, formazan crystals were formed at the bottom of each well. The spent medium along with suspension of cultured cells was carefully removed (avoiding disturbance of formazan crystals). Added 100 µl of DMSO (Dimethyl sulphoxide) to each well and mixed thoroughly to dissolve the dark blue formazan crystals. The plates were read using ELISA reader at 540 nm against the reference wavelength of 630 nm and cell proliferation expressed as splenocytes stimulation index was calculated as:

$$\text{Splenocytes proliferation index (SPI)} = \frac{\text{A540nm in the presence of mitogen}}{\text{A540nm in the absence of mitogen}}$$

3.7.2. Phagocytosis

Murine peritoneal macrophages were used to determine the percent phagocytosis in the presence of peptides (Hay and Westwood, 2008).

Reagents

Antibiotic stock solution: Dissolved 0.15 g DMEM, 100 mg penicillin, 100 mg streptomycin and 3 mg amphotericin in 10 ml of distilled water and filter sterilized through 0.22 µm Millex-GV filters (Millipore) and stored at 4^0C until used.

DMEM Ham's F12 media (without phenol red): Prepared by dissolving DMEM (15.6 g), sodium bicarbonate (1.2 g), antibiotic solution (1% from stock) and fetal bovine serum (FBS, 10%) in 1 liter of autoclaved distilled water. The pH of the resulting solution was adjusted to 7.2 - 7.4 with 1 N NaOH and filter sterilized through 0.22 µm Millex-GV filters (Millipore). The prepared media was distributed in small aliquots and stored at 4^0C.

Yeast cell preparation: Dried Baker's yeast (*Saccharomyces cerevisiae*) cells were obtained from Dairy Microbiology Division, NDRI. Yeast beads were dissolved in DMEM and sonicated gently to disrupt clumps. Then the cell number was adjusted to $1x10^6$ cells/ml and stored at 4^0C until used.

Peptides (5 mg/ml): Four peptides (B, C, F and G) were dissolved in DMEM medium.

Procedure

Collection of peritoneal fluid

Macrophages were collected from the peritoneal cavity of mice using DMEM Ham's F12 (without phenol red). Using scissors and forceps the outer skin of mice was cut and injected with 5 ml of DMEM into the peritoneal cavity using a syringe carefully After injection, peritoneum was gently massaged to dislodge any attached cells into the DMEM media. Maximum peritoneum fluid was collected and transferred in to the fresh tubes and kept it on ice till used.

Isolation of macrophages and phagocytosis

The collected cell suspensions were transferred to a 30mm culture petri dish and incubated in a humidified atmosphere at 5% CO_2 in air at 37°C for 2 hr to allow attachment of adherent cells according to Kumangi *et al.* (1979). After incubation, non-adherent cells were removed with washing and the adherent macrophages ($1x10^6$ cells/ml) were cultured in DMEM Ham's F12 media for 2 hr. The peptides dissolved in DMEM media (10 ng, 100 ng, 1 µg, 10 µg, 100 µg and 1000 µg/ml respectively) were added to the cells and incubated for an additional 2 hr. After incubation, 100 µl of yeast cell suspension ($1x10^9$ cells/ml) was added and kept it for 1 hr. Finally, cells were stained with May-Grunwald stain freshly diluted with Giemsa buffer (1:2) for 5 min and washed with buffer and air dried. Cells were observed at

1000X magnification in an oil immersion microscope. Percent phagocytosis was calculated by counting the number of macrophages attached to engulfed yeast cells per hundred macrophages.

3.7.3. Cytokine secretion

Splenocytes were collected and cultured in RPMI-1640 medium in the presence of peptides as described above in section 3.7.1. After incubation, the cell suspensions from each well were centrifuged at 1800 g for 5 min and supernatants were collected and stored at -80^{o}C till estimation of IFN-ɣ, IL-4, IL-10 and TGF-β.

Reagents

Coating buffer (10X): Diluted 10X coating buffer to 1X concentration with distilled water.

Capture antibodies against IFN-ɣ/IL-4/IL-10/TGF-β (250X): Antigen-affinity purified monoclonal antibodies specific for IFN-ɣ/ IL-4/IL-10/ TGF-β were diluted 250 times in coating buffer.

Wash Buffer (PBS/T): 0.05% Tween-20 in PBS (10 mM, pH 7.4)

Assay Diluent (5X): Diluted 5X assay diluent to 1X concentration with distilled water.

Standards for IFN-ɣ/IL-4/IL-10/TGF-β: Working concentrations of recombinant standard proteins (15-1000 pg/ml for IFN-ɣ; 7-500 pg/ml for IL-4; 15-250 pg/ml for IL-10; 7- 500 pg/ml for TGF-β) were prepared by serial dilutions of respective standards in 1X assay diluent.

Detection antibodies against IFN-ɣ/ IL-4/IL-10/ TGF-β (250X): Biotin conjugated anti mouse IFN-ɣ/IL-4/IL-10/TGF-β antibodies were diluted 250 times in 1X assay diluent.

Detection Enzyme (250X): Working Avidin-HRP conjugate (1X) was prepared by dissolving 48 µl of enzyme (250X) in 12 ml of 1X assay diluent.

Substrate solution: 1X TMB.

Stop Solution (2N H_2SO_4): Prepared by slowly adding 5.32 ml of H_2SO_4 to 94.67 ml of distilled water.

Procedure

Levels of interleukins in the supernatant of cultured splenocytes (IFN-ɣ, IL-4, IL-10 and TGF-β) were estimated by using commercially available ELISA kits (eBioscience, San Diego, CA, USA) according to manufacturer's protocol. Briefly, NUNC Maxisorp 96 well plates were coated with 100 µl of 1X capture antibody in 1X coating buffer and incubated for overnight at 4°C. The wells were aspirated and then washed five times with PBS/T (wash buffer) allowing time for soaking between each

wash (1 minute). The plates were blotted on absorbent paper to remove any residual buffer. After the last wash, free binding sites were blocked by the addition of 200 µl of blocking buffer in each well and incubated at room temperature for 1 hr. After incubation, wells were aspirated and again washed five times with wash buffer with intermediate soaking and blotting. This was followed by addition of serially diluted standard proteins (IFN-ɣ/IL-4/IL-10/TGF-β) in duplicate to appropriate wells to prepare standard curve. The splenocytes culture supernatants diluted six times for IFN-ɣ, seven times for TGF-β, ten times for IL-10 and undiluted sample for IL-4 estimation were added (100 µl/well) in duplicate to respective wells while wells with only assay diluent were kept as blanks. The plates were then incubated for 2 hr at room temperature followed by washing with wash buffer for five times. Subsequently, 100 µl/well of respective biotin conjugated detection antibodies against IFN-ɣ/TGF-β/IL-4/IL-10 were added in all wells. The plates were again incubated for 1hr at room temperature. The wells were aspirated and washed thrice with in between soaking and blotting after which avidin conjugated detection enzyme (100 µl/well) was added. Plates were incubated at room temperature for 30 min followed by aspiration and washing seven times. TMB substrate solution (100 µl/well) was now added in each well and plates were incubated in dark at room temperature for 15 min to allow colour development. Finally, stop solution (50 µl) was added in each well and plates were read at 450 nm.

3.8. Cell culture study

Cytotoxic assessment of peptides

Antibiotic stock solution: Dissolved 0.15 g DMEM, 100 mg penicillin, 100 mg streptomycin and 3 mg amphotericin in 10 ml of distilled water and filter sterilized through 0.22 µm Millex-GV filters (Millipore) and stored at 4^0C until used.

DMEM Ham's F12 media: Prepared by dissolving DMEM (15.6 g), sodium bicarbonate (1.2 g), antibiotic solution (1% from stock) and fetal bovine serum (FBS, 10%) in 1 liter of autoclaved distilled water. The pH of the resulting solution was adjusted to 7.2 - 7.4 with 1 N NaOH and filter sterilized through 0.22 µm Millex-GV filters (Millipore). The prepared media was distributed in small aliquots and stored at 4^0C.

Caco-2 cell culture: The human colon adenocarcinoma cell line, Caco-2 was cultured in filter sterilized DMEM containing FBS (10%) and antibiotic solution (1% from stock) in a humidified atmosphere at 37^0C in a 5% CO_2 incubator. Cells were

cultured in 25 and 75 cm^2 cell culture flasks. For experimental purposes, cells were cultured in 96 well plates ($1X10^6$ cells/well) and were allowed to attach for overnight before treatment with peptides. After incubation, cells were treated with the peptides dissolved in DMEM at different concentrations (10 ng,100 ng,1 µg,10 µg,100 µg and 1000 µg/ml respectively) and incubated for an additional 24 hr. Finally, cell viability and membrane integrity was determined using following methods.

3.8.1. MTT assay

Cell viability was assessed by using MTT method that assesses the ability of the cell's to convert MTT into visible formazan crystals using succinate dehydrogenase (Mosmann,1983).

Reagents

MTT solution (5 mg/ml)

Phosphate buffer saline (PBS; 10 mM, pH 7.4)

DMSO (Dimethyl sulphoxide)

Peptides (5 mg/ml): Four peptides (B, C, F and G) were dissolved in DMEM medium

Procedure

After pre-treatment of cells with peptides at various concentrations (10 ng to 1 mg/ml), culture supernatant was discarded from each well and cells were rinsed with PBS (10 mM, pH 7.4) followed by the addition of 90 µl of DMEM and 10 µl of MTT (5 mg/ml). After 4 hr of incubation at 37 ^{0}C, the medium was replaced with DMSO (100 µl/well) to solubilise the formazan crystals and the absorbance was determined at 570 nm.

3.8.2. Neutral red assay

Neutral red assay was carried out using the protocol described previously by Borenfreund and Puerner (1984). Live cells take up the neutral red, which is concentrated within the lysosomes of the cells.

Reagents

Phosphate buffer saline (PBS; 10 mM, pH 7.4)

Neutral red (50 µg/ml): Dissolved 2 mg of neutral red in 40 ml of FBS free DMEM media.

Formal-calcium solution: Prepared by mixing 10 ml of 40% formaldehyde, 10 ml of 10% anhydrous calcium chloride and 80 ml of water.

Acetic acid - Ethanol mixture: Mixed 1% glacial acetic acid in 100 ml of 50% ethanol.

Peptides (5 mg/ml): Four peptides (B, C, F and G) were dissolved in DMEM medium

Procedure

After 24 hr peptide incubation, culture supernatant was discarded from each well and cells were washed twice with 100 µl of PBS (10 mM, pH 7.4). 200 µl of neutral red was added to each well and incubated it for 4 hr in dark at 37^0C in 5% CO_2 incubator. After the incubation, cells were washed with 100 µl of formal-calcium for one time to remove unincorporated neutral red dye. Finally, 200 µl of acetic acid and ethanol mixture was added to each well and kept it for 15 min before taking the absorbance at 540 nm.

3.8.3. LDH assay

LDH is a cytosolic enzyme, under normal conditions that is not secreted outside the cell but leaks into the culture medium upon damage to cell membranes. The assay used was based on the reduction of pyruvate to lactate in the presence of LDH with parallel oxidation of NADH. The formation of NAD from NADH in the above reaction results in a change in absorbance at 340 nm (Bergmeyer and Gawehn, 1978).

Reagents

Phosphate buffer (0.2 M, pH 7.4): It was prepared by mixing 19.0 ml of 0.2 M NaH_2PO_4 (3.12 g of NaH_2PO_4 in 100 ml distilled water) and 81.0 ml of 0.2 M Na_2HPO_4 (3.56 g of Na_2HPO_4 in 100 ml distilled water).

$NADH_2$ (3.5 mM): Dissolved 12.41 mg of $NADH_2$ in 5 ml of distilled water.

Sodium pyruvate (21 mM): Dissolved 23.1 mg of sodium pyruvate in 10 ml of phosphate buffer (0.2 M, pH 7.4).

Peptides (5 mg/ml): Four peptides (B, C, F and G) were dissolved in DMEM medium

Procedure

Caco-2 cells were cultured in 96 well plate ($1x10^6$ cells/well) and kept overnight for surface attachment in CO_2 incubator. Peptides at various concentrations (10 ng to 1 mg/ml) were added in to respective wells for 24 hr incubation. Then, culture medium was collected from each well and centrifuged for 5 min at 3000 rpm in order to obtain a cell free supernatant. The reaction mixture containing 2.7 ml of phosphate buffer (0.2 M pH 7.4), 100 µl of sodium pyruvate (21 mM) and 100 µl of $NADH_2$ (3.5 mM) was mixed and incubated at 37^oC for 10 min. After incubation added 100 µl of culture supernatant to the reaction mixture and change in absorbance with time was observed at 340 nm wavelength. One unit of enzyme was defined as the amount of enzyme which oxidized one nano mole of $NADH_2$ to NAD per minute at 25^0C.

3.9. H_2O_2 induced oxidative stress model

H_2O_2 mediated cytotoxicity in the Caco-2 cells was determined by using method of Gu *et al.* (2012).

Reagents

Phosphate buffer saline (PBS; 10 mM, pH 7.4)

Hydrogen peroxide (H_2O_2, 0.2 to 4 mM): Just before the use, concentration of H_2O_2 was determined using $KMnO_4$ titration method (Klassen *et al.,* 1994). DMEM without FBS was used for preparing H_2O_2 solution.

MTT solution (5 mg/ml)

Procedure

Caco-2 cells were seeded ($1x10^6$ cells/wells) in a transparent 96-well plate and incubated overnight with DMEM for their attachment. The adhered Caco-2 cells were treated with 100 µl of H_2O_2 having 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2, 2.5, 3, 3.5 and 4 mM concentration respectively for 10 hr. After incubation, cells were washed with PBS (10 mM, pH 7.4) in each well and viability was determined by using MTT method (Mosmann,1983) as described previously in section 3.8.1.

3.10. Protective effect of peptides against H_2O_2 induced oxidative stress

Protective activity of peptides against H_2O_2 induced oxidative stress was determined by following the method of Gu *et al.* (2012).

3.10.1. Cell viability by MTT assay

Reagents

Phosphate buffer saline (PBS; 10 mM, pH 7.4)

Hydrogen peroxide (H_2O_2, 1.5 mM): Prepared in DMEM without FBS.

MTT solution (5 mg/ml)

Peptides (5 mg/ml): Four peptides (B, C, F and G) were dissolved in DMEM medium.

Procedure

Caco-2 cells were seeded ($1x10^6$ cells/well) in a 96-well plate and incubated for overnight. Cells were treated with various concentrations of individual peptides (10 ng, 100 ng,1 µg,10 µg,100 µg and 1 mg/ml respectively) dissolved in DMEM for 24 hr. After peptides pre-treatment, 100 µl of 1.5 mM H_2O_2 (IC_{50} of H_2O_2) prepared in FBS free DMEM media was added to the cells and incubated for an additional 10 hr followed by washing of cells in PBS (10 mM, pH 7.4). Finally, cell viability was determined by using MTT assay (Mosmann,1983) as described previously in section (3.8.1).

3.10.2. Intracellular ROS estimation by fluorescence

Effect of peptides on the levels of intracellular ROS production in the presence of H_2O_2 in Caco-2 cells was quantified using the method of Cathcart *et al.* (1983).

Reagents

Phosphate buffer saline (PBS; 10 mM, pH 7.4)

Hydrogen peroxide (H_2O_2, 1.5 mM): Prepared in FBS free DMEM medium.

DMSO (dimethyl sulphoxide)

DCFH-DA (2',7'-dichlorofluorescin diacetate) stock solution (1 mM): Prepared by dissolving 2.5 mg of DCFH-DA dye in 5 ml of DSMO.

DCFH-DA working solution (10 µM): Prepared by adding 50 µl of the above stock solution in to the 5 ml of PBS (10 mM, pH 7.4).

Peptides (5 mg/ml): Four peptides (B, C, F and G) were dissolved in DMEM medium.

Procedure

Caco-2 cells were cultured in a 96 well plate ($1x10^6$ cells/well) and incubated for overnight in CO_2 incubator. After incubation, cells were treated with different concentrations of peptides dissolved in DMEM (10 ng, 100 ng, 1 µg, 10 µg, 100 µg and 1000 µg/ml respectively) for 24 hr followed by 10 hr incubation with 1.5 mM H_2O_2 prepared in FBS free DMEM media. Thereafter, cells were incubated with 50 µl of 10 µM DCFH-DA for 60 min in the dark. Then, the cells in each well were washed twice with 100 µl of PBS (10 mM, pH 7.4) and resuspended again in 200 µl of PBS (10 mM, pH 7.4). The qualitative analysis of ROS generation was done using a fluorescence microscope. DCFH-DA fluorescence intensity was examined using microplate reader at 485 nm excitation and 535 nm emission wavelengths.

3.10.3. Measurement of oxidative products

Effect of peptides on oxidative products such as MDA and protein carbonyls were determined using Caco-2 cell lysate whereas, culture supernatant was used to estimate nitric oxide levels.

After 24 hr of pre-incubation with peptides, the cells were exposed to H_2O_2 (1.5 mM) for 10 hr. Then, Caco-2 cells were washed with PBS (10mM, pH 7.4) and harvested and centrifuged for 5 min at 1500 rpm. The cell supernatant was used for nitric oxide estimation while cell pellet obtained was sonicated on ice in 3 ml of cold phosphate buffer (50 mM pH 7.0) for 15 sec followed by centrifugation at 10,000 g for 15 min at 4^0C. Cell lysate supernatant obtained was used for the estimation of MDA and protein carbonyl levels.

3.10.3.1. Malondialdehyde (MDA)

Malondialdehyde (MDA), a TBA reactive compound level has been estimated spectrophotometrically according to Uchiyama and Mihara (1978) with some modifications.

Reagents

TBA (Thiobarbituric acid; 0.375%): Dissolved 0.375 g of TBA in 100 ml of hot distilled water

Trichloro acetic acid (TCA, 5%): Dissolved 5.0 g of TCA in 100 ml of distilled water.

HCL (0.25 N)

Reagent mixture: Prepared by mixing above all three reagents in 1:1:1 ratio.

Procedure

To 2 ml of reagent mixture (TBA+TCA+HCL) 1 ml of cell lysate was added and mixed well. The reaction mixture was then boiled in a boiling water bath for 45 min followed by cooling at room temperature. Subsequently, it was centrifuged at 3000 rpm for 10 min at room temperature. Finally, supernatant was collected and absorbance was read at 535 nm against blank. MDA levels (nmol/ml) were determined from the standard curve prepared by using 3,3,3,3-tetraethoxy propane (TEP) (range from 5-40 nmol/ml) and expressed as nmol/ml of cells.

3.10.3.2. Protein carbonyls

The protein carbonyl content in the cell lysate was assessed according to the method of Levine *et al.* (1990).

Reagents

Potassium phosphate buffer (5 mM), pH 7.4:

Solution A: Dissolved 0.44 g of Na_2HPO_4 in 500ml of distilled water

Solution B: Dissolved 0.34 g of KH_2PO_4 in 500ml of distilled water

Mixed solution A and B and adjusted the pH to 7.4

HCl (2 M)

Streptomycin sulphate stock solution (10%): Dissolved 10 g of streptomycin sulphate in 100 ml of potassium phosphate buffer (50 mM, pH 7.2)

2,4-dinitro phenylhydrazine (DNPH; 10 mM): Dissolved 19.8 mg of DNPH in 10 ml of 2 M HCl

Trichloro acetic acid (TCA; 20%): Dissolved 20 g of TCA in 100 ml of distilled water.

Guanidine hydrochloride (6 M): Dissolved 5.7 g of guanidine hydrochloride in 10 ml of 20 mM potassium phosphate buffer and pH adjusted to 2.3 with trifluoroacetic acid.

Ethanol-Ethylacetate solution : Prepared by mixing ethanol and ethyl acetate in 1:1 ratio.

Procedure

In order to remove nucleic acids from cell lysate to avoid their interference, streptomycin sulphate (10% stock solution made in 50 mM potassium phosphate buffer, pH 7.2) was added at a final concentration of 1% in the sample and incubated for 15 min at room temperature followed by centrifugation at 6000 g for 10 min at 4^0C. Finally, supernatant was collected and used it to determine the protein carbonyl content as described.

Carbonyl analysis

Briefly, 500 µl of cell lysate was added to sample tube and control tube. Added 500 µl of DNPH to sample tube and 500 µl of 2 M HCl to control tube and incubated in the dark for one hour at room temperature. Vortexed the both tubes briefly at every 15 min during incubation. After incubation, added 1 ml of 20% TCA to both tubes and vortexed. Placed the tubes on ice and incubated for 5 min followed by centrifugation (10,000 g) for 10 min at 4^0C. Discarded the supernatant and resuspended the pellets in 10% TCA and kept on ice for 5 min. Centrifuged the tubes (10,000 g) for 10 min at 4^0C. Then, discarded the supernatant and resuspended the pellets in 1 ml of ethanol and ethyl acetate solution (1:1), vortexed thoroughly and again centrifuged at 10,000 g for 10 min at 4^0C. Repeated this step for two more times. After the final wash, resuspended the protein pellets in 500 µl of guanidine hydrochloride and vortexed. Centrifuged (10,000 g) the tubes for 10 min at 4^0C to remove any of the leftover debris. Finally, transferred 220 µl supernatant from the sample tube and 220 µl supernatant from control tube to the two wells of 96 well plate and measured the absorbance at the wavelength of 360 nm. Protein carbonyl content was calculated using molar absorption coefficient (DNPH) of 22000 $M^{-1}cm^{-1}$ and expressed as nmoles of dinitrophenylhydrazone carbonyl adduct per mg of protein.

3.10.3.3. Nitric oxide (NO)

Total nitric oxide production in culture supernatant was determined by the method of Miranda *et al.* (2001). Nitrate present in the sample was reduced to nitrite using vanadium chloride, which was then estimated using Griess reagent.

Reagents

$NaNO_2$ (200 µM): Dissolved 69 mg of $NaNO_2$ in 100 ml of distilled water

1M HCL

Vanadium chloride (VCl_3; 50 mM): Dissolved 400 mg of vanadium chloride in 50 ml of 1 M HCl

Griess reagent (Sigma, USA)

Procedure

After peptides pre-treatment followed by H_2O_2 (1.5 mM) incubation as described above, the culture supernatant was collected from each well and used to determine the nitric oxide levels. Briefly, 100 µl of vanadium chloride and 50 µl of Griess reagent were added to 100 µl of culture supernatant and incubated at 37^0C for 40 min. The absorbance was measured at 550 nm and the concentration of nitrite (indicator of nitric oxide) was calculated from the standard curve generated from serial dilutions of $NaNO_2$ (range from 1 to 20 nmol in DMEM) and values were expressed as nmol/ml of cells.

3.10.4. Antioxidative enzymes

Antioxidative enzymes such as catalase, superoxide dismutase and glutathione peroxidise activity was determined in the cell lysate which was prepared after 24 hr peptides incubation followed by 10 hr H_2O_2 treatment as described above.

Catalase (CAT)

Catalase activity was determined spectrophotometrically (Aebi,1984).

Reagents

Potassium phosphate buffer (50 mM, pH 7.0):

Solution A: Dissolved 4.45 g Na_2HPO_4 in 500 ml of distilled water.

Solution B: Dissolved 3.40 g KH_2PO4 in 500 ml of distilled water.

Mixed solution A and B and pH was adjusted to 7.0.

H_2O_2 (30 mM): Just before the use, concentration of H_2O_2 was determined using titration method (Klassen *et al.,* 1994). Dissolved 170 µl of 30% H_2O_2 in 50 ml of potassium phosphate buffer (50 mM, pH 7.0) to obtain OD of 0.50.

Procedure

The reaction mixture contained 100 µl of cell lysate, 400 µl of H_2O_2 and volume made to 3 ml with phosphate buffer. The reaction was initiated with the addition of H_2O_2 and decrease in the absorbance at 240 nm due to the decomposition of H_2O_2 was observed. Initial absorbance was approximately A = 0.500 and the decrease in

the absorbance was followed for 1 min. The enzyme activity was determined by using an extinction coefficient of $39.4M^{-1}$ cm^{-1} and expressed as units/mg of protein, where unit enzyme activity is one µmole of H_2O_2 consumed/min and calculated as:

$$\frac{\Delta A/min \times volume\ of\ assay\ mixture\ (3\ ml) \times dilution\ factor \times 1000}{Volume\ of\ sample\ (ml) \times 0.0394 \times protein\ concentration\ (mg/ml)}$$

Superoxide dismutase (SOD)

The SOD activity was estimated spectrophotometrically (Marklund and Marklund, 1974). The inhibition of pyrogallol auto-oxidation by superoxide dismutase is a measure of enzyme activity in this method.

Reagents

DTPA-Tris-HCL buffer (50 mM Tris - 1 mM DTPA, pH 8.2): Dissolved 605 mg of Tris and 39.33 mg of diethylenetriaminepentaacetic acid (DTPA) in 80 ml of distilled water and pH adjusted to 8.2 with 1 N HCL and volume made up to 100 ml.

Pyrogallol (2 mM): Dissolved 25.2 mg of pyrogallol in 100 ml of 10 mM HCL.

Procedure

The rate of auto-oxidation of pyrogallol was taken from the increase in absorbance at 420 nm against a reference cuvette containing 3 ml DTPA-Tris-HCl buffer (1 mM DTPA in 50 mM Tris, pH 8.2) using Specord 200 double beam UV/visible spectrophotometer (Analytikjena, Germany). The volume of pyrogallol (400 µl) in the absence of superoxide dismutase, at which increase in absorbance (0.02 min^{-1}) was selected for the reaction. Reaction mixture contained 100 µl of cell lysate, 400 µl of pyrogallol and DTPA-tris-HCl buffer to make total volume to 3 ml. Absorbance was read against blank containing sample and DTPA-tris-HCl buffer without pyrogallol. Percent inhibition was calculated using the following equation.

$$\%\ inhibition\ of\ pyrogallol\ auto\text{-}oxidation = [1-(\Delta A/\Delta A_{max})] \times 100$$

Where, ΔA= Absorbance change due to pyrogallol auto-oxidation in the sample reaction system

ΔA_{max} = Absorbance change due to pyrogallol auto-oxidation in the control (without cell lysate)

The amount of enzyme that inhibited auto-oxidation of pyrogallol by 50% was defined as one unit of enzyme. The activity of SOD was expressed as units/mg protein.

$$1/a \times dilution\ factor\ /\ protein\ concentration\ in\ the\ sample\ (mg/ml)$$

Where, a= Volume of the sample (ml) that inhibits auto-oxidation of pyragallol by 50% in one min.

Glutathione peroxidase (GPx)

GPx activity was determined spectrophotometrically using the method of Paglia and Valentine (1967). GPx was assessed by utilizing excess of glutathione reductase that couples the rate of oxidation of NADPH to reaction of the peroxidase reaction with cumene hydroperoxidase and glutathione (reduced).

Reagents

Potassium phosphate buffer (50 mM, pH 7.0)

EDTA (10 mM): Dissolved 2.9 mg of EDTA in 10 ml of potassium phospthate buffer

NaN_3 (10 mM): Dissolved 65 mg of NaN_3 in 10 ml of potassium phospthate buffer

NADPH (2 mM): Dissolved 16.6 mg of NADPH in 10 ml of potassium phospthate buffer

Glutathione reductase: 100 U/ml in potassium phospthate buffer

Glutathione (reduced) (10 mM): Dissolved 30.73 mg of reduced glutathione in 10 ml of potassium phospthate buffer

Cumene hydroperoxide (1.5 mM): Dissolved 28.5 µl in 10 ml of potassium phospthate buffer.

Procedure

The reaction mixture (0.8 ml) containing 390 µl of potassium phosphate buffer, 100 µl of EDTA, 100 µl of NaN_3, 100 µl of NADPH, 10 µl of glutathione reductase, 100 µl of glutathione (reduced) was mixed with 100 µl of cell lysate and incubated at room temperature for 5 min. Then, added 100 µl of cumene hydroperoxide to the reaction mixture to initiate the reaction. Absorbance was taken against blank containing phosphate buffer (50 mM, pH 7.0). Oxidation of NADPH was monitored for 5 min by change in absorbance at 340 nm. The enzyme activity was calculated using extinction coefficient of NADPH (6.22×10^3 $M^{-1}cm^{-1}$), where unit enzyme activity is one nmole of NADPH oxidized per minute per mg of protein and calculated as:

$$\frac{\Delta A/min \times \text{volume of assay mixture (1ml)} \times \text{dilution factor}}{\text{Volume of sample (0.1ml)} \times 6.22 \times \text{protein concentration in sample (mg/ml)}} \times 1000$$

3.10.5. Expression of oxidative stress associated genes

Effect of peptides on Nrf-2 and Keap1 genes expression in Caco-2 cells was assessed by qRT-PCR.

3.10.5.1. Isolation of total RNA from Caco-2 cells

After 24 hr pre-incubation of Caco-2 cells with peptides followed by H_2O_2 treatment for 10 hr in 6-welled tissue culture plate, cells were washed with PBS (10 mM, pH 7.4) for one time and homogenized/sliced by adding 1 ml of TRI reagent to each well. The suspended cells were kept in DEPC treated centrifuged tube and stored at -20^{0}C for further use.

Reagents

TRI reagent (Sigma)

DNAse and RNase free chloroform (Sigma)

DNAse and RNase free isopropanol (Sigma)

Ethanol (75 %) (S. D. Fine Chem. Ltd, Mumbai)

Nuclease free water (Thermo scientific)

Di Ethyl Pyro Carbonate (DEPC) (0.1 %): 1 ml of DEPC was added to 999 ml of double distilled water and stirred overnight for complete solublization of DEPC. The solution was used to keep plastic wares free of RNase.

Procedure

Total RNA from liver cells was isolated by the single-step method of Chomzynski and Sacchi, (1987). The TRI reagent added samples were initially kept at room temperature for 5 minutes. Then 200 µl of chloroform was added to the samples and kept at room temperature for 5 minutes again. After vigorous shaking for 15 seconds, the milky solution so formed was centrifuged at 12,000 g for 15 min at 4^{o}C. The upper aqueous layer was carefully taken and added with 500 µl of chilled isopropanol. The contents were mixed gently and kept on ice for 10 min. The solution was centrifuged at 12,000 g for 10 min at 4^{o}C and the supernatant was discarded without disturbing the RNA pellet. The pellet was washed with 1 ml of 75% ethanol by centrifuging at 10,000 g for 5 min at 4^{o}C. Ethanol was decanted and the tube was left open in a laminar flow hood for 5 min to dry the pellet. RNA pellet was then dissolved in 20 µl of nuclease free water.

3.10.5.2. Assessment of the quantity, quality and purity of RNA

The quality of isolated RNA was assessed on 1.5% agarose gel electrophoresis by following the method of Sambrook *et al.* 1989.

Reagents

Tris borate EDTA buffer (TBE 10X): Dissolved 10.8 g Tris-base, 0.74 g Di sodium EDTA and 7.5 g boric acid in 60 ml distilled water and pH adjusted to 8.3 with 1 N NaOH. The volume was made up to 100 ml with distilled water. Filtered and stored at 4°C. It was further diluted 10 times with distilled water before use.

Agarose (1.5%): Dissolved 0.6 g of agarose in 40 ml of 1X *TBE buffer.*

Ethidium bromide (10 mg/ml): Dissolved 1.5 µl ethidium bromide (10 mg/ml) in 30 ml 1X TBE buffer before use to prepare working concentration (0.5 µg/ml).

Gel loading dye, 6X (Thermo Scientific): Diluted (1:5) with RNA samples.

Procedure

Dissolved 0.6 g of agarose in 40 ml of 1X TBE buffer by melting until a clear transparent solution appeared. It was allowed to cool (~50^{0}C) and then added 1.5 µl of ethidium bromide to it. The melted solution was then poured into a casting tray containing a comb (5.2 cm long, 4 mm wide) and was allowed to solidify, resulting into a matrix. The gel solidified within 20-30 min and was ready to use. The gel was submerged in a horizontal electrophoresis tank (Genei, Bangalore) containing 350 ml of 1X TBE buffer. The comb was carefully removed and 5 µl of isolated RNA samples were mixed with 1 µl of 6X gel loading buffer and dispensed carefully into the wells. Immediately after loading the samples, the system was connected with an electric power supply unit (Atto Model- AE 8750). Electrophoresis was carried out at a constant voltage of 100 V at room temperature. The gel was constantly monitored and migration was judged by observing the movement of the dye present in the loading buffer. When the dye front reached at the bottom of gel (~60 min), the electric current was switched off. After electrophoresis, the gel was examined under ultraviolet trans-illuminator and photographs were taken by gel documentation system (Alpha-Imager gel documentation system).

3.10.5.3. Preparation of cDNA using reverse transcriptase (RT)

The RT reaction was carried out to prepare complementary DNA (cDNA) for amplification of genes Nrf-2 and Keap1.

Reagents

Total RNA

RevertAid^{TM} First strand cDNA synthesis kit (thermoscientific, India)

Procedure

Reverse transcription reaction was performed by adding total RNA (1 µg), random hexamer and oligo dT primers to DEPC treated water (table 3.1) and incubating them at 65^0C for 10 min in thermocycler (GenePro, Bioer). Then the reaction mixture was chilled on ice and spun down. RNAase inhibitor, reaction buffer, dNTP and *RevertAid*^{TM} reverse transcriptase were then added to it and incubated for 10 min at 25^0C followed by 42^0C for 30 min and the reaction was terminated by heating at 95^0C for 3 min in thermocycler itself. The prepared cDNA was stored at -20^0C till further use. Final confirmation of successful cDNA synthesis was done by checking the amplification of a house keeping gene (GAPDH) by RT-PCR.

Table 3.1. Reaction mixture of cDNA preparation

S.No.	Components of RT Reaction	Volume
1	RNA	1.0 µg
2	Random hexamer	0.5 µl
3	Oligo dT primer	0.5 µl
4	10 mM dNTP mix	2.0 µl
5	RNase inhibitor	1.0 µl
6	RevertAid^{TM} RT	2.0 µl
7	5 X Reaction buffer	4.0 µl
8	Total volume (made by DEPC treated water)	20 µl

Table 3.2. Primer sequences for amplification of genes

Name of the gene	Name of the primer	Sequence (5' to 3')	Amplicon length (bp)
Nrf-2	Nrf-2 F.P Nrf-2 R.P	5' ATCCATTCCTGAGTTACAGTGTCT3' 5' TCTGTCAGTTTGGCTTCTGGA 3'	90
Keap1	Keap1 F.P Keap1 R.P	5' AGACGTGGACTTTCGTAGCC3' 5' CCAGGAACGTGTGACCATCA 3'	111
GAPDH	GAPDH F.P GAPDH R.P	5' GCACCGTCAAGGCTGAGAAC3' 5' TGGTGAAGACGCCAGTGGA3'	138

3.10.5.4. Quantification of mRNA expression by qRT-PCR

cDNA was subjected to relative quantification of Nrf-2, Keap1 and GAPDH by real-time PCR.

Reagents

Primers: Primers specific for Nrf-2, Keap1 and GAPDH were custom synthesized from Eurofins, Bangalore, India. The sequence of primers is given in table 3.2.

Real Time Thermocycler (7500 Fast- Real Time PCR, Applied Biosystems)

SYBR Green I reaction mixture: composition given in table 3.3.

Table 3.3. Reaction components and concentrations required for real time PCR

S. No.	Reaction components	Stock concentration	Volume (µL)	Final Concentration
1	Nuclease free water	-	10.5	-
2	Forward primer	10 µM	0.5	0.2 µM
3	Reverse primer	10 µM	0.5	0.2 µM
4	PCR master mix	2X	12.5	1X
5	Template DNA	1 µg/20 µl	1.0	0.05 µg/25 µL
6	Total		25.0	

Procedure

The reaction volume of 10 µl contains 1 µl of diluted cDNA (1:2), 5 µl of SYBR Green I master mix (Roche Diagnostics, Indiana pols, IN, US) and 0.5 µM of each primer (0.5 µl each) and 3 µl of nuclease free water. The concentration of amplified cDNA in each sample was calculated relative to that of GAPDH. After RT-PCR amplification, a dissociation analysis was performed to ensure that only one product is amplified in each PCR and to rule out the presence of primer dimers (if any) in all samples in Real Time Thermocycler (7500 Fast- Real Time PCR, Applied Biosystems, Foster City, California, USA). The results were analyzed using “Thermo cycler firm ware, version G2.10” software. The program used in Real time PCR is given in Table 3.4.

Table 3.4 Reaction program of real time PCR for gene amplification

	Programme Name	Steps	Temperature	Time
Stage 1				
		Step 2	95°C	10 sec
Stage 2	Amplification (40 cycles)	Denaturation	95°C	15 sec
		Annealing and extension	60°C	1 min
Stage 3	Melt curve	Step 1	95°C	15 sec
		Step 2	60°C	1 min
		Step 3	95°C	30 sec
		Step 4	60°C	1 min
Stage 4	Cooling		4°C	Pause/Infinite

3.10.5.5. Analysis of relative target gene expression

Generation of quantitative data by real-time PCR is based on the number of cycles required for optimal amplification generated fluorescence to reach a specific threshold of detection (the quantification cycle). The relative expression ratio of the target gene was tested for significance as per method given by Livak and Schmittgen (2001).

$\Delta C_T(R) = C_T(R) - C_T(HKG)$

$\Delta C_T(T) = C_T(T) - C_T(HKG)$

$\Delta\Delta C_T = \Delta C_T(T) - \Delta C_T(R)$

$F = 2^{-\Delta\Delta CT}$

Where, C_T – Threshold cycle

$C_T(R)$ – Threshold cycle for reference in negative control gene sample

$C_T(T)$ – Threshold cycle for target in treatment gene sample

C_T (HKG) – Threshold cycle of housekeeping gene in sample

ΔC_T – Difference between Threshold cycle

F = Fold induction

3.10.6. Western blotting of transcription factor Nrf-2

Effect of peptides on the activation and translocation of transcription factor Nrf-2 in Caco-2 cells has been assessed. For this purpose cells were grown in 6-welled culture plates using DMEM media.

Reagents

HCL (6M)

Stacking gel buffer (Tris-HCL, 0.5 mM, pH 6.8): Dissolved 6.05 g of Tris in 100 ml of distilled water and pH was adjusted to 6.8 using 6 M HCL and stored at 4^0C.

Separating gel buffer (Tris-HCL, 1.5 mM, pH 8.8): Dissolved 18.17 g of Tris in 100 ml of distilled water and pH was adjusted to 8.8 with 6 M HCL and stored at 4^0C.

Acrylamide - Bis acrylamide solution (30%): Dissolved 29 g of Acrylamide and 1 g of Bis acrylamide in 100 ml of distilled water and filtered with whatmann no.1 filter paper and stored at 4^0C.

SDS solution (10%): Dissolved 10 g of SDS in 100 ml of distilled water and stored at room temperature.

Bromophenol blue (0.5%): Dissolved 125 mg of Bromophenol blue in 25 ml of distilled water and stored at 4^0C.

APS (10%): Dissolved 100 mg of APS in 1 ml of distilled water.

TEMED (Tetramethylethylenediamine): Used as such (commercial preparation)

Glycerol

Separating gel (12%):

Composition: 30% Acryl/ Bisacrylamide - 6.0 ml
1.5M Tris HCL (pH 8.8) - 3.75 ml
10% SDS - 150 µl
Distilled water - 5 ml
10% APS - 75 µl (prepared freshly)
TEMED - 10 µl

Total volume: 15 ml

Stacking gel (4.5%)

Composition: 30% Acryl/ Bisacrylamide - 600 µl
0.5 M Tris HCL (pH 6.8) -1 ml
10% SDS - 40 µl
Distilled water- 2.4 ml
10% APS - 20 µl (prepared freshly)

TEMED - 5 µl

Total volume: 4 ml

Loading dye:

Composition: Distilled water- 3.5 ml

0.5 M Tris-HCL (pH 6.8) - 1.5 ml

Glycerol- 2.5 ml

10% SDS- 2 ml

0.5% Bromophenol blue- 200 µl

Total volume: 9.7 ml

Electrode buffer: Prepared by dissolving 6.06 g Tris (25 mM), 14.415 g glycine (192 mM) and 1g SDS (0.1%) in 1000 ml of distilled water and stored at 4^0C.

Blotting buffer: Prepared by dissolving 12.12 g Tris (50 mM), 57.66 g NaCl (384 mM), 0.2 g SDS (0.01%) and 400 ml of methanol and volume was made up to 2000 ml with distilled water and stored at 4^0C.

Tris Buffered Saline with Tween-20 (TBS-T): Prepared by dissolving 4.84 g Tris (20 mM), 17.53 g NaCl (150 mM) and 2 ml of Tween-20 (0.1% v/v) in 1900 ml with distilled water. The pH of the buffer was adjusted to 7.6 with 6 M HCL and the volume was made up to 2000 ml with distilled water and stored at 4^0C.

Primary antibody: Nrf-2 C-20 rabbit polyclonal IgG (Santa Cruz Biotech)

Secondary antibody: Anti-Rabbit IgG-Peroxidase antibody produced in goat (Sigma)

Procedure

Preparation of cell lysate

After 24 hr pre-treatment with peptides followed by 10 hr H_2O_2 treatment, Caco-2 cells were washed with PBS in each well and scraped the cells followed by centrifugation at 1500 rpm for 5 min to collect the cells. After centrifugation, discarded the supernatant and cell pellet was dissolved in 200 µl of CER (Cytoplasmic Extraction Reagent containing PMSF, BOASTER, USA) and vortexed at maximum speed for 5 sec to suspend pellets. Incubated the tubes on ice for 30 min followed by centrifugation at 12,000 g at 4^0C for 5 min. Immediately pipetted the supernatant (containing cytoplasmic proteins) into a pre-cooling tube without touching the precipitate to avoid contamination of cytoplasmic proteins and stored at -80^0C till used. To the remaining precipitate 50 µl of NER (Nuclear Extraction Reagent containing PMSF, BOASTER, USA) was added and vortexed at maximum speed for 15 seconds to suspend pellets, kept back on the ice and vortexed for 15-

30 sec each 1-2 min in following 30 minutes. Centrifuged at 12,000-16,000 g at 4^0C for 10 min. Supernatant (containing nuclear proteins) was collected immediately into a new pre-cooling tube and stored at -80^0C till used. The supernatants (both cytoplasmic and nucleus) were analysed for protein content using the lowry method as described in section 3.12.

SDS-PAGE

The gel cast was assembled with 1.5 mm spacers and tested with distilled water for leakage. Once the cast was assembled and tested for leakage, 12 % separating gel was prepared and immediately transferred into the cast and allowed to set for 15 min. The stacking gel was prepared and was immediately overlaid on the separating gel and the gel comb was inserted making sure no bubbles were in the wells. This was allowed to set for 15 min, then the comb was removed. The gel assembly was taken out of the casting apparatus and put into the gel tank, electrode buffer (25 mM Tris base, 192 mM Glycine, 0.1 % SDS) was then poured into the tank so that the buffer level was above the top of the gel. Cell lysate aliquots were removed from -80 ºC and taken the volumes corresponding to 30 µg of protein to the eppendorf tubes. Added 2 µl of 2-mercaptoethanol (50%) and 4 µl of bromophenol blue (0.1%) to each tube. Vortexed the tubes for 10 sec followed by boiling in water bath at 100^0C for 3 min. Cooled the samples at RT for 15 min and centrifuged at 10,000 g for 1 min to remove any insoluble material and loaded in to the wells of stacking gel. Pre-stained molecular weight marker was added in one well to check the movement and also the transfer efficiency after transblotting. The electrophoresis was run at constant voltage of 80V until the tracking dye reaches the bottom of the gel.

Western blotting

Equilibration of gel and activation of PVDF membrane

After electrophoresis, the gel sandwich was disassembled and stacking gel portion was removed and a small piece from the lower left-hand corner of the gel was cut to remember the orientation of the gel. The gel was equilibrated immediately in transfer buffer for 30 min at room temperature. The transfer buffer was changed at least once during equilibration. While the gel was equilibrating, a piece of the PVDF membrane was cut about 1 cm larger than the gel. The membrane was placed in 100% methanol for 30 sec only to activate the positive charges of the membrane. Immediately, the membrane was transferred to glass dish containing distilled water and kept for 5 min to remove methanol. Initially, the membrane will float on water

and move in a zigzag motion if the positive charges have been activated properly. Subsequently the membrane was placed in transfer buffer for 5 min. A small piece from the lower left-hand corner of the membrane (glossy surface up) was cut to remember the orientation of the membrane.

Assembly of transblot apparatus and running the blot

The transfer sandwich was assembled under transfer buffer taken in a rectangular clean tray to minimize trapping of air bubbles. The required gel cassette holder duly opened, Scotch-Brite pads (2 per sandwich) and the Whatmann 3 MM chromatography papers (8 pieces per sandwich, cut in the same dimension as of the Scotch-Brite pad) were immersed in transfer buffer. The transfer sandwich was assembled in the following order from bottom to top (from cathode to anode side):

1) Black plastic support on bottom
2) Scotch-Brite pad (1No.)
3) 3 MM filter papers (4 Nos.)
4) Separating gel: here, the gel was placed on filter paper in reversed manner so that the cut edge at bottom was then on the right-hand side.
5) PVDF membrane: here, the membrane was also reversed to have glossy surface down facing the gel and the cut edge at bottom was then on the right hand side matching the cut edge of the gel
6) 3 MM filter papers (4 Nos.)
7) Scotch-Brite pad (1 No.)
8) White plastic support on top.

The sandwich was closed and kept in a transfer tank filled with chilled (4^0C) transfer buffer up to the brim of the electrode assembly. The lid was placed on the tank and the transfer was carried out with 30V for 1 hr subsequently the voltage was increased to 40V and was run for 3 hr. At the end of the transfer period, the power was turned off, the power cords were disconnected and the apparatus was disassembled carefully. Protein transfer efficiency was assessed by analysing the transfer of pre-stained molecular weight marker on the PVDF membrane.

Blocking

The membrane was washed with distilled water and the non specific binding sites of the membrane were blocked by incubating the membrane in 5% skim milk powder (non fat dried milk) prepared in TBS-T for 5 hr at 4^0C.

Primary antibody incubation

Next day, the membrane with the blocking solution was removed from the refrigerator and allowed to equilibrate to room temperature for 1 hr. The membrane was rinsed briefly for two times for 30 sec with TBS-T and incubated with primary antibody (Nrf-2 C-20 rabbit polyclonal IgG, 1 :10000) prepared in TBS-T for overnight at 4^0C. The membrane was removed from primary antibody solution and washed four times with TBS-T for 15 min with gentle agitation.

Secondary antibody incubation

The membrane was removed from the last wash solution and incubated with enzyme conjugated secondary antibody (Anti-Rabbit IgG- Peroxidase antibody produced in goat, 1:10000) prepared in TBS-T for 2 hr at room temperature with intermittent gentle agitation. The membrane was removed from the secondary antibody solution and washed eight times with TBS-T for 15 min with gentle agitation.

After last washing, removed the membrane and kept on plastic sheet. Added ECL chemiluminescent reagent (Immobilon^{TM} Western chemiluminescent HRP substrate, Millipore) on to the PVDF membrane by taking luminal and peroxidase reagents in 1:1 ratio. Incubated for 3-5 min and discarded the ECL reagent from the membrane. Kept the PVDF membrane in a zip lock and used it for the detection of protein bands with TYPHOON instrument (GE Healthcare Bio-Science Pvt. Ltd., Hongkong).

3.11. Bioavailability and transepithelial transport study

Seeding of cells in transwell plate

For bioavailability and transepithelial transport studies, 6-welled polyester culture plates with transwell permeable inserts with smallest pore size of 0.4 µm were used. Caco-2 cells were seeded on the apical chamber of each well in 6 welled transwell culture plate at the density of $3x10^4$ cells/well along with 0.5 ml of DMEM media such that the total volume was 1.5 ml per well. Likewise, 1.5 ml of medium was added to the basal chamber and plates were kept at 37ºC in a CO_2 incubator. Culture attained confluence within 6 to 7 days and were kept for 15 more days for differentiation.

3.11.1. Integrity checking

After 21 days of culture, the formation of intact and confluent monolayer was monitored by measuring the diffusion of phenol red through the monolayer (Jovov *et al*., 1991). For that, the medium used for the growth was removed, both monolayer and basal chamber were washed carefully with 1 ml of PBS (10 mM, pH 7.4) and after washing 1.5 ml of DMEM containing phenol red (without serum and antibiotic)

was added on the apical chamber. The basolateral chamber was filled with 1.5 ml of PBS (phenol red free), and the plate was incubated for 1 hr in CO_2 incubator. The diffusion of phenol red across the monolayer was determined by measuring the absorbance of 100 µl of basolateral solution at 558 nm with microplate reader.

3.11.2. Bioavailability studies

3.11.2.1. Saturation concentration of peptides during transport

Reagents

Hank's Balanced Salt Solution (HBSS) pH 7.2-7.4: Used as such (Commercial preparation)

Peptides: Two peptides (C and G) were dissolved in HBSS solution.

Protocol

After assessing the integrity of monolayer using phenol red dye method, washed both apical and basal chambers of transwell plate with 1 ml of HBSS. After washing, added 1.5 ml of HBSS on both apical and basal chambers and incubated for 30 min in CO_2 incubator. Thereafter, except for control well, a portion of HBSS was replaced with respective peptides solution (C or G) such that the final concentration of the solution on apical chamber was 100, 200, 300, 400 and 500 µg/ml in respective wells. At zero min, 300 µl of supernatant was collected from both apical and basal chambers and the plate was again incubated for 60 min in CO_2 incubator. After incubation, the remaining 1.2 ml of supernatant was collected from both apical and basal chambers and stored at -20^0C for further analysis.

3.11.2.2. Saturation time of peptides during transport

Reagents

Hank's Balanced Salt Solution (HBSS) pH 7.2-7.4: Used as such (Commercial preparation)

Peptides: Peptides C (300 µg/ml) and G (400 µg/ml) were dissolved in HBSS solution.

Protocol

After checking the leaking of phenol red through Caco-2 cell monolayer, the wells were washed three times with 1 ml of HBSS on both apical and basal chambers. Each well was incubated with 1.5 ml of HBSS on both apical and basal chambers for 30 min at 37ºC, 5% CO_2. After incubation, a portion of HBSS was replaced with peptide C and peptide G so that the final concentration of peptides was either 300 µg/ml or 400 µg/ml on apical chamber was same in each well. The plates were

incubated for different time period i.e. 0, 5, 15, 30, 60 and 90 min. Supernatants were collected from both apical and basal chambers at the end of different time period and stored at -20^{0}C for further analysis.

3.11.3. Transport route studies

To determine the route for the peptides transport, various inhibitors (Gly-Pro, Cytochalasin D, Wortmannin) were used along with the peptides under the present study.

Reagents

Hank's Balanced Salt Solution (HBSS) pH 7.2-7.4: Used as such (Commercial preparation)

Phosphate buffer solution (PBS; 10 mM, pH 7.4): Autoclaved and 0.22 µm filter sterilised.

Dimethyl sulfoxide (DMSO)

Gly-Pro (20 mM): Dissolved in HBSS

Cytochalasin D (1 µg/ml): Dissolved in DMSO.

Wortmannin (1 µM): Dissolved in DMSO.

Protocol

Before experiment, the integrity of the cell monolayer was analysed using the phenol red test as mentioned above in section 3.11.1. Both apical and basal chambers were washed with 1 ml of PBS (10 mM, pH 7.4) and Incubated with 1.5 ml HBSS for 30 min. After incubation, except for control well, a portion of HBSS on apical chamber was replaced with respective peptides solution (C and G at 300 and 400 µg/ml respectively) along with respective inhibitors (1 µM wortmannin, 10 µM Gly- Pro and 1 µg/ml cytochalsin D) for transepithelial transport studies. The plates were again incubated for 90 min in CO_2 incubator and 300 µl of supernatants were collected from both apical and basal chambers at zero min, while the remaining 1.2 ml of supernatants were collected after 90 min at the end of the experiment and stored at -20^{0}C.

3.11.4. Reverse Phase- High Performance Liquid Chromatography (RP- HPLC)

3.11.4.1. Preparation of samples for RP- HPLC

The supernatants collected from the apical and basal chambers were lyophilized and analyzed by RP-HPLC. All the reagents for RP-HPLC were prepared using HPLC grade water and solvents. Water used for preparation of solvents was membrane filtered (0.22 µm PVDF, Millipore, SA, France) and degassed using Millipore filtration

assembly till complete removal of air bubbles was observed. Then the solvents were tightly capped and sealed with paraffin for carriage and were immediately used.

Reagents

Solvent A: 0.1 % trifluoroacetic acid in water

Solvent B: 0.1 % trifluoroacetic acid in water+ 90 % acetonitrile+ 9.9 % HPLC grade water

3.11.4.2. Preparation of standard curve

Standard curves for peptide C, G and Bradykinnin was prepared by using 25, 50 and 100 µg/ 20 µl respectively. Peptides solutions were prepared by using HPLC grade water.

Operating conditions

System: Waters 32 HPLC system

Pumps: 515 HPLC pump

Software: Millenium32 software

Detector: 2487 dual λ absorbance detector

Column: C18 Spherisorb ®, ODS 4.6 X 250 mm 5 µm, Particle size, 300 Ao pore size

Guard column: ODS2, 10 mm × 1.6 mm

Flow rate used: 1 ml/min

Volume injected: 20 µl

Wave length: 220 and 280 nm

3.11.4.3. HPLC programme

The programme followed for analytical RP-HPLC is given in table 3.5

Table. 3.5. Analytical HPLC programme

S. No.	Time (min)	Solvent A (%)	Solvent B (%)
1	0.01	98.0	2.00
2	30.00	35.0	65.00
3	33.00	0.00	100.0
4	38.00	0.00	100.0
5	40.00	98.0	2.00
6	50.00	100.0	0.00

Protocol

The RP-HPLC column (C18 - Sepharose 10 x 0.64 cm) was washed with Solvent B for 15 minutes and equilibrated in solvent A till base line gets stabilized. Then 20 µl of standard Peptides C, G and Bradykinnin were injected through the injector loop into the column using Hamilton syringe. The detection of peptides was performed on dual wavelength i.e. 220 and 280 nm. The retention time of the standard peptides were recorded. Then the lyophilised samples prepared were applied on analytical RP-HPLC to detect the peaks corresponding to peptide C, G and Bradykinnin standards (Figure 2.1). Qualitative and quantitative determination of peptides in the samples was performed by comparison using standard equation of standard curve.

3.12. Protein estimation by Lowry's method

The concentration of protein in various samples was estimated by using the method of Lowry *et al.* (1951).

Reagents

Solution A (0.5% Copper sulphate): Dissolved 50 mg copper sulphate in 10 ml of distilled water.

Solution B (1.0% Sodium potassium tartrate): Dissolved 100 mg of Sodium potassium tartrate in 10 ml of distilled water.

Solution C (2.0% Sodium carbonate in 0.1 N NaOH): Dissolved 0.2 g of NaOH in 50 ml distilled water. To this 1 g of sodium carbonate was added.

Lowry's Reagent: Prepared by mixing solutions A, B and C in the ratio of 1:1:48.

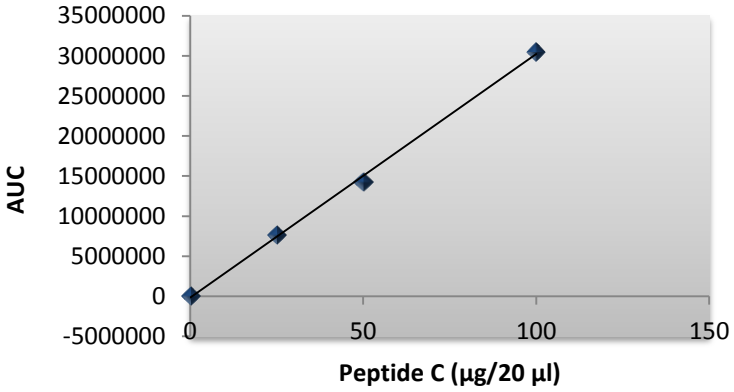
Bovine Serum Albumin (BSA, 5 mg/ml)

Folin Ciocalteu's phenol reagent (2 N)

Procedure

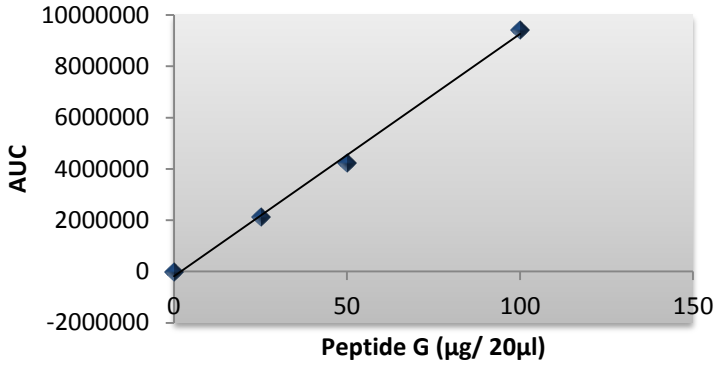
Different aliquots (10-100 µl) of standard BSA solution (5 mg/ml) and samples (10 µl) were added in clean and dry test tubes. The volume of each test tube was made to 100 µl with distilled water. To these, 5 ml of Lowry's reagent was added, mixed well and allowed to stand at room temperature for 15 min. Then 0.5 ml of 2N Folin and Ciocalteu's phenol reagent was added to each test tube, mixed well and kept at 37^0C for 30 min in dark. The absorbance in each tube was read at 630 nm in Specord 200 double beam spectrophotometer (Analytik Jena, Germany) after adjusting the blank zero. The standard curve was plotted between amount of BSA (µg) and its absorbance for estimation of protein in samples.

a)



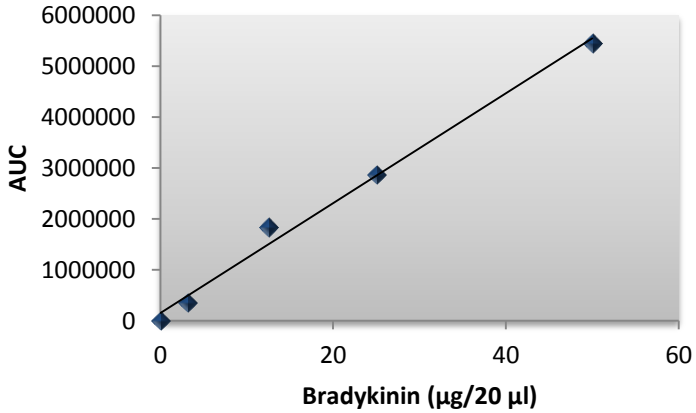
Y=30401x-16859
R^2=0.998

b)



Y=94490x-18257
R^2=0.996

c)



Y = 10786X+15193
R^2 = 0.991

Figure 2.1. Standard curves of peptides by RP-HPLC with C_{18} Coloum

(a) Peptide C
(b) Peptide G
(c) Bradykinin

3.13. Statistical analysis

The results were analyzed using GraphPad Prism (Version 5.01) software. The statistical analysis was done using analysis of variance (ANOVA) and the Tukey test was used to separate the means ($P<0.05$) which were considered statistically significant.

Chapter 4

Results

Results

Ten buffalo casein derived peptides namely A to J were selected in the current analysis to determine their role in the oxidative stress and immunomodulation. This has been performed by screening the peptides initially by estimating their free radical scavenging and immunomodulatory properties by using chemical and *ex vivo* murine model system respectively. After screening, the four selected peptides (B, C, F and G) were further evaluated for their antioxidative property under *in vitro* conditions by using Caco-2 cell lines in the presence of H_2O_2 induced oxidative stress. Whereas, *ex vivo* murine splenocytes were used to assess the immunomodulation potential of selected peptides through estimating cytokine levels in the supernatant of cultured cells. In addition, bioavailability and transport route studies were also conducted for the two peptides (C and G) across the intestinal epithelial membrane using Caco-2 cell monolayer.

4.1 Sequence of casein derived peptides with bioactive potential

In the present study, ten peptides (A to J) were custom synthesised based upon amino acid sequences obtained from buffalo casein (table 4.1). Eight of these peptides (A to G and I) were derived from antioxidative or immunomodulatory peptides available in literature from bovine casein using ClustalW similarity analysis of bovine sequences with buffalo sequences. Wherever the substitution in amino acid was made based upon buffalo sequence is also highlighted by underline as shown in table 4.1.Thesequence of remaining two peptides (H and J) were obtained from buffalo casein itself using trypsin-pepsin and pepsin enzymatic hydrolysis respectively based upon ongoing research work in our laboratory on osteogenic peptides.

4.2. Screening of peptides for overlapping antioxidative and immunomodulatory properties

The selected ten peptides were screened for evaluating their overlapping antioxidative and immunomodulatory properties. Three chemical methods such as ABTS assay, ORAC assay and linoleic acid model system were used to analyse the antioxidative capacity of peptides. On the other hand, *ex-vivo* murine splenocytes and peritoneal macrophages were used to determine their immunomodulatory capacity.

Table 4.1. List of buffalo casein derived peptides used under present study

Peptide	Peptide sequence in literature	Peptide derived frombuffalo casein sequence	Source	Reported activity	Reference
A	VKEAMAPK	VKEAMAPK	Bovine β-cn f(98-105)	Antioxidative	Rival *et al.*, 2001b
B	AVPYPQR	AVPYPQR	Bovine β-cn f(177-183)	Antioxidative	Rival *et al.*, 2001b
C	YFYPEL	YFYPQL	Bovine αS1-cn f(144-149)	Antioxidative	Suetsuna*et al.*, 2000
D	VLPVPEK	VLPVPQK	Bovine β-cn f(170-176)	Antioxidative Bone health promotion	Rival *et al.*, 2001b
E	KVLPVPEK	KVLPVPQK	Bovine β-cn f(169-176)	Antioxidative	Rival *et al.*, 2001b
F	YQQPVLGPVR	YQEPVLGPVR	Bovine β-cn f(193-202)	Immunomodul-Atory	Meisel and Schlimme ,1994.
G	LLY	LLY	Bovine β-cn f(191-193)	Immunomodul-atory	Berthou*et al.*,1987
H	Obtained from buffalo casein by trypsin-pepsin hydrolysis	NAVPITPTL	Bovine α-S2 f(115-123)	Bone health promotion	Reddi*et al.*, 2016 (Laboratory)
I	QEVLNE	QGVLNE	Bovine α-S1 f(28-33)	Anti microbial	Alli*et al.*, 1998
J	Obtained from buffalo casein by pepsin hydrolysis	VLPVP	Bovine β-cn f(170-175)	-	Reddi*et al.*, 2016 (Laboratory)

4.2.1. Oxygen radical absorption capacity (ORAC) assay

ORAC assay measures the oxidative degradation of the fluorescent molecule (fluorescein) after being mixed with free radical generator azo compound such as AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride). Trolox, a water soluble vitamin E analogue was used as standard in the assay and the antioxidative capacity of the each peptide was expressed as trolox equivalents (TEAC) in μM. The results of oxygen radical absorption capacity of peptides are shown in table 4.2. Peptide A showed strongest ($p<0.001$) inhibitory effect on the decay of fluorescence induced by

AAPH (168±1.15 µM TEAC)only at higher concentration such as 1mg/ml than its lower concentrations (10ng to 100µg/ml).In case of peptide B, as compared with lower doses (10ng to 10µg/ml), remarkable ($p<0.01$) radical scavenging property was observed at its higher doses such as 100µg/ml (165.8 ± 4.65 µM TEAC) and 1mg/ml (255.6 ± 10.6 µM TEAC) respectively. Likewise, three peptides (C, F and G) also exhibited strong ($p<0.01$) radical absorption capacity at 100µg/ml and 1mg/ml of their amounts used respectively. Whereas, there remaining five peptides including D, E, H, I and J did not show respective radical scavenging property by the ORAC method on using their different concentrations in this assay.

Table 4.2. Oxygen radical absorption capacity of peptides

Peptide	Peptide sequence	TEAC (µM)					
		Concentration of peptide					
		10 ng/ml	100 ng/ml	1 µg/ml	10 µg/ml	100 µg/ml	1 mg/ml
A	VKEAMAPK	6.05±1.5	9.05±0.2	13±0.2	15.30±0.4	33.80±3.7	168±1.1***
B	AVPYPQR	15.45±0.9	14.40±0.2	7.25±1.0	27.7±2.4	165.8±4.6**	255±10.6***
C	YFYPQL	7.35±0.0	14.25±0.1	18.85±0.1	43.85±1.4	155±12.8***	215±10.3***
D	VLPVPQK	10.90±0.0	11.25±0.6	8.40±0.2	9.90±0.4	10.25±0.3	14.80±0.5
E	KVLPVPQK	8.55±0.2	8.55±0.0	16.60±0.5	19.20±0.5	17.70±0.1	18.20±0.7
F	YQEPVLGP-VR	11.45±0.4	10.80±0.0	11.60±1.0	21.4±1.7	83±7.7**	229±11.8***
G	LLY	15.05±1.3	15.65±4.3	12.80±0.5	53.7±10.1	199±3.9***	242±18.2***
H	NAVPITPTL	6.65±0.6	8.65±0.6	5.85±1.8	4.35±0.3	5.20±1.5	12.8±2.8
I	QGVLNE	6.5±0.8	5.35±0.6	7.85±0.1	7.35±1.6	7.65±0.3	13.8±3.1
J	VLPVP	18.15±1.1	19±1.3	21.6±0.6	26.4±0.8	32±1.0	51.7±0.5

Values are mean ± SEM (n=3). Asterisks representing the significance difference (p**<0.01, p***<0.001)

4.2.2. ABTS free radial scavenging assay

ABTS method also measures the relative ability of peptides to scavenge ABTS free radicals generated by reacting with a strong oxidizing agent such as potassium persulfate. In this method trolox was used as standard and results were expressed as trolox equivalents (TEAC) in µM. The results of ABTS free radical scavenging

assay are shown in table 4.3. In this assay, peptides B and C showed an efficient ($p<0.01$) ABTS radical scavenging property respectively in a concentration dependent manner. In case of peptide F and G, much higher effects ($p<0.001$) were observed at higher concentrations (100µg/ml and 1mg/ml) respectively than their lesser amounts (10ng to 10 µg/ml)used. Peptide J also showed intermediate type of radical scavenging activity ranging from 127-157.5µM TEAC irrespective of its amount used. The remaining five peptides (A, D, E, H and I) showed no significant radical scavenging property by ABTS method.

Table 4.3. ABTS radical scavenging property of peptides

TEAC (µM)							
Peptide	**Peptide Sequence**	**Concentration of peptide**					
		10 ng/ml	**100 ng/ml**	**1 µg/ml**	**10 µg/ml**	**100 µg/ml**	**1 mg/ml**
A	VKEAMAPK	2.50±0.5	3±0.00	9.50±0.5	16±1.0	20.50±0.5	26±1.0
B	AVPYPQR	9.68±2.0	15.16±2.2	53.3±4.3	90.6±6.6*	164.8±5.2**	393±7.7***
C	YFYPQL	18.50±3.5	44.5±1.5	95±5.0	119±3.0**	197±10.0***	435±2.5***
D	VLPVPQK	17.0±3.0	19.5±0.5	37±7.0	35±5.0	42±7.0	56±0.0
E	KVLPVPQK	7.05±1.9	13.6±3.4	21.2±0.8	38.00±2.0	36.3±0.6	55.5±4.5
F	YQEPVLGP-VR	59±16.0	57±0.0	75±1.5	90±6.0	277±3.0***	399±4.5***
G	LLY	36.50±7.5	46±1.0	47.5±11.5	59±13.0	294±6.5***	421±3.0***
H	NAVPITPTL	13±0.0	14±0.0	15±3.0	17±3.0	24.50±4.5	22.0±2.0
I	QGVLNE	18±4.0	22.50±0.5	32±5.0	37.5±1.5	42.50±3.5	56.5±0.5
J	VLPVP	127.0±2.0	128.5±1.5	128±6.0	128±3.0	141±1.0	157.5±7.5

Values are mean ± SEM (n =3). Asterisks representing the significance difference (p**<0.01, p***<0.001)

4.2.3. Linoleic acid based oxidation model system

By using linoleic acid model system, the inhibitory capacity of peptides towards linoleic acid auto-oxidation was investigated. A rapid increase in the auto-oxidation of linoleic acid was observed in the control where no peptide was added and therefore its oxidation reached a very high value after 140 hr (table 4.4). The addition of peptide A at the concentration of 100µg/ml was found to exhibit extraordinarily

($p<0.01$) inhibitory activity (62.13%) on linoleic acid oxidation after 140 hr of incubation as compared to control. Likewise, significant ($p<0.05$) inhibitory response (43.98%)was also observed during the presence of peptide B. Peptides D and E on using 100 µg/ml also showed reduction in oxidation of linoleic acid by 36.61% and 13.6% respectively after 140 hr of treatment.

Table 4.4. Inhibitory effect of peptides on linoleic acid oxidation

Absorbance at 500 nm									
Peptide	**Peptide Sequence**	**Time (hr)**							
		0	**20**	**40**	**60**	**80**	**100**	**120**	**140**
	Control	0.111± 0.001	0.116± 0.003	0.134± 0.001	0.310± 0.002	0.549± 0.004	0.728± 0.001	0.769± 0.003	0.852± 0.004
A	VKEAMAPK	0.104± 0.000	0.087± 0.024	0.112± 0.012	0.150± 0.035	0.165± 0.032	0.229± 0.029	0.251± 0.039	0.300± 0.030***
B	AVPYPQR	0.109± 0.002	0.107± 0.005	0.117± 0.017	0.162± 0.036	0.162± 0.036	0.222± 0.024	0.255± 0.035	0.333± 0.021***
C	YFYPQL	0.114± 0.001	0.101± 0.014	0.139± 0.005	0.244± 0.044	0.508± 0.052	0.623± 0.055	0.694± 0.036	0.709±0 .051
D	VLPVPQK	0.107± 0.002	0.111± 0.001	0.118± 0.018	0.203± 0.045	0.297± 0.039	0.422± 0.054	0.502± 0.048	0.540± 0.052**
E	KVLPVPQK	0.107± 0.002	0.072± 0.027	0.116± 0.016	0.250± 0.024	0.511± 0.022	0.631± 0.053	0.699± 0.041	0.736± 0.047**
F	YQEPVLG-PVR	0.107± 0.002	0.116± 0.003	0.133± 0.001	0.305± 0.002	0.535± 0.001	0.635± 0.001	0.737± 0.003	0.785±0 .002
G	LLY	0.107± 0.002	0.084± 0.028	0.111± 0.022	0.258± 0.047	0.498± 0.042	0.647± 0.047	0.699± 0.041	0.750±0 .039
H	NAVPITPTL	0.108± 0.002	0.084± 0.028	0.125± 0.015	0.201± 0.050	0.503± 0.028	0.665± 0.044	0.697± 0.048	0.734±0 .054
I	QGVLNE	0.108± 0.000	0.101± 0.102	0.128± 0.010	0.260± 0.046	0.501± 0.049	0.687± 0.019	0.719± 0.030	0.741± 0.052
J	VLPVP	0.109± 0.001	0.089± 0.028	0.116± 0.018	0.283± 0.027	0.505± 0.047	0.685± 0.031	0.698± 0.052	0.741± 0.052

Values are mean ± SEM (n =3). Asterisks representing the significance difference (p**<0.01, p***<0.001)

While, in case of remaining six peptides (C, F, G, H, I and J) little inhibitory effect (11.1%, 8.87%, 7.8%, 8.5%, 7% and 7.63% respectively) was observed on linoleic acid oxidation than control where no peptide was incubated with linoleic acid.

4.2.4. Splenocytes proliferation index (SPI)

In order to assess the role of peptides in immunomodulation, *ex-vivo* murine model was used to estimate the effect of peptides on splenocytes proliferation.

Table 4.5. Effect of peptides on splenocytes proliferation

Splenocytes proliferation index (SPI)							
Peptide	**Peptide Sequence**	**Concentration of peptide**					
		10 ng/ml	**100 ng/ml**	**1 µg/ml**	**10 µg/ml**	**100 µg/ml**	**1 mg/ml**
Negative control		**- 1.00±0.00[a]**					
Positive control-1.70±0.019[c]							
A	VKEAMAPK	1.00± 0.05[a]	1.05± 0.11[a]	1.0± 0.02[a]	1.12± 0.02[a]	1.16± 0.07[a]	1.14± 0.07[a]
B	AVPYPQR	0.607± 0.02[b]	0.678± 0.01[b]	0.733± 0.03[b]	0.846± 0.02[a]	0.892± 0.04[a]	0.860± 0.06[a]
C	YFYPQL	0.600± 0.04[b]	0.645± 0.07[b]	0.672± 0.06[b]	0.845± 0.07[a]	1.13± 0.05[a]	1.25± 0.06[a]
D	VLPVPQK	0.625± 0.008[b]	0.729± 0.05[b]	0.708± 0.04[b]	0.844± 0.08[a]	1.07± 0.04[a]	1.12± 0.04[a]
E	KVLPVPQK	0.811± 0.08[a]	0.924± 0.02[a]	0.874± 0.04[a]	0.979± 0.008[a]	1.008± 0.03[a]	1.17± 0.0[a]
F	YQEPVLG-PVR	0.499± 0.006[b]	0.513± 0.006[b]	0.575± 0.031[b]	0.645± 0.032[b]	0.703± 0.023[b]	0.616± 0.027[b]
G	LLY	0.521± 0.05[b]	0.548± 0.02[b]	0.573± 0.02[b]	0.716± 0.01[b]	0.778± 0.05[b]	0.692± 0.09[b]
H	NAVPITPTL	0.423± 0.04[b]	0.501± 0.008[b]	0.519± 0.005[b]	0.527± 0.01[b]	0.687± 0.01[b]	0.747± 0.03[b]
I	QGVLNE	0.474± 0.03[b]	0.521± 0.02[b]	0.525± 0.01[b]	0.522± 0.01[b]	0.674± 0.007[b]	0.706± 0.023[b]
J	VLPVP	1.023± 0.07[a]	0.881± 0.02[a]	0.923± 0.04[a]	1.092± 0.06[a]	0.947± 0.018[a]	1.0± 0.06[a]

Values are mean ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

Spleen is the major site of immune responses to the antigens because of its key role in the formation of B and T-lymphocytes involved in the innate and adaptive immune systems of the body. Effect of peptides on the proliferation of cultured splenocytes is shown in table 4.5. As compared to negative control, addition of ConA (5 µg/ml), a T-cell mitogen remarkably ($p<0.001$) stimulated the proliferation of splenocytes in the positive control. Whereas, the incubation of spleen cells with peptides B,C or D at their lower concentrations ranging from 10 ng to 1 µg/ml were found to considerably

suppress ($p<0.01$) the proliferation as compared to their higher amounts used as well as negative control. Similarly, four peptides (F, G, H and I) remarkably ($p<0.01$) suppressed the splenocytes proliferation index regardless of their amount (10 ng to 1 mg/ml) used. On the other hand, peptides A, E and J did not show any changes in the splenocytes proliferation index.

4.2.5. Phagocytosis

Similar to splenocytes proliferation, estimation of phagocytic activity by murine peritoneal macrophages is an important parameter of immunomodulation which is an important innate immune response for the destruction of pathogens by a type of white blood cells. The results of impact of casein derived peptides on phagocytosis by peritoneal macrophages are shown in table 4.6. Addition of peptides B, F or I at 100 µg/ml and 1 mg/ml of respective doses were found to remarkably ($p<0.01$) enhance the percent phagocytosis of yeast cells by murine peritoneal macrophages as compared to control. While peptide H was noticed to show this effect at an additional dose of 10 µg/ml also. Likewise, enhanced phagocytosis was also shown ($p<0.01$) by G peptide on addition of 100 ng to 1 mg/ml in cultured macrophages but, peptide C brought remarkable ($p<0.01$) effect only at the dose of 1 mg/ml. The remaining four peptides (A, D, E and J) did not have any effect on phagocytosis as compared to control. Representative photographs of macrophages showing engulfed yeast cells are shown plate 4.2.

The comparative visualisation of peptides with antioxidative and immunomodulatory properties for final selection is shown by heat plot in plate 4.1. It was prepared by in-house scripts in our software. Based upon the above screening, four peptides (B, C, F and G) having overlapping activities of free radical scavenging and immunomodulation were finally selected for their further cellular evaluation.

Table 4.6. Effect of peptides on phagocytosis of murine peritoneal macrophages

% Phagocytosis							
Peptide	**Peptide Sequence**	**Concentration of peptide**					
		10 ng/ml	**100 ng/ml**	**1 µg/ml**	**10 µg/ml**	**100 µg/ml**	**1 mg/ml**
Control	44[a]						
A	VKEAMAPK	36.5±1.5[a]	40.5±1.5[a]	41.5±3.5[a]	44.5±2.5[a]	46±3.0[a]	45.0±0.5[a]
B	AVPYPQR	44.5±0.5[a]	46.5±0.5[a]	45.0±1.0[a]	50.0±1.0[a]	53.5±1.5[b]	60.5±1.5[b]
C	YFYPQL	46.5±1.5[a]	47.5±1.5[a]	48.0±1.0[a]	48.0±0.0[a]	48.5±0.5[a]	56.0±1.0[b]
D	VLPVPQK	37±1.0[a]	37±2.0[a]	36±2.0[a]	41.5±3.5[a]	48±2.0[a]	50±4.0[a]
E	KVLPVPQK	47.5±1.5[a]	48.5±0.5[a]	52±4.0[a]	55.5±3.5[a]	53±2.0[a]	57.5±1.5[a]
F	YQEPVLGP-VR	52.5±2.5[a]	44.5±1.5[a]	50.5±0.5[a]	53.5±1.5[a]	66.5±0.5[b]	73.5±2.5[b]
G	LLY	52±3.0[a]	62±3.0[b]	71.5±2.5[b]	67.5±1.5[b]	75±3.0[b]	77.5±1.5[b]
H	NAVPITPTL	49.5±1.5[a]	49.5±2.5[a]	48.5±1.5[a]	56.5±2.5[b]	68.5±2.5[b]	71±1.0[b]
I	QGVLNE	38.0±1.0[a]	40.5±2.5[a]	50.5±1.5[a]	53±2.0[a]	56.5±1.5[b]	74±2.0[b]
J	VLPVP	48.5±2.5[a]	51±3.0[a]	45±1.0[a]	47±2.0[a]	45.5±1.5[a]	47.5±1.5[a]

Values are mean ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

4.3. Assessment of cellular response of potential casein peptides *in vitro*

The human colorectal adenocarcinoma cell line, Caco-2 was used for the purpose of *in vitro* studies, this cell line was first developed by the Sloan-Kettering Institute for Cancer Research through research conducted by Dr. Jorgen Fogh (Fogh and Trempe, 1975). When cultured under specific conditions the Caco-2 cells become differentiated and polarized such that their phenotype, morphologically and functionally resembles the enterocytes lining the small intestine. Plate 4.3 represents the images of Caco-2 cells on different days of culture. Antioxidative potential of the above four selected casein peptides (B, C, F and G) was determined in Caco-2 cells against H_2O_2 induced oxidative stress.

4.3.1. Cytotoxicity of peptides

Initially, the cytotoxic effect of the selected peptides (B, C, F and G) on Caco-2 cell viability was determined by using MTT, neutral red and LDH assays respectively.

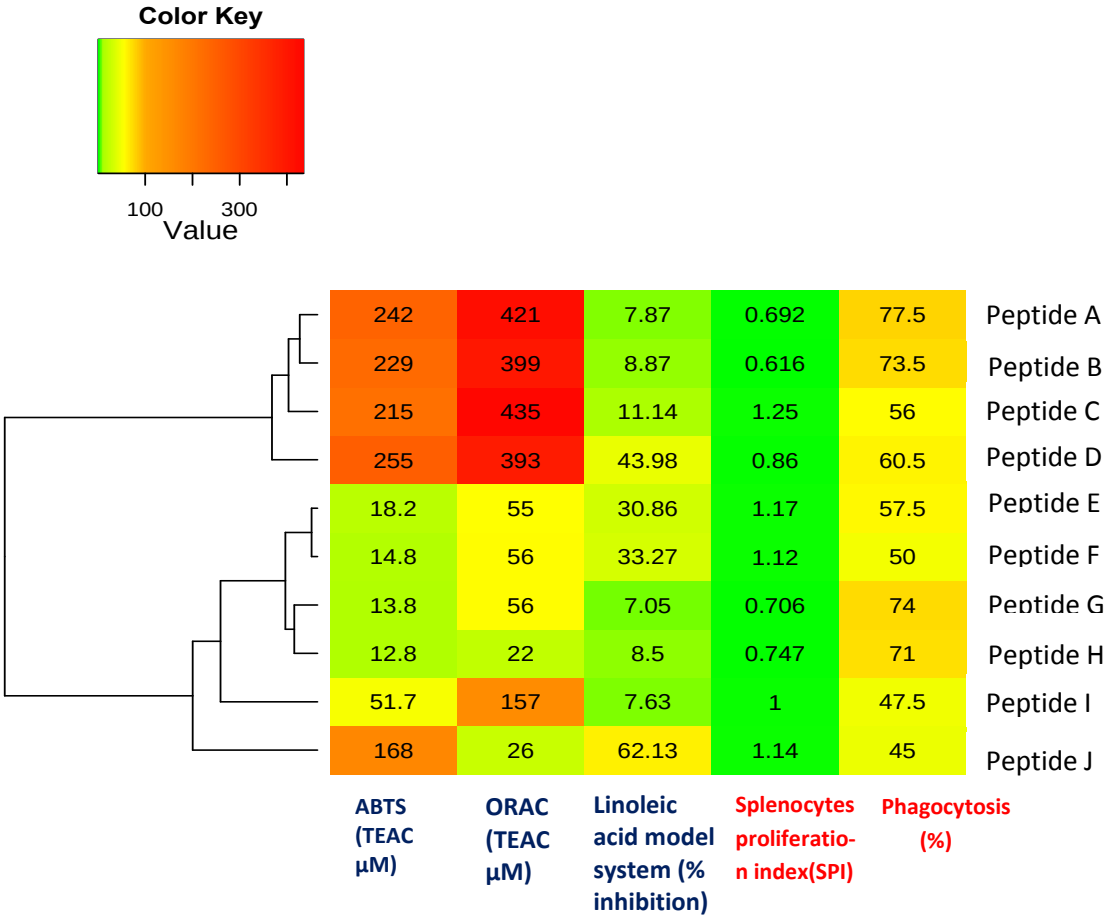
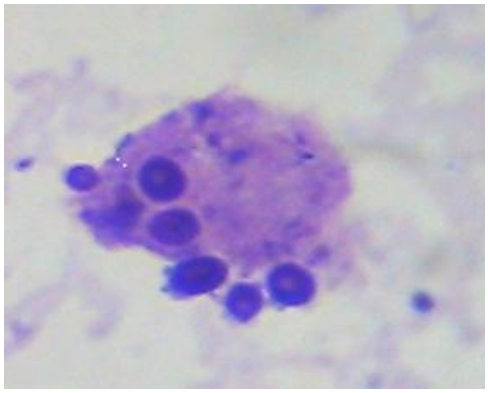


Plate 4.1. Heat plot depicting comparative evaluation of antioxidative and immunomodulatory potential of peptides.



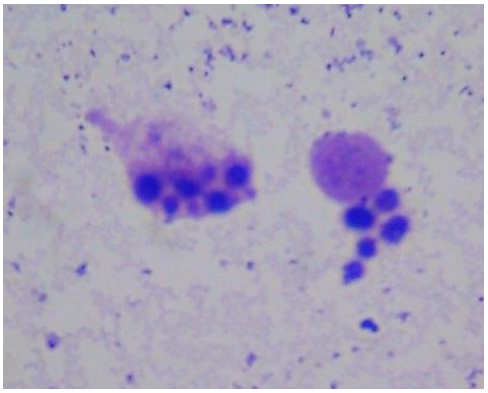


Plate 4.2. Representative photographs macrophages (1000X) showing adhered and engulfed yeast cells

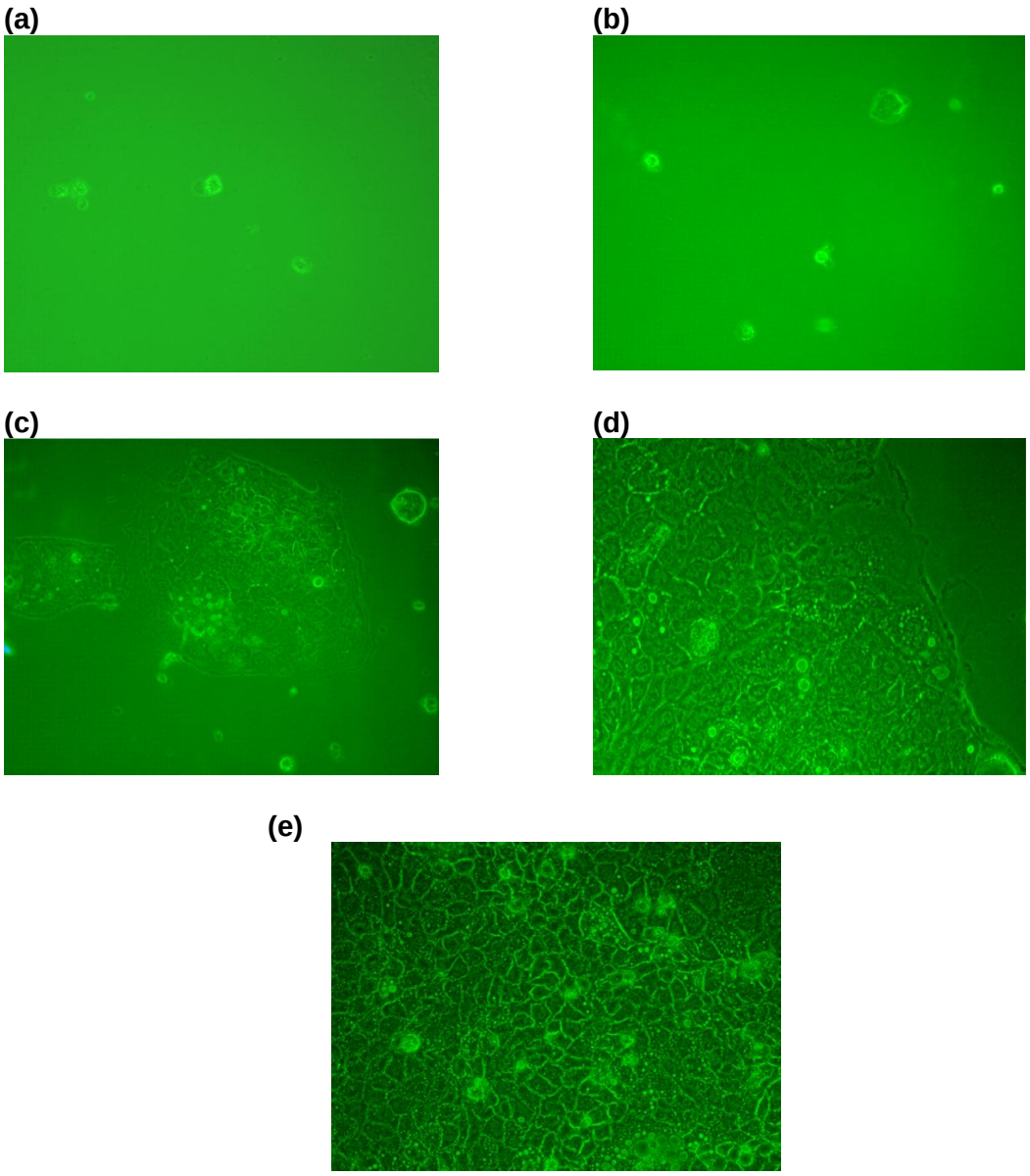


Plate 4.3. Representative photographs of Caco-2 cells under phase contrast inverted microscope (400X) on different days of culture
a: Day 1
b: Day 2
c: Day 3
f: Day 5
e: Day 7 Fully confluent cell monolayer

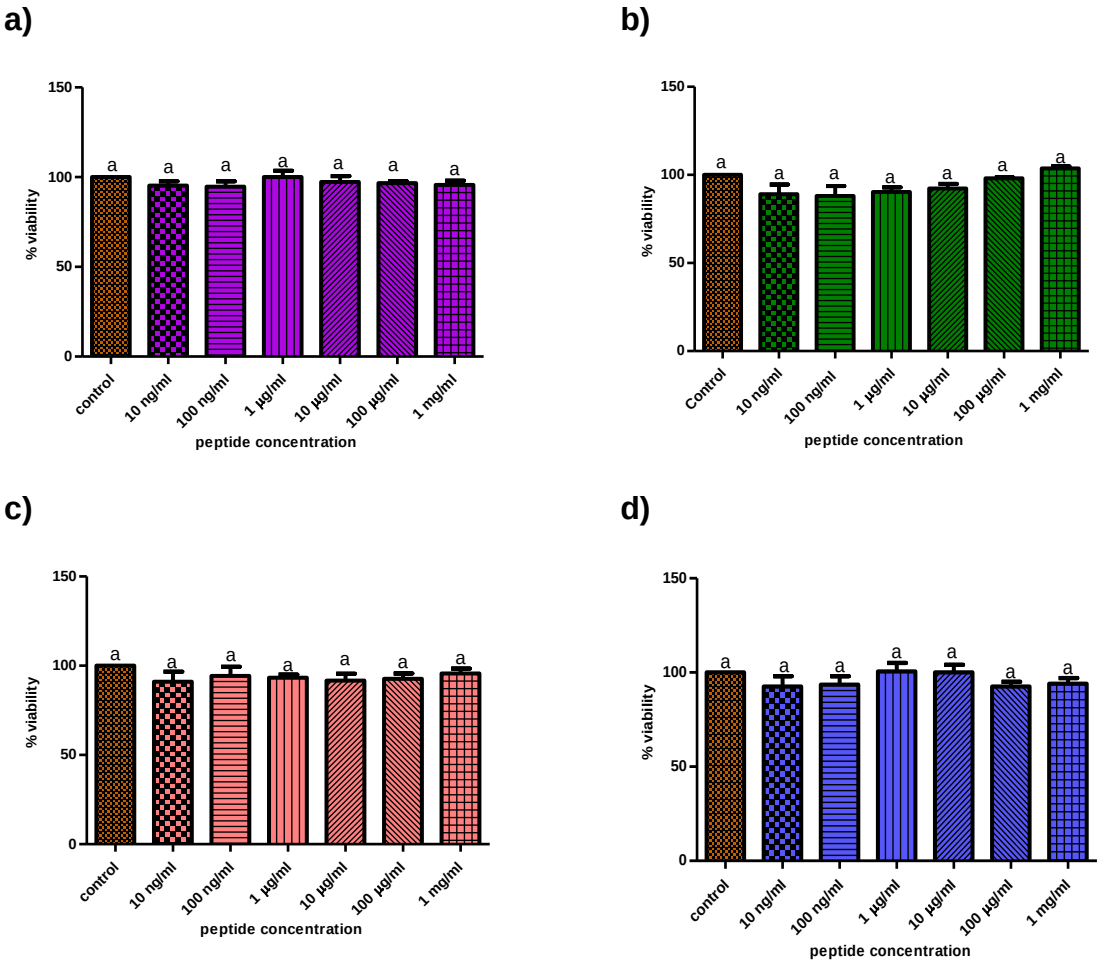


Figure 4.1. Cytotoxic effect of casein derived peptides on cell viability by MTT assay
a: Peptide B
b: Peptide C
c: Peptide F
d: Peptide G
The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

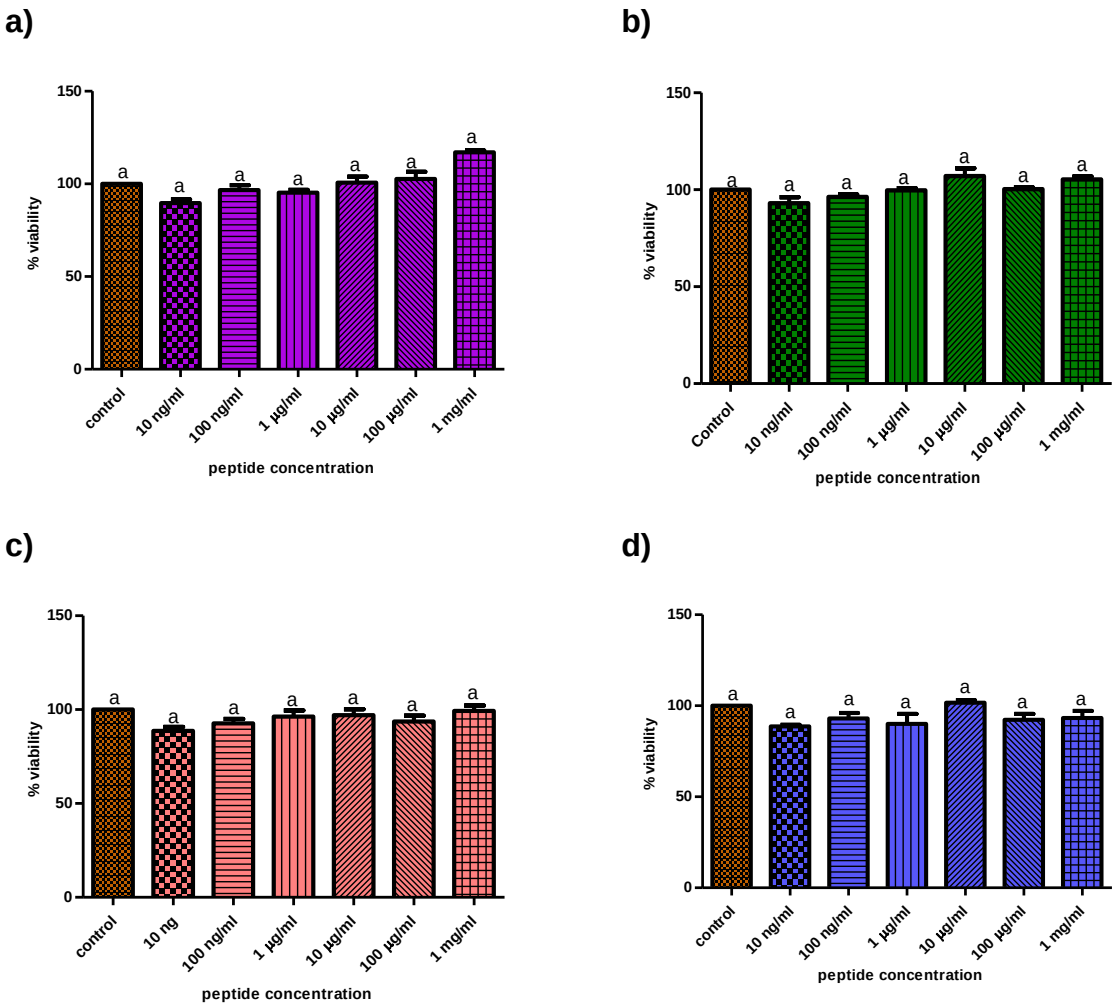


Figure 4.2. Cytotoxic effect of casein derived peptides on cell viability by neutral red assay

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

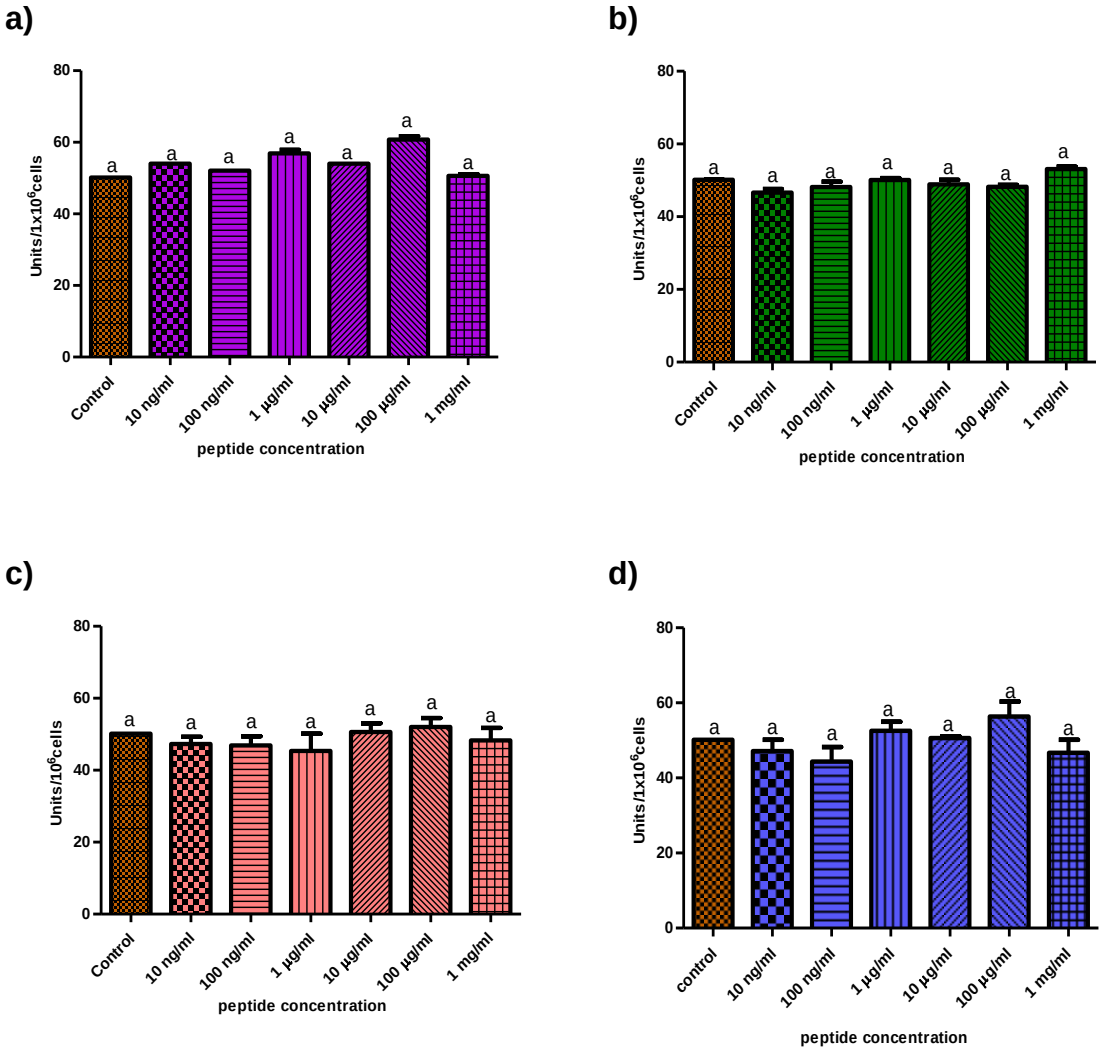


Figure 4.3. Cytotoxic effect of casein derived peptides on cell viability by LDH assay

a: Peptide B
b: Peptide C
c: Peptide F
d: Peptide G

The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

Cells supplemented individually with peptides B, C, F and G at their respective doses ranging from 10 ng to1 mg/ml for 24 hr did not affect cell viability than control where no peptide was added as determined by MTT assay (figure 4.1). Similar results were also confirmed by neutral red assay (figure 4.2) and LDH enzyme assay (figure 4.3). Based upon the above three cytotoxic assays, it was concluded that the presence of casein peptides at different doses in cultured cells had no toxic effects on the viability and membrane integrity of Caco-2 cells. Therefore, various concentrations of peptides ranged from 10 ng to 1 mg/ml are safe to be used in further studies.

4.3.2. H_2O_2 induced oxidative stress model in Caco-2 cells

Caco-2 cells were exposed to different concentrations of H_2O_2 ranging from 0.2 mM to 4 mM for 10 hr and the cell viability was determined by using MTT assay. As depicted in figure 4.4 and plate4.4, a significant ($p<0.05$) decrease in the cell viability was initiated at the minimum dose of 0.3mM, after that a concentration dependent decline was observed in the viabilityof Caco-2 cells as compared to control.It was observed that the addition of 1.5 mM H_2O_2,considerably ($p<0.01$) decreased the cell survivability to about 50% and there was a gradual reduction in the survivability to 27% on addition of 4mM H_2O_2.Based upon these results, 1.5 mM H_2O_2 concentration (IC_{50}) was selected to induce the oxidative stress in Caco-2 cells for further experiments.

4.3.3. Protective effect of peptides on cell viability against H_2O_2 induced stress

The results of protective effect of peptides on cell viability against H_2O_2 induced stress by MTT assay are shown in figure 4.5. It was observed that cells pre-incubated with the peptidesC and G at respective higher concentrations such as 100 µg/ml and 1 mg/ml were found to remarkably ($p<0.01$) increase the cell viability as compared to H_2O_2 control (figure 4.5b and d).The pictorial presentation of protective effect by peptide C and G were also shown on Caco-2 cells morphology at plate 4.5 and 4.6. While no such protective effect wasdetectedat their lower doses ranging from 10 ng to 10 µg/ml.On the other hand peptides B and F did not show increased cell viability significantly against H_2O_2 induced oxidative stress regardless of their respective amounts used in cell culture (figure 4.5a and c).

4.3.4. Inhibitory effect of peptides on ROS generation

In order to further confirm the protective effect of four selected peptides (B,C,F and G) against H_2O_2 induced oxidative stress, the inhibition of ROS generation was also evaluated by DCFH-DA (2',7'-dichlorfluorescein-diacetate) fluorescent dye.

Theresults are depicted in figure 4.6.DCFH-DA is a well-established compound to detect and quantify the intracellular ROS. As compared to negative control (NC), addition of H_2O_2 remarkably($p<0.001$) increasedtheROS production(2.51 fold)in the Caco-2 cells due to oxidative stressgeneration. While, 24 hr pre-treatment of cells with the peptides B and F at respective higher doses (1 µg to 1 mg/ml) were found to significantly reduce ($p<0.01$) the increased ROS levels as compared to H_2O_2 control (figure 4.6a and c).Likewise, such inhibitory response ($p<0.01$) was also observed by the addition of peptidesC and G on addition of respective amounts ranged from 10 µg to 1 mg/ml (figure 4.6b and d).

4.3.5. Effect of peptides on H_2O_2 induced oxidative products

A. Malondialdehyde (MDA)

Exposure of Caco-2 cells to IC_{50} concentration of H_2O_2 (1.5 mM) for 10 hr remarkably ($p<0.001$) increased the MDA levels (1.72 fold) as compared to negative control (NC). While, the pre-treatment of cells with peptide B considerably ($p<0.01$) reduced the elevated MDA levels induced by H_2O_2 in a concentration dependent manner (figure 4.7a). Likewise, the addition of peptides C, F or G were also detected to suppress ($p<0.01$) the formation of MDA, irrespective of their dose used (10 ng to 1 mg/ml) as compared to H_2O_2 control (figure 4.7b-d).

B. Protein carbonyl content

Addition of H_2O_2 to the Caco-2 cells significantly ($p<0.001$) increased the protein carbonyl levels (5.07 fold) due to oxidative stress generation as compared to negative control (NC) (figure 4.8). Whereas, cells pre-supplemented with the three peptides (B,F or G) were observed to decrease ($p<0.05$) the protein carbonyl content as compared to H_2O_2 control regardless of their amount used (10 ng to 1 mg/ml) (figure 4.8a,c and d). However, in case of peptide C, a significant ($p<0.05$) decrease in protein carbonyl levels was observed on using 1 mg/ml of peptide than H_2O_2 control (figure 4.8b).

C. Nitric oxide

Nitric oxide, an important molecule involved in the formation of highly reactive peroxynitrite ($ONOO^-$) by reacting with super oxide radicals (O_2^-) during oxidative stress.In current analysis, no significant changes were observed in the levels of nitric oxide in the culture supernatant after addition of H_2O_2 as compared to negative control.Likewise, treatment of cells with any ofthefour selectedpeptides (B,C,F or

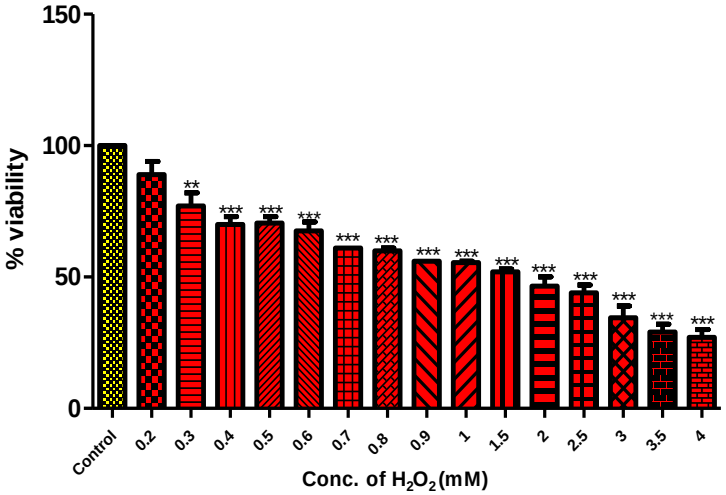


Figure 4.4. Standardisation of H_2O_2 level (IC_{50}) for inducing oxidative stress Values are expressed as means ± SEM (n=3). Asterisks representing the significance difference (p**<0.01, p***<0.001)

(a) **(b)**

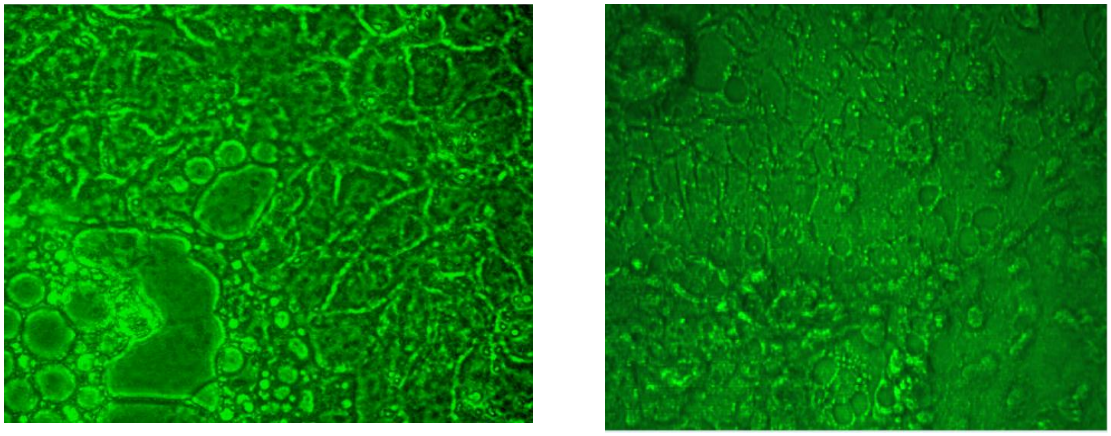


Plate 4.4. Representative photographs showing H_2O_2 induced changes in morphology of Caco-2 cells under phase contrast inverted microscope (400X).
a: Cells without H_2O_2
b: Cells with H_2O_2

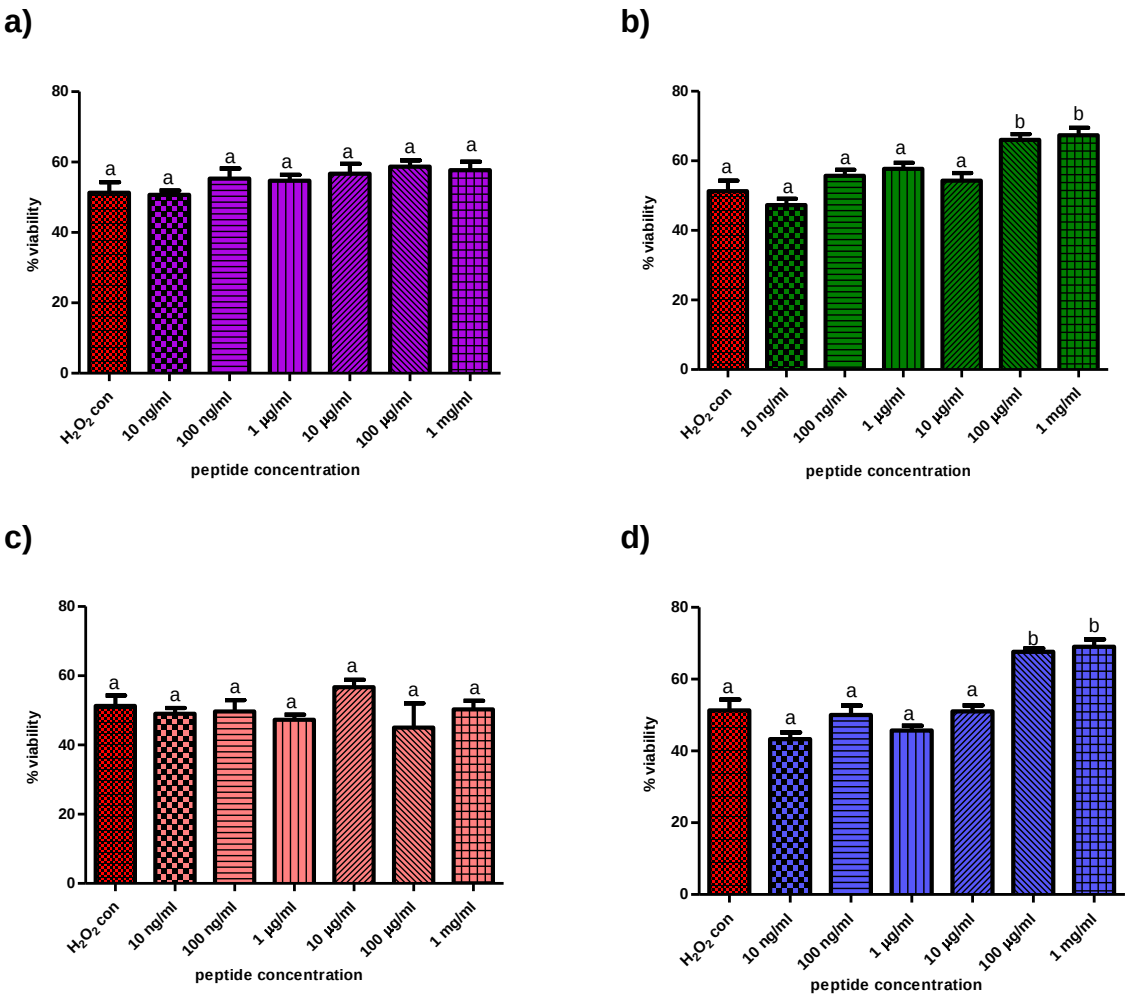


Figure 4.5. Protective effect of peptides on cell viability by MTT assay under H_2O_2 induced oxidative stress

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p< 0.05$).

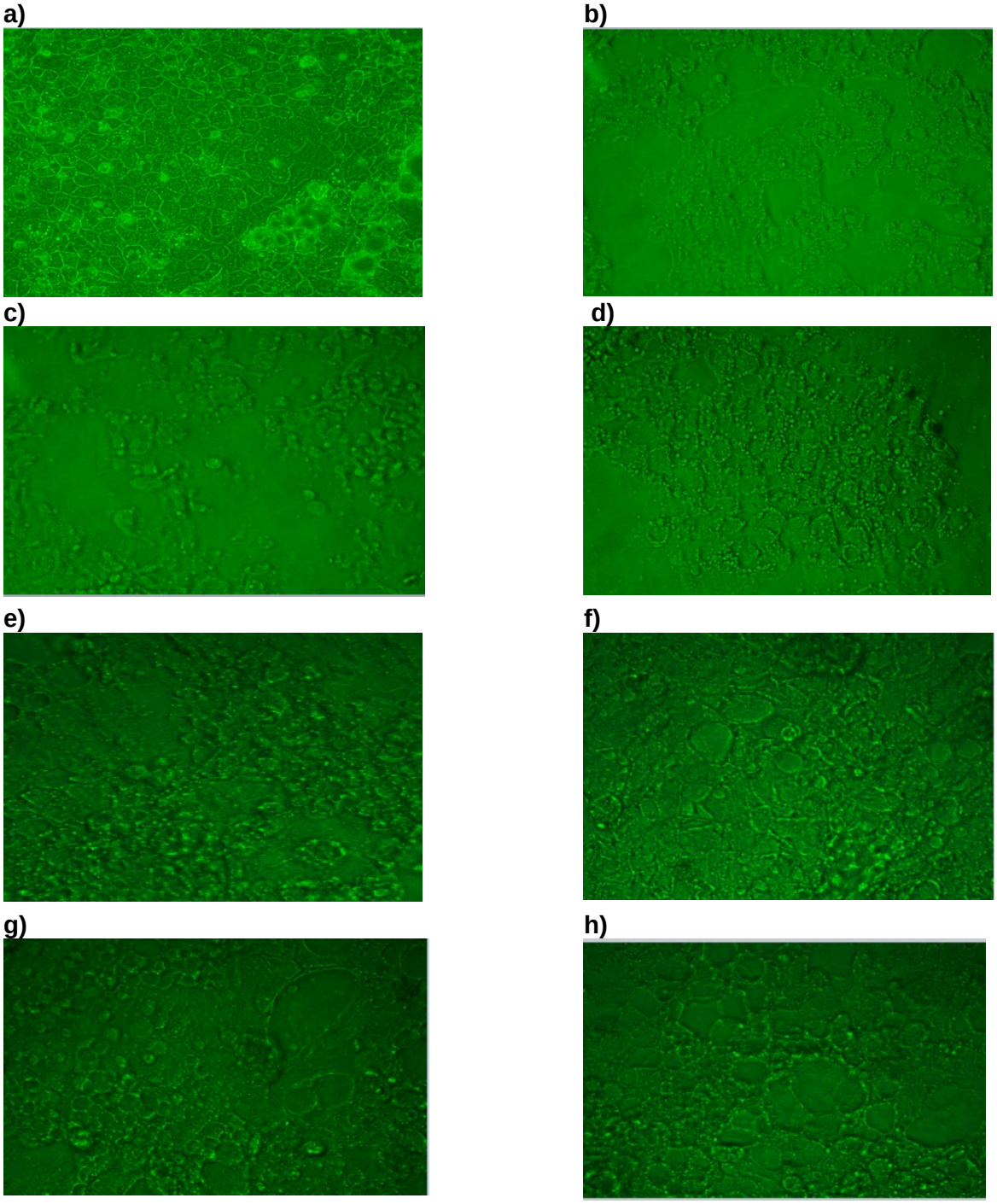


Plate 4.5. Pictorial presentation depicting the effect of peptide C on the morphology of viable Caco-2 cells under phase contrast microscope (400X)

a: Negative control without peptide and H_2O_2

b: H_2O_2 control with 1.5mM H_2O_2 without peptide

c: 10 ng/ml of peptide and 1.5mM H_2O_2

d: 100 ng/ml of peptide and 1.5mM H_2O_2

e: 1 µg/ml of peptide and 1.5mM H_2O_2

f: 10 µg/ml of peptide and1.5mM H_2O_2

g: 100 µg/ml of peptide and 1.5mM H_2O_2

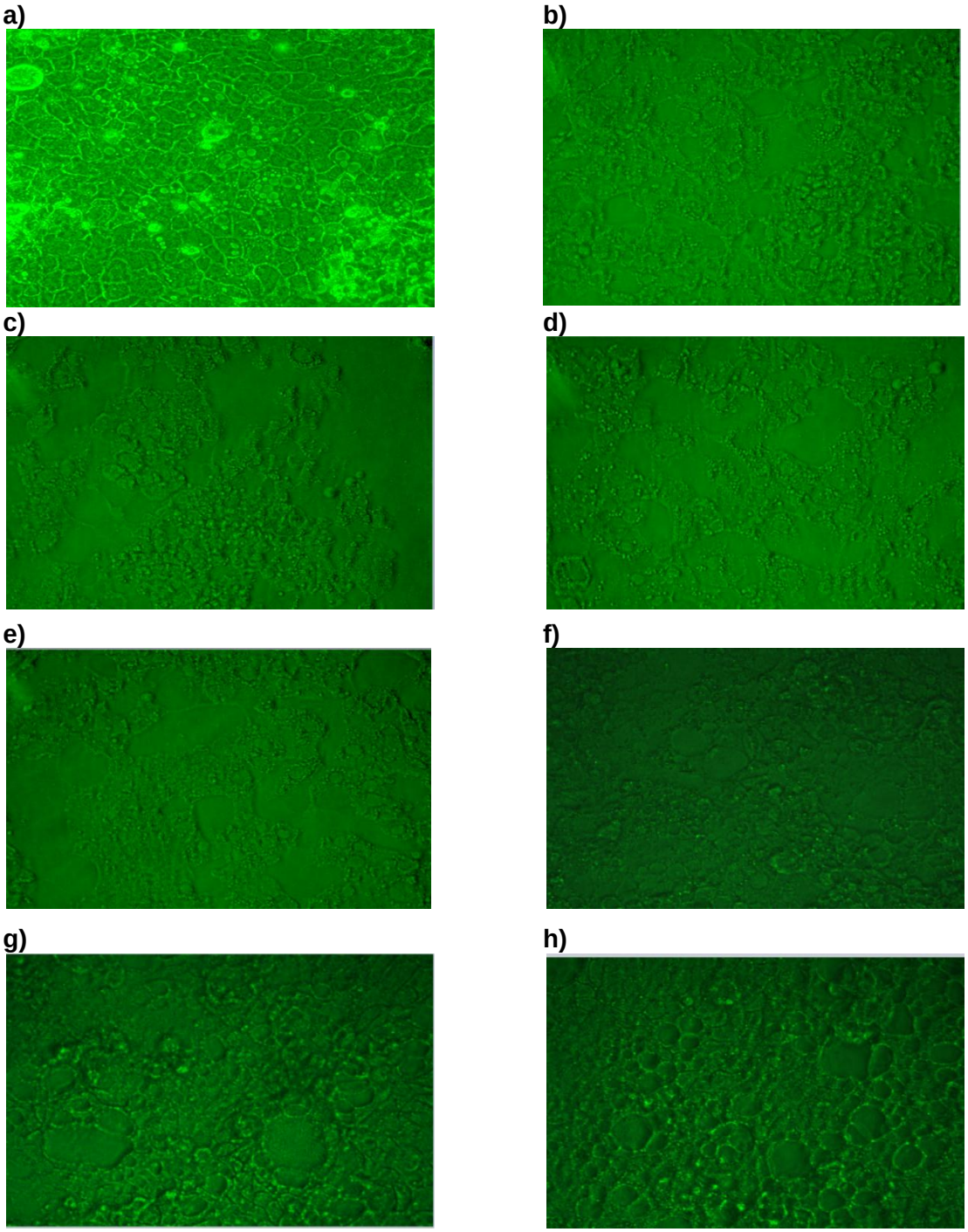


Plate 4.6. Pictorial presentation depicting the effect of peptide G on the morphology of viable Caco-2 cells under phase contrast microscope (400X)
a: Negative control without peptide and H_2O_2
b: H_2O_2 control with 1.5mM H_2O_2 without peptide
c: 10 ng/ml of peptide and 1.5mM H_2O_2
d: 100 ng/ml of peptide and 1.5mM H_2O_2
e: 1 µg/ml of peptide and 1.5mM H_2O_2
f: 10 µg/ml of peptide and1.5mM H_2O_2
g: 100 µg/ml of peptide and 1.5mM H_2O_2

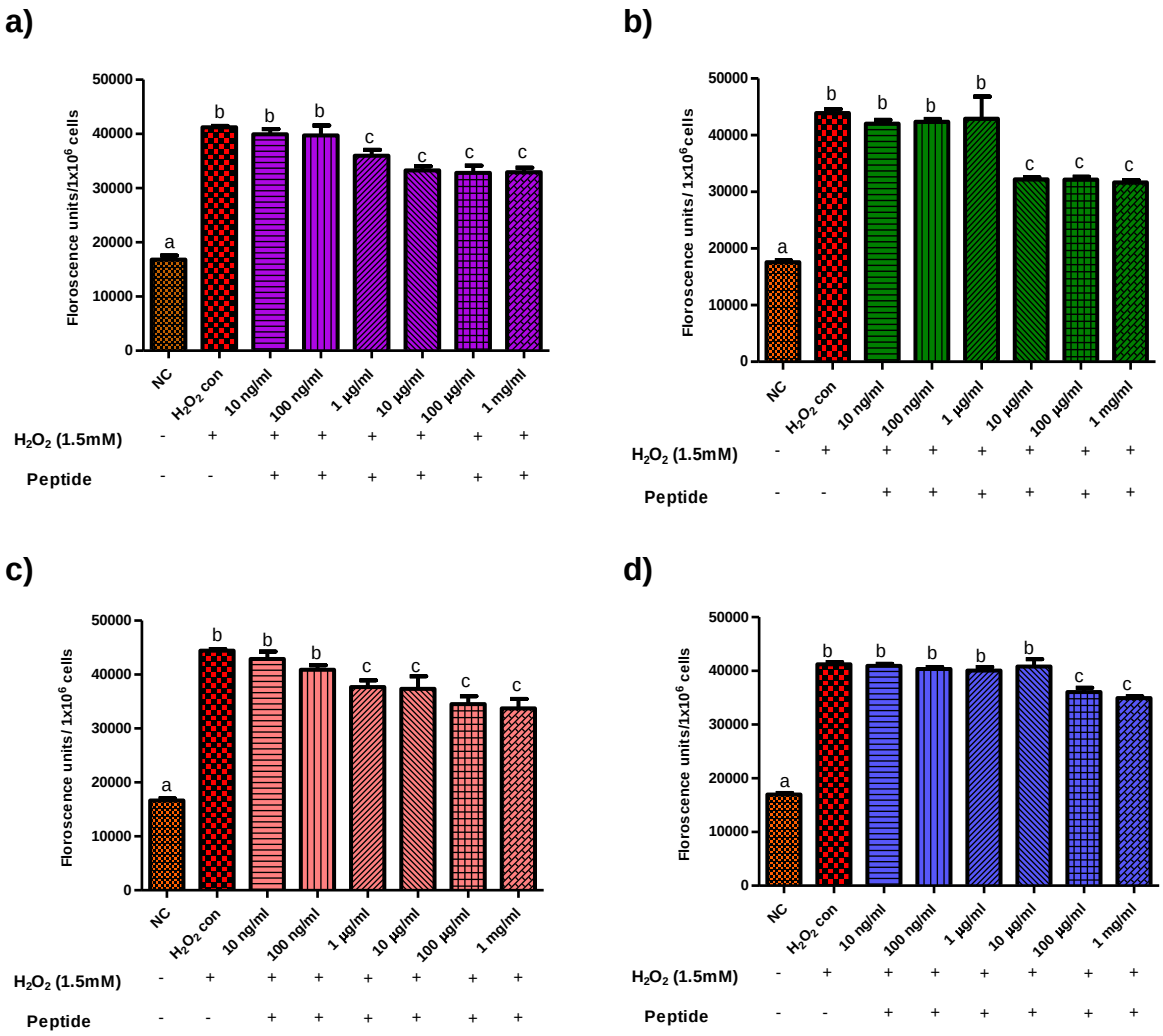


Figure 4.6. Inhibitory effect of peptides on ROS generation

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

NC: Negative control. The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

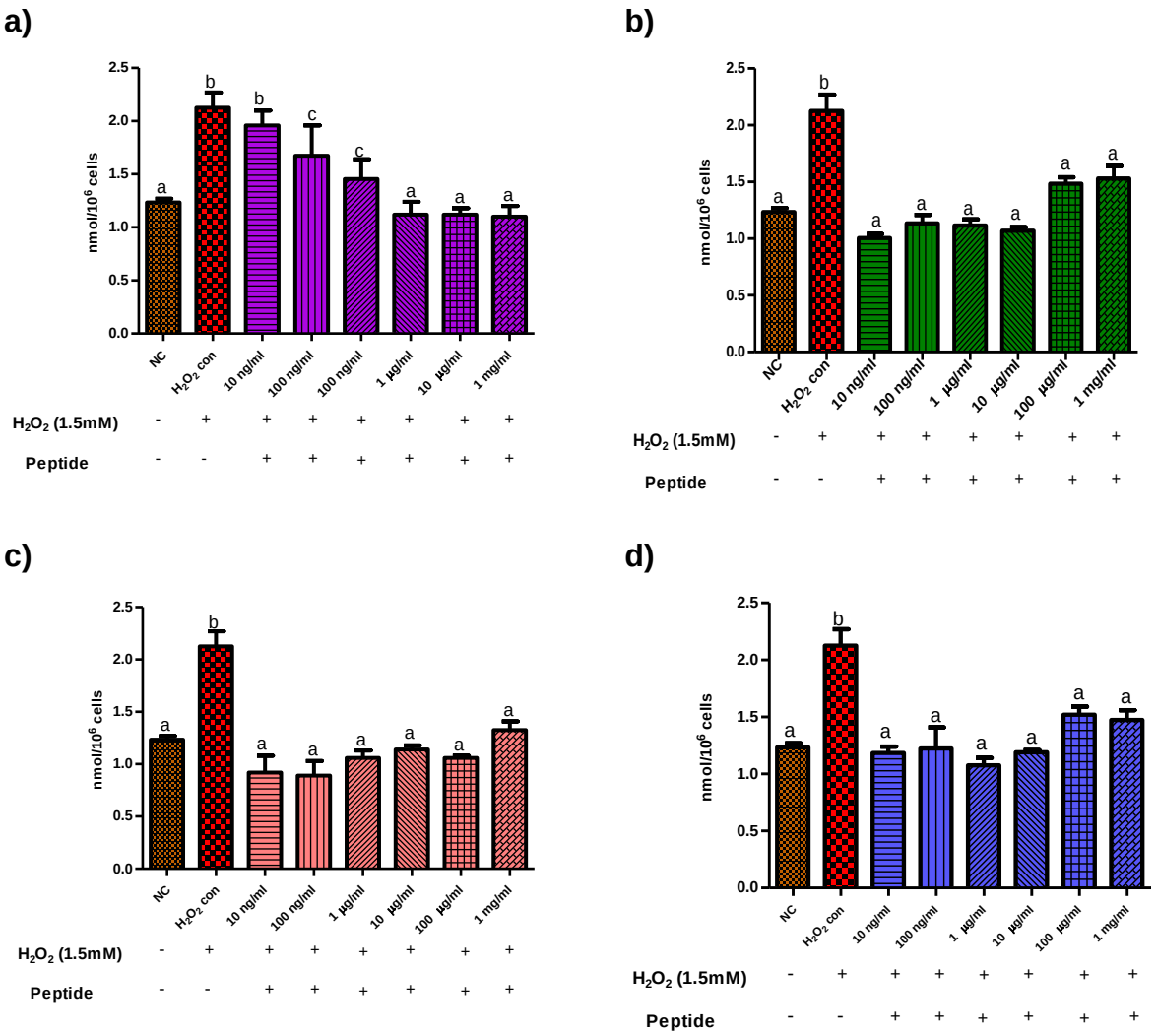


Figure 4.7. Inhibitory effect of peptides on H_2O_2 induced MDA formation

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

NC: Negative control. The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

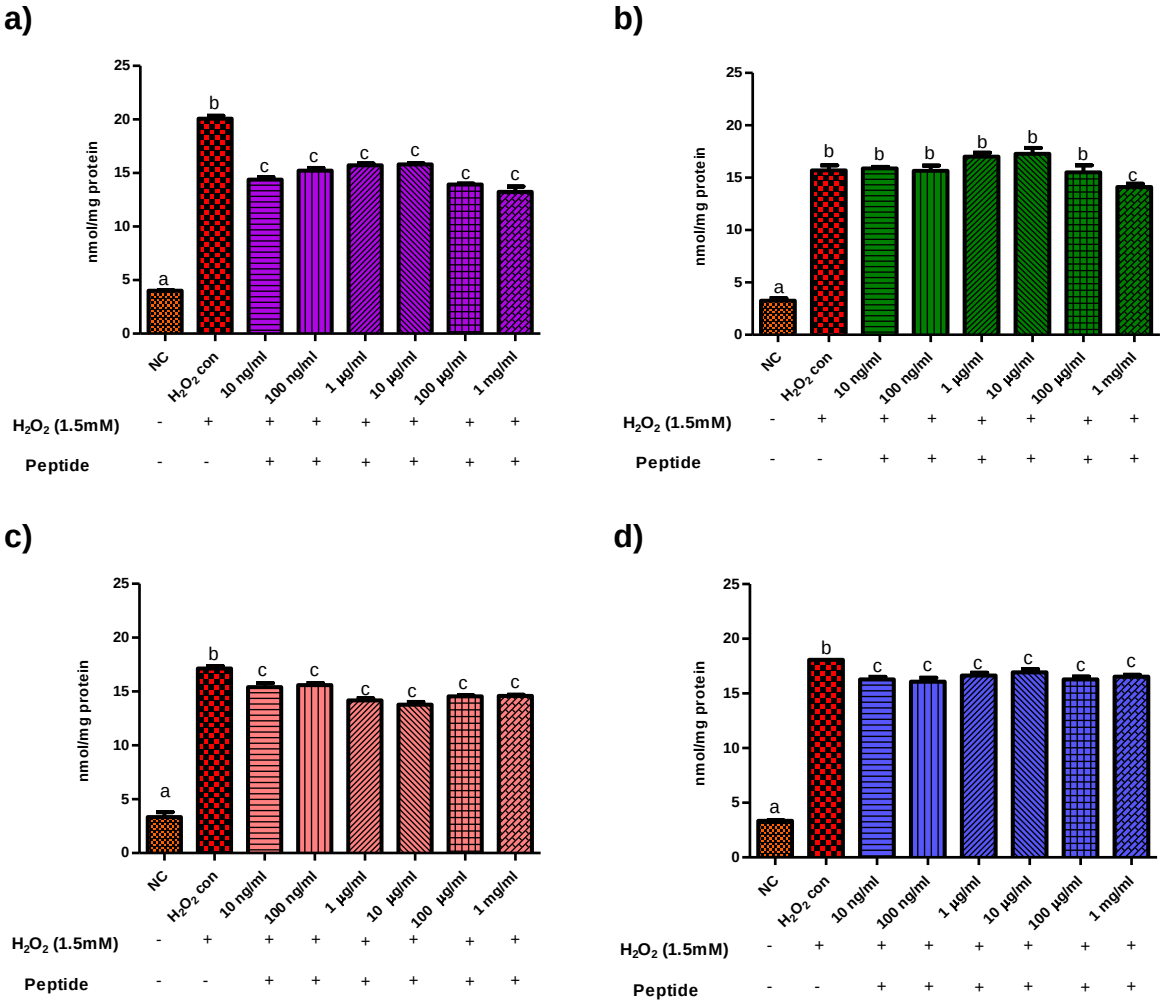


Figure 4.8. Inhibitory effect of peptides on H_2O_2 induced protein carbonyl formation

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

NC: Negative control. The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

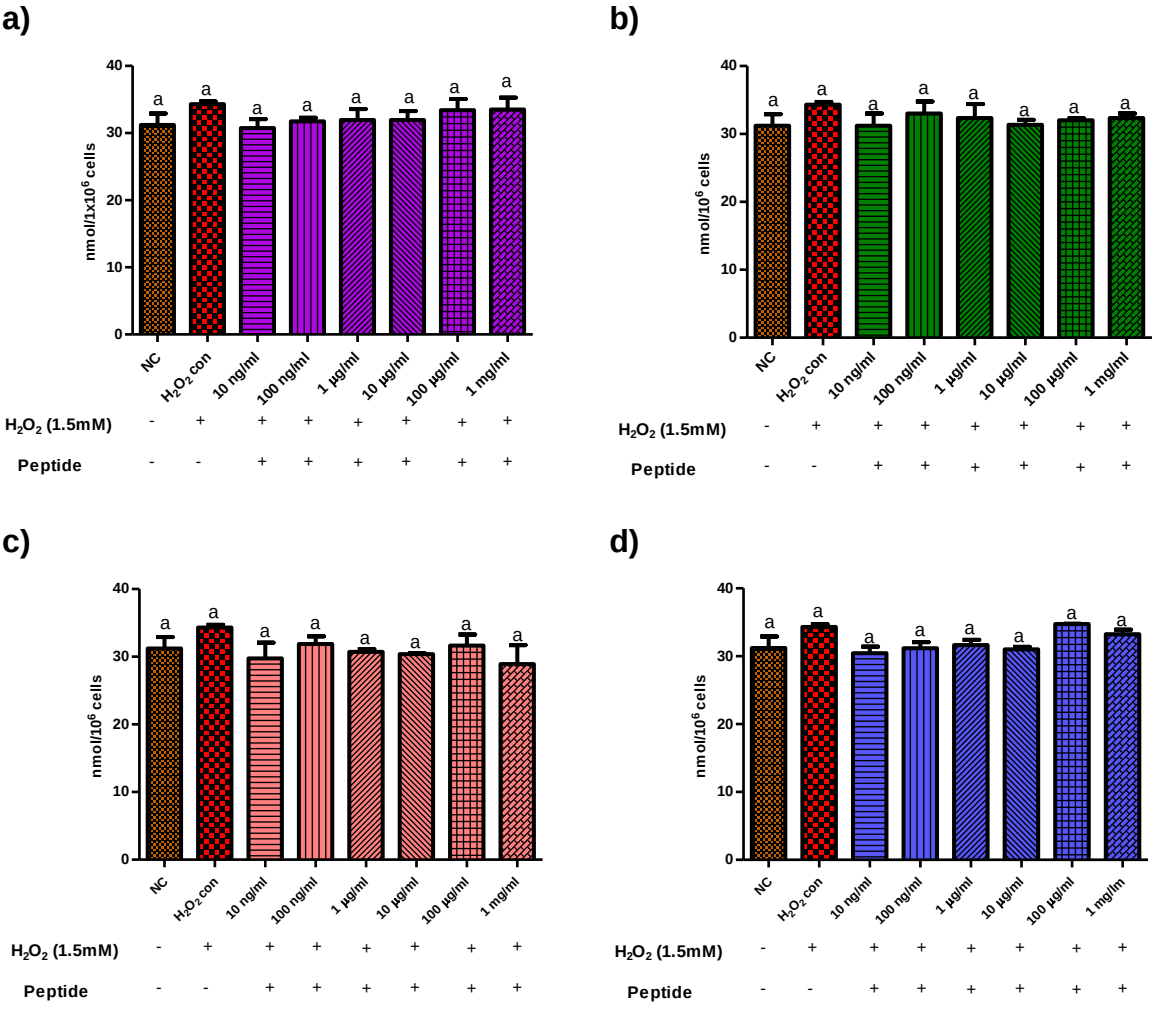


Figure 4.9. Effect of peptides on H_2O_2 induced nitric oxide production

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

NC: Negative control. The values are expressed as means $\pm$ SEM (n=3). Means with different alphabets indicate significant difference ($p< 0.05$).

G) at different concentrations (10 ng to 1 mg/ml) did not show any significant changes in the nitric oxide secretion in to the culture medium (figure 4.9).

4.3.6. Effect of peptides on antioxidative enzymes

A. Catalase (CAT)

Catalase, an crucial enzyme in protecting the cell from oxidative damage caused by free radical generation. Effect of casein derived peptides on the activity of catalase is depicted in figure 4.10. Addition of 1.5 mM H_2O_2 to the Caco-2 cells considerably ($p<0.01$) enhanced the activity of catalase (23% increase) due to oxidative stress generation than negative control (NC). Whereas, cells pre-supplemented with respective peptides B, C, F and G for 24 hr, were observed to further increase its activity remarkably ($p<0.01$) independent of their doses used as compared to H_2O_2 control.

B.Superoxide dismutase (SOD)

SOD is an important antioxidant defence enzyme present in nearly all living cells exposed to oxygen. In current investigation, similar to the catalase, cells treated with H_2O_2 significantly ($p<0.05$) stimulated (22% increase) the activity of superoxide dismutase than negative control. Cells pre-incubated with different concentrations (10 ng to1 mg/ml) of peptide C additionally increased its activity remarkably ($p<0.01$) as compared to H_2O_2 control (figure 4.11b). However, in case of peptides B, F or G no such significant changes were observed than H_2O_2 control (figure 4.11a,c and d).

C.Glutathioneperoxidase (GPx)

Glutathione peroxidase, another important antioxidative enzyme involved in scavenging and inactivating hydrogen and lipid peroxides, also protects the body against oxidative stress. As presented in figure 4.12, similar to CAT and SOD, a considerable ($p<0.01$) increaseinGPx activity (38% increase) was also observed after addition of 1.5 mM H_2O_2 than negative control (NC). Further significant ($p<0.05$) increase in its activity was noticed by treatment with two peptides (C and F) regardless of their respective doses used for the treatment as compared to H_2O_2 control (figure 4.12b and c). Though, no much variation was noticed in the case of peptides B and G treatment than H_2O_2 control (figure 4.12a and d).

D. Ratios of CAT/SOD and GPx/SOD

Changes in CAT/SOD and GPX/SOD ratios are strong indicators of antioxidant cellular response than individual enzyme activities. As depicted in figure 4.13 and 4.14,no significant changes were observed in CAT/SOD and GPx/SOD ratios after

H_2O_2 treatment as compared to negative control. Whereas cells incubated with respective peptides B and G at 100 ng to 1 mg/ml and peptide C at 1 µg to 1 mg/ml concentration respectively were found to increase ($p<0.01$) the ratio of CAT/SOD than H_2O_2 and negative controls. However, treatment with peptide F did not show such changes in CAT/SOD ratio (figure 4.13c). On the other hand GPx/SOD ratio has insignificant variations on treatment with the selected four peptides B, C, F and G individually at different doses ranged from 10 ng to 1 mg/ml as compared to H_2O_2 control though the increase was found to be considerably higher than negative control on using respective peptides C, F and G (figure 4.14b-d).

4.3.7. mRNA expression of oxidative stress related genes

Effect of peptides (B,C,F and G) on the expression of Nrf-2 and Keap1 genes in Caco-2 cells are shown in figure 4.15 and 4.16. It was observed that oxidative stress generation in the cellswith H_2O_2 significantly ($p<0.01$) stimulated the expression of Nrf-2, a key transcription factorresponsible for the induction of antioxidative enzymes synthesis during oxidative stress as compared to negative control. The pre-treatment of Caco-2 cells with four selected peptides (B, C, F and G) before H_2O_2 induced oxidative stress either brought Nrf-2 mRNA expression back to normal levels by bringing it closer to negative control (NC) or suppressed its expression. The significant suppression in Nrf-2 expression occurred in case of supplementing peptide B or C at higher amounts ranged from 10µg/ml to 1mg/ml (figure 4.15a and b). On the other hand pre-treatment of Caco-2 cells with peptide G significantly ($p<0.01$) suppressed the Nrf-2 expression at lower amounts (10 ng to 10 µg/ml) while higher amounts (100 µg to 1 mg/ml) supplementation brought the expression back to normal levels (figure 4.15d). Treatment with peptide F at various concentrations (10 ng to 1 mg/ml) did not show anysuppression of Nrf-2 gene though expression is reduced than H_2O_2 treated cells (figure 4.15c). In case of Keap1, an oxidative stress sensor which functions to regulate the proteosomal degradation of Nrf-2, no effects were found in its expression in the cells of H_2O_2 control as compared with negative control. Similarly, no such variations were recorded by the pre-treatment with four selected peptides (B, C, F and G) at their respective doses (10 ng to 1 mg/ml) (figure 4.16a-d).

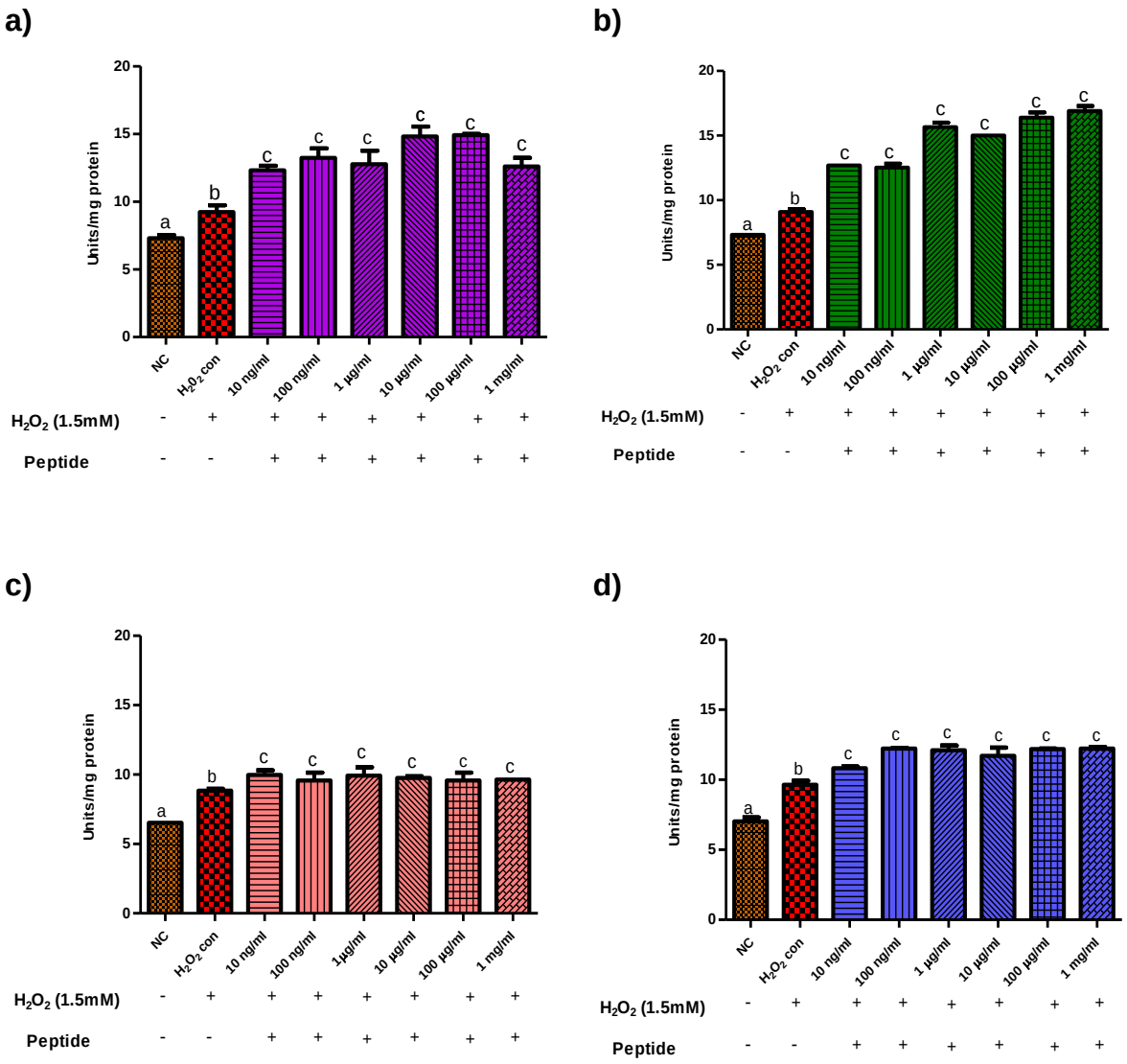


Figure 4.10. Effect of casein derived peptides on catalase (CAT) activity of Caco-2 cells

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

NC: Negative control. The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

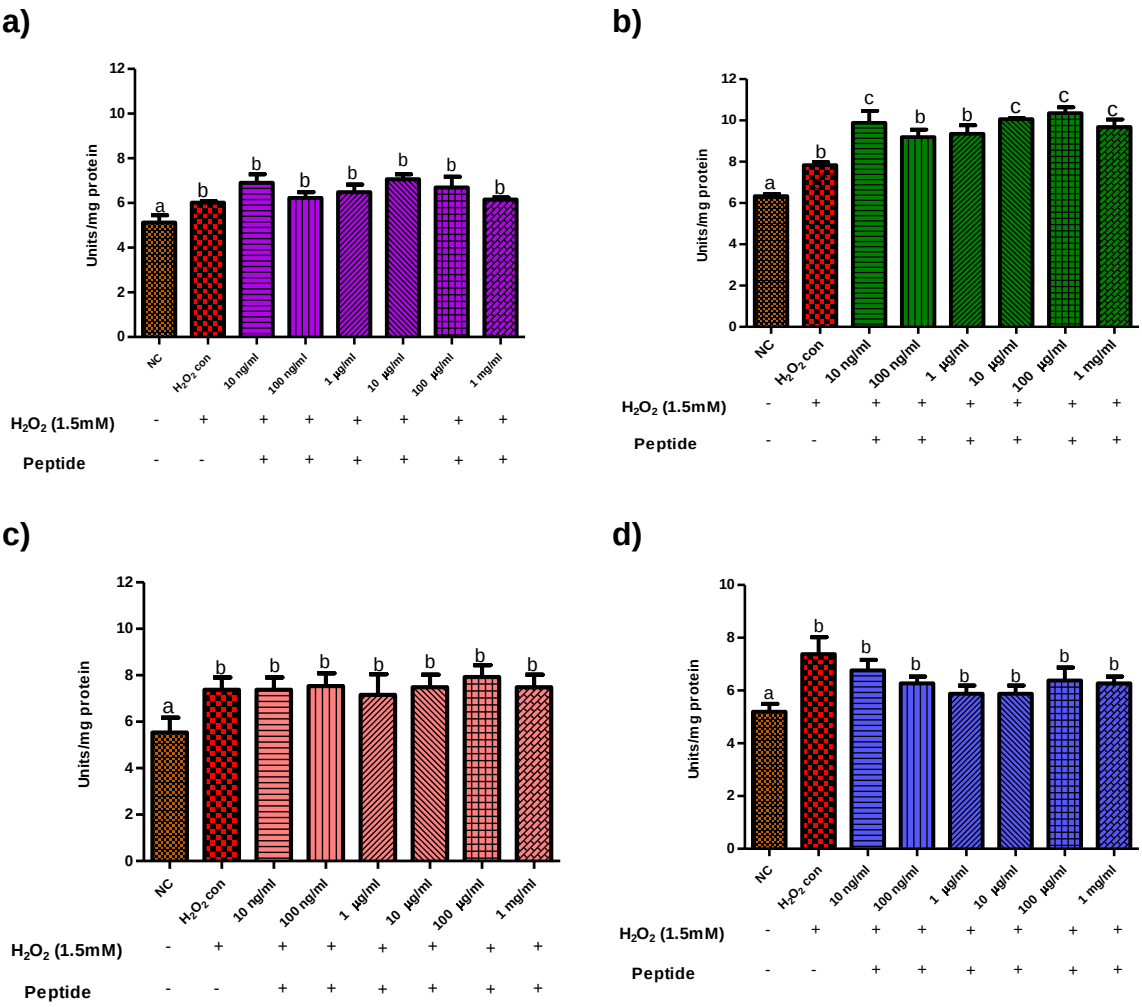


Figure 4.11. Effect of casein derived peptides on superoxide dismutase (SOD) activity of Caco-2 cells

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

NC: Negative control. The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

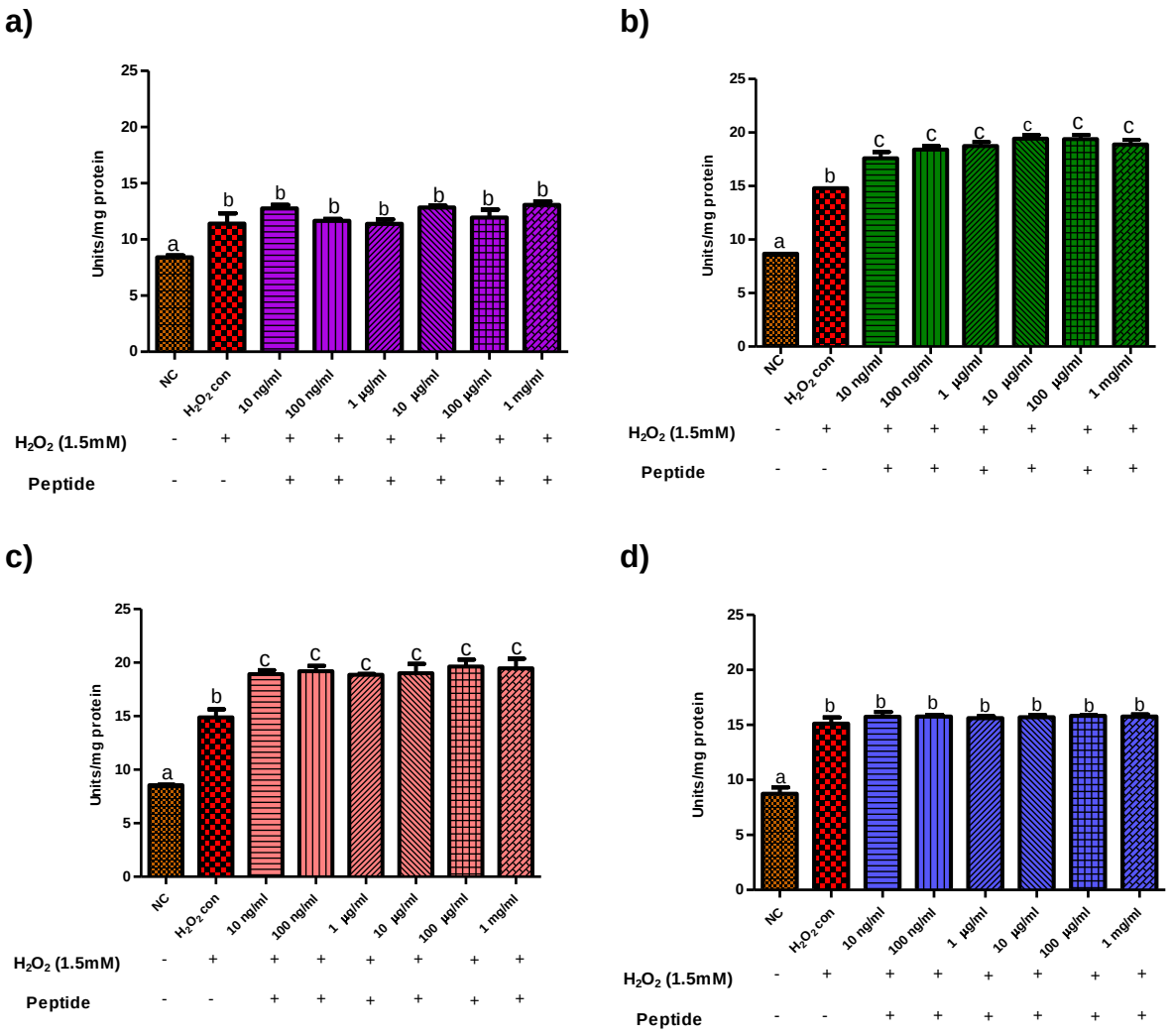


Figure 4.12. Effect of casein derived peptides on glutathione peroxidase (GPx) activity of Caco-2 cells

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

NC: Negative control. The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

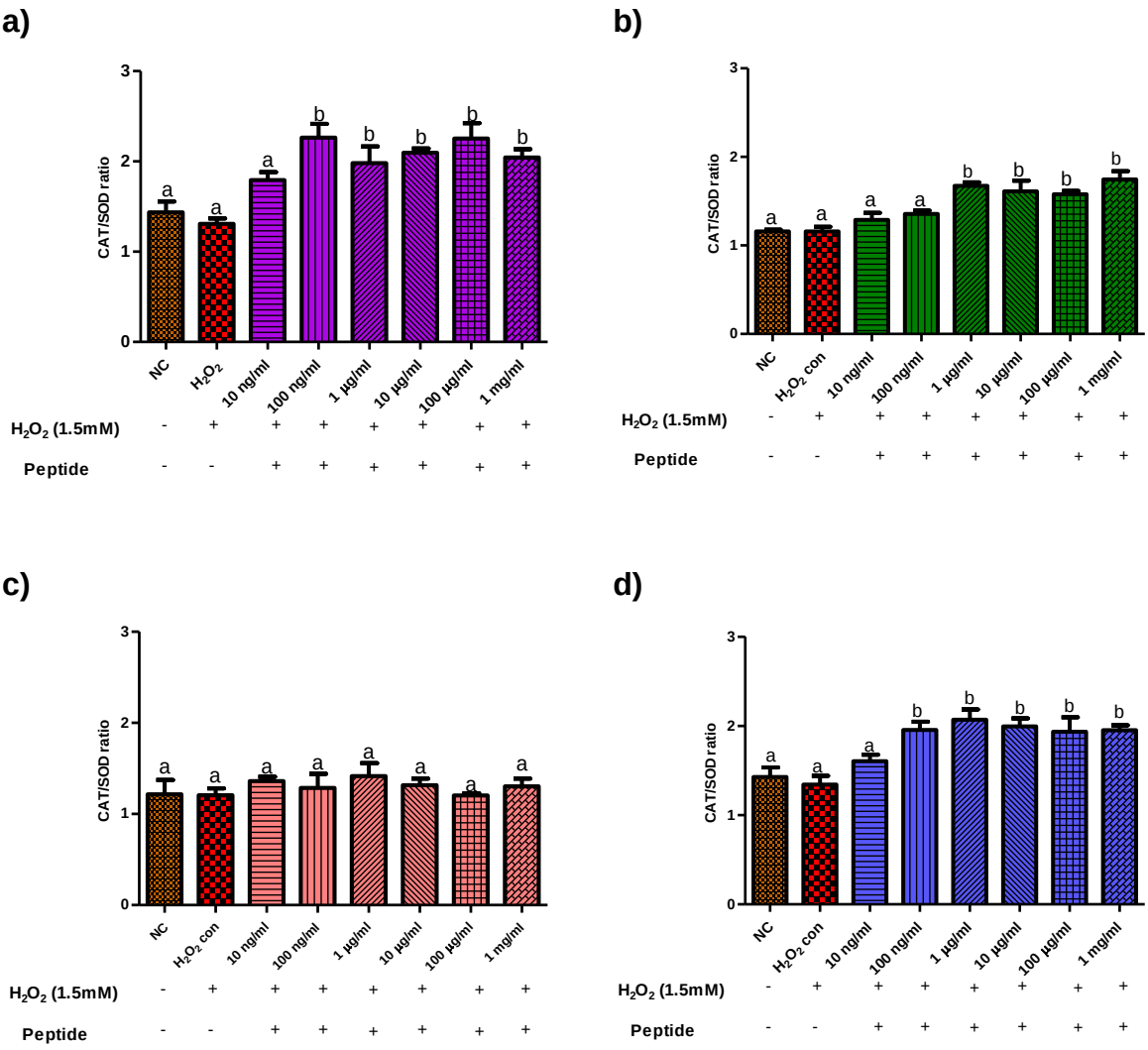


Figure 4.13. Effect of casein derived peptides on CAT/SOD ratio in Caco-2 cells
a: Peptide B
b: Peptide C
c: Peptide F
d: Peptide G
NC: Negative control. The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

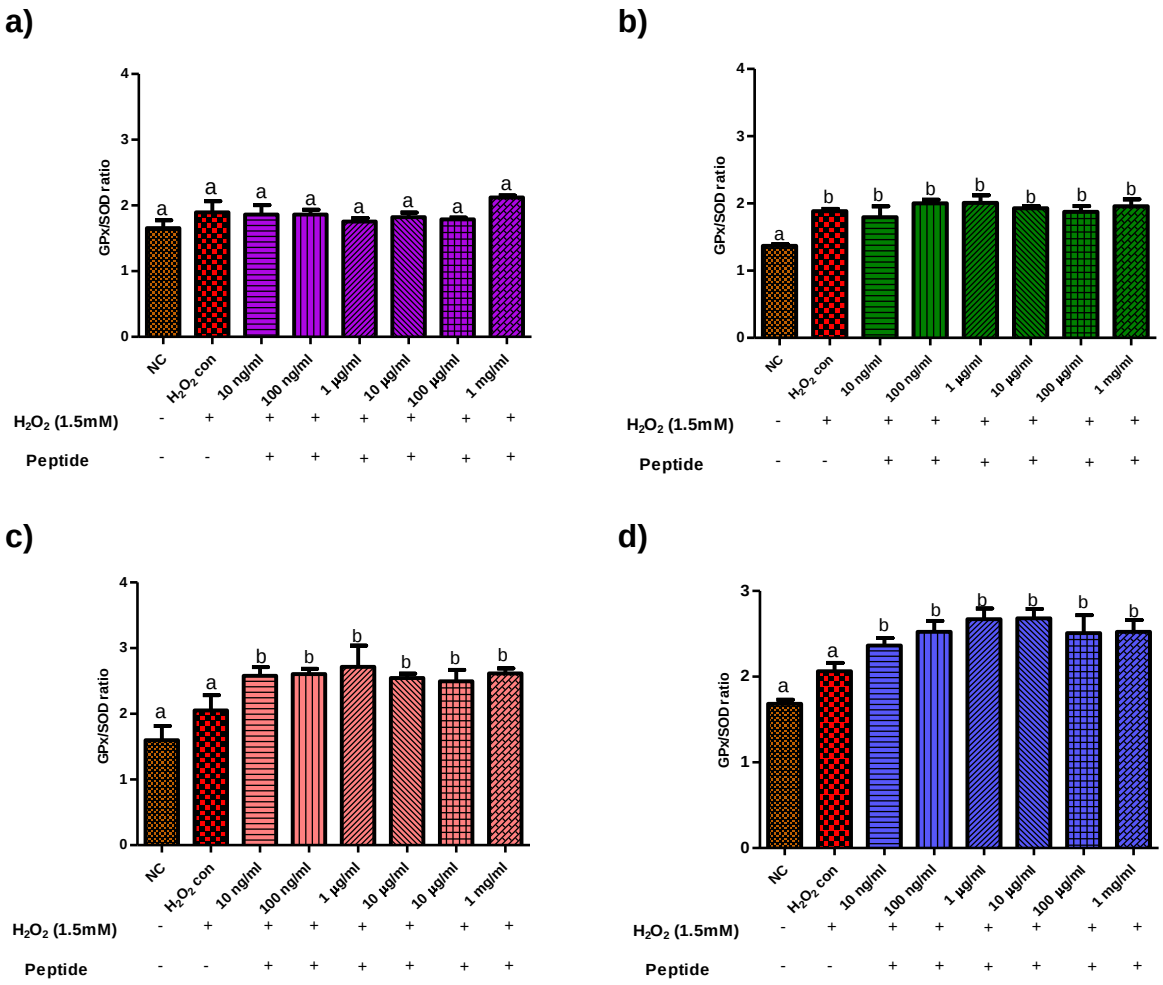


Figure 4.14. Effect of casein derived peptides on GPx/SOD ratioinCaco-2 cells

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

NC: Negative control. The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

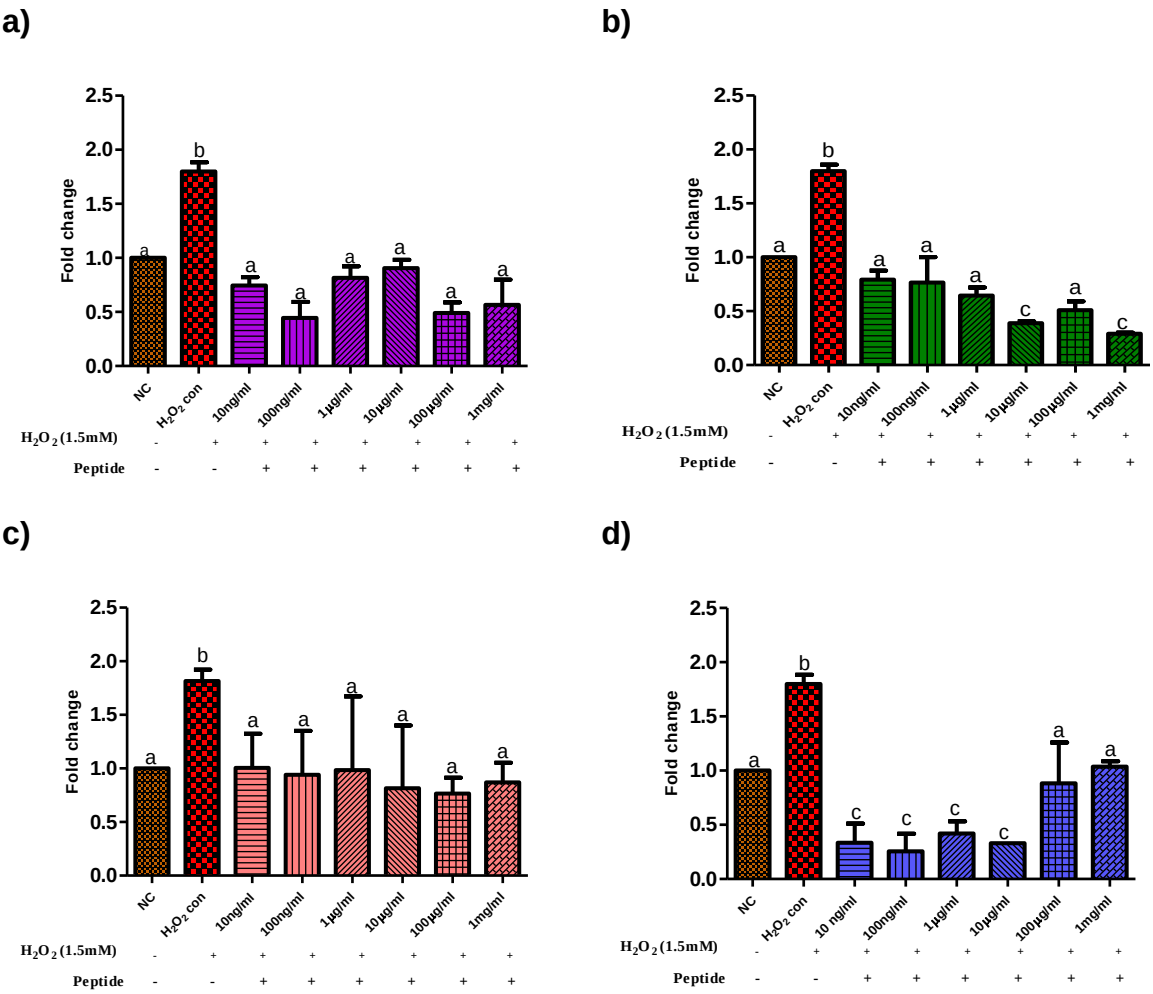


Figure 4.15. Effect of casein derived peptides on Nrf-2 mRNA expression in Caco-2 cells

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

NC: Negative control. The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

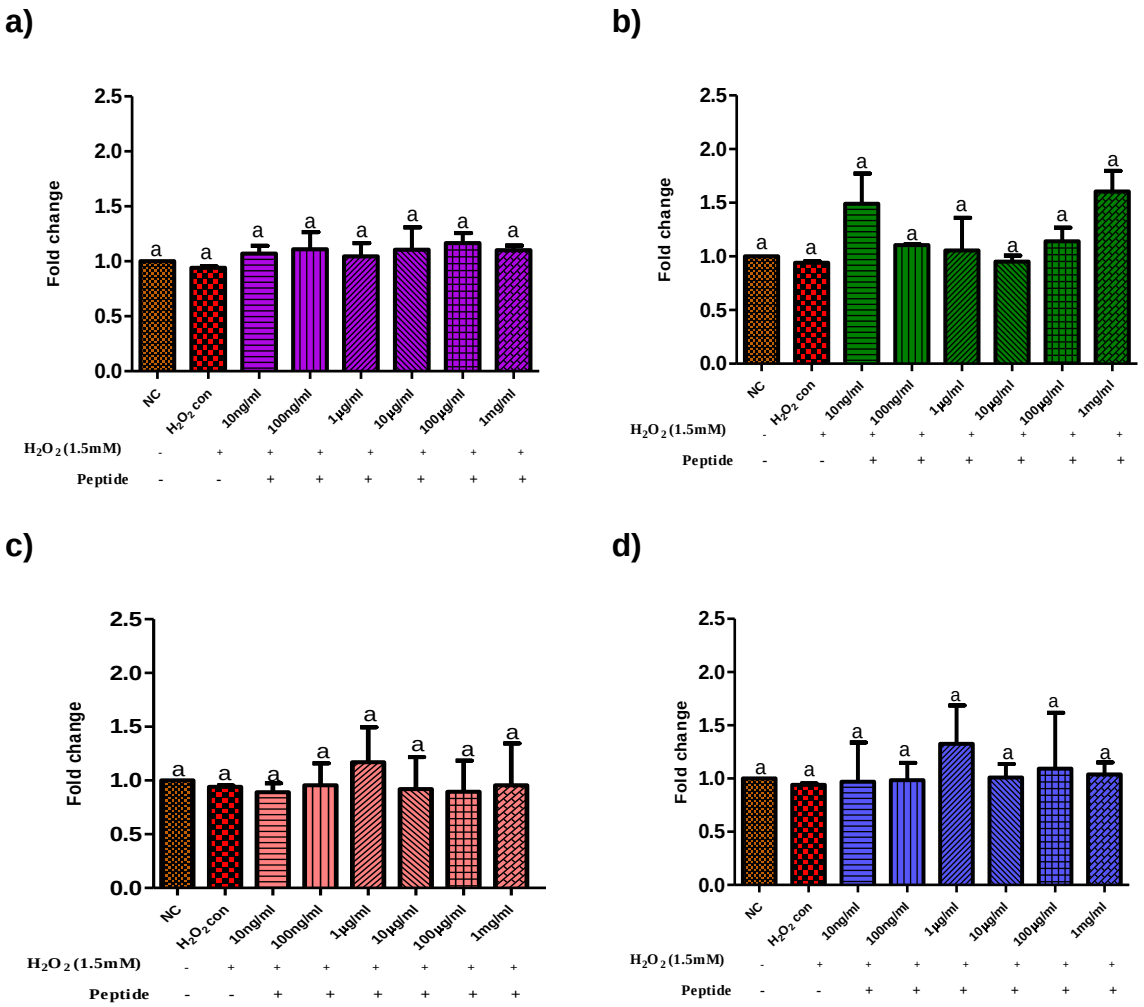


Figure 4.16. Effect of casein derived peptides on Keap-1 mRNA expression in Caco-2 Cells

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

NC: Negative control. The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

4.3.8. Western blotting analysis of Nrf-2 transcription factor

The results of pre-treatment with peptides on the activation and translocation of Nrf-2 transcription factor in the presence of H_2O_2 induced oxidative stress are shown in plate 4.7. Treatment with H_2O_2 appeared to increase the translocation of activated Nrf-2 from cytoplasm to nucleus. Two of the selected peptides (hexa peptide: C and tri peptide: G) were also found to induce the activation and translocation of Nrf-2 from cytoplasm to the nucleus even more than H_2O_2 and negative controls. The remaining two peptides (B and F) did not show much variation in translocation of Nrf-2 factor.

4.3.9. Cytokine release during immunomodulation

Effect of peptides on the secretion of cytokines in the supernatant of murine cultured splenocytes was determined in order to assess their active role in immunomodulation. Secretion of four cytokines (IFN-γ, IL-10, IL-4 and TGF- β) in tothesplenocytes culture medium was studied in the current analysis as they are among the best markers of pro/anti-inflammatory reactions. The incubationof all the respective four selected peptides (B,C,F and G) for 24 hr at various concentrations (10 ng/ml to 1 mg/ml) were detected to remarkably reduce ($P<0.001$) the levels of IFN-γ, a pro-inflammatory cytokine in the supernatant of cultured spleen cells as compared to control (figure 4.17). On the other hand, supplementation of cultured cells with peptides C or F were found to significantly ($p<0.01$) increase the secretion of IL-10, an anti-inflammatory/ regulatory cytokine in the culture medium independent of their respective doses used (figure 4.18b and c). Similar effect ($p<0.01$) was also observed in case of peptides B and G at 10 µg to 1mg/ml and 100 ng to1 mg/ml concentrations respectively (figure 4.18a and d). Likewise, an another anti-inflammatory/ regulatory cytokine, IL-4 was also elevated($p<0.01$) in the culture supernatant by treatment with peptide C on addition of higher doses ranging from 1 µg to1 mg/ml (figure 4.19b) contrary to the absence of any significant changes with peptidesB, F and G as compared to control (figure 4.19a,b and d). Incubation of spleen cells with peptides F and G (10 ng to 1 mg/ml) also significantly ($p<0.05$) up regulated the secretion of TGF-β, a regulatory cytokine in the medium of cultured splenocytes (figure 4.20c and d). However, no such variations were observed in case of the other two peptides (B and C) (figure 4.20a and b).

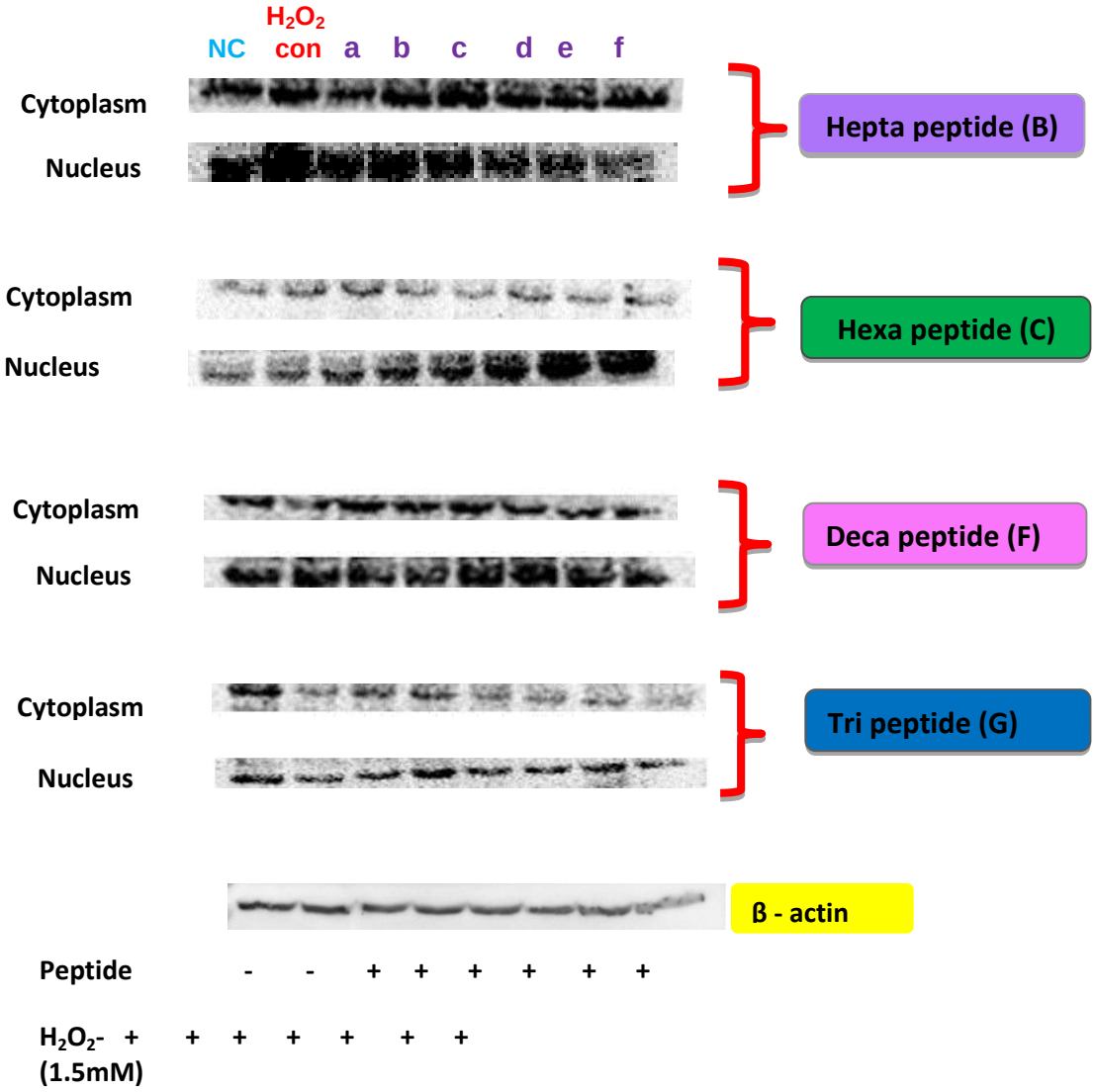


Plate 4.7. Effect of peptides on the activation and translocation of Nrf-2 in Caco-2 cells by western blotting analysis
NC = Negative control
a = 1 mg/ml
b = 100 µg/ml
c = 10 µg/ml
d = 1 µg/ml
e = 100 ng/ml
f = 10 ng/ml

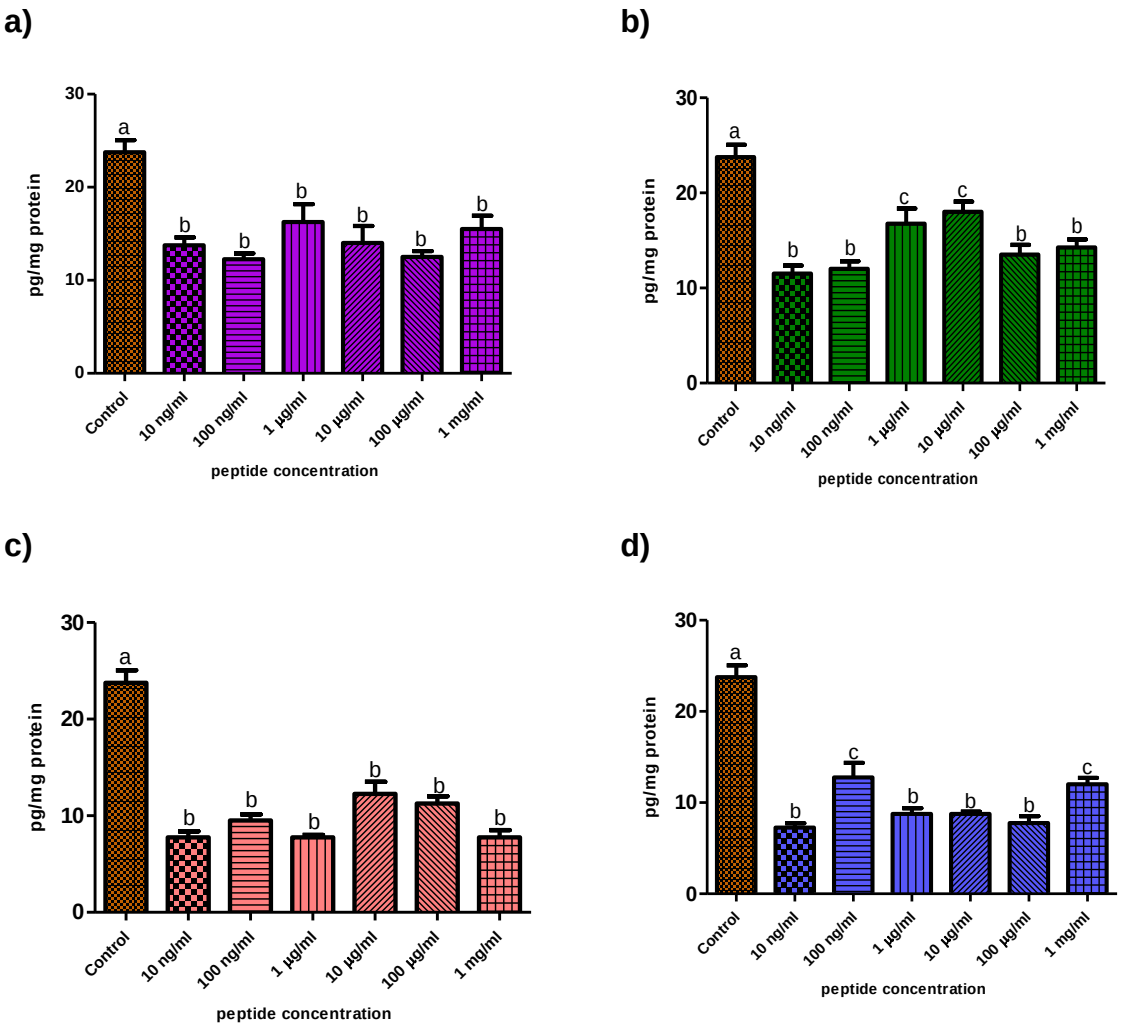


Figure 4.17. Effect of casein derived peptides on IFN-γ secretion in the supernatant cultured spleen cells

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

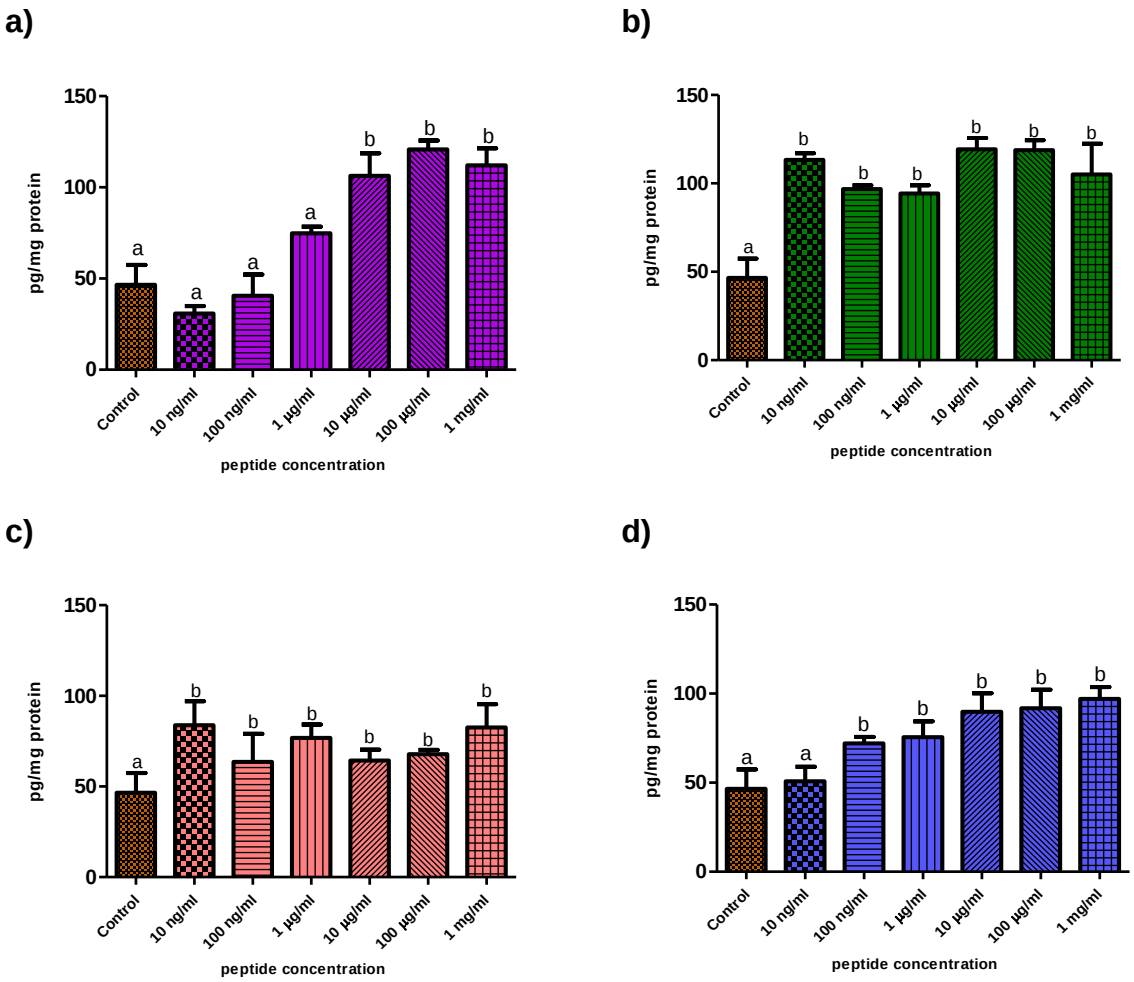


Figure 4.18. Effect of casein derived peptides on IL-10 secretion in the supernatant cultured spleen cells

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

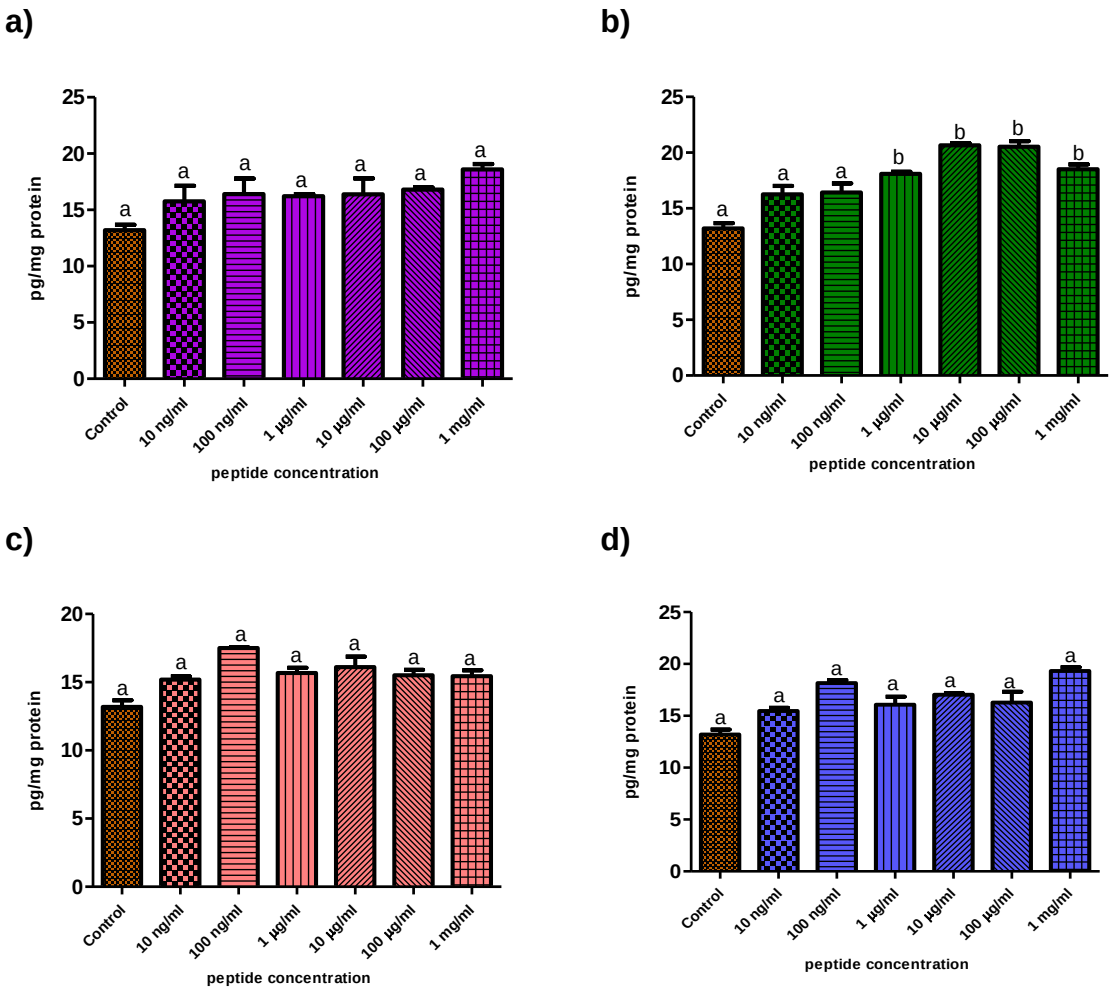


Figure 4.19. Effect of casein derived peptides on IL-4 secretion in the supernatant cultured spleen cells

a: Peptide B
b: Peptide C
c: Peptide F
d: Peptide G

The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

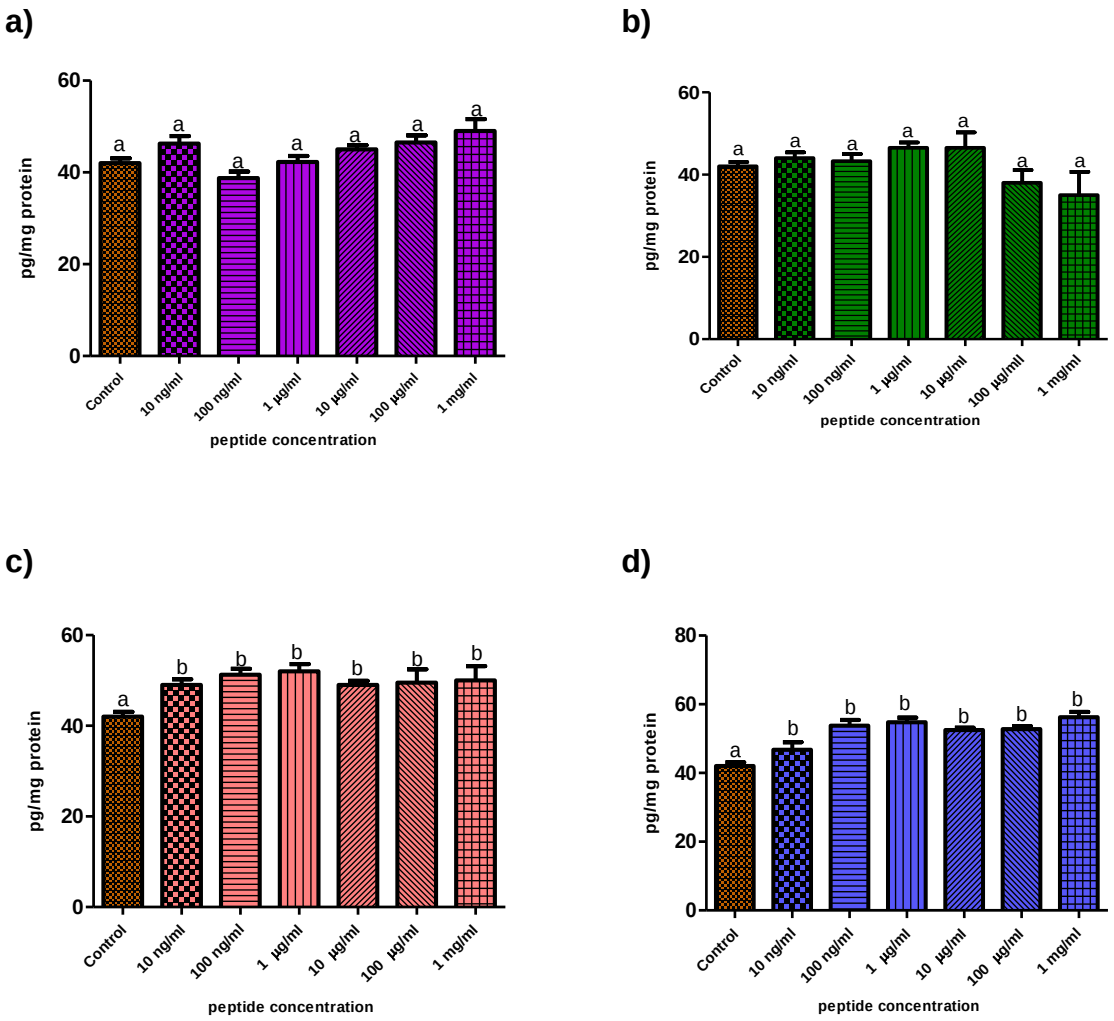


Figure 4.20. Effect of casein derived peptides on TGF-β secretion in the supernatant cultured spleen cells

a: Peptide B

b: Peptide C

c: Peptide F

d: Peptide G

The values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$).

4.4. Bioavailability and transepithelial transport of potential bioactive peptides with overlapping antioxidative and immunomodulatory activities

The physiological effects of bioactive peptides depend on their ability to reach in an active form to their target organs. This mainly depends upon the resistance capacity of peptides to gastrointestinal and brush border membrane peptidases, and absorption through the intestinal epithelium (Segura-Campos *et al.,*2011). In the current analysis, out of four, two peptides (hexa peptide: C and tri peptide: G) were selected for transepithelial transport studies using Caco-2 cells. Peptide C was chosen based upon its ability to protect cells against oxidative damage with immunomodulatory properties as evidenced in the results of previous objective. Likewise, the other peptide selected in present study has also overlapping activities of antioxidation and immunomodulation in addition to its short length (tri peptide) nature which may be responsible to cross epithelial barrier in intact form more effectively based upon previous findings of researchers. Caco-2 cells grown on the apical chamber of six welled transwell plate (plate 4.8) became confluent monolayer within 6-7 days and the cells got differentiated in another 15 days for performing trans-epithelial transport studies. Plate 4.8d shows the differentiated Caco-2 cell monolayer after 21 days of culture on the transwell insert. Caco-2 cells grown on insert having less than 2% of leakage of phenol red were used for the assessment of bioavailability and transport studies.

4.4.1. Transport of bioactive peptides

Peptide C (hexa peptide), peptide G (tri peptide) and bradykinin (nona peptide) were eluted from C_{18} column of RP-HPLC with retention time of 17.76,13.89 and 25.75 minutes respectively using water acetonitrile solvents (27.3, 35.71, 52.5 % respectively) as shown in (figure 4.21).

Concentration dependent transport of peptides (C and G) is shown in figure 4.24. In both cases incubation of different amounts of peptides (100 µg to 500 µg/ml) on apical chamber did not appear in basal chamber within one minute of their addition (figure 4.22 and 4.23). However transport of peptides appeared to increase across the intestinal epithelial membrane from apical to basal solutions in a span of 60 min with increase of amount of peptide in apical chamber. In case of peptide C, 300 µg/ml in apical chamber observed to be saturated limit above which no further increase in peptide in basal chamber was observed (figure 4.24a). Likewise, peptide G appeared to the maximal amount in basal chamber after incubating 400 µg/ml in the upper chamber (figure 4.24b).

The transport of peptides C and G across the epithelial membrane as a function of incubation time are shown in figure 4.25 and 4.26 using their saturating amounts (300 µg/ml and 400 µg/ml) respectively. In case of peptide C, a gradual increase in the flux of peptide in a time dependent manner was observed up to 30 min of incubation from apical to basal chamber (figure 4.28a). Later no further increase was observed even at 90 min of incubation. On the other hand, peptide G transported maximally by 4 minutes of incubation and passage appeared saturated on later time intervals (figure 4.28b). During this study it was also observed that peptide G disappears very rapidly from the apical chamber than peptide C. Recovery of peptide C from apical chamber was 22, 15.6, 1.17 and 0.94% respectively with in 1, 5, 15 and 30 minutes of its initial addition respectively and no peptide was determined after 60 min of incubation. On the other hand, peptide G recovered and 18,19,18,16 and 3% respectively from apical chamber after 1, 2,3 and 4 minutes of its addition and after 5 min of incubation no peptide was quantified.

4.4.2. Bradykinin

Bradykinin (RPPGFSPFR), was used as a model peptide to compare the bioavailability of bioactive peptides because of its lower transport rate (0.1%) and resistance capacity to hydrolysis by membrane peptidases (Shimizu *et al.,* 1997). In present study, bradykinin was used as control peptide at the concentration of 400 µg/ml on the apical surface. Within 1 min of incubation, a single peak was observed on the apical surface at retention time of 25.15 minute however, no such peak was found in the basal surface (figure 4.27a) within one minute of incubation. However, after 60 min of incubation, bradykinin appeared as a single peak in the basal surface indicating its apical to basal flux in the intact form (figure4.27b).

4.4.3. Comparison between the bioavailability of peptides

Comparative analysis of bioavailability of peptides is shown in figure 4.29. It was observed that the transport of peptide G (tri peptide) was found to be 1.72 ± 0.22% (6.9 µg/ml) from apical to the basal surface in its intact form which was remarkably more ($p<0.01$) form than the peptide C (hexapeptide) which showed transport of about 0.91 ± 0.41% (2.75 µg/ml)without any hydrolysis as analysed by RP-HPLC. Whereas, in case of bradykinin (control peptide), the transport recorded was 0.17 ±0.02% (0.69 µg/ml) without any hydrolytic product was tremendously less ($p<0.001$) as compared to peptide C and peptide G (table 4.7).

4.4.4. Route of peptide transport across the epithelial membrane

In order to evaluate the route for the transport of peptides across Caco-2 cell monolayer, different inhibitors were used which inhibit the flux of peptides from apical to the basal surface. Figure 4.30 representing the effect of cytochalacin D, Gly-Pro and wortmannin on the transport of peptides across Caco-2 cell monolayer. Supplementation of cells with wortmannin, an inhibitor of transcytosis pathway to the apical surface at 1 μM concentration, significantly ($p<0.001$) decreased the flux of peptide C (hexa peptide) and bradykinin to the basal surface as compared to control which was observed to be 35% and 63% respectively of the control (figure 4.30a and c). Whereas the presence of cytochalacin D, a disruptor of tight junctions between the cells and allows more peptide to transport at the dose of 1 μg/ml. Under present investigation, cytochalacin D did not show any changes in the transport of the respective three peptides (hexa peptide: C, tri peptide: G and bradykinin) from apical to basal surface. Although, the addition of Gly-Pro, a competitive inhibitor for peptide transporter PepT1 at 10 mM concentration remarkably ($p<0.01$) decreased the transport of tri peptide (G) from apical to the basal surface by 38% as compared to control (figure 4.30b).

Table 4.7. Bioavailability and transport route of peptides C, G and bradykinin

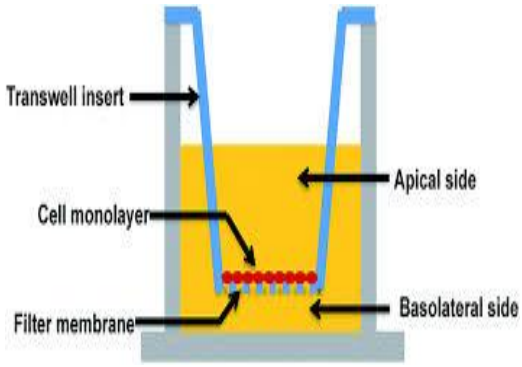
Peptide	Conc. of peptide added on apical surface (μg/ml)	Peptide conc. in basal surface (μg/ml)	Bioavailability (%)	Transport route
Peptide C (Hexa peptide)	300	2.75 ± 0.72	0.91± 0.04	Transcytosis
Peptide G (Tri peptide)	400	6.9 ± 0.90	1.72 ± 0.22	PepT1 mediated
Bradykinin (Nona peptide)	400	0.69 ± 0.01	0.17 ± 0.02	Transcytosis

Values are mean ± SEM (n=3).

a)

b)

c)



d)

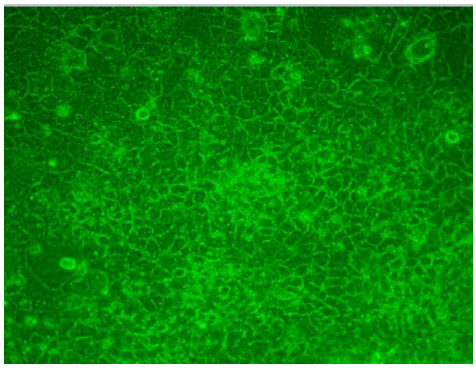
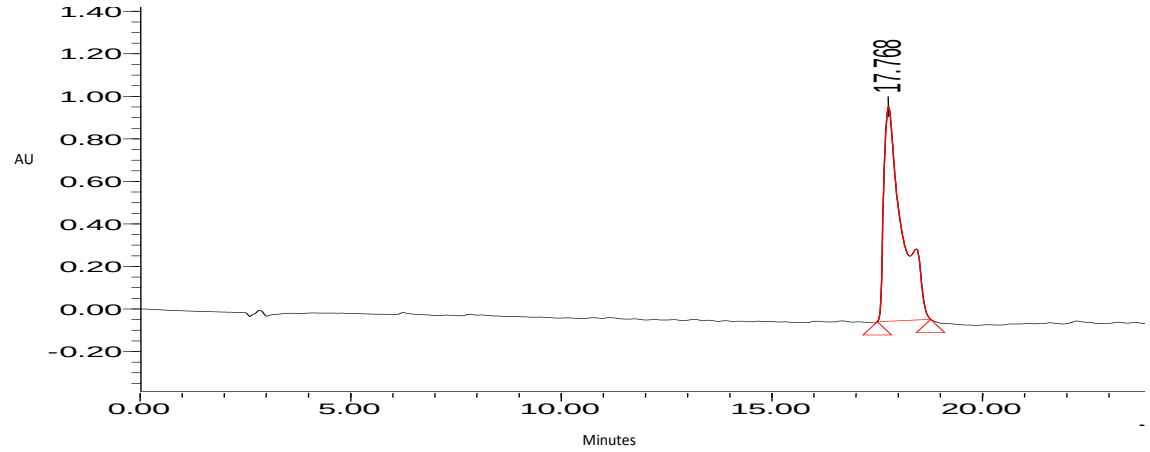
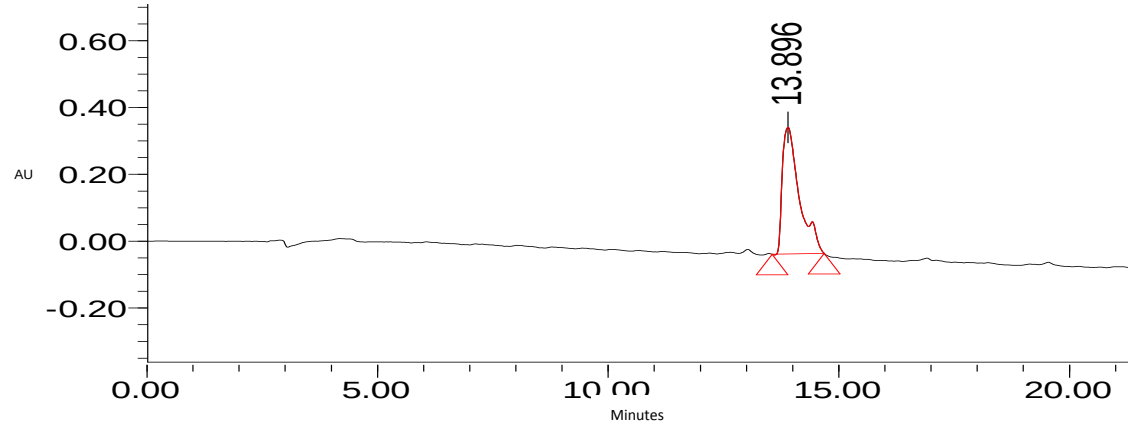


Plate 4.8. a: Six-welled transwell plate with culture inserts
b:Transwell plate with Caco-2 cells on apical chamber and culture medium in apical and basal chambers
c: Structure of a single transwell with apical and basal compartments
d: Fully confluent 21 day old Caco-2 culture on transwell insert

a)



b)



c)

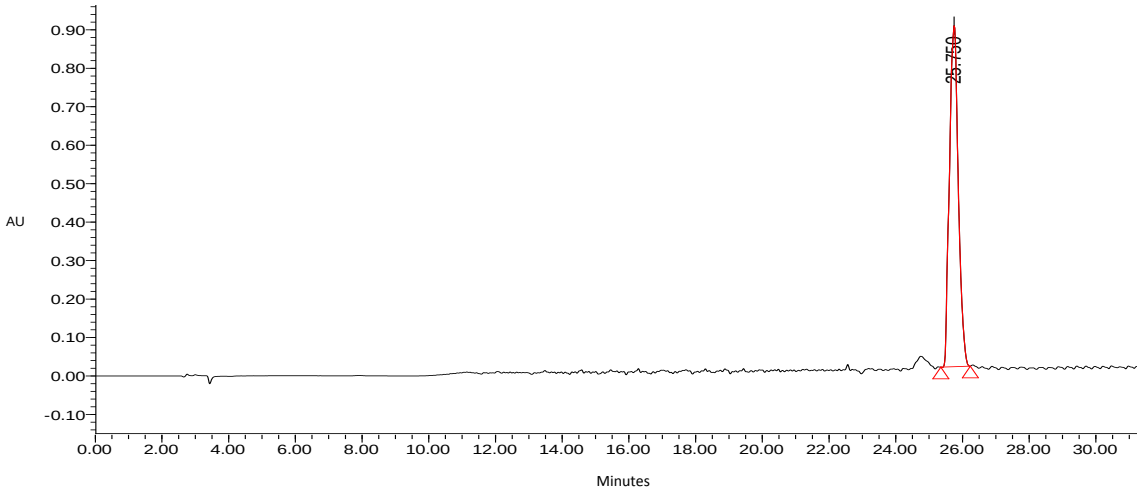


Figure 4.21. Chromatogram of standard peptides on C_{18} HPLC column

a:Peptide C (Hexa peptide)

b:Peptide G (Tri peptide)

c: Bradykinin (Nona peptide)

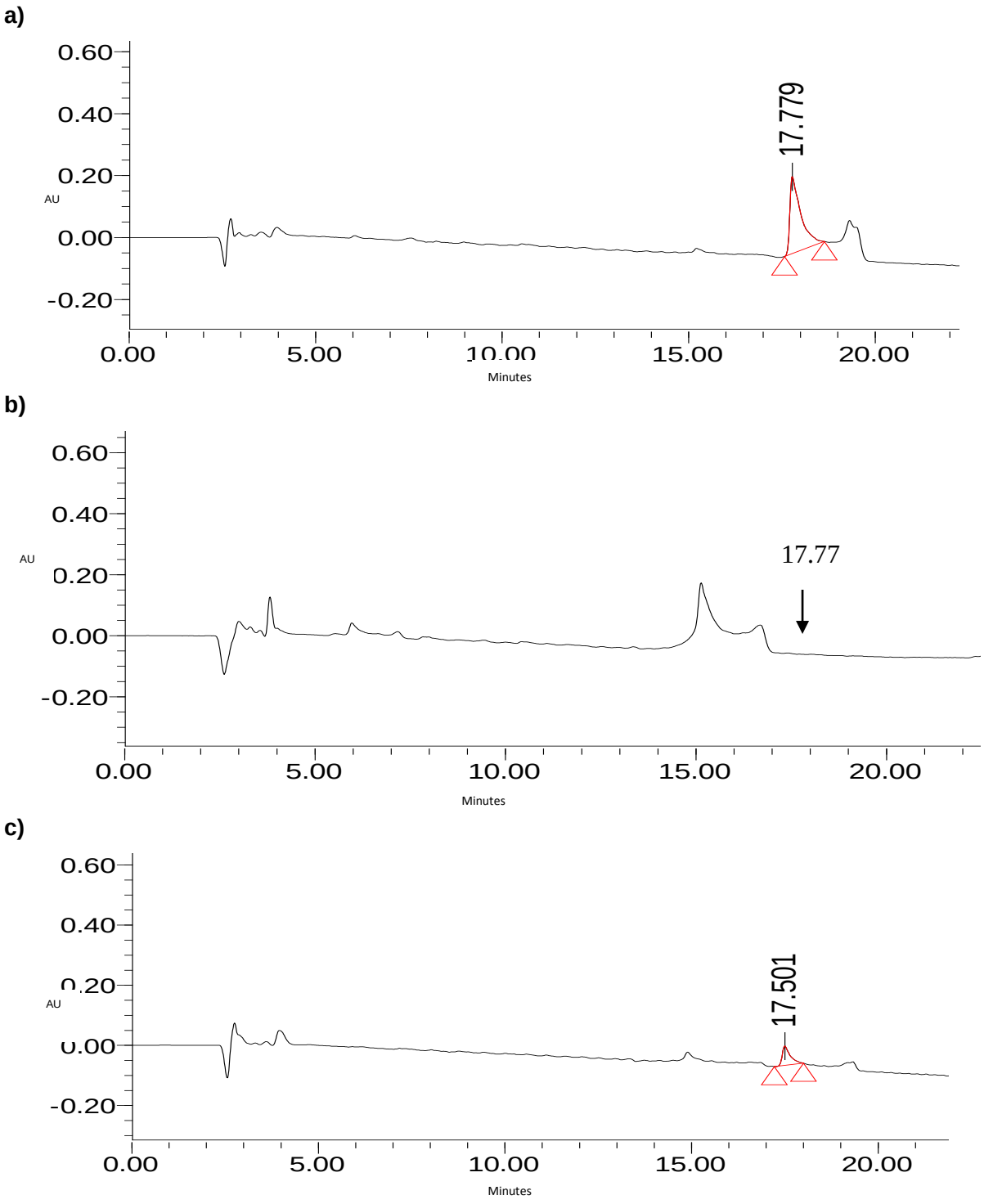


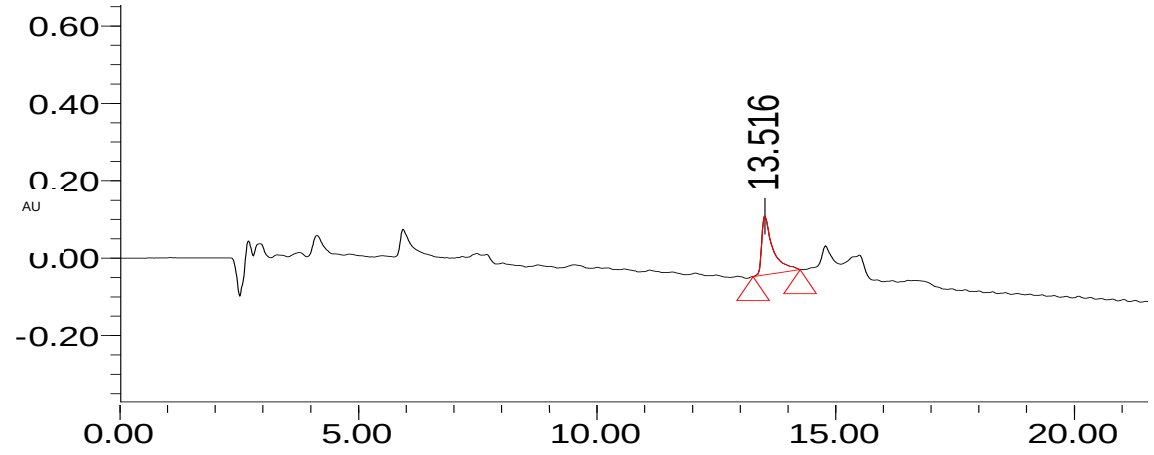
Figure 4.22. RP-HPLC **c**hromatogram of peptide C (Hexa peptide) during concentration dependent (300µg/ml) transepithelial transport

a: Apical solution with in one min of incubation

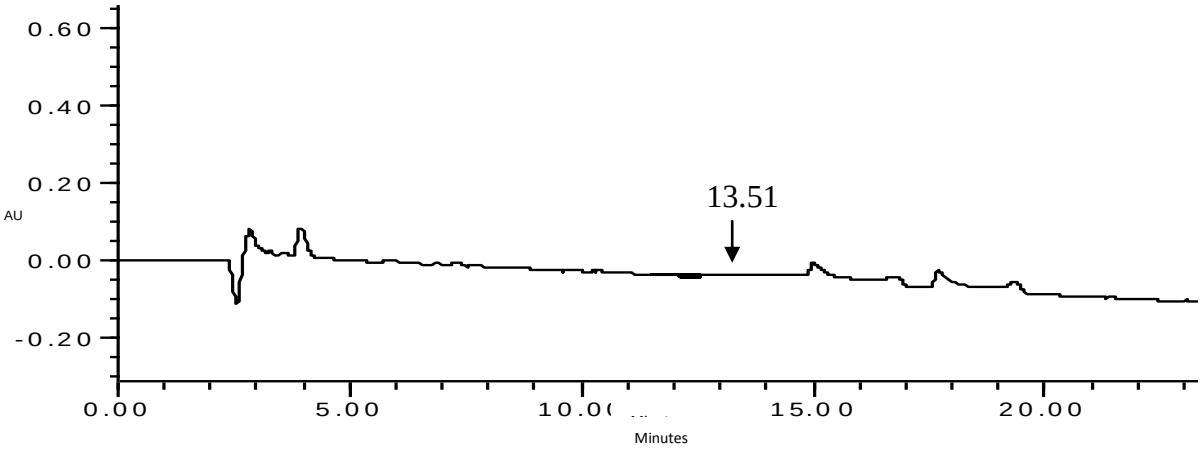
b: Basal solution with in one min of incubation

c: Basal solution after 60 min of incubation

a)



b)



c)

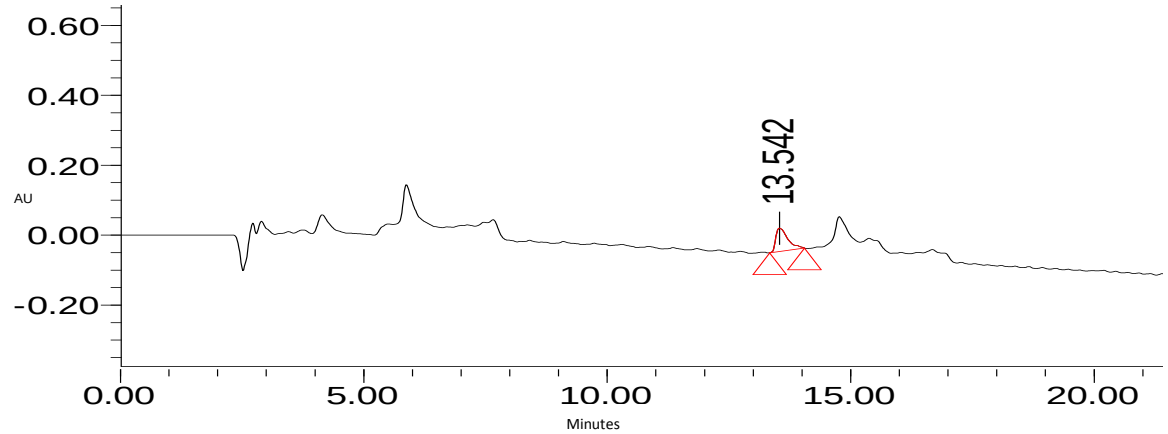


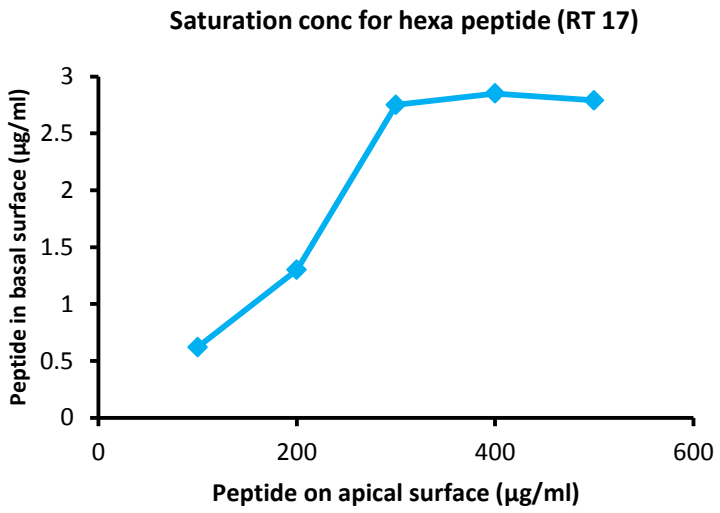
Figure4.23. RP-HPLC chromatogram of peptide G (Tri peptide) during concentration dependent (400µg/ml) transepithelial transport

a: Apical solution with in 1 min of incubation

b: Basal solution with in 1 min of incubation

c: Basalsolution after 60 min of incubation

a)



b)

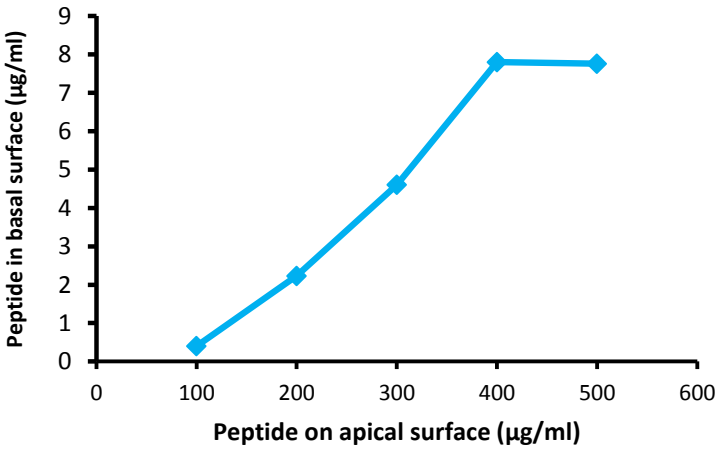


Figure 4.24. Concentration dependent transport of peptides across Caco-2 cell monolayer during 60 minutes

a: Peptide C (Hexa peptide)

b: Peptide G (Tri peptide)

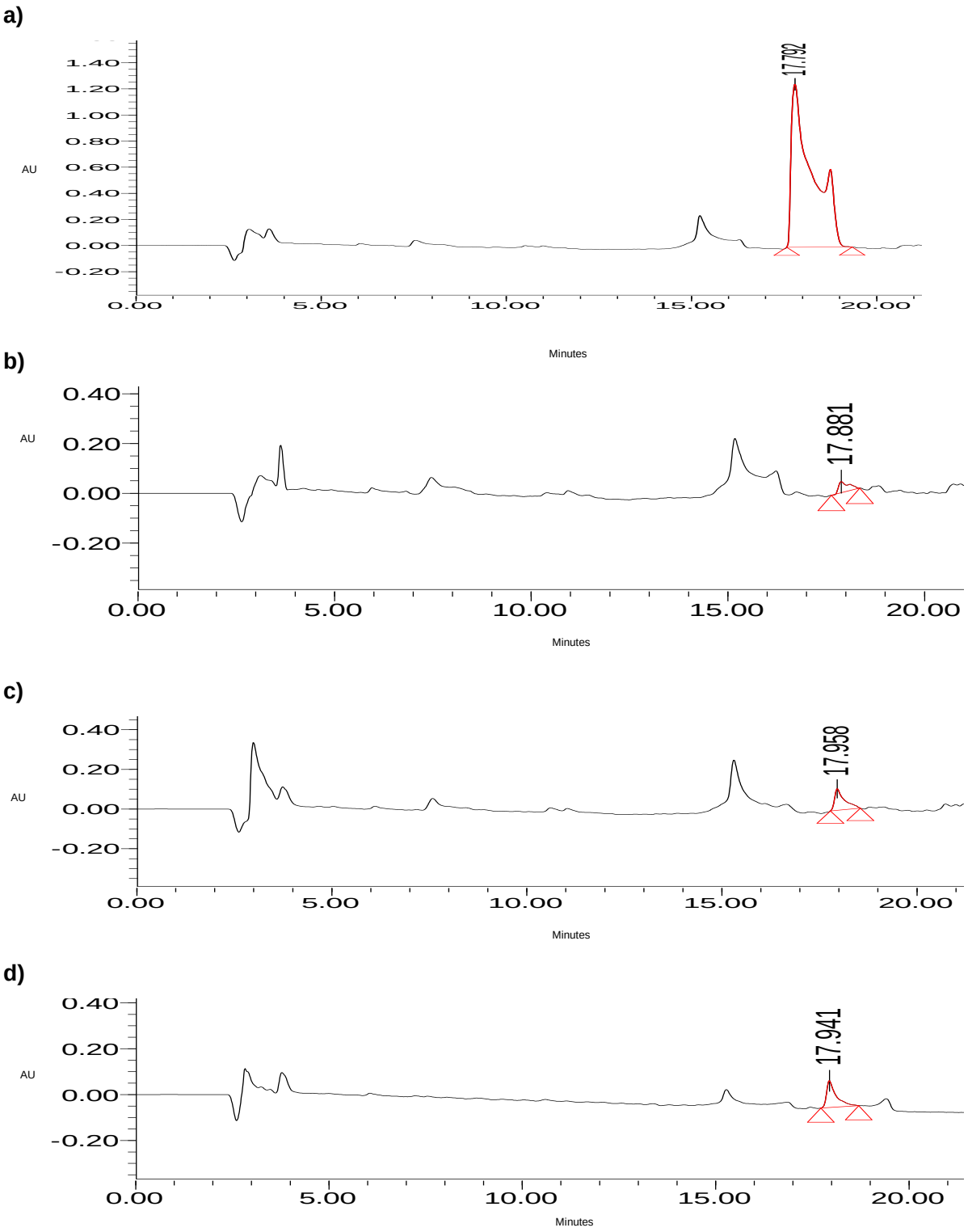


Figure 4.25. RP-HPLC chromatogram of peptide C (Hexa peptide) using 300 µg/ml and showing time dependent transepithelial transport
a: Apical solution within 1 min of incubation time
b: Basal solution after 5 min of incubation
c: Basal solution after 15 min of incubation
d: Basal solution after 60 min of incubation

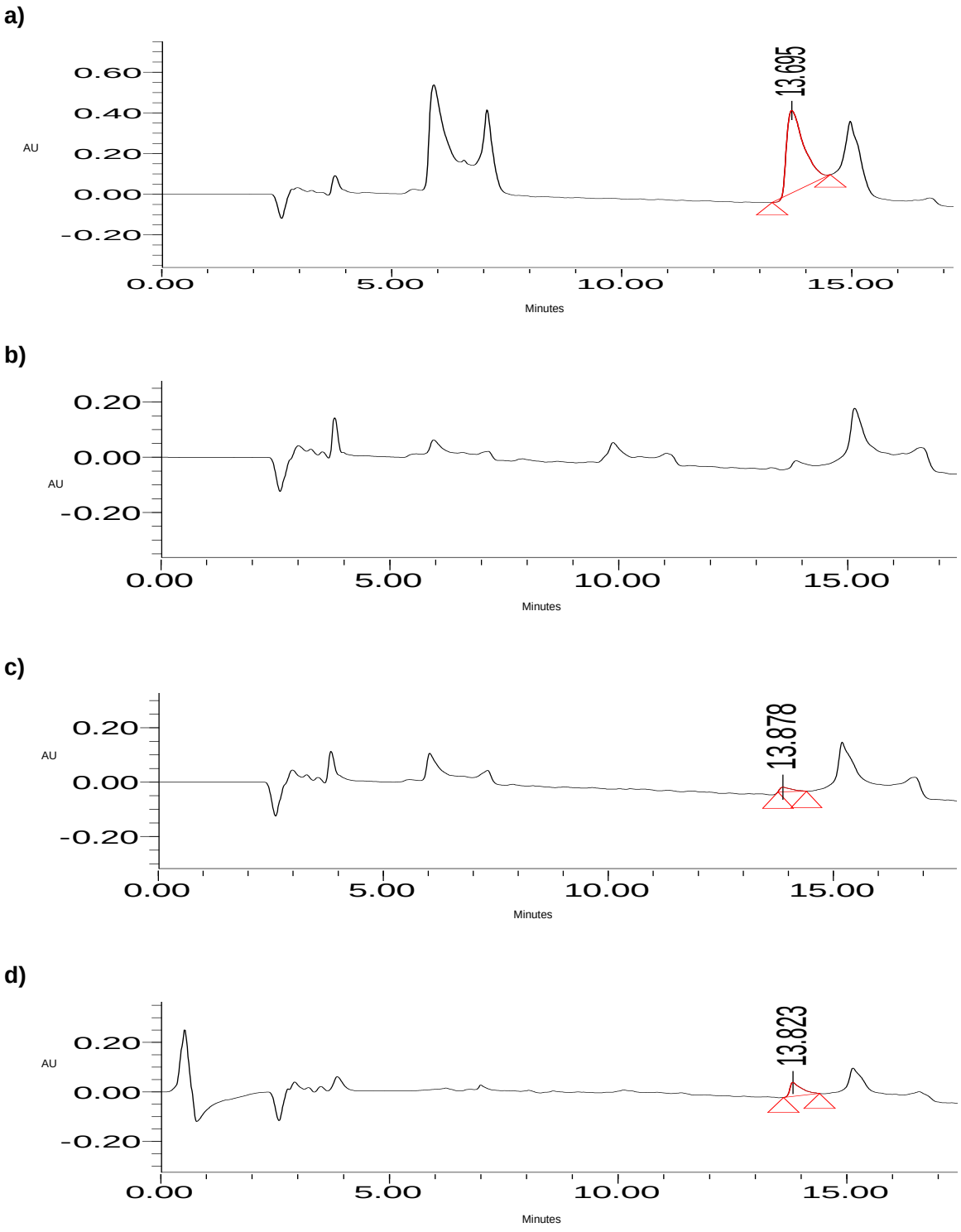
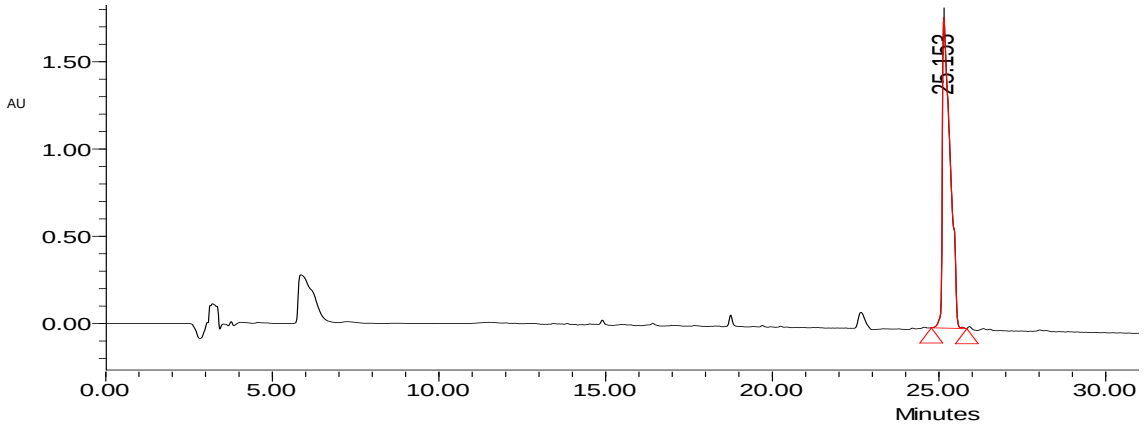


Figure 4.26. RP-HPLC chromatogram of peptide G (Tri peptide) using 400 µg/ml and showing time dependent transepithelial transport

a: Apical solution within 1 min of incubation
b: In basal solution after 5 min of incubation
c: In basal solution after 15 min of incubation
d: In basal solution after 60 min of incubation

a)



b)

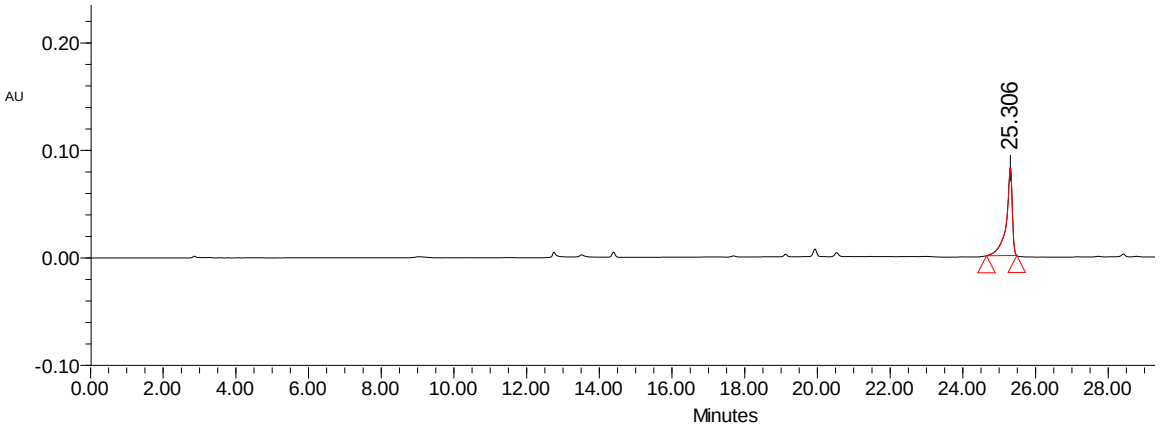
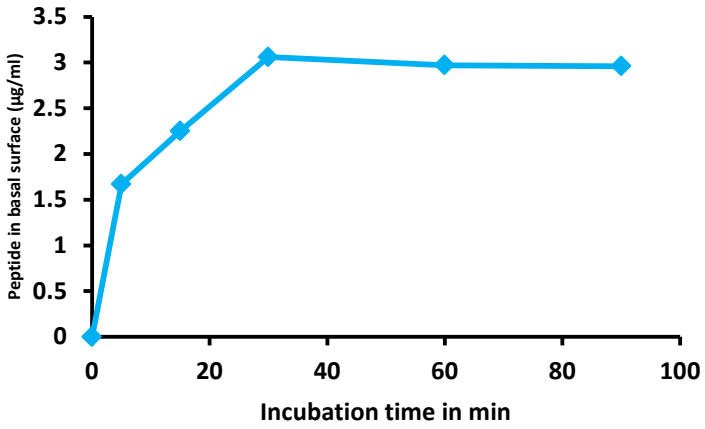


Figure 4.27. RP-HPLCchromatogram of bradykinin using 400 µg/ml and showing transepithelial transport
a: Apical solution with in one min of incubation time
b: Basal solution after 60 min of incubation of time

a)



b)

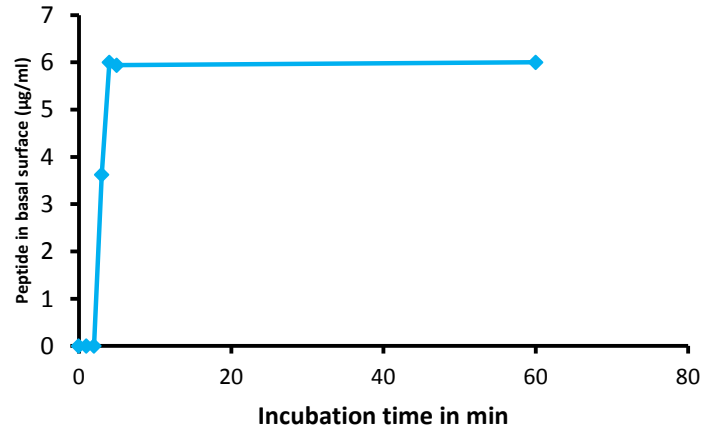


Figure 4.28. Time dependent transport of peptides across Caco-2 cell monolayer
a: Peptide C(Hexa peptide)
b: Peptide G (Tri peptide)

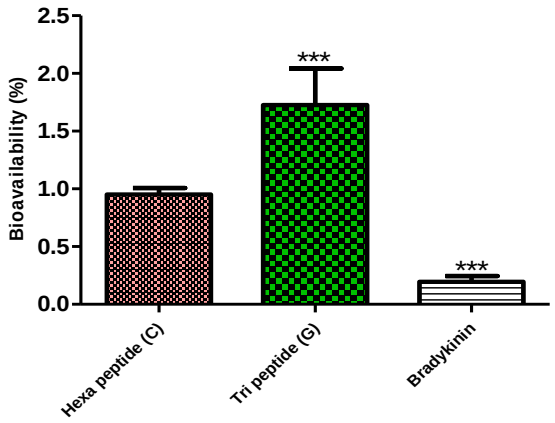


Figure 4.29. Bioavailability (%) of peptide C, peptide G and bradykinin
Values are expressed as Mean ±S.E.M (n=3). Asterisks representing the significancedifference (p***<0.001)

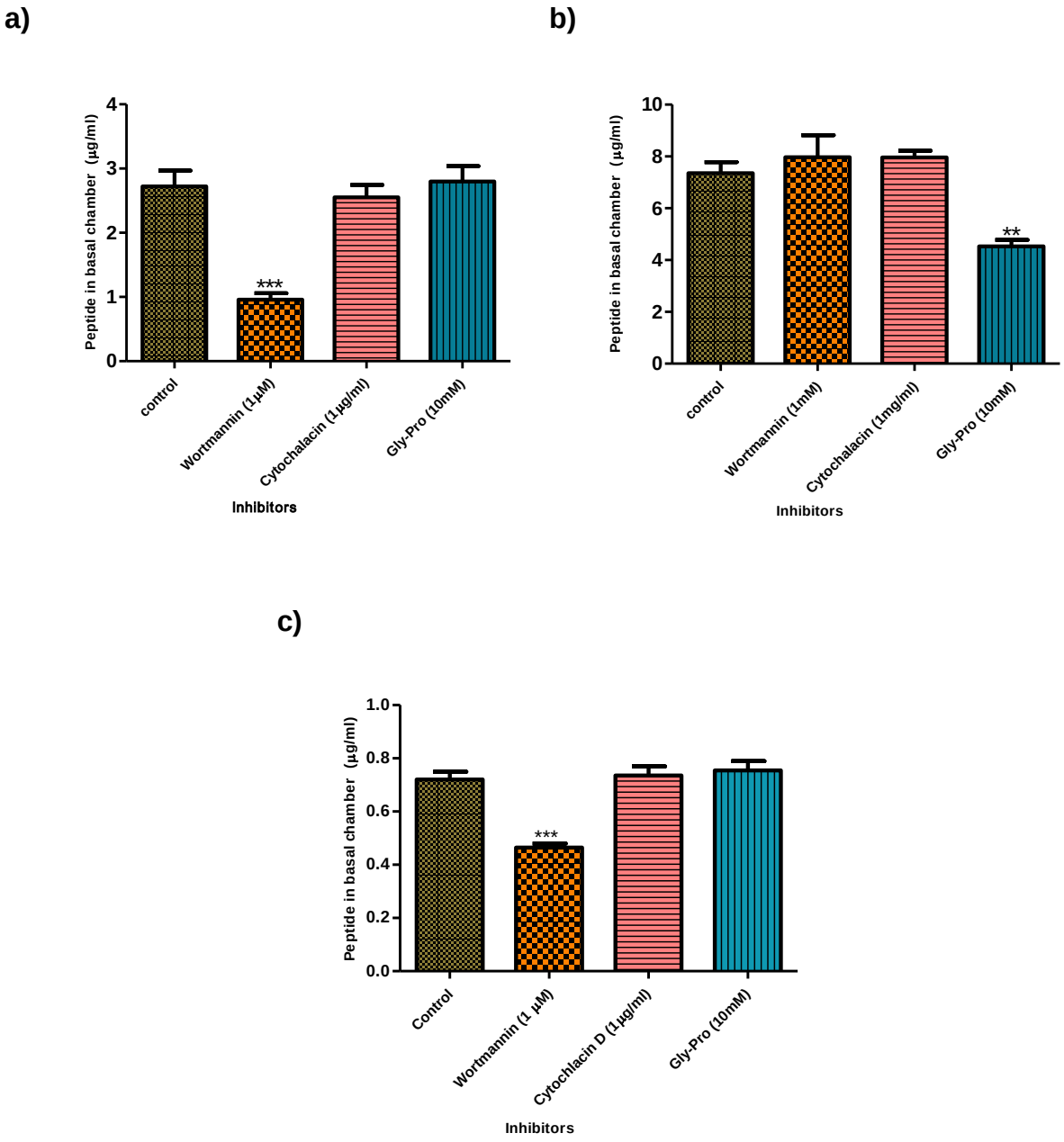


Figure 4.30. Effect of Gly-Pro, cytochalacin D and wortmannin on apical to basal transport of peptides across Caco-2 cell monolayer.
a:Peptide C (Hexa peptide)
b: Peptide G (Tri peptide)
c: Bradykinin (Nona peptide)

Values are expressed as Mean±S.E.M (n=3). Asterisks representing the significance difference (p**<0.01, p***<0.01) with control.

Chapter 5

Discussion

Discussion

Progression of life style associated diseases is mainly associated with the oxidative stress which can be considered an imbalance between the ratio of reactive oxygen species (ROS) production and degradation which leads to the alteration and activation of various signalling pathways of inflammatory reactions results in the destruction of the cell (Maeda and Omata, 2008; Montecucco *et al.,* 2011; Sell and Eckel, 2009). Overlapping compounds with antioxidative and immunomodulatory properties may reverse the adverse effects of such oxidative stress linked inflammatory conditions. Current studies have shown that among the food sources, milk contains various biologically active peptides with various overlapping activities. Phelan *et al.* (2009) reported that milk casein derived hydrolysates contained both antioxidative and anti-inflammatory effects under *in vitro* conditions. Hydrolysates generally contain numerous types of peptides with different bioactivities making them difficult to use for prophylactic and specific therapeutics. Hence, detailed basic studies are needed to determine the efficacy of products with characterisation of peptides under various experimental model systems. Therefore, under present study detailed evaluation of milk casein derived peptides was carried out under *in vitro* and *ex vivo* environments for determining their overlapping antioxidative and immunomodulation potentials.

5.1 Screening of peptides for free radical scavenging and immunomodulatory properties:

Initially the free radical scavenging property of casein derived ten peptides (A to J) by using six different concentrations ranging from 10 ng to 1 mg/ml has been carried out by using three chemical methods, which quantifies the hydrogen atom donating capacity of peptides. Peptides A, B, C, F and G at higher doses such as 100 µg and 1 mg/ml showed strongest inhibitory effect on peroxyl radical induced oxidation in ORAC assay. Similarly at the same concentrations, four peptides (B, C F and G) exhibited sufficiently high $ABTS^{\cdot+}$ free radical scavenging ability in ABTS method. In addition, peptides A, B, D and E were found to significantly scavenge the lipid peroxides which were produced during linoleic acid oxidation. The results in our findings are in agreement with the previous observations for the free radical

scavenging property of casein derived peptides and hydrolysates which was determined only by chemical methods (Bezerra *et al.,* 2013; Hernandez- Ledesma *et al.,* 2005). Antioxidative peptides containing aromatic amino acid residues (Trp and Tyr) in the sequence have strong radical scavenging activities due to the labile hydrogen atom (Saito *et al.,* 2003). Presence of Tyr amino acid in the peptides B, C, F and G might be responsible for their high free radical absorption capacity in chemical methods. On the other hand, peptides played an active role in immunomodulation under *ex vivo* studies in mice through splenocytes proliferation and phagocytosis by peritoneal macrophages. A considerable suppressive effect was observed on the murine splenocytes proliferation by the addition of lower doses (10 ng to 1 µg/ml) of peptides B, C and D. Similar response was also noticed in case of peptides F, G, H and I regardless of their concentration used. Previous studies also confirmed that depending on the concentration, various peptides were found to stimulate or suppress the proliferation of splenocytes. For example, Kayser and Meisel, (1996) who found that β-casomorphin-7 (BCM-7) had suppressive effect at lower doses (10^{-12} to 10^{-8} M) and stimulatory effect at higher doses (10^{-6} to 10^{-4} M) on splenocytes proliferation. In addition, peptides B, C, F, G, H and I also remarkably enhanced the percent phagocytosis in murine peritoneal macrophages. These findings for stimulatory effect of peptides on the phagocytosis in this study are in line with the experiments conducted by Parker *et al.* (1984) who determined the stimulatory effect of two peptides derived from human casein VGPIPY (Val-Gly-Pro-Ile-Pro-Tyr) and GLF (Gly-Leu-Phe) on the phagocytosis of sheep red blood cells at 1 µg/ml and 10 µg/ml respectively.

By using the above screening analysis, out of ten, four peptides (B, C, F and G) were chosen which exhibited maximum overlapping free radical scavenging as well as immunomodulation properties. However, only the chemical methods which were used under the current investigation for assessing radical scavenging capacity are not sufficient to fully justify the antioxidative potential of peptides. Whereas, the immunomodulaory parameters such as splenocytes proliferation and phagocytosis did not provide the detailed information about the pro/ anti-inflammatory nature of the peptides. Therefore, in present analysis, the antioxidative potential of peptides was further assessed under *in vitro* conditions by using human Caco-2 cell lines in the presence of H_2O_2 induced oxidative stress. While *ex vivo* murine model was

preferred to determine the immunomodulatory effect of peptides through estimating cytokine secretion in the supernatant of cultured spleen cells.

5.2 Assessment of cellular response of potential casein peptides *in vitro*

The human colon carcinoma cell line, Caco-2 has received much attention in recent years for its use as an *in vitro* model system of the intestinal epithelium. These cells were originally established from a well differentiated colon adenocarcinoma and under culture conditions they spontaneously differentiated, both structurally and functionally, into cells resembling mature enterocytes (Pinto, 1983). For the purpose of *in vitro* analysis we used Caco-2 cells after incubating with different concentrations ranging from 10 ng to 1 mg/ml of the four selected casein peptides (B, C, F and G) individually for 24 hr and found that the presence of the all the four peptides at their respective doses did not impair the cell viability and membrane integrity, indicating that the peptide concentrations selected for the study has no cytotoxic effect upon the cell viability. Such non toxic effect of casein peptides on Caco-2 cell viability was also observed by the earlier observations (Jing and Kitts, 2004) also. H_2O_2, a most valuable exogenous free radical generator was used to establish an oxidative stress induced cell injury model to investigate the antioxidative effect of compounds in Caco-2 cells. H_2O_2 is the least active ROS, its stability confers the ability to cross biological membranes and thereby to target a wide range of intracellular and extracellular sites (Fatokun *et al.,* 2006). Treatment with 1.5 mM H_2O_2 for 10 hr provoked a significant decrease in cell viability by 50% (IC_{50}) and also increased the ROS generation than negative control which is in line with previous observations of (García-Nebot *et al.,* 2014). The addition of higher concentrations of C and G peptides (100 µg/ml and 1 mg/ml) showed the capability to protect the cell viability against H_2O_2 induced oxidative cell death and also significantly inhibited the ROS production in Caco-2 cells. Although the other two peptides (B and F) did not display such protective effect on the cell viability but, they have been found to significantly abolish the H_2O_2 stimulated ROS generation in the cells at their higher amounts (1 µg to 1 mg/ml) used. This inhibitory effect of peptides on ROS formation strongly supported the direct free radical scavenging capacity of peptides which was determined by the chemical methods previously. These results in our findings are in agreement with the previous studies for the preservative effect of casein derived peptides on Caco-2 cell viability against H_2O_2 induced oxidative damage (Garcia-

Nebot *et al.,* 2011). The inhibitory response of casein peptides against ROS induction in the current analysis is also supported by the earlier findings of Cheng *et al. (2*015) who found the preventive effect of casein glycomacropeptide hydrolysate (GHP) on the intracellular ROS generation against H_2O_2 induced stress. In addition to inhibit the ROS production, 24 hr incubation of Caco-2 cells with peptides B, C, F and G were also observed to sufficiently decrease the levels of lipid and protein oxidation markers such as MDA and protein carbonyls respectively in response to oxidative stress (1.5 mM H_2O_2 for 10 hr). Similarly, antioxidative peptides supplementation to the *in vitro* cultured cells are also shown to suppress the MDA and protein carbonyl levels in various experimental studies (Zhao *et al.,* 2014; Nagasawa *et al.,* 2001; Jongberg *et al.,* 2009). Antioxidant agents reduce the oxidative stress in the cells may directly by scavenging the ROS or by activating antioxidative enzymes or by stimulating the detoxification mechanisms within cells, resulting in ROS removal. As enzymatic antioxidant systems, including CAT, SOD and GPx play an important role in protection against the deleterious effects of oxidative stress during disease conditions (Rahman, 2007). In the present investigation, treatment with all the four peptides (B, C, F and G) independently at different doses (10 ng to 1 mg/ml) in Caco-2 cells significantly enhanced catalase activity in the presence of H_2O_2, a main target substrate for the catalase enzyme. It strongly indicated the oxidative stress clearance capacity of peptides towards H_2O_2 induced stress generation in the cells. The increased catalase activity by peptides treatment in our study is also consonance with the previous observations reported for the stimulatory effect of casein hydrolysates on catalase activity in jurkat cells (Phelan *et al.,* 2009). Addition of peptide C to the cultured Caco-2 cells further increased the activity of SOD, an another antioxidative enzyme in the cellular system irrespective of its dose was used. In addition to affecting the catalase and SOD activities, cells treated with various doses of peptides C and F (10 ng to 1 mg/ml), considerably elevated the activity of GPx, an important enzyme in the redox cycle responsible for the reduction of H_2O_2.This stimulatory action of casein derived peptides on the activity of antioxidative enzymes clearly indicating the protective role of peptides against stress induced cellular oxidative damage. Further, cell sensitivity to a free radical attack was also suggested to depend on the relationship between the ratio of CAT/SOD and GPx/SOD rather than on absolute amounts of individual antioxidative enzyme (Michiels *et al.,* 1994). Increase in the ratio of CAT/SOD under

present investigation in the presence of peptides B, C and F, additionally established their ability to cope with H_2O_2 induced oxidative stress. Therefore the coordinative action of the four selected casein peptides (B, C, F and G) with the antioxidative enzymes may efficiently detoxify the adverse effects of oxidative stress in the cells.

Studies have reported that some antioxidants protect the cells through mechanisms involving the Nrf2 signalling pathway (Kobayashi & Yamamoto, 2006). This pathway is mainly responsible for the induction of antioxidative enzymes synthesis during oxidative stress conditions. Thus, we further examined the levels of Nrf2 and Keap1 mRNA expression in H_2O_2 treated cells in the presence of different doses (10 ng to 1 mg/ml) of casein peptides B, C, F and G. Pre-treatment of Caco-2 cells with the three peptides (B, C and G) considerably suppressed the mRNA expression of Nrf-2 than the H_2O_2 control cells without affecting the expression levels of Keap1, a protein involves in the degradation of Nrf-2 under normal cellular conditions. On the other hand, two peptides (C and G) were observed to facilitate the activation and translocation of Nrf-2 transcription factor from cytoplasm to the nucleus of the Caco-2 cells in response to H_2O_2 induced oxidative stress, which was assessed by using western blotting analysis. This stimulatory effect of peptides on Nrf-2 translocation further supporting the increased activities of antioxidative enzymes in response to the stress conditions induced with H_2O_2 as previously discussed above. Most of the previous studies also suggested that antioxidative compounds enhanced the antioxidative enzyme activities through Nrf-2 signaling mechanism (Jeong *et al.,* 2005; Hwang *et al.,* 2011). Casein hydrolysate derived from the glycomacropeptide (GHP) was found to induce the Nrf2 mediated heme oxygenase-1 (HO-1) gene expression in macrophages (RAW 264.7 cell line) against H_2O_2 induced stress (Cheng *et al.,* 2015). Curcumin (diferuloymethane), a yellow pigment from *Curcuma longa* with antioxidative property was found to induce the expression of HO-1 enzyme through the activation and translocation of Nrf-2 in the rat liver cells (Farombi *et al.,* 2008). Sulforaphane, a naturally occurring iso thiocyanate with promising chemopreventive activity was observed to increase the nuclear accumulation of Nrf-2 and up-regulation of *HO-1* expression in human branchial epithelial cells (Lee and Lee, 2011).

In addition to the antioxidative property, peptides played an active role in immunomodulation by *ex vivo* studies in mice by affecting the cytokine secretion in to

the supernatant of cultured splenocytes. Addition of all the four casein peptides (B, C, F and G) to the cultured spleen cells were found to significantly reduce the secretion of proinflammatory cytokine (IFN-γ) and enhanced the production of regulatory/anti-inflammatory cytokines (IL-10,TGF-β and IL-4) in the culture medium. The anti-inflammatory nature of the peptides was also supported by their suppressive effect on splenocytes proliferation in the previous objective. Thus, all these observations in conjunction clearly indicate the potential anti-inflammatory effect of peptides on immune system. These findings have also been corroborated by earlier researchers who found the anti-inflammatory property of casein/whey hydrolysates containing peptides produced by enzymatic hydrolysis on activated macrophages and respiratory and intestinal epithelial cells (Nielsen *et al.,* 2012; Piccolomini *et al.,* 2012; Iskandar *et al.,* 2013). All these observations strongly supporting the overlapping antioxidative and anti-inflammatory potentials of casein peptides (B, C, F and G). This overlapping activities of peptides in our results are in line with the preceding studies carried for the antioxidative and anti-inflammatory nature of various compounds. Root extracts of Angelica Dahurica showed antioxidative and anti-inflammatory abilities by stimulating antioxidative enzymes (CAT and SOD) and by inhibiting nitric oxide production in a dose-dependent manner in lipopolysaccharide-treated RAW264.7 cells (Pervin *et al.,* 2014). Various dietary ingredients such as curcumin, epigallocatechin gallate (EGCG), astaxanthin and lutein were also observed to have the preventive effect on oxidative stress generation and inflammatory conditions under various *in vitro* and *in vivo* model systems (Kant *et al.,* 2014; Pae & Wu, 2013; Yuan *et al.,* 2011; Rhone & Basu, 2008; Riccioni *et al.,* 2012).

5.3. Bioavailability and transepithelial transport of potential bioactive peptides with overlapping antioxidative and immunomodulatory activities

Implementation of peptides potential on biological effects depends largely on their ability to remain intact until reaching the target organ (Sienkiewicz *et al.,* 2009). Most peptides of more than three amino acids are extracellularly hydrolyzed by enzymes in the brush border membrane of the intestinal epithelium. Whereas, di and tri peptides, in contrast can be absorbed intact and hydrolyzed later (Segura-Campos *et al.,* 2011). In the current investigation, two peptides (C: hexa peptide and G: tri peptide) were selected for the bioavailability and transepithelial transport studies by

using Caco-2 cell monolayer cultures in the transwell plate. For the purpose of comparative study, bradykinin (nona peptide) was used as control peptide as its bioavailability was already established and is also found to shows maximum resistance capacity to brush border membrane peptidases (Shimizu *et al.,* 1997). Both of the peptides (C and G) studied appeared to show their transport in the intact form across Caco-2 cell membrane. However, after the addition of peptide C (hexa peptide) at its saturated concentration (300 µg/ml) to the apical chamber, only 2.57 µg/ml of peptide was recovered in the basal chamber intact at 30 min of incubation time, the remaining peptide either hydrolysed by membrane peptidases on the apical chamber or metabolised by epithelial cell during transcytosis. On the other hand, incubation of cells with the peptide G (tri peptide) at 400 µg/ml of its saturated dose on the apical surface, only 6.9 µg/ml was detected to be transported to the basal surface in intact form and a rapid decrease in its recovery was also noticed on apical chamber within a span of 4 min of incubation time. This degradative effect of membrane associated peptidases during the transport of peptides is also in agreement with the earlier findings of Ding *et al.* (2015) for the penta peptide (RVPSL) transport across the Caco-2 cell membrane. Bioavailability studies carried by Sienkiewicz *et al.* (2009) for bovine BCM-5 and BCM-7 (derived from β-casein) also supporting our results in the present study. On the other hand, bradykinin, which was used as control peptide showed resistance to membrane peptidases gave a single peak both at the apical and basal chambers as was in accordance with the findings of Shimizu *et al.* (1997). Peptide G was found to have higher bioavailability of about 1.72% (1.81 fold) than the peptide C which reached only 0.95% in basal chamber while, bradykinin had least bioavailability of 0.17%.

After assessing the bioavailability, the route for the transport of the casein derived peptides (C and G) across Caco-2 cell membrane was evaluated in the presence of specific inhibitors such as Wortmannin, Cytochalacin D and Gly-Pro which inhibit the transport of peptides through transcytosis, paracellular transport and PepT1 mediated transport mechanisms respectively (Ding *et al.,* 2014). Transport of di and tri peptides across the intestinal membrane is mainly mediated by Peptide transporter 1(PepT1) which is competitively inhibited by the presence of Gly-Pro (Nielsen and Brodin, 2003). Whereas the large peptides having more than three amino acids which can not be transported by PepT1 therefore they can be

translocated from apical to the basal surface though transcytosis mechanism, which involves membrane invagination and vesicle internalization of the compound (Sai *et al.,* 1998). In addition, para cellular transport pathway is also involved in the transport of peptides through the tight junctions between the cells (Sun *et al.,* 2009). In the present analysis, peptide C (hexa peptide) and bradykinin (nona peptide) were found to be transported through transcytosis transport pathway. These observations are also supported by the previous findings conducted for the transport of oligo peptides (4 to 16 amino acids) across Caco-2 cell membrane (Shimizu *et al.,* 1997; Regazzo *et al.,* 2010). On the other hand peptide G (tri peptide) transport was significantly inhibited by the presence of competitive inhibitor Gly-Pro suggesting its transport through transcytosis mechanism. These results in the current analysis are in line with the studies of Satake *et al.* (2002) who found the transport route of the tri peptide (VPP) through PepT1 mediated transport mechanism across Caco-2 cell monolayer.

Thus current investigation clearly establishes the overlapping antioxidative and immunomodulatory potential of four casein derived peptides (B,C,F and G) to varying extent using different experimental model systems with adequate bioavailability of the two of the peptides (C and G) studied across the human intestinal epithelial cells.

Chapter 6

Summary and conclusion

Summary and Conclusion

The present study was carried out to determine the overlapping antioxidative and immunomodulatory potentials of buffalo casein derived peptides under *in vitro* and *ex vivo* environments. In the first objective, ten peptides (A to J) were screened for their free radical scavenging capacity by using three chemical methods (ABTS assay, ORAC assay and linoleic acid model system) and immunomodulation property by using splenocyte proliferation index assay and phagocytosis of murine peritoneal macrophages under *ex vivo* conditions. Four peptides (B, C, F and G) were selected out of ten, based on their strong free radical scavenging and immunomodulatory properties and further evaluated them under cellular conditions in the second objective. Bioavailability and trasepithelial transport studies across the membrane were also performed for the two of peptides (C and G) having better overlapping antioxidative and immunomodulatory potential out of four in the third objective using Caco-2 cell monolayer. The results are summarized below.

Ten oligopeptides were custom synthesised after obtaining their sequence either from literature available on bovine or unpublished data from our laboratory and then changing amino acid wherever required according buffalo casein sequence based upon clustalW sequence similarity analysis.

6.1 To screen buffalo casein peptides for antioxidative and immunomodulatory activity

- Out of ten peptides (A to J), four peptides (B, C, F and G) at higher concentrations such as 100 µg/ml and 1 mg/ml strongly ($p<0.01$) absorbed the oxygen free radicals as compared to their lower doses (10 ng to 10 µg/ml) in the ORAC assay. Similarly, such efficient ($p<0.001$) radical absorption capacity was also shown by the peptide A at 1 mg/ml of its dose used. Though no significant activity was found in case of peptides D, E, H, I and J.
- In ABTS method, peptides B and C showed significant ($p<0.01$) concentration dependent radical scavenging ability. Whereas, two peptides (F and G) only at higher amounts (100 µg/ml and 1 mg/ml) displayed extraordinary ($P<0.01$) radical absorption ability in this method. However no significant scavenging property was found by the addition of peptides A, D, E, H, I and J.
- As compared to control in which no peptide was added, four peptides (A, B, D and E) at 100 µg/ml of dose exhibited strong ($p<0.01$) inhibitory effect

(62.13%, 43.98%, 33.27% and 30.86**%** respectively) on the oxidation of linoleic acid. While, the remaining peptides (C, F, G, H, I and J) displayed very less inhibitory response of about 11.1%, 8.87%, 7.8%, 8.5%, 7% and 7.63% respectively against linoleic acid oxidation.

- Under *ex vivo* conditions, supplementation of murine splenocytes with T- cell mitogen, Con A in the positive control remarkably ($p<0.001$) enhanced the proliferation than negative control in which no mitogen and peptide was added. While spleen cells incubated with three peptides (B, C and D) individually at their respective doses (10 ng to 1 µg/ml) considerably ($p<0.01$) suppressed the proliferation as compared to negative control. Similarly, such significant effect ($p<0.01$) was also observed by the peptides F, G, H and I independent of their amount added to the cells. Whereas, no statistical changes were observed in case of peptides A, E and J at their various amounts (10 ng to 1 mg/ml) used.
- On the other hand, as compared to control, murine peritoneal macrophages treated with higher concentrations such as 100 µg/ml and 1 mg/ml of three peptides (B, F and I) notably ($p<0.01$) enhanced the percent phagocytosis. Likewise, such stimulatory response on phagocytosis was also shown by peptide G at 100 ng to 1 mg/ml and peptide H at 10 µg to 1 mg/ml of dose used. However, no considerable effect was noticed by the peptides A, D, E and J.

6.2. To investigate the cellular response of potential casein peptide(s) *in vitro*

- Under *in vitro* conditions, extracellular supplementation of human intestinal adenocarcinoma cells, Caco-2 with different concentrations (10 ng to 1 mg/ml) of four selected peptides (B, C, F and G) separately in the culture medium for 24 hr did not showed significant changes in the cell viability as compared to control as assessed by MTT and neutral red assays.
- No statistical variations were observed in the LDH activity than control after incubation of cells with the peptides B, C, F and G individually at their respective doses (10 ng to 1 mg/ml) for 24 hr.
- Addition of 1.5 mM concentration of H_2O_2 to the Caco-2 cells for 10 hr remarkably ($p<0.01$) reduced the viability of Caco-2 cells to 50% (IC_{50}) in MTT assay as compared to negative control in which no H_2O_2 was added.

- Caco-2 cells treated with peptides C and G at higher doses (100 µg/ml and 1 mg/ml) considerably ($p<0.01$) increased the cell viability than H_2O_2 control, but, no such significant changes were observed by treatment with peptides B and F at their different amounts (10 ng to 1 mg/ml) used.
- Addition of H_2O_2 drastically ($p<0.001$) elevated the production of ROS (2.51 fold) in the cells than negative control. While cells pre-incubated with two peptides (B and F) at 1 µg to 1 mg/ml of amount respectively, showed strong ($p<0.01$) tumbling effect on the increased ROS levels as compared to H_2O_2 control. Similar inhibitory response was also detected ($p<0.01$) in the presence of peptides C and G at their higher doses ranging from 10 µg to 1 mg/ml used.
- Oxidative stress generation in Caco-2 cells due to H_2O_2 treatment (1.5 mM for 10 hr) remarkably ($p<0.001$) increased the levels of lipid and protein oxidation markers such as MDA (1.7 fold) and protein carbonyls (5.07 fold) respectively than negative control.
- A concentration dependent decrease ($p<0.01$) in MDA levels were observed by the pre-supplementation of cells with peptide B. Also, such drastical ($p<0.01$) dipping effect was also noticed by the peptides C, F, and G regardless of their concentration used.
- Increased protein carbonyl levels due to H_2O_2 induced stress were also suppressed significantly ($p<0.05$) by the addition of various amounts (10 ng to 1 mg/ml) of peptides B, F and G for 24 hr while, treatment with peptide C also showed such inhibitory effect ($p<0.05$) only at 1 mg/ml of its dose used.
- No statistical variations were noticed in nitric oxide levels in the supernatant of cultured cells after treatment with H_2O_2 as compared to negative control. Likewise, no changes were also detected by the supplementation of the four selected peptides (B, C, F and G).
- Oxidative stress generation in the cells with IC_{50} of H_2O_2 (1.5 mM for 10 hr) considerably ($p<0.01$) stimulated the activities of antioxidative enzymes such as catalase, SOD and GPx respectively than negative control.
- Pre treatment of Caco-2 cells with four selected peptides (B, C, F and G) individually at their respective amounts (10 ng to 1 mg/ml) for 24 hr, extremely ($p<0.01$) enhanced the activity of catalase as compared with H_2O_2 control.

- Cells supplemented with peptide C was found to remarkably ($p<0.01$) elevate the activity of SOD independent of its concentration used than H_2O_2 control, but no such changes were observed by treatment with different doses (10 ng to 1 mg/ml) of peptides B, F and G.
- A noticeable increase ($p<0.05$) was detected in the GPx activity by the addition peptides C and F at various amounts (10 ng to 1 mg/ml) as compared to H_2O_2 control. While, in case of peptides B and G, no such significant variations were observed.
- H_2O_2 treatment (1.5 mM) for 10 hr in the Caco-2 cells did not affect the ratio of CAT/SOD and GPx/SOD as compared to negative control. Whereas, cells pre-incubated with two peptides (B and G) at their higher doses ranging from 100 ng to 1 mg/ml considerably ($p<0.01$) enhanced the ratio of CAT/SOD than H_2O_2 control. Similar effect was also found by the peptide C at 1 µg to 1 mg/ml of its concentration used but, no such significant changes were noticed in case of peptide F. On the other hand, as compared to H_2O_2 control, addition of all the four selected peptides (B, C, F and G) at different doses (10 ng to 1 mg/ml) for 24 hr showed no statistical alterations in GPx/SOD ratio.
- As compared with the negative control, addition of H_2O_2 (1.5 mM for 10 hr) significantly ($p<0.01$) increased the mRNA expression of Nrf-2 in the Caco-2 cells. Whereas, pre-treatment with peptides B and C remarkably ($p<0.01$) reduced its expression at their lower doses ranging from 10 ng to 1 mg/ml which was further suppressed ($p<0.001$) on using higher amounts (10 µg to 1 mg/ml) in case of peptide C. Likewise, peptide G at lower doses (10 ng to 1 µg/ml) also found to have such reducing effect ($p<0.01$) on the expression of Nrf-2.
- Oxidative stress generation in the Caco-2 cells did not affect the Keap1 mRNA expression as compared to negative control. Similarly, such non considerable effect was also detected by the incubation with all the four selected peptides (B, C, F and G) at their various concentrations (10 ng to 1 mg/ml).
- As compared to negative and H_2O_2 controls, cells supplemented with peptides C and G at lower doses (10 ng to 1 µg/ml) were observed to stimulate the activation and translocation of Nrf-2 transcription factor from cytoplasm to the

nucleus of the cells. However, no such responses were noticed in the case of B and F-peptides.

- Under *ex vivo* conditions for determining the immunomodulatory property, the four selected peptides (B, C, F and G) at different doses (10 ng to 1 mg/ml) tremendously ($p<0.001$) reduced the levels of pro-inflammatory cytokine (IFN-γ) in the supernatant of cultured spleen cells than negative control.
- The secretion of IL-10, a regulatory/ anti inflammatory cytokine had been up regulated ($p<0.01$) by the peptides C and F irrespective of their concentration used while higher amounts of peptide B (10 µg to 1 mg/ml) and G (100 ng to 1 mg/ml) showed rising effect ($p<0.01$) was observed only at their higher amounts ranging from and respectively.
- Incubation of splenocytes with peptides F and G significantly ($p<0.01$) enhanced the release of TGF-β, an another regulatory/ anti-inflammatory cytokine in to the culture supernatant at their different doses ranging from 10 ng to 1 mg/ml as compared to control. While, no statistical changes were found in case of peptides B and C.
- Supplementation of spleen cells with peptide C at the dose of 1 µg to 1 mg/ml was noticed to increase IL-4 levels considerably ($p<0.01$) than negative control. Whereas, no such significant variations were noticed by the use of their peptides (B, F and G) at their various amounts (10 ng to 1 mg/ml) used.

6.3. To evaluate bioavailability and transepithelial transport of potential anti-oxidative and immunomodulatory peptide(s)

- As analysed by RP-HPLC, addition of peptide C (hexa peptide) and peptide G (tri peptide) at different concentrations (100,200,300,400 and 500 µg/ml) to the Caco-2 cells on the apical chamber for 60 min gave single peaks at 17 and 13 minutes of retention time corresponding to their standard respectively in the basal chamber and showed the saturation in peptide transport at 300 and 400 µg/ml respectively.
- In case of peptide C, the rapid increase in the transport to the basal chamber on using 300 µg/ml on apical side was observed upto 30 min, after that transport reached to saturation. On the other hand, peptide G transport in basal chamber obtained saturated levels within a span of 4 min upon incubating 400 µg/ml of it on apical side.

- Addition of 400 µg/ml dose of bradykinin, which was used as control peptide on the apical surface, showed resistance to the enzymatic degradation and gave a single peak at 25 minutes of elution time corresponding to its standard in the basal chamber after 60 min of incubation.
- The addition of wortmannin, an inhibitor of transcytosis showed remarkable ($p<0.001$) decrease in the flux of peptide C (hexa peptide) and bradykinnin in the basal chamber as compared to the control which was 35% and 63% respectively of the transport in control.
- Supplementation of cells with Cytochalacin D, a disruptor of tight junctions between the cells did not affect the transport of peptides C and G as well as bradykinin to the basal surface as compared to control.
- Incubation of cells with Gly-Pro which competes for peptide transporter PepT1, considerably ($p<0.01$) decreased the flux of peptide G (tri peptide) from apical to the basal chamber by 38%.
- Bioavailability was estimated to be more for the peptide G (1.72%) followed by peptide C (0.95%) and it was minimum for the bradykinin (control peptide) (0.17%) and the main route of transepithelial transport for peptide C and bradykinin was observed to be transcytosis while for peptide G it was detected to be through PepT1 mediated mechanism.

6.4. Conclusion

- Out of ten buffalo casein derived peptides studied, four peptides (B,C,F and G) were found to have strong overlapping antioxidative and immunomodulatory potentials based upon cellular assays.

- Bioavailability studies carried on two peptides (hexa peptide: C and tri peptide: G) on intestinal cell line (Caco-2 cells) showed their sufficient transport of 0.95% and 1.72% across the epithelial membrane in their intact form through transcytosis and PepT1 mediated mechanisms respectively.

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