EFFECT OF *SYZYGIUM CUMINI* IN GLUCOSE INDUCED CARDIAC STRESS

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(A) Introduction

I. Importance of the present study

The prevalence of diabetes is rapidly increasing day by day and has reached epidemic proportions worldwide. It is found to be associated with various micro and macrovascular complications such as coronary artery diseases and other cardiovascular disorders [1, 2]. The stress generated in hyperglycemia lead to two to four fold increase in cardiovascular diseases as compared to the non-diabetic groups [3, 4]. In developing countries, this ratio will occur more than double till the end of 2030 [5] and will become a leading cause of substantial morbidity and mortality among people. This pathological condition generates an imbalance between reactive oxygen species (ROS) production and the antioxidant defense system, thereby increasing ROS mediated oxidative stress on cardiac cell [6, 7].

The state of glucose induced oxidative stress not only disrupts the activity of cellular metabolism but also regulate extracellular matrix in cells and degrades structural collagens (mainly type IV collagen) leading to cardiac remodelling that involves molecular, cellular, and interstitial changes which ultimately modulate the size, shape and function of the heart. Cardiac failure is an outcome of the cascade of such events and contributes to modulation of the extracellular matrix (ECM) [8, 9]. Gelatinases, primarily MMP-2 (Gelatinase A- 72 kDa) and MMP-9 (Gelatinase B- 92 kDa) are most characterized matrix metalloproteinases in cardiac system [10]. The central role of MMPs in ECM remodeling makes them an attractive drug target during diabetic cardiomyopathies [11, 12]. A number of MMP inhibitors have been developed over the past few years, however, their design, synthesis, development and testing has been a big challenge due to their toxic side effects and no satisfactory results are obtained till date as they fail in clinical trials [13]. To identify new MMP inhibitors having less toxicity and more specificity is therefore of utmost importance [14]. In addition, drugs like metformin and pioglitazone prescribed by clinical practitioners against diabetes create more problems in the longer run in different organs eg. cardiovascular system, eyes, kidneys and nerves [15]. Therefore, there is a great need for the development of an effective, safe and cost effective therapy against diabetes associated cardiac complications.

Plants are natural source of phytochemicals, which have the potential to act against various diseases through their multifold properties [16, 17]. Recently, there is a great deal of interest

observed among scientists in identifying the safe and cheaper source of antioxidants that hold health promoting therapeutic potential.

At the same time, these plant based polyphenols are found to be less toxic and their marked effects in the prevention of oxidative stress have been well documented [18]. Plant based therapies are in trend from the ancient time. Various plants and plant products as shown in **Table 1** were known to have protective activities in various pathological conditions [19-22]. However the exact mechanism of their action is poorly defined.

Syzygium cumini (L.) skeels, (Myrtaceae) is found to be important in Ayurveda and Unani medicine to fight against diabetes. The plant is evergreen in nature with fruits rich in iron, calcium, phosphorus, minerals, vitamin-C, sodium, potassium and carotene and are known to useful in suppressing oxidative stress [23, 24]. *S. cumini* leaves have been used extensively for treatment of diabetes, stomach ache, leucorrhoea, fever, constipation gastropathy, dermopathy and possess antibacterial, anthelmintic, hypoglycemic and anti-diarrhoeal activities attributed to the presence of phytochemicals [25-27]. We selected *Syzygium cumini* for our study due to its antidiabetic nature and with an intention to explore its cardioprotective properties under glucose stress. The present study was designed to carry out analysis of bioactive components and anti glycoxidative properties in pulp and seeds of *S. cumini* and enriched extract was selected to investigate the cardioprotective potential under *in vitro* hyperglycemic conditions.

Many herbal products despite of their significant potential in *in vitro* systems failed to replicate similar efficiency in *in vivo* models due to their poor lipid solubility and size, resulting in poor absorption leading to poor bioavailability. To counter this issue we also synthesized silver nanoparticles (SNPs) by green methods using *S. cumini* methanol seed extract as reducing agent. SNPs have recently been studied for their antidiabetic potential where a significant reduction in blood glucose level was observed in diabetic rats and also found to be beneficial in delayed diabetic wound healing [28-30]. Our hypothesis is that, silver nanoparticles containing *S. cumini* phytoneutrients may pass blood brain barrier and be less toxic to human cardiac cells (dose & time dependent manner).

Table 1: List of Indian medicinal plants and their known therapeutic potency

S. No	Plants (Botanical Name)	Main Phytomolecules	Antidiabetic activity	Cardioprotective activity during diabetic stress	Anti Apoptotic activity	Anti matrix metallo proteinae activity	Molecular mechanisms
1	Bitter gourd (*Momordica charantia*)	Flavonoids	Yes	X	X	X	X
2	Tulsi (*Ocimum sanctum*)	Flavonoids, Polyphenols	Yes	X	X	X	X
3	Pomegranate (*Punica granatum*)	Anthocynins, Flavonoids, Tannins	Yes	X	X	X	X
4	Raimunia (*Lantana camara*)	Alkaloids, Terpenoids, Phenolics	Yes	X	X	X	X
5	Barbados nut (*Jatropha curcas*)	Phenolics, Flavonoids, Saponins	Yes	X	X	X	X
6	Geloy (*Tinospora cordifolia*)	Alkaloidal constituents	Yes	X	X	X	X
7	Chinese Aloe (*Aloe vera*)	Aloe emodin, Aloin	Yes	X	X	X	X
8	Amaltas (*Cassia fistula*)	Xanthenes, Flavans, Flavonols	Yes	X	X	X	X
9	***Haldi (*Curcuma longa*)**	**Curcuminoids, curcumin**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
10	***Jamun (*Syzygium cumini*)**	**Flavonoids, Phenols**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
11	***Arjun (*Terminalia arjuna*)**	**Alkaloidal constituents**	Yes	Yes	X	X	X
12	Garlic (*Allium sativum*)	Allicin, Tannins, Flavonoids	Yes	X	X	X	X
13	Kudampuli (*Garcinia combogia*)	(-) hydroxyl citric acid	Yes	X	X	X	X

14	Neem (*Azadirachta indica*)	Alkaloids, Saponins, Glycosides, Flavonoids	Yes	X	X	X	X
15	Melon (*Cucumis trigonus*)	Alkaloids, Lipids	Yes	X	X	X	X
16	Sharifa (*Annona squamosa*)	Anonaine, Benzyltetrahydro-isoquinoline	Yes	X	X	X	X
17	***Green tea (*Camellia sinensis*)**	**Lysine, Saponins, flavonoids**	**Yes**	**Yes**	**Yes**	**X**	**X**
18	Olive (*Olea europia)*	Oleuropenin, Mono and di unsaturated	Yes	X	X	X	X
19	Tejpatta (*Cinnamonan tamala*)	Cinnamic acid, flavonoids	Yes	X	X	X	X
20	Grapes (*Vitis vinifera*)	Phenolic compounds such as, catechins	Yes	X	X	X	X

- **Characterized by our group**

Table 1 shows list of some Indian medicinal plants exhibiting biological activities as observed from literature and studies conducted in our laboratory. Some of the plants such as *T. arjuna* were reported to be cardioprotective under glucose stress but was found to be failed in clinical trial and also their mechanism is not well characterized. Based on such comparative analysis, we selected *S. cumini*, suitable for our study as being a well known antidiabetic, it's cardioprotective, antiapoptotic and anti-MMP activity under glucose induced cardiac stress were not explored earlier. *S. cumini* is non toxic and taken as a dietary supplement. Most interestingly, we conducted our study on nonconsumed fruit part, the seeds of *S. cumini* that may provide a cost effective, safe and natural therapy in future against glucose induced cardiac stress.

(B) Origin of the proposed work

Diabetes is found to be commonly associated with cardiovascular diseases causing significant morbidity and mortality. Many antidiabetic drugs have cardiovascular side effects too. Cardiac toxicity believed to be a multi-factorial process and leads to cardiomyocytes death as

terminal downstream events. In diabetes, the levels of free radicals increase drastically, thereby disturbing the equilibrium between free radical productions and antioxidant capability which ultimately lead to cardiac failure. Based on the growing demands of natural products and considering the disadvantages associated with synthetic drugs, the study stimulates the usage of natural products. Reports have demonstrated that anti-oxidant natural substances including herbal medicines could inhibit the diabetic cardiomyopathies by inhibition of ROS generation. Hence *S. cumini* as an antidiabetic plant, may represent a promising source for protecting cardiac cells against the diabetic cardiomyopathy. Use of crude extracts rather than isolated compounds could be an approach to increase the efficacy of the therapy. To take into account the increasing diabetic population and associated cardiac malfestations, there is an upsurge to develop a safe and less toxic therapy for long-term relief. The proposed study is therefore designed to investigate the effect of *S. cumini* against glucose induced cardiac stress.

(C) The relevance and expected outcome of the proposed study

Proposed work aims to study the screening of cardioprotectants from traditionally used Indian medicinal plant, *Syzygium cumini* which would contribute to study the molecular mechanism of bioactive molecules and to develop therapeutic antioxidative and cardioprotective strategies against diabetic cardiomyopathies, a major cause of mortality worldwide.

(D) Key Questions

The key questions of the proposed studies were-

1. What are the various bioactive components in *Syzygium cumini*?
2. Which is the most enriched extract of *Syzygium cumini*?
3. What is the optimised glucose dose and time for generating stress on cardiac myocytes?
4. What is the safe *Syzygium cumini* dose for treatment of stressed cardiomyocytes?
5. Is the well known antidiabetic agent *S. cumini*, a persuasive cardioprotectant too?
6. Is there any effect of *Syzygium cumini* on extracellular matrix components*?*
7. Does *S. cumini* act as a therapeutic target for MMP inhibition under glucose induced cardiac stress?
8. What is the effect of *S. cumini* on inflammatory cytokines and apoptotic proteins?

9. Can we increase the bioavailability of most enriched *Syzygium cumini* extract?
10. Do silver nanoparticles synthesised using *S. cumini* methanol seed extract efficiently suppress glucose induced stress on cardiac myocytes?

(E) Objectives of the study

In order to answer these questions, the present study was designed to characterize *S. cumini* for the presence of phyochemicals and to analyze its cardioprotective potential under glucose induced stress. The main objectives of the study were-

Objective 1: Screening of *Syzygium cumini* pulp and seed extracts for their anti-glycoxidative potential and detailed characterization of selected *S. cumini* extracts

Objective 2: To investigate the effect of *S. cumini* methanol seed extract (MSE) on glucose induced cardiac stress

Objective 3: To evaluate the effect of *S. cumini* methanol seed extract (MSE) on extra cellular matrix components

Objective 4: Characterization of *S. cumini* silver nanoparticles (ScSNPs) and analyzing their cardioprotective potential on glucose induced stress

Based on the above objectives, the thesis is structured as follows:

Chapter 1- Introduction

Chapter 2- Review of literature

Chapter 3- Discusses about screening *of Syzygium cumini* pulp and seed extracts for their anti-glycoxidative potential and detailed characterization of selected *S. cumini* extracts

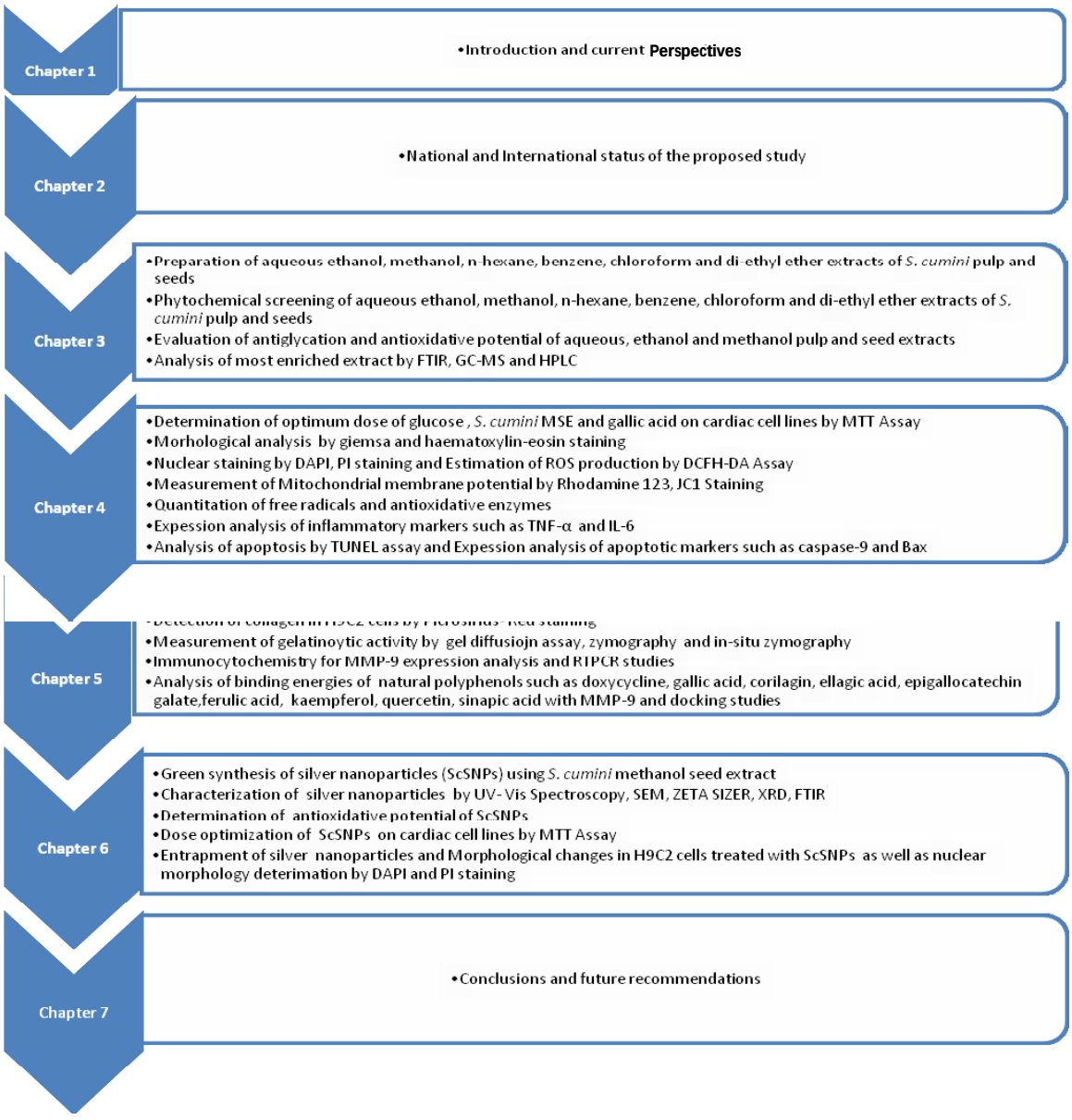
Chapter 4 – Contains study conducted to investigate the effect of *S. cumini* methanol seed extract (MSE) on glucose induced cardiac stress

Chapter 5 – Demonstrates the experiments and results performed to evaluate the effect of *S. cumini* methanol seed extract (MSE) on extra cellular matrix components

Chapter 6 – Includes study to characterize the *S. cumini* silver nanoparticles (ScSNPs) and analyzing their effect on glucose induced cardiac stress

Chapter 7 – Discusses the conclusion part of the research work, limitations and future Recommendations

(F) Design of Study



(G) Summary of thesis content

Chapter 1 Represents the introduction and the current perspectives of the proposed work.

Chapter 2 Describes the review of literature on national and international status of diabetes associated cardiac malfunction and the limitations of the existing therapies.

Chapter 3: Screening *of Syzygium cumini* pulp and seed extracts for their anti glycoxidative potential and detailed characterization of selected *S. cumini* extracts

This section deals with the introduction, material and methods, results, discussion and conclusions of the objective 1**.** The comparative analysis of various *S. cumini* extracts were performed to find out the most enriched extract. Initially, *S. cumini* fruits were collected and authenticated by a botanist. The seeds and fruits were separately examined for the presence of phytocontents. Aqueous and organic solvents pulp and seed extracts prepared in ethanol, methanol, diethyl ether, n-hexane, benzene and chloroform were screened for the presence of tannins, flavonoids, terpenoids, glycosides, phenol, alkaloids, steroids, saponins, reducing monosaccharide, anthraquinones and proanthocyanidins. Aqueous (ASE), ethanol (ESE) and methanol seed extracts (MSE) as well as aqueous (APE), ethanol (EPE) and methanol pulp extracts (MPE) showed the presence of most of the phytochemicals in higher amount as compared to diethyl ether (DSE; DPE), n-hexane (HSE; HPE), benzene (BSE; BPE) and chloroform (CSE; CPE) extracts. Therefore we limited our study towards the aqueous, ethanol and methanol pulp and seed extracts and performed a comparative analysis to explore most enriched extract of *S. cumini*.

For that, *firstly*, anti-glycoxidative potential of *S. cumini* seeds and fruit pulp was evaluated by antiglycation [31] as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH) [32], 2,2'-azino-bis(3 ethylbenzothiazoline-6-sulphonic acid) ABTS [33], Nitric Oxide (NO) [34], hydrogen peroxide (H_2O_2) [35] and superoxide anion (O_2^-) [36] assays. At 1 mg/ml concentration, methanol seed extract showed highest antiglycation (79 ± 1.00%) and scavenging potential for DPPH (60.87 ± 0.06%), ABTS (84.86 ± 2.18%), NO (72.87 ± 1.18%), H_2O_2 (75.65 ± 1.45%) and O_2^- (71.53 ± 2.04%) as compared to methanol pulp extract which showed antiglycation potential (59 ± 1.32%) and inhibition potential for DPPH (59.76 ± 0.50%), ABTS (81.61 ± 1.37%), NO (68.33 ± 1.33%), H_2O_2 (70.19 ± 2.38%) and O_2^- (64.19 ± 1.43%) respectively. Hence, our study illustrated that among aqueous, ethanol and methanol seed and pulp extracts,

methanol extracts showed highest scavenging for such free radicals. On further comparison between methanol pulp and seed extracts, highest antioxidative potential was observed for MSE.

Increasing evidences in both experimental and clinical studies suggest that free radicals formed disproportionately during diseases lead to increased lipid peroxidation. We therefore investigated the effect of *S. cumini* pulp and seed extracts on non enzymatic peroxidation of lipids by measuring the levels of malondialdehyde (MDA), which is produced based on the acid-catalyzed decomposition of lipid peroxides. The Ferric thiocyanate method indicates the amount of peroxide in the initial stages of lipid peroxidation whereas the thiobarbituric acid method shows the amount of peroxide in the secondary stage of lipid peroxidation [37, 38]. Among all the six extracts, highest peroxide inhibition potential was monitored for methanol seed extract. The ability of the methanol extracts to significantly suppress lipid peroxidation was due to the antioxidant activities of its phenolic components, known to act as free radical scavengers. Methanol seed extract showed the efficient hampering of peroxides than that of pulp extract.

Further study was conducted with aqueous, ethanol and methanol seed extracts to confirm the most potent extract. FTIR analysis showed highest peak intensity 3250-3450 cm^{-1} for polyphenolic OH group, 3000 cm^{-1} for aromatic C-H stretching, 2547-2973 cm^{-1} for C-H stretching, 3250-3450 cm^{-1} for primary aliphatic amines, Below 700 cm^{-1} for aromatic C-H out of plane deformation bands, 1000-1200 cm^{-1} for C-O single bonds, 1620 cm^{-1} for carbonyl groups [C=O] from the polyphenols in *S. cumini* MSE as compared to ESE and ASE. Subsequently, GC-MS analysis was carried out because of the high sensitivity and resolving power for the separation of mixtures and showed that MSE is enriched maximally with phyto components having antioxidant, antibacterial and anti-inflammatory activities as compared to rest. We confirmed the results by HPLC analysis that MSE is enriched with gallic acid (polyphenol). The gallic acid peak intensity and area was found to be highest in MSE as compared to ESE and ASE.

Conclusion:

This section concluded that *S. cumini* seed extracts contain maximal phytoconstituents which have higher antiglycation, antioxidative and lipid peroxides inhibition potential as compared

to *S. cumini* fruit pulp extracts. Also it was observed that methanol extract of *S. cumini* seeds is nutritionally and therapeutically most enriched as compared to ethanol and aqueous extracts.

Chapter 4: To investigate the effect of *S. cumini* methanol seed extract (MSE) on glucose induced cardiac stress

This chapter confers about the introduction, material, methods, results, discussion and conclusion of objective 2, where the cardioprotective effect of *S. cumini* methanol seed extract on glucose induced H9C2 cells was investigated. The cell lines used in this study were Heart-derived H9C2 cardiomyoblast cells and obtained from the National centre for cell science (NCCS), Pune, India. H9C2 cells has been proved to be efficient *in vitro* model for studying the cardiac stress as it reflects the similar responses to those observed in primary cardiac myocytes. Optimized doses for glucose, *S. cumini* MSE and gallic acid were found to be 4.5 mg/ml, 9 µg/ml and 3.4 µg/ml respectively by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. H9C2 cells were treated with optimal dose of glucose and *S. cumini* MSE. Gallic acid was taken as positive control for the study as *S. cumini* seeds were found to be rich in gallic acid derivatives. The entire study was carried out in six experimental groups. (a) Control (Untreated) Cells (b) Cells induced with 4.5 mg/ml glucose (GI) (c) Glucose-induced cells treated with *S. cumini* MSE (9 µg/ml) (GI + MSE) (d) MSE treated cells (MSE) (e) Glucose-induced cells treated with gallic acid (3.4 µg/ml) (GI + GAL) (f) Gallic acid treated cells (GAL). The treatments were given for 48 hrs. Various biochemical, fluorescence based and molecular assays were conducted next to confirm the cardioprotective potential of *S. cumini* MSE in glucose stressed cells.

In this regard, *firstly*, morphological assessment was done by nonstaining and staining methods such as Haematoxylin-eosin, giemsa staining. Nuclear morphology was detected by 4',6-diamidino-2-phenylindole (DAPI) and propidium Iodide (PI) staining. Glucose induced cells showed increase in cellular and nuclear size, however MSE reduced the increase in cell size upto control in glucose stressed cardiac cells. In order to measure the intracellular ROS level, DCFH-DA staining and FACS analysis was performed and MSE treatment showed the decrease in ROS level which was found to be elevated in glucose induced cells. The

scavenging of NO and H_2O_2 radicals and the activity of three biologically important antioxidative enzymes such as Catalase (CAT), Superoxide dismutase (SOD) and Glutathione S transferase (GST) were evaluated in glucose induced and MSE treated stressed cells. Alteration in mitochondrial membrane potential was determined by Nonyl acridine orange, Rhodamine 123 and JC-1 staining. Our data suggests that glucose-mediated cardiac stress is leading to change in mitochondrial membrane potential, suggesting mitochondrial-mediated pathway critical for progressive stress and MSE repaired the cells from mitochondrial dysfunction.

Tumor Necrosis Factor-α- and Inter leukin-6 play critical role in myocardial ischemia and heart failure. We further measured the expression of proinflammatory cytokines (TNF-α, IL-6) and apoptotic markers (caspase-9, Bax) in the above described experimental sets. Cells treated with glucose showed about 2 fold and 2.4 fold increase in TNF-α and IL-6 mRNA expression respectively and MSE treatment reduced it considerably. Stressed cells treated with Gallic acid also decreased the expression upto the control. The increase in expression of such markers indicated the hyperglycemia induced stress in cardiac cells and MSE treatment suppressed the expression. MSE also significantly reduce the glucose induced caspase-9 and Bax expression levels comparable to control.

Conclusion: Our study revealed that increase in oxidative stress due to ROS, mitochondrial stress, upregulaton in inflammatory and apoptotic markers in diabetic cardiomyopathy results in myocardial remodelling *in vitro* and MSE is having ability to significantly reduce these ROS-mediated events, further minimizing the glucose-mediated cardiac stress.

Chapter 5: To evaluate the effect of *S. cumini* methanol seed extract (MSE) on extra cellular matrix components

This chapter deals with the introduction, material and methods, results, discussion and conclusion of objective 3, where we explored the role of *S. cumini* as a natural inhibitor against gelatinase A & B activity in hyperglycemic conditions. Gelatinases (Matrix metalloproteinase- 2 & 9) are critical proteins responsible for ECM remodeling in diabetes. Previous reports stated that it remains latent in healthy hearts but induced during diabetic cardiomyopathies, causing extensive degradation of ECM and matrix turnover associated

cardiac abnormalities and heart failure [39]. MMP-9 promoter is highly conserved and contains nuclear factor-kappa B binding site, a key transcription factor for the production of MMP-9 known to be activated by various proinflammatory cytokines [40]. Immunoflurorescence studies revealed the localization of NF-κB from cytoplasm to nucleus in glucose induced cells whereas MSE treated glucose induced cells reduced its translocation in the nucleus. Gallic acid also showed inhibition of NF-κB localization inside the nucleus. Among ECM components, collagen is a major determinant of the modulated myocardial structural integrity and gives mechanical strength, stiffness and toughness to the vasculature [41]. Cardiac stress causes the imbalance between its synthesis and degradation and leads to malfunction in collagen turnover. We found in our studies the twofold increase in collagen content in glucose induced cells while MSE treatment reduced it considerably. Gallic acid also showed the decrease in collagen content nearby control. To explore the role of *S. cumini* MSE on ECM remodeling, total cell proteins from the above described experimental groups were isolated to analyze MMP-2 & 9 activities in all the sets. Zymography and disc diffusion assay showed the twofold increase in gelatinolytic activity of MMPs in glucose induced cells whereas MSE and gallic acid treatment inhibits the activity upto control. *In situ zymography* further validated a significant increase in the intensity of fluorescence by formation of FITC-Gelatin conjugated product in glucose stressed cells, however weak signal was detected on MSE exposure, whereas MMP-2 found to be constitutively expressed.

As a potential therapeutic target, MMP-9 activity was also screened at molecular and protein levels. For this, semiquantitative RT-PCR was conducted with the similar groups. MMP-9 expression was found to be increased in stressed cells whereas MSE treatment significantly decreased the expression levels comparable to control. This was confirmed by qRT-PCR which showed a 2 fold increase upon glucose treatment and MSE significantly reversed the upregulation of MMP-9 mRNA. These results indicate that *S. cumini* has a potential to suppress MMP-9 expression in glucose stressed cardiacmyocytes. Immunocytochemistry study clearly indicated the upregulation in the expression of MMP-9 upon glucose treatment whereas MSE or gallic acid treated stressed cells reduced MMP-9 protein expression to nearby control levels which further validate the results obtained in above mentioned assays. Hence our study highlights that MSE had an inhibitory effect on MMP-9 activity which is upregulated during *in vitro* hyperglycemic condition.

S. cumini is found to be enriched with various polyphenols such as corilagin, ellagic acid, epigallocatechin gallate, ferulic acid, gallic acid, kaempferol, quercetin and sinapic acid etc. For further confirming MMP as a natural inhibitor of MMPs, docking studies were conducted by AutoDock Vina. The binding energies of *S. cumini* polyphenols with MMP-2 & 9 elucidated the strong interaction of these molecules with MMPs. However, Corilagin was found to have maximum affinity of -8.0 and -8.6 Kcal/mol for MMP-2 and MMP-9 respectively among all the polyphenols and interestingly gallic acid showed the least binding energy. It was found that all the polyphenols docked on Zn^{2+} metal binding site (consensus sequence HEBGHxLGLxHS) on three histidine residues (His226, His230 and His236) of the catalytic domain of MMP-2 & 9. Our docking results suggest that *S. cumini* may exert its effect by blocking the substrate binding site of MMPs. Doxycycline, a FDA approved MMP inhibitor showed binding energy of -6.8 and -7.1 Kcal/mol for MMP-2 & 9 correspondingly and was used as a positive control. Our docking results prove that *S. cumini* polyphenols can serve as a safe, and efficient MMP-9 inhibitor parallel to Doxycycline and have potential to suppress the high glucose induced cardiac stress *invitro*.

Conclusion: Our study concluded that *Syzygium cumini* may employ as a natural matrix metalloproteinase-9 inhibitor in glucose stressed cardiac cells

Chapter 6: Characterization of *S. cumini* silver nanoparticles (ScSNPs) and analyzing their effect on glucose induced cardiac stress

This chapter describes the introduction, material and methods, results, discussion and conclusion of objective 4**.** The role of silver nanoparticles of methanol seed extract of *S. cumini* in glucose induced cardiac cells was examined. Recently silver nanoparticles of plant extracts were found to be efficient against diabetes. However some reports stated the toxicity of chemically synthesized nanoparticles in different organs such as heart, kidney, liver etc. than that of 'green' method. Therefore "green" methods for the synthesis of silver nanoparticles have increasingly become a topic of interests as conventional chemical methods are expensive and have toxicity issues. Green synthesis of nanoparticles is ecofriendly, cost effective and less toxic as it uses natural molecules as reducing agents. We therefore synthesized silver nanoparticles using *Syzygium cumini* methanol seed extract which is found to be most enriched in phytonutrients, and had strong antioxidative potential.

To check their toxicity, we tested them in our glucose induced cardiac *in vitro* models. For that, we have synthesized silver nanoparticles of methanol seed extract of *S. cumini* (ScSNPs) by green method to evaluate their effect on glucose induced cardiac stress. The synthesized silver nanoparticles were characterized by UV-Vis spectra, SEM, Zeta sizer, XRD and FTIR analysis. Colour change of the extract (dark brown) gave an approximate idea about the synthesis of nanoparticles. UV-Vis spectra showed sharp peak intensity in UV range at 416 nm. The size of these nanoparticles was also investigated by SEM analysis and zeta sizer. To determine the crystallographic structure, XRD was conducted and the pattern showed different intensity peaks in the whole spectrum of 2θ values with a scanning rate of $1°$ per minute. The diffraction peaks were consistent with the standard JCPDS database (No. 04-0783 for ScSNPs) and specifically indexed to a face centered cubic (FCC) crystal structure. FTIR analysis showed the highest peak intensity for 1000-1200 cm-1 for C-O single bonds and below 700 cm^{-1} for aromatic C-H out of plane deformation bands, confirmed the abundance of functional groups and polyphenols. Our characterization studies corroborated the formation of silver nanoparticles and their structural information to evaluate its effect on glucose induced cardiac stress. Antioxidative potential of these ScSNPs was also analyzed by DPPH and ABTS assays. We found that ScSNPs had higher antioxidative potential than *S. cumini* MSE. This study showed that ScSNPs can be used as a potential radical scavenger against cardiotoxicity caused by high glucose induced free radicals.

To assess the effect of ScSNPs in cardiac cell lines against glucose induced stress, $20\ \mu g/ml$ dose of ScSNPs was selected by MTT assay. Further, we conducted our study in four groups. (i) Control (ii) Glucose induced (GI) (iii) Glucose induced + silver nanoparticles of *S. cumini* MSE (GI+ ScSNPs) (iv) Silver nanoparticles of *S. cumini* MSE alone (ScSNPs). SEM analysis showed the entrapment of silver nanoparticles in cells. Morphological analysis revealed the protection due to ScSNPs in glucose stressed cardiacmyocytes. No toxicity was observed in ScSNPs alone treated cells. Nuclear staining was performed by DAPI and PI stains to observe the changes in nuclear morphology and the study showed the reversal of stress from glucose on ScSNPs treatment.

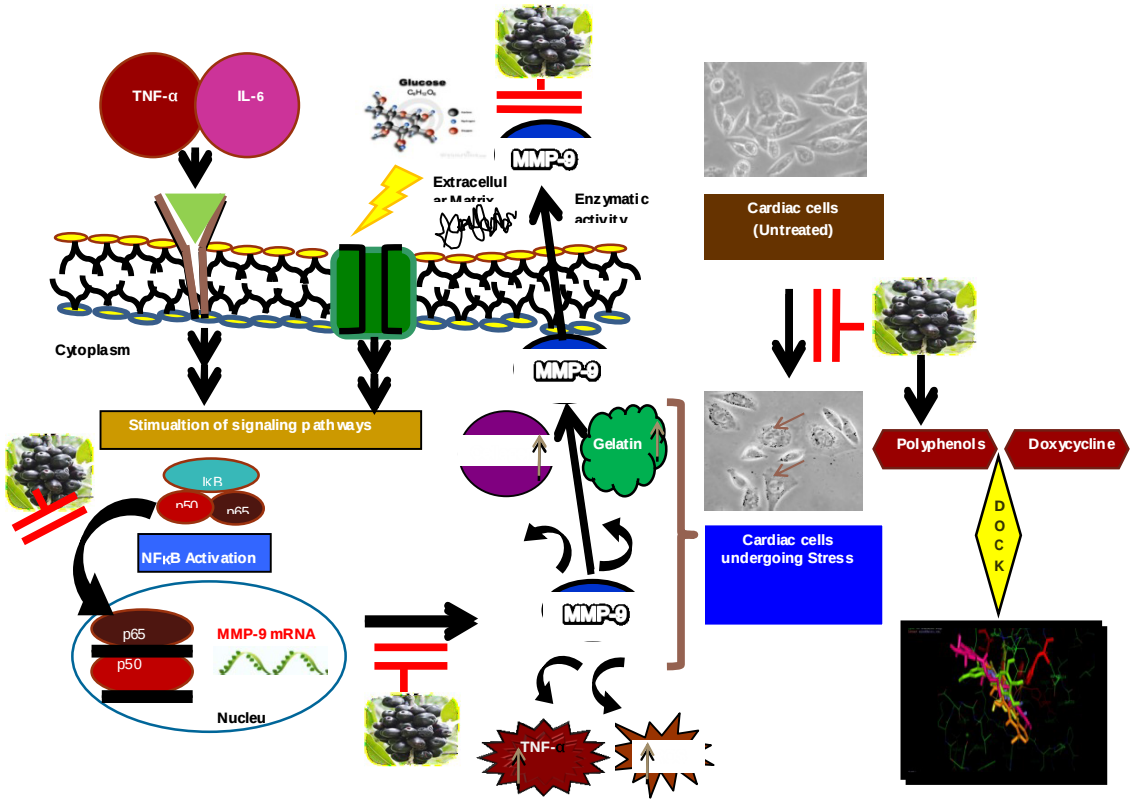
Conclusion: Our study proved the antioxidant and non-toxic nature of *S. cumini* silver nanoparticles. Hence, these ScSNPs may possess greater bioavailability and may be well utilized in cardiac protection against glucose mediated stress.

Chapter 7: Thesis Conclusions/ Future recommendations

Here we summarize the conclusions made in the entire study. This section includes the limitations and future recommendations of the present study. It has been concluded from our research that *S. cumini* has the potential to alienate the effects of cardiac stress in hyperglycemic condition. This establishes the dual effective role of *S. cumini* as anti-diabetic and cardioprotective. Our study suggests that in order to develop potentially therapeutic compounds, the bioactive molecules have to be isolated from *S. cumini* that represent potential source of molecules of significant relevance for developing novel drugs for treating and /or controlling the *in vitro* diabetic cardiomyopathy.

Future perspectives

- Validation of *S. cumini* cardioprotective potential in primary rat cardiacmyocytes and *in vivo* models
- Proposing the potential use of any natural polyphenols such as corilagin, quercetin, epigallocatechin gallate etc. as an MMP inhibitor shall be advantageous in terms of its cost, safety and availability.
- In depth understanding of *S. cumini* silver nanoparticles to enhance its pharmacological potential
- In future, novel therapeutic strategies can be designed as certain barriers like bioavailability and poor absorption may limit its activity.



Proposed model: *S. cumini* MSE, a natural inhibitor against glucose induced MMP-9 mediated cardiac stress by inhibiting nuclear localization of NF-κB, ROS overproduction and targeting inflammatory markers.

BIBLIOGRAPHY

[1] Cade W. T., "*Diabetes-related microvascular and macrovascular diseases in the physical therapy setting*", *Physical Therapy*, vol. 88, pp. 1322-1335, 2008.

[2] Fuller J. H., Stevens L. K., Wang S. L., "*Risk factors for cardiovascular mortality and morbidity: the WHO mutinational Study of vascular disease in diabetes*", *Diabetologia,* vol. 44, pp. S54-64, 2001.

[3] Chavali V., Nandi S. S., Singh S. R., Mishra P. K., "*Generating double knockout mice to model genetic intervention for diabetic cardiomyopathy in humans*", *Methods in Molecular Biology*, vol. 1194, pp. 385-400, 2014.

[4] Sowers J. R., Epstein M., Frohlich E. D., "*Diabetes, hypertension, and cardiovascular disease: an update*", Hypertension, vol. 37, pp. 1053-1059, 2001.

[5] Osei K., "*Global epidemic of type 2 diabetes: implications for developing countries*", *Ethnicity and Disease,* vol. 13, pp. S102-S106, 2003.

[6] Blokhina O., Violainen E., Fagerstedt K. V., "*Antioxidants, oxidative damage and oxygen deprivation stress: A review*", *Annals of Botany,* vol. 91, pp. 179-194, 2003.

[7] Umadevi S., Gopi V., Simna S. P., Parthasarathy A., Yousuf S. M. J., Elangovan V., "*Studies on the cardio protective role of gallic acid against AGE-induced cell proliferation and oxidative stress in H9C2 (2-1) cells*", *Cardiovascular Toxicology*, vol. 12, pp. 304-311, 2012.

[8] Li Y. Y., Mc Tiernan C. F., Feldman M., "*Interplay of matrix metalloproteinases, tissue inhibitors of metalloproteinases and their regulators in cardiac matrix remodelling*", *Cardiovascular Research*, vol. 46, pp. 214-224, 2000.

[9] Spinale F. G., "*Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function*", *Physiological Reviews*, vol. 87, pp. 1285-1242, 2007.

[10] Guo J., Jie W., Shen Z., Li M., Lan Y., Kong Y., Guo S., Li T., Zheng S., "*SCF increases cardiac stem cell migration through PI3K/AKT and MMP-2/9 signaling*," International Journal of Molecular Medicine, vol. 34, pp. 112-118, 2014.

[11] Fingleton B., "Matrix metalloproteinases as valid clinical targets," *Current Pharma Design*, vol. 13, pp. 333-346, 2007.

[12] Fingleton B., "MMPs as therapeutic targets--still a viable option?," *Semin Cell Developmental Biology*, vol. 19, pp. 61-68, 2008.

[13] Coussens L. M., Fingleton B., Matrisian L. M., "*Matrix metalloproteinase inhibitors and cancer: trials and tribulations*", *Science*, vol. 295, pp. 2387-2392, 2002.

[14] Tu G., Xu W., Huang H., Li S., "*Progress in the development of matrix metalloproteinase inhibitors,*" *Current Medicinal Chemistry*, vol. 15, pp. 1388-1395, 2008.

[15] Panicker G. K., Karnad D. R., Salvi V., Kothari S., "*Cardiovascular risk of oral antidiabetic drugs: Current evidence and regulatory requirements for new drugs*," *Journal of the Association of Physicians of India*, vol. 60, pp. 56-61, 2012.

[16] http://www.traffic.org/medicinal-plants.

[17] Pandey K. B., Rizvi S. I., "*Plant polyphenols as dietary antioxidants in human health and disease*", Oxidative Medicine Cellular Longevity, vol. 2, pp. 270-278, 2009.

[18] Bouayed J., Bohn T., "*Exogenous antioxidants- Double-edged swords in cellular redox state. Health beneficial effects at physiologic doses versus deleterious effects at high doses*", Oxidative Medicine Cellular Longevity, vol. 3, pp. 228-237, 2010.

[19] Chen H., Yao Ke., Nadas J., Bode A. M., Malakhova M., Oi N., Li H., Lubet R. A., Z. Dong. "*Prediction of molecular targets of cancer preventing flavonoid compounds using computational methods*", Plos One, vol. 7, e38261, 2012.

[20] Wang N., Wang Z., Mo S. L., Loo T. Y., Wang D. M., Luo H. B., Yang D. P., Chen Y. L., Shen J. G., Chen J. P., "*Ellagic acid, a phenolic compound, exerts anti-angiogenesis effects via VEGFR-2 signaling pathway in breast cancer*", Breast Cancer Research Treatment, vol. 134, pp. 943-955, 2012.

[21] Kohli S., Chhabra A., Jaiswal A., Rustagi Y., Sharma M., Rani V. "*Curcumin suppresses gelatinase B mediated norepinephrine induced stress in H9C2 cardiomyocytes*", *Plos One*, vol. 8: e76519, 2013.

[22] Filipiak K., Hidalgo M., Silvan J. M., Fabre B., Carbajo R. J., Pineda-Lucena A., Ramos A., de Pascual-Teresa B., de Pascual-Teresa S., "*Dietary gallic acid and anthocyanin cytotoxicity on human fibrosarcoma HT1080 cells. A study on the mode of action*", *Food Function*, vol. 5, pp. 381-389, 2014.

[23] Gowri S., Vasantha K., "*Phytochemical screening and antibacterial activity of Syzygium cumini leaves extracts*," International Journal of pharm Tech Research, vol. 2, pp. 1569-1573, 2010.

[24] Ravi K., Sekar D. S., Subramanian S., "*Hypoglycemic activity of inorganic constituent of Eugenia jambolana seed on streptozotocin induced diabetes in rats*", Biological Trace Element Research, vol. 99, pp. 145-155, 2004.

[25] Mohamed A. A., Ali S. I., El-Baz F. K., "*Antioxidant and antibacterial Activities of crude extracts and essential oils of Syzygium cumini leaves*", PLoS One, vol. 8, e60269, 2013.

[26] Bhandary M. J., Chandrashekar K. R., Kaveriappa K. M., "*Medical ethnobotany of the siddis of Uttara Kannada district, Karnataka, India*," Journal of Ethnopharmacology, vol. 47, pp. 149-158, 1999.

[27] Ayyanar M., Subash-Babu P., "*Syzygium cumini* (L.) *Skeels: A review of its phytochemical constituents and traditional uses*," Asian Pacific Journal of Tropical Biomedicine, vol. 2, pp. 240-246, 2012.

[28] Tang J., Xiong L., Zhou G., Wang S., Wang J., Liu L., Li J., Yuan F., Lu S., Wan Z., Chou L., Xi T. "*Silver nanoparticles crossing through and distribution in the blood-brain barrier in vitro*," Journal of Nanoscience and Nanotechnology, vol. 10, pp. 6313-6317, 2010.

[29] Alkaladi A., Abdelazim A. M., Afifi M., "*Antidiabetic activity of zinc oxide and silver nanoparticles on streptozotocin-induced diabetic rats*", International Journal of Molecular Sciences, vol. 15, pp. 2015-2023, 2014.

[30] Mishra M., Kumar H., Tripathi K., "*Diabetic delayed wound healing and the role of silver nanoparticles*", Digest Journal of Nanomaterials and Biostructures, vol. 3, pp. 49-54, 2008.

[31] McPherson J. D., Shilton B. H., Walton D. J. "*Role of fructose in glycation and cross-linking of proteins,*" Biochemistry, vol. 27, pp. 901-907, 1988.

[32] Brand-Williams W., Cuvelier M. E., Berset C., "*Use of a radical method to evaluate antioxidant*", Lebensm-Wiss U-Technology, vol. 28, pp. 25-30, 1995.

[33] Re R., Pellegrini N., Proteggente A., Pannala A., Yang M., Rice-Evans C., "*Antioxidant activity: Applying an improved ABTS radical cation decolorization assay*", Free Radical Biology and Medicine, vol. 26, pp. 1231-1237, 1999.

[34] Garrat D. C., "*The quantitative analysis of drugs*", 3rd ed. Japan: Chapman and Hall Publishers; 1964.

[35] Ruch R. J., Cheng S. J., Klaunig J. E., "*Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from chinese green tea*", Carcinogens, vol. 10, pp. 1003-1008, 1989.

[36] Nishimiki M., Rao N. A., Yagi K., "*The occurrence of super-oxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen*", Biochemical and Biophysical Research Communication, vol. 46, pp. 849-853, 1972.

[37] Saha K., Lajis N. H., Israf D. A., Hamzah A. S., Khozirah S., Khamis S., Syahida A., "*Evaluation of antioxidant and nitric oxide inhibitory activities of selected Malaysian medicinal plants*", Journal of Ethnopharmacology, vol. 92, pp. 263-267, 2004.

[38] Rahmat A., Kumar V., Fong L. M., Endrini S., Sani H. A., "*Determination of total antioxidant activity in three types of local vegetables shoots and the cytotoxic effect of their ethanolic extracts against different cancer cell lines*", Asia Pacific Journal of Clinical Nutrition, vol. 12, pp. 292-295, 2003.

[39] Mishra P. K., Chavali V., Metreveli N., Tyagi S. C., "*Ablation of MMP-9 induces survival and differentiation of cardiac stem cells into cardiomyocytes in the heart of diabetics: a role of extracellular matrix*," Canadian Journal of Physiology and Pharmacology, vol. 90, pp. 353-360, 2012.

[40] Tung W. H., Tsai H. W., Lee I. T., Hsieh H. L., Chen W. J., Chen Y. L., Yang C. M., "*Japanese encephalitis virus induces matrix metalloproteinase-9 in rat brain astrocytes via NF-κB signaling dependent on MAPKs and reactive oxygen species*", Brazilian Journal of Pharmacology, vol. 161, pp.1566-1883, 2010.

[41] Wess T. J., "*Collagen fibrillar structure and hierarchies. In Fratzl. P. (ed.). Collagen structure and mechanics*", New York, Springer, LCC, 2008.

AUTHOR'S PUBLICATIONS

1. **Neha Atale**, Sahil Gupta, Umesh Chandra Yadav, Vibha Rani (2014). Cell-death assessment by fluorescent and nonfluorescent cytosolic and nuclear staining techniques. Journal of Microscopy 255(1):7-19. Impact factor- 2.15, Indexed in SCOPUS, PUBMED H Index -76, H5 Index- 29.

2. **Neha Atale**, Khushboo Gupta, Vibha Rani (2014) Protective effect of Syzygium cumini against pesticide-induced cardiotoxicity. Environmental Science Pollution Research 21(13):7956-7972. Impact factor-2.757, Indexed in SCOPUS, PUBMED, H5 Index- 36.

3. **Neha Atale**, Mainak Chakraborty, Sujata Mohanty, Susinjan Bhattacharya, Darshika Nigam, Manish Sharma, Vibha Rani (2013). Cardioprotective role of Syzygium cumini against glucose- induced oxidative stress in H9C2 cardiac myocytes. Cardiovascular Toxicology 13(3):278-289. Impact Factor- 2.060, Indexed in SCOPUS, PUBMED, H Index- 32, H5 Index- 19.

4. **Neha Atale**, Vibha Rani (2013). GC-MS analysis of bioactive components in the ethanolic and methanolic extract of Syzygium cumini. International Journal of Pharma and Bio Sciences 4(4):296–304. Impact factor-2.958, Indexed in SCOPUS, GOOGLE SCHOLAR, International indexed Journal.

5. **Neha Atale**, Astha Jaiswal, Aastha Chhabra, Umang Malhotra, Shrey Kohli, Sujata Mohanty, Vibha Rani (2011). Phytochemical and antioxidant screening of Syzygium cumini seed extracts: A comparative study. Journal of Pharmacy Research. 4(12): 4530-4532. Impact factor- 2.667 TM (India), JPR: BioMedRx:An International indexed Journal

6. **Neha Atale**, Vibha Rani. Antiglycoxidation potential of *Syzygium cumini* seeds: An *in vitro* study (Communicated)

7. **Neha Atale**, Hitesh Kumar Jaiswal, Vibha Rani. *Syzygium cumini*: A natural matrix metalloproteinase-9 inhibitor in glucose stressed cardiac cells Submitted in journal of functional food (Under revision)

8. **Neha Atale**, Vibha Rani. Green synthesis and characterization of silver nanoparticles of S. cumini methanol seed extract and their effect against glucose induced cardiac stress (Manuscript under preparation).

Publications as a co-author

9. Aditi Jain, **Neha Atale**, Shrey Kohli, Susinjan Bhattacharya, Manish Sharma, Vibha Rani (2015) An assessment of norepinephrine mediated hypertrophy to apoptosis transition in cardiac cells: A signal for cell death. Chemico-Biological Interactions 225:54-62 (Impact factor- 2.982 indexed in SCOPUS, PUBMED) H Index-79, H5 Index- 45.

10. Umang Malhotra, Astha Jaiswal, Aastha Chhabra, **Neha Atale**, Vibha Rani (2012). Computational structural and functional characterization of protein family: Key for the hidden mystery. Journal of Pharmacy Research 5(7):3643-3649. Impact factor- 2.667TM (India), JPR: BioMedRx: An International indexed Journal.

11. Deepika Dogra, Suchit Ahuja, Shruti Krishnan, Shrey Kohli, Anand Ramteke, **Neha Atale**, Vibha Rani (2011). Phytochemical screening and antioxidative activity of aqueous extract of Indian Camellia sinensis. Journal of Pharmacy Research. 4(6):33-35. Impact factor- 2.667TM (India), JPR: BioMedRx : An International indexed Journal.

Book Chapter:

Vibha Rani, Surymya Asthana, Mohit Vadhera, Umesh Chand Singh Yadav, Neha Atale "Tools and techniques to measure oxidative stress" in "free radicals in human health & disease" Springer publisher Ltd. Doi 10.1007/978-81-322-2035-0-4, 2015.

Conference Publications:

Participation in International Conferences:

1. Attended "International Conference on "Bioproduts from Natural Resources" in The Department of Biotechnology at Jaypee Institute of Information Technology (JIIT), NOIDA, in association with Scientity Inc., 3 February, 2011.

2. Oral presentation in "International Conference on Recent Advances in Ayurvedic pharmaceutics (ICRAAP- 11)" organized by Dept. of Rasa Shastra, Faculty of Ayurveda, Institute of medical sciences, Banaras Hindu University, 14-15 October 2011.

3. Poster presentation in "International Conference on Genes Ggenetics & Genomics: Today & Tomarrow- Human concerns –ISHG-2012" organized by Panjab University, Chandigarh, 3-5 March 2012.

4. One day International Symposium on "Evolutions 2013 – Evolving Solutions in Protein Interaction Analysis" in Leela palace, New Delhi, 17 May, 2013.

5. Abstract Published in International Conference (ISC -2012) International science congress association- 2012, 8-9 Dec 2012.

6. One day International Symposium on "Evolutions 2014 – Evolving solutions in protein interaction analysis" in Leela palace, New Delhi, 13 May, 2014.

7. Poster Presentation and Abstract Published in The Department of Biotechnology at Jaypee Institute of Information Technology (JIIT), NOIDA, in association with Scientity Inc., organized an International Conference "Bioproducts and the OMICS Revolution", March 16-17, 2013.

8. Two days International Conference "XII Annual Meeting of the Indian Section: International society for heart research" 14-15 March 2015 in JNU, New Delhi.

Participation in National Conferences:

1. Poster presentation in AICTE sponsored National Seminar "Industry Expectations from Pharmacy College" organized by ITS Paramedical (pharmacy) College Ghaziabad, 5-6 August 2011.

2. Best Oral presentation award in "National Seminar on Transcriptomics: A Recent Era" organized by BCS –Insilico biology, Lucknow (India), 7 April 2012.

3. National Workshop and training programme on BDFACSCalibur- 3-5 October 2012.

4. One day National Scientific event organized by Eppendorf, New Delhi, 8 March 2013.

5. One day National IPR Workshop organized by IPR and Patenting Activities Committee, Jaypee Institute of Information Technology, Noida, 23 August 2014.

6. Best Oral presentation award in one day National conference BIOGENESIS-IV organized by Department of Biotechnology, IILM, Grater Noida, 29-30 July 2015.