CHAPTER VII

7. IN VITRO PHARMACOLOGICAL ACTIVITIES OF *Myxopyrum serratulum*

A. W. Hill (MS) AND *Nilgirianthus ciliatus* Nees (NC) ETHANOLIC EXTRACTS AND GELATIN ENCAPSULATED *Myxopyrum serratulum* A. W. Hill (GMSN) AND *Nilgirianthus ciliatus* Nees NANOPARTICLES (GNCN)

7.1. INTRODUCTION

In India traditional medicines have been used for many centuries by a substantial proportion of the populations (Amit Pandey, Parul Singh, 2011). World Health Organization (WHO) estimated that 80% of the populations of developing countries rely on traditional medicines, mostly plant drugs, for their primary health care needs (Prabakaran M et al., 2011). Medicinal plants play an important role in the discovery of novel drugs used in modern medicines (D. Jeya Sheela et al., 2012). Pharmacological research on the medicinal properties of phytochemicals has become mandatory to establish the claimed medicinal properties of herbs (Jayashree T et al., 2011). *In vitro* mechanism based screening of herbal medicine is mandatory in the initial phases of plant drug research before taking them to *in vivo* study to evaluate their efficacy.

7.2. Materials

DPPH (1, 1-diphenyl-2-picryl hydrazyl) and other chemicals used for antioxidant activity were procured from Sigma chemicals. L6 myotubes and 3T3L1 preadipocytes were procured from National Centre for Cell Science, Pune. MTT-(3-(4, 5-dimethyl thiazol -2-yl)-5-diphenyl tetrazolium bromide was obtained from SRL chemicals. Phosphate buffered saline and antibiotics were obtained from Gibco Invitrogen. Foetal bovine serum and Dulbecco’s Modified Eagles Medium were
obtained from Lonza. Rosiglitazone, Insulin and Glucose were obtained from Sigma Aldrich. Glucose oxidase - peroxidase (GOD-POD) kit was obtained from Accurex Biomedical Pvt. Ltd.

7.3. ANTIOXIDANT ASSAYS

7.3.1. DPPH radical scavenging activity assay: (Brand Williams W et al., 1995)

DPPH (1, 1-diphenyl-2-picryl hydrazyl) method is the most accepted model for evaluating the free radical scavenging activity of any new drug. The DPPH assay method is based on the reduction of DPPH, a stable free radical (purple color) to the non radical form DPPHH (yellow color) in the presence of hydrogen donating antioxidants. The degree of decolorization (yellow color) depends on the number of electrons captured; decolorization indicates the scavenging potential of the antioxidant compounds

Different concentration of ethanolic extracts of MS, NC, GMSN2 and GNCN3 mixed with 1 ml of 0.3 mM methanolic DPPH solution. The mixture was shaken vigorously and incubated for 30 min in the dark at room temperature and the absorbance was measured at 517 nm. Ascorbic acid was used as a positive control.

Degree of DPPH radical scavenging activity was expressed as

\[
\text{Percentage of inhibition} = \frac{A \text{ control} - A \text{ sample}}{A \text{ control}} \times 100
\]

Where A control is the absorbance of the DPPH alone and A sample is the absorbance of DPPH with different concentration of the test samples.
7.3.2. Nitric oxide scavenging assay: (Garrat, 1964 & B.N.Panda et al., 2009).

Nitric oxide scavenging activity can be estimated by the use of Griess Illosvoy reaction. At physiological pH (7.2), sodium nitroprusside in aqueous solution generates NO. Under aerobic conditions, NO reacts with oxygen to produce stable products (nitrate and nitrite) which can be determined using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions which was measured at 540 nm using UV spectrophotometer.

The reaction mixture containing sodium nitroprusside (10 mM) in phosphate buffered saline and different concentrations of MS, NC, GMSN2 and GNCN3 in ethanol were incubated at 30°C for 2 h. The same reaction mixture without the extract but the equivalent amount of ethanol served as the control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H₃P0₄ and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with Naphthylethylenediamine dihydrochloride was immediately read at 540 nm. Inhibition of nitrite formation by the plant extracts, nanoparticles and ascorbic acid were calculated relative to the control using the formula

\[
\text{% of inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100
\]

Where \( A_{\text{control}} \) is the absorbance control without the sample and \( A_{\text{sample}} \) is the absorbance of different concentrations of test samples.
7.4. In vitro antidiabetic activities

7.4.1. Cell Culture Procedure

L6 myoblasts and 3T3L1 preadipocytes were cultured in DMEM with 10% foetal bovine serum (FBS) and supplemented with penicillin (120 units/ml), streptomycin (75 µg/ml), gentamycin (160 µg/ml) and amphotericin B (3 µg/ml) at 37°C in 5% carbon dioxide atmosphere. For differentiation, the L6 myoblasts were transferred to DMEM with 2% FBS for 4 days, post confluence. The level of differentiation was measured by observing the multinucleated cells. 3T3L1 preadipocytes were grown in 24 well plates until 2 days post-confluence and the cells were induced by the differentiation medium (combination of 0.5 mM/l of IBMX, 0.25 µM/l of DEX and 1 mg/l of insulin in DMEM medium with 10 % FBS) to differentiate into adipocytes. Three days after induction, the differentiation medium was replaced with medium containing 1 mg/ml insulin alone. After 2 days, the medium was subsequently replaced again with fresh culture medium (DMEM with 10 % FBS), the extent of differentiation was established by observing the formation of multinucleation in cells.

7.4.2. Cytotoxicity Assay

MTT assay is a colorimetric technique used for measuring energetic cell metabolism by determining the activity of mitochondrial succinate dehydrogenase enzyme (T. Mosmann, 1983). It is based on the reduction of water soluble yellow tetrazole compound by mitochondrial succinate dehydrogenase enzyme into an insoluble violet formazan crystal.

L6 myoblasts & 3T3-L1 preadipocytes were seeded at a density of 1x10⁴ in a 96 well plate and allowed to attach to the well plate for 24 h. Cells were treated with
different increasing concentrations of NC, MS, GMSN2 and GNCN3. At 48 h following plating, the cells were incubated with 100 µl of fresh medium supplemented with MTT solution (0.25 mg/ml) for 45 min. The supernatant culture medium was aspirated and the insoluble formazan crystals were dissolved in DMSO for at least 2 h in the dark. MTT reduction was quantified by measuring the absorbance at 570 nm using a microplate reader. The effect of plant extract and nanoparticles on cell viability was expressed using the formula

\[
\text{Percent viability} = \left( \frac{\text{OD of untreated cells} - \text{OD of treated cells}}{\text{OD of untreated cells}} \right)
\]

7.4.3. Glucose Uptake Assay

Glucose uptake activity of NC, MS, GMSN2 and GNCN3 was measured in differentiated L6 cells. After starvation, the cells were treated with insulin, MS, NC, GMSN2 and GNCN3. These will bind to the receptors on the surface of the cells. These triggered the translocation of glucose transporters to the cell surface. Then treat it with 2 deoxy-D-glucose, so the glucose will enter into the cell. Glucose uptake rate was measured by using glucose oxidase peroxidase method and determine effect of MS, NC, GMSN2 and GNCN3 on the glucose uptake activity.

Glucose uptake activity was determined in differentiated L6 cells by non radioactive method described by Pareek et al (2009). The ability of plant extract to induce glucose uptake was measured in two different ways ie, Glucose uptake in absence of insulin (extract alone) and in presence of insulin (extract with insulin). Differentiated cells were serum starved for 5 h and were incubated with different non toxic concentrations of extract, nanoparticles, standard drug and added glucose (1M), incubated at 37°C for 30 minutes and then cells were either stimulated with 10 nM
insulin or left untreated for 20 min. After experimental incubation, cells were rinsed once with Krebs Ringer phosphate buffer (KRB) solution and were subsequently incubated for 15 min in KRB buffered solution. The glucose uptake was terminated by washing the cells thrice with ice cold KRP buffer solution. Cells were subsequently lysed by freezing and thawing thrice and an aliquot of cell lysates were used to measure the cell associated glucose. Glucose uptake was measured as the difference between the initial and final glucose content in the incubated medium by GOD-POD method (Trinder, 1969).

One ml of reagent was mixed with 10 µl of sample and incubated for 10 min at 37°C, within 60 min the absorbance of sample and standard was measured at 510 nm against reagent blank. The time interval from sample addition to measured time must be exactly same for standard, control and sample.

7.4.4. Antiadipogenic assay

Adipogenesis is the process of differentiation of different cell types into adipocytes. Adipocytes accumulate triglycerides, in the form of lipid droplets which can be measured by Oil Red O staining.

3T3-L1 preadipocyte was cultured in maintenance medium comprised of DMEM containing 10% FBS and antibiotics. 3T3L1 preadipocytes were induced by the combination of IBMX, DEX and insulin to differentiate into adipocytes (day 0). After induction for 72 h, the differentiation medium was replaced with 10% FBS–DMEM containing 1 mg/l insulin for 48 h (day 5). The medium was replaced again with fresh culture medium for 48 h (day 7). The degree of differentiation of the cells was investigated by adding different concentration of MS, NC, GMSN2 and GNCN3 ranging from 15.6 µg/ml to 250 µg/ml from day 0, a period of time which covered the
entire induction and post induction stages. Alternatively, preadipocytes were maintained with fresh FBS–DMEM for the whole induction period. Oil red O staining assay was performed at the end of the induction period to monitor the degree of differentiation. Photomicroscopic evaluation was also carried out for the comparison of triglyceride accumulation.

**Oil red o staining**

3T3-L1 adipocytes were washed twice with phosphate buffered saline at pH 7.4 and fixed with 10% formalin for 30 min. Cells were rinsed with deionized water and then stained with oil red O (0.25% w/v in 60 % Isopropanol) at room temperature for 30 min. Finally, the dye retained in the 3T3-L1 cells was extracted with isopropanol and quantified by measuring the absorbance at 540 nm using microplate reader (Huang, 2006). The relative lipid contents were calculated from

\[
\text{Percentage of lipid content} = \left(\frac{\text{OD of sample} - \text{OD of non-differentiated control}}{\text{OD of untreated control} - \text{OD of non-differentiated control}}\right) \times 100
\]

**7.5. RESULTS AND DISCUSSION**

**7.5.1. Antioxidant activity**

Antioxidants are the substances which have the properties of breaking free radical chain reaction. Due to their scavenging activity, antioxidants are useful for the management of various diseases. Many medicinal plants contain extensive quantity of phytochemical compounds exhibiting antioxidant properties. The free radical scavenging properties of MS, NC, GMSN2 and GNCN3 was evaluated by DPPH and nitric oxide scavenging assay methods. These two methods are relatively stable free radical methods for determining the antioxidant properties of herbal drugs. In both
methods, the ethanolic extracts and its nanoparticles exhibited antioxidant properties in a dose dependent manner.

DPPH is a stable, synthetic radical that does not disintegrate in water, methanol, or ethanol. The free radical scavenging activities of extracts depend on the ability of antioxidant compounds to lose hydrogen and the structural conformation of these components (Fukumoto and Mazza, 2000). DPPH radicals solution prepared in methanol is converted into DPPH-H (diphenylhydrazine) molecule in the presence of an antioxidant agent which leads to discoloration. The discoloration of the DPPH therefore reflects the radical scavenging activity of the extracts. In DPPH radical scavenging assay method, MS and GMSN2 showed relatively strongest inhibition of DPPH radical with 90.93 and 95.17% respectively, While NC and GNCN3 exhibited 76.09 and 83.81% of DPPH radical inhibition respectively at 1000 µg/ml (Figure 7.1). The standard ascorbic acid showed 96.51% scavenging potential at 100 µg/ml. The results of MS and GMSN2 were comparable with the standard ascorbic acid. The higher percentage of inhibition represents excellent antioxidant potential. In DPPH assay method, GMSN2 exhibited very good antioxidant activity with lowest IC50 value 99.40 µg/ml.

The antioxidant potency of MS, NC, GMSN2 and GNCN3 was measured by nitric oxide scavenging assay method. In this method, nitric oxide or reactive nitrogen species formed during their reaction with oxygen or with superoxides. These compounds are responsible for altering the structural and functional behavior of many cellular components. Incubation with sodium nitroprusside in phosphate buffer saline at 25°C for 2 h resulted in linear time-dependent nitrite production, which is reduced by the ethanolic extracts of MS, NC, GMSN2 and GNCN3. This may be due to the
antioxidant principles in the extract, which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. The scavenging potential was increased with an increased concentration of MS, NC, GMSN2 and GNCN3. The maximum nitric oxide scavenging activity was observed in GMSN2 with 79.63 % compared to MS which showed 72.89 % inhibition at 1000 µg/ml (Figure 7.2). The IC\textsubscript{50} values were 238.95 µg/ml and 375.11 µg/ml for GMSN2 and MS respectively. The IC\textsubscript{50} value of NC was 748.28 and GNCN3 was 399.28 µg/ml. The antioxidant property implies that it is capable of donating hydrogen atom in a dose dependent manner because the antioxidant substance having the ability to reduce ROS by donating the hydrogen atom. It is important to be noted that MS and GMSN2 had greater inhibition comparative to NC and GNCN3 but less than ascorbic acid, it showed 82.31% inhibition of NO at 100 µg/ml concentration. Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Rice-Evans \textit{et al.}, 1997; Jorgensen \textit{et al.}, 1999)

Antioxidant compounds are usually in the phenolic form. The antioxidant properties of phenolic compounds originate from their properties of proton loss, chelate formation and dismutation of radicals. The larger quantity of phenolic compounds in the MS extract may be the causative factor towards the excellent antioxidant property. Since the phenolic compounds are the primary antioxidants or free radicals terminators due to the presence of hydroxyl groups, which function as hydrogen donor. The study results suggest that MS and GMSN2 showed excellent antioxidant activity. The radical scavenging effect of the ethanolic extract of MS and its nanoparticles GMSN2 may be due the presence of larger quantity of total phenolic compounds. It is proved that MS ethanolic extract has high phenolic contents and so
correlated the antioxidant activity than NC. Many studies in the literature present positive correlations between the quantity of phenolic compounds and the antioxidant activities (Sagar and Singh, 2011 & Liu et al., 2009). Hence this excellent antioxidant activity could be responsible for other pharmacological activities like antidiabetic, anticancer and hepatoprotective.

Figure 7.1. DPPH scavenging effect of MS, NC, GMSN2 and GNCN3

Results were expressed as Mean±SEM

(Standard drug: Ascorbic acid – 96.26% at 100µg/ml)
Results were expressed as Mean±SEM

(Standard drug: Ascorbic acid – 82.31% at 100µg/ml)

7.5.2. Cytotoxicity studies

Micro culture tetrazolium assay method was used to evaluate the non toxic concentration of NC, MS, GNCN3 and GMSN2 on L6 cells and 3T3L1 cells. It measures the metabolic activity by measuring the mitochondrial activity through enzymatic reaction on the reduction of MTT to formazan. The amount of formazan crystals produced is directly proportional to the number of active cells. In the present study, the ethanolic extract of NC, MS and its nanoparticles GNCN3, GMSN2 was screened for its cytotoxicity against L6 myoblasts and 3T3 L1 preadipocytes at different concentration from 7.8 to 1000 µg/ml. The cell viability profile after treated with NC, MS, GNCN3 and GMSN2 is presented in figure 7.3 and 7.4. The results showed that the percentage of cell viability was decreased with increasing concentration of GMSN2 and GNCN3.
Figure 7.3: MTT assay results of NC and GNCN3 in L6 Myotubes and 3T3 L1 preadipocytes

When the doses were increased, the cells viability reduced and there was a least viability at a dose of 1000 µg/ml. IC\textsubscript{50} values of MS was 894.74 µg and 714.97 µg.

Figure 7.4: MTT assay results of MS and GMSN2 in L6 Myotubes and 3T3 L1 preadipocytes

When the doses were increased, the cells viability reduced and there was a least viability at a dose of 1000 µg/ml. IC\textsubscript{50} values of MS was 894.74 µg and 714.97 µg.
\( \mu g \) (Figure 7.4) in L6 and 3T3L1 cells respectively. IC\(_{50}\) value of NC was 724.23 \( \mu g \) and 508 \( \mu g \) for L6 cells and 3T3L1 cells respectively. Altogether, the present study demonstrated that the ethanolic extract do not cause any toxic effect up to 500 \( \mu g/ml \). The effective concentration of extracts was 500 \( \mu g/ml \); it indicates that the myoblasts and adipocytes tolerated the plant extracts very well at a concentration of 500 \( \mu g/ml \). Therefore the concentration below 500 \( \mu g/ml \) was used in the subsequent assays since myoblasts and preadipocytes revealed optimal tolerance for it.

After treatment with GMSN2 and GNCN3 on 3T3L1 cells, 70% of the 3T3L1 cells remained viable at a concentration of 1000 \( \mu g/ml \). At the same concentration of GMSN2 and GNCN3 killed 20% of L6 cells. MTT assay revealed that GMSN2 and GNCN3 do not exhibit any cytotoxic effect on 3T3L1 and L6 cells with the IC\(_{50}\) value > 1000 \( \mu g/ml \). The less toxicity of GMSN2 and GNCN3 at higher concentration confirming the safe nature of GMSN2 and GNCN3. Therefore the concentration below 1000 \( \mu g/ml \) was used in the subsequent assays since L6 myotubes and 3T3 L1 preadipocytes revealed optimal tolerance for it.

### 7.5.3. **In vitro** antidiabetic studies

The influence of NC, MS, GNCN3 and GMSN2 on basal and insulin stimulated glucose uptake in L6 cell lines was investigated by non radioactive method. In the present study we have established for the first time that NC, MS, GNCN3 and GMSN2 have insulin like effects in L6 cells which is a typical target tissue of insulin and plays significant roles in the maintenance of glucose homeostasis. The results showed that the extracts and nanoparticles significantly enhanced glucose uptake in L6 myoblasts in a concentration dependent manner.
comparable to the effects of standard Insulin 100 nM (injectable antidiabetic) and Rosiglitazone 50 µM (oral antidiabetic) and the best dose exhibiting maximum activity was found to be 125 µg/ml.

Under basal condition NC, MS, GNCN3 and GMSN2 increases the glucose uptake rate at all concentrations over control. Glucose uptake of NC in L6 cells was increased by 102.30, 107.85, 113.54, 121.67, 134.05 at 7.8, 15.6, 31.25, 62.5, 125 µg/ml respectively over control. Glucose uptake of GNCN3 in L6 cells was greater than that of NC, GNCN3 induced the glucose uptake by 109.51, 118.09, 126.32, 148.33, 169.69 % at 7.8, 15.6, 31.25, 62.5, 125 µg/ml respectively over control. GMSN2 increased glucose uptake to 119.14 % at a concentration of 7.8 µg/ml and induced maximal glucose uptake of 259.41 % at 125µg/ml over control. This effect was greater than that of effect produced by the extract MS (Figure 7.5). The level of GMSN2 induced glucose uptake was greater than that induced by GNCN3. The results were compared with standard drug rosigilitazone. It enhances glucose uptake by 263.63 % over control at 50µM.

The extracts NC and MS, nanoparticles GNCN3 and GMSN2 were also examined with insulin to confirm any synergistic effects but the results showed that the NC, MS, GNCN3 and GMSN2 do not have any synergistic effects with insulin. NC stimulates the glucose uptake in L6 cells by 212.60 % over control when used with insulin. In the same way MS stimulates the glucose uptake by 286.35 % over control when used with insulin (Figure 7.6). Insulin induced a glucose uptake from 259.41 % to 329.40 % and 169.69 % to 254.68 % over control for GMSN2 and GNCN3 respectively. This may be either by elevating the synthesis of the insulin independent (basal) glucose transporter GLUT – 1 or by increasing expression or
translocation of the insulin dependent glucose transporter GLUT 4. The results of the present study clearly indicated that NC, MS, GNCN3 and GMSN2 stimulates glucose uptake under in vitro conditions. This may be due to its effects on the various receptors located in the skeletal muscle L6 cell line. MS and GMSN2 showed more glucose uptake activity in both in vitro methods when compared to the NC and GNCN3.

Figure 7.5: Basal Glucose uptake assay of MS, NC, GMSN2 and GNCN3 in L6 myoblasts.

The extracts MS and NC and its nanoparticles GNCN3 and GMSN2 were tested without insulin at various concentrations (Control 100%). All the data were expressed as means ± SEM of three independent experiments.
Figure 7.6: Insulin stimulated Glucose uptake assay of MS, NC, GMSN2 and GNCN3 in L6 myoblasts.

The extracts MS and NC and its nanoparticles GNCN3 and GMSN2 were tested with insulin at various concentrations (Control 100%). All the data were expressed as means ± SEM of three independent experiments.

**Antiadipogenisis Assay**

Antiadipogenic effect of NC, MS, GNCN3 and GMSN2 was examined in 3T3 L1 cells. Adipogenesis is the process of differentiation of preadipocytes into mature adipocytes, during this process cells undergo a series of processes which include cell growth arrest, alteration of cell shape, synthesis fatty acids, accumulate triglycerides and secrete a wide variety of hormones and cytokines (Suzuki K & Kono T, 1980). The intracellular lipid droplet accumulation is commonly monitored as a general marker for determining adipogenic effect. The amount of lipid accumulated in the 3T3 L1 adipocytes subsequent treatment with MS, NC, GNCN3 and GMSN2 was calculated in terms of the absorbance of the oil red O dye extracted from stained cells.
The results showed that adipogenesis was substantially inhibited by both extracts and nanoparticles in a dose dependent manner (Figure 7.8 and 7.9). The results of the study showed that the plant extracts MS and NC having the ability to reduce lipid accumulation in 3T3 L1 adipocytes when compared to untreated cells, expressing antiobesity activity which is an attractive property for an antidiabetic drug. The inhibitory effects of MS and NC on lipid accumulation during adipogenesis were dose dependent. MS and NC reduced the lipid accumulation by 47.70 % and 51.17 % respectively at 250 µg/ml. GMSN2 treatment strongly reduces the intracellular lipid accumulation by 12.48 and 74.8 % at 15.6 µg/ml and 250 µg/ml concentrations respectively. Treatment of multiple concentration of GNCN3 decreases the lipid content in 3T3L1 adipocytes and showing 57.3 % of inhibition at 250 µg/ml concentration (Figure 7.7). Among those treated, GMSN2 showed strongest anti adipogenic effect in 3T3L1 differentiation than GNCN3, suggesting antiobesity property which is a desirable property for an antidiabetic drug.

Figure 7.7: Anti Adipogenesis assay of NC and MS and its Nanoparticles GNCN3 and GMSN2 in 3T3 L1 preadipocyte

All the data were expressed as means ± SEM of three independent experiments.
A number studies have revealed that natural phytochemical compounds like resveratrol, berberine, genistein inhibit adipogenesis by inhibiting preadipocyte proliferation and suppressing lipid accumulation (Yeo CR et al., 2011). Epicatechins, catechins and tannins are the most important active anti-oxidant constituents found in the medicinal plants to enhance the glucose uptake and anti-adipogenesis in differentiated adipocytes (Rayalam et al., 2008, Deuschlander M et al., 2011).
In both *in vitro* antioxidant and antidiabetic methods, MS and GMSN2 showed excellent activities when compared to the NC and GNCN3. However, *in vivo* studies were carried out to substantiate the *in vitro* results by employing different *in vivo* models for their effective utilization as therapeutic agents. So MS and GMSN2 were selected for further study to confirm the antidiabetic effect in high fat diet induced diabetes in C57BL/6J mice.

**Figure 7.9: Effect of MS and GMSN2 on fat droplet formation in 3T3-L1 cells.** Preadipocytes were differentiated with different concentrations of MS and GMSN2 for 8 days, after 72 hours of exposure stained with Oil red O dye and examined under a light microscope. (a) Preadipocytes (b) control untreated (c) Differentiation induced + low dose of MS (d) Differentiation induced + low dose of GMSN2 (e) Differentiation induced + high dose of MS (f) Differentiation induced + high dose of GMSN2
7.6. REFERENCES


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