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COMPARATIVE SCREENING AND CHARACTERIZATION OF ANTIOSTEOPOROTIC ACTIVITY OF *SESBANIA GRANDIFLORA* (L) POIR CRUDE LEAF EXTRACT AND ITS NANOPARTICLE FORMULATION ON SAOS-2 CELL LINES

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Keywords:

Osteoporosis, *Sesbania grandiflora*, Saos2, Alkaline phosphatase, Antioxidant, Nanoparticles

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ABSTRACT: Increasing severity of the disease osteoporosis in postmenopausal women with long term exposure to pharmacotherapy has spurred interest in the development of using food as medicine and phyto-pharmacotherapy. We examined the effect of n-hexane crude leaves extract and its nanoparticle formulation of *Sesbania grandiflora* in cell proliferation and bone mineralization at different concentrations in Saos-2 cell line. Osteogenic differentiation and cell viability of Saos-2 cells were quantified by monitoring alkaline phosphatase activity and MTT assay. HPLC analysis confirms the presence of bioactive compound α tocopherol. It was found that the concentrations 10 µg & 20 µg/100 µL significantly induced cell proliferation ($p<0.05$), and the expression of ALP ($p<0.01$) was not inhibited by the presence of the bioactive compound. Our data suggest that *S. grandiflora* nanoparticles affect the Saos-2 cell health in higher dose concentration and will be a good natural herbal medicine candidate for the treatment of osteoporosis at lower dose concentration. Further research is required to gain insight into the plant bioactive compounds and the molecular mechanism involved in their mode of action for safe and effective exploitation.

INTRODUCTION: Bone is a complex dynamic vascular mineralized tissue that serves as an attachment site for muscles and tendons. It protects internal organs and plays a vital role as a mineral reservoir. Bone integrity depends mainly on complex coupled activity of osteoclast bone resorption and osteoblastic bone formation [1].

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Osteoporosis is a silent systemic skeletal disorder distinguished by diminished bone density, strength, and debilitating fractures [2]. This is mainly due to imbalances between bone resorption and formation due to increased osteoclastogenesis activity than osteoblastogenesis activity.

The amount of bone formed during every remodeling cycle decreases with age in both sexes. The pharmacotherapy for osteoporosis is usually focused on increasing the estrogen level of bone remodeling. The mechanisms involve many aspects, such as stimulating parathyroid hormone (PTH), inducing the expression of OPG (osteoprotegerin), decreasing cytokines levels like IL-1, 4, 6 and

supplementing calcium, phosphorus in bones, to inhibit the proliferation of osteoclast, inducing osteoclast apoptosis and to increase the proliferation and differentiation of osteoblast [3]. Calcium supplementation alone offers trivial advantage on enhancing bone mineral density through postmenopausal life [4]. The hormone therapy given to postmenopausal women for osteoporosis is controversial because of an increase in the risk of endometrial and breast cancer, cardiovascular disease, *etc.* [5] Estrogen, bis-phosphonates, calcitonin, calcium products anabolic steroids are used as effective medications; however, each of them has established some side effects [6]. Therefore, cost-effective and safe anabolic agents that prevent osteoporosis and promote the proliferation of osteoblasts are needed. The application of traditional knowledge in exploration of lead compounds for the treatment of many diseases is a valuable approach to find a safe therapy.

Many medicinal plants have long been used in the treatment of osteoporosis in many countries. These natural medicines derived from plants have fewer to no side effects, low cost, and fairly long-term effectiveness than synthesized drugs [7]. *Sesbania grandiflora* (L) poir, commonly known as Agathiin India, is used in traditional medicine systems as a potent antibiotic, anti-helminthic, anti-tumor, and contraceptive properties [8-10]. It is also rich in calcium and possesses vitamin E isomers, tocopherols, and tocotrienols, which are bioactive compounds responsible for antioxidant activity. Osteoblasts are characterized by their ability to synthesize type I collagen protein and inducing the mineralization of the matrix via secretion of alkaline phosphatase (ALP) like enzymes.

Hence, ALP activity is considered to be one of the most significant markers of osteoblastic activity *in-vitro*. A number of *in-vitro* model systems have been proposed; among them, Saos-2 is considered the most promising cell line because it expresses all phenotypes of osteoblastic cells. Nanotechnology involves the tailoring of materials at the infinitesimal level to achieve unique properties for the desired applications [11]. This plant contains numerous chemical constituents that usually exert their therapeutic effects through different pathways, and they will have different targets.

Thus, it helps in combating osteoporosis pathogenesis in a more robust way. The rapid development of nanotechnology has opened the possibility of controlling and manipulating structures at the molecular level and has led to the creation of novel surface architectures and materials.

Several nano-oriented approaches are being intended in order to optimize the technological aspects of drugs. The use of these processes like nanosuspension has dramatically enhanced dissolution rates *in-vitro* and bio-availabilities *in-vivo* of many drugs. Various studies have indicated that this plant possesses various phytochemicals, particularly phytoestrogens, which can provide a positive effect on bone.

Based on the above medicinal properties, the objective of the present study is to investigate and compare the ability of the *S. grandiflora* leaf extract and SGE nanoparticles to promote the proliferation of human osteoblast-like model cells Saos-2, thereby inducing bone formation *in-vitro*. The proliferative potential was studied with the help of a standard assay for cell proliferation MTT and ALP.

MATERIALS AND METHODS:

Collection of Leaves: Fresh *Sesbania grandiflora* leaves were collected from local farmers. The plant was botanically identified and confirmed by the Department of Agriculture and rural development studies, Agricultural College and research institute, Madurai Campus. The leaves were washed in distilled water, air-dried 26 ± 2 °C for 5-6 days & pulverized, and stored at 5 °C until further use.

Preparation of Leaf Extracts: Powdered leaves of 200 g were extracted with 500 mL of n-hexane in Soxhlet's apparatus at 60-80 °C for 48 h. The samples were then filtered and centrifuged for 20 min at 3000 g, and the supernatant was dried in a rotary evaporator and weighed. After successive extraction, the solvent was evaporated through a rotary evaporator and freeze-dried. The plant extract was freeze-dried and stored at -70 °C [12].

Phytochemical Screening of *Sesbania Grandiflora*: Preliminary phytochemical screening was carried out using aliquots of n-hexane leaf extract of *Sesbania grandiflora* to identify the

secondary metabolites present as per the standard procedures [13, 14].

Analysis of Plant Bioactive Compounds using HPLC: The powdered extract (3 g) was prepared in HPLC grade Hexane, and the sample was sonicated using an ultrasonicator for 10 min. Then the extract was filtered with Whatman no.2 filter paper and injected into the HPLC column. The analyses were performed in Agilent-infinity 1260, an instrument using a LICHROSORB silica gel Si-60 C-18 Column (25 × 0.4 cm/ 5 µm).

The mobile phase is 0.5% isopropanol in hexane at a flow rate of 1 mL/min; the injection volume for all samples was 40 µL; the wavelength is set at 290 nm [15]. This qualitative and quantitative analysis was performed in triplicate. The alpha-tocopherol was used as standards.

Formulation of Nanosuspension of *S. grandiflora* Extract: To prepare nanosuspension of *S. grandiflora*, nano precipitation technique should be followed with a slight modification of a previously reported process [16]. Dissolve 5 g of *S. grandiflora* extract in 30 mL of acetone and ethanol (3:1) by sonication at 20 W for 30 s.

The resulting solution is then slowly injected (1 mL min^{-1}) with a syringe connected to a thin teflon tube, into 50 mL water containing polyvinyl alcohol (PVA) 1.5% w/v with continuous magnetic stirring at 1000 rpm. The resulting emulsion obtained is then diluted in 100 mL PVA solution (0.2% w/v in water) in order to minimize coalescence, and the mixture was continuously stirred (500 rpm) for 6 h at room temperature to allow solvent evaporation and nano-particle formation. The resulting nano-suspension is subsequently cooled down to −18 °C and freeze dried16.

Morphological Study and Characterization: The morphology can be studied using AFM. A thin film of the sample prepared on a glass on the slide was allowed to dry for 5 min, and the slides were then scanned with AFM (Nanosurf, Switzerland Product: BT 02218). Fourier transform-infrared (FT-IR) analysis was performed to identify the possible biomolecules present in compounds. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in the annotated spectrum. One drop of sample placed between the plates of sodium chloride. This was performed using the instrument Nicolet-380 FT-IR, Thermo Electron Corporation, resolution 4 cm^{-1}.

Antioxidant Activity using 2, 2 – Diphenyil – picrylhydrazyl DPPH Method: Aliquots of extract of 10 µg – 100 µg dissolved in dimethyl sulfoxide (DMSO) were plated out in duplicate in a 96-well microtiter plate [17]. The volume is adjusted to 100 µL by adding methanol, the methanolic DPPH (10 µM) solution of 250 µL was added to the test samples, and methanol was used to control test samples in control well. The plate was shaken for 2 min and incubated for 20 min in darkness at 37 ºC, in a water bath. The percentage of decolorisation was obtained spectrophoto-metrically at 517 nm. The percentage of radical scavenging activity is expressed as the inhibition percentage and is calculated using the following formula:

% radical scavenging activity = (Control OD – Sample OD / Control OD) × 100

Procurement and Maintenance of Saos-2 Cell Line: Saos-2 cell line was obtained from NCCS, Pune, India. The cells were cultured in Mc Coy's 5A modified medium in a humidified atmosphere (5% CO_2 95% air) at 37 °C containing 1% anti-microbial and antifungal solution, supplemented with 15%FBS. Upon reaching sub-confluency, the cells were loaded in 96 well cell culture plates for culturing for 48 h in 5 increasing concentration (10 µg, 20 µg, 30 µg, 40 µg, and 50 µg/100 µl). After 48 hours, MTT and ALP assays were carried out to understand the effect of the plant extract on osteoblastic cells.

Cell Viability Test MTT Assay: Saos-2 Cell incubated in Mc coy's 5A modified medium was loaded in 96 well cell culture plate (1.2×10^5 cells/mL) and cultured for 48 h in 5 increasing concentration of SGE leaves extract and SGE nanoparticles (10 µg, 20 µ g, 30 µg, 40 µg and 50 µg / 100 µl). MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium stock solution (10 µL /100 µL medium) was added to the wells, and plates were incubated at 37 °C for 4 h. Subsequently, 0.05% DMSO was added and measured colori-metrically at 570 nm. 0.1% Triton X-100 treated well was used as a positive control. Optical density

in comparison to the control cells, was plotted as cell viability. % of Cell viability was calculated as follows:

% Cell Viability = mean Sample OD/ mean Control OD × 100

Alkaline Phosphatase Assay: Cellular ALP activity was determined by the release of 4-NP from 4-NPP. Alkaline Phosphatase Assay Kit is designed to measure ALP activity directly in biological samples without pre-treatment. The improved method utilizes p-nitrophenyl phosphate that is hydrolyzed by ALP into a yellow-colored product (maximal absorbance at 405 nm) [18]. The rate of the reaction is directly proportional to the enzyme activity. A subconfluent monolayer culture was trypsinized, and cells of 1.2×10^5 cells/mL were seeded in multi-well cell culture plates. The cells were serum-starved for 24 H. To the serum-starved cells 10 µg-50 µg of crude plant leaf extract of SGE & nanoparticle of SGE were added to each well and incubated for 48 h. The reaction was stopped by washing the cells with PBS thrice and lysed in 150 µL of 0.1% Triton X-100. The concentration was measured at 405 nm.

Analysis of Hemolysis Activity: Fresh human blood was collected from healthy donor using 5 mL sterile syringe with needle by venipuncture. The blood collected was transferred into a clean sterile centrifuge tube containing Ethylenediaminetetra acetic Acid (EDTA) (3 mg / 5 mL of blood). The blood sample is centrifuged using the bench centrifuge for 5 min at 3000 rpm. The supernatant was discarded using Pasteur pipette, and the packed cells were washed five times with PBS (pH 7.2) [19]. The washed erythrocytes were suspended in PBS at 2% v/v and stored at 40 °C until use.

The hemolytic assay was conducted by serial dilution of extracts 10 µg to 50 µg (crude aqueous extract and nanoparticles of SGE) using U-shaped bottom microtiter plates with 2% v/v of the erythrocyte's suspension in PBS.

A 75 µL of prepared diluted PBS extract was added to microtiter wells. After dilution 25 µL of treated erythrocyte was added to each of the wells and incubated for 30 min at room temperature for the sedimentation patterns to take place. The sedimentation patterns of erythrocyte suspension in the undisturbed plate were read at 405nm after incubating for 30 min at room temperature to determine the titer value.

0.1% Triton-X 100 was used as a positive control, and PBS was used as a negative control. A positive pattern will be indicated by the appearance of full hemolysis as a big circular spot of red solution surrounded by a small clear zone.

A negative pattern will be indicated by the appearance of a small uniform spot of erythrocytes at the bottom of the well, surrounded by a big concentric clear zone.

Statistical Analysis: Data obtained from the cell viability, and ALP studies were analyzed using the student's T-test at $p<0.05$ and $p<0.01$.

Results:

Phytochemical Screening: The results of phytochemical screening of different extracts are shown in **Table 1**. Phytochemical screening revealed the presence of carbohydrates, alkaloids, flavonoids, glycosides, tannins, steroids, proteins, terpenoids in n-hexane extract.

TABLE 1: PHYTOCHEMICAL SCREENING OF N-HEXANE SGE

S. no.	Phytochemical Test	Reagents used	Interference for the presence of Phytochemicals	Results
1	Alkaloid test	Mayer's reagent, Draggendrof's reagent	Appearance of reddish-brown precipitate	Presence
2	Flavonoid test	Alkaline reagent test	Formation of yellow color	Presence
3	Glycoside's test	Ammonia solution, Benedict's reagent	Orange red precipitate	Presence
4	Tannin's test	Ferric chloride	Formation of green color	Presence
5	Terpenoid test	Chloroform	Reddish brown Color	Presence
6	Protein's test	Xanthoproteic test	Formation of Yellow color	Presence
7	Carbohydrate test	Benedict's reagent	Formation of deep blue color	Presence
8	Steroid test	Chloroform test	Blue color steroidal ring at interphase	Presence

Atomic Force Microscopy Analysis of SGE Nanoparticles: The AFM results indicated that the surface topology of the synthesized nanoparticles was the predominantly spherical shape of different size. **Fig. 1** shows the representative AFM image recorded. This microscopic study has been employed to characterize the size, shape and morphologies of formed nanoparticles. From the images, it is evident that the morphology of the nanoparticles of SGE is spherical in shape with a smooth surface. It is because PVA helps to impart spherical and smooth surfaces on nanoparticles. The average particle size analyzed from the AFM images is observed to be 60-80 nm **Fig. 1A**. The height of the nanoparticles was measured to be only 30.5 nm, as shown in the line analysis **Fig. 1B**.

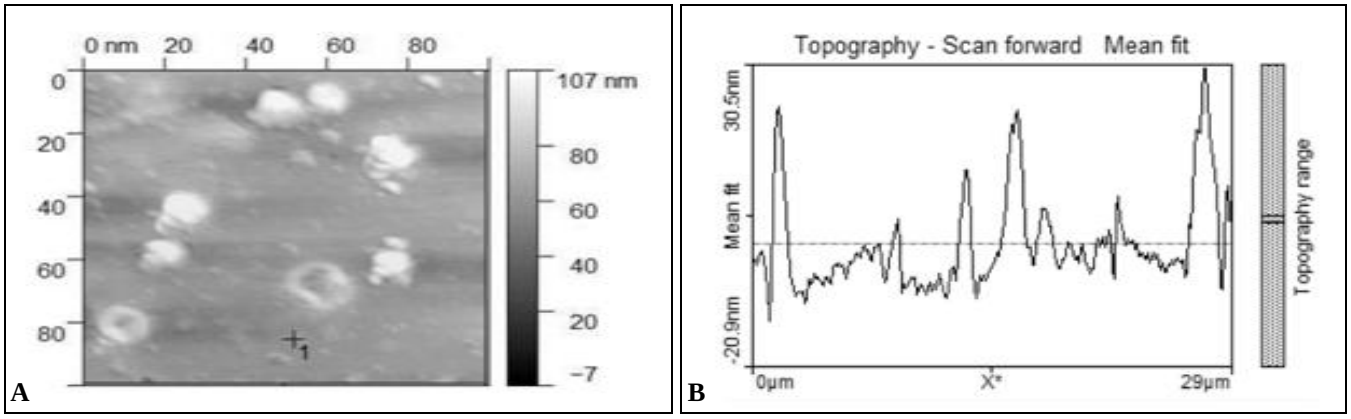

FIG. 1: (A) AFM IMAGE OF SGE NANOPARTICLES. (B) LINE ANALYSIS SHOWING THE PARTICLE SIZE AND HEIGHT

High-Performance Liquid Chromatography Analysis: The HPLC-DAD analysis of *S. grandiflora* leaves and nanoparticle extracts revealed the presence of ascorbic acid and flavonoid derivatives. **Fig. 2A** & **B** shows the HPLC chromatogram of the crude plant extract and SGE nanoparticles. Generally, β and δ- tocopherol levels in *S. grandiflora* leaf samples are lower than those of alpha and gamma-tocopherol. The peak 1 area detected at 290 nm corresponds to the standard peak area of α tocopherol recorded. The peak area around 2 min and 4.4 min retention times *i.e.,* peak 2 and 3 belong to the co-extracted fatty acids such as oleic acid, linoleic acids or ascorbic acids and γ tocopherol [20]. The lead and secondary bioactive compounds like ascorbic acid and gamma tocopherols identified in HPLC are in good correlation with the FTIR report, which shows the interaction of functional groups like phenols and carboxylic acid. Attention was given to these peaks because they do interfere with the tocopherols peak. When comparing α tocopherol peak (peak 1) the SGE nanoparticles have no interference in its separation than in SGE leaf extract. The results obtained in this work can be compared with those in other literature because it could be a useful guide in identifying lead compounds in Sesbania other than tocopherol.

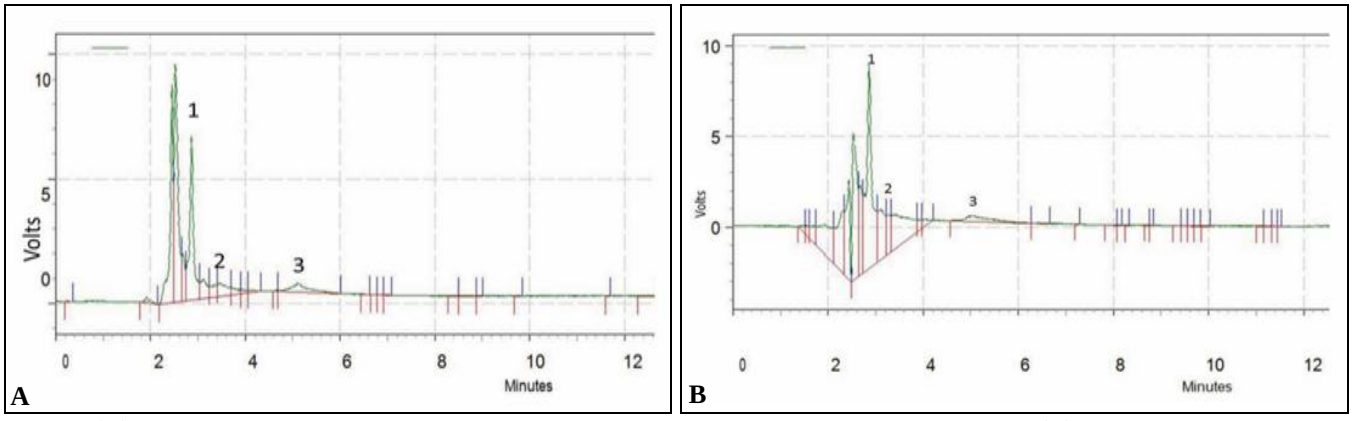

FIG. 2: (A) HPLC ANALYSIS OF *SESBANIA GRANDIFLORA* LEAF EXTRACTS. (B) HPLC ANALYSIS OF NANOSUSPENSION OF SGE

FTIR Analysis: FTIR measurement was carried to identify the possible biomolecules in *Sesbania grandiflora* leaf extracts which are responsible for drug activity leading to efficient stabilization of the nanoparticles. **Table 2** shows the identified functional groups that are more characteristic of the peaks.

The absorption peak at 3437.0, 1660, 1017.6 cm^{-1} are more prominent and mainly responsible for phenols, carboxylic acid, and aromatic amines. This result showed a positive correlation with HPLC peaks proving that the eluted bioactive compound is tocopherol, ascorbic acid, and gallic acid. It is confirmed that the presence of alkanes is mainly because of the stabilizer polyvinyl alcohol we used in the nanoparticle synthesis process.

TABLE 2: FUNCTIONAL GROUPS IDENTIFIED FROM THE OBSERVED FTIR PEAKS

Peak Values	Functional Group
3437.0	Phenols H- bonded
2997.1	Alkanes =C-H stretch
2357.2	Alkanes C-H stretch
1660.0	C=O Stretch Carboxylic acid
1434.3	C-H Bend Alkanes
1311.3	C-N Stretch Aromatic Amines
1017.6	Aliphatic Amines C-N stretch
950.8	=C-H Bend Alkenes
698.1	C (triple bond) C-H Bend Alkynes

Determination of Antioxidant Activity DPPH Method: In the DPPH radical scavenging assay, antioxidants react with DPPH and convert it to the yellow-colored, α-diphenyl-β-picryl hydrazine. The degree of discoloration indicates the radical-scavenging potential of the sample. The highest dose-dependent antioxidant activity in plant extract was observed in 70 µg/100 µL, whereas in SGE nanoparticle it was observed in 10 µg/100 µL. By comparing the results of *S. grandiflora* leaf extracts and SGE nanoparticle *in-vitro* antioxidant assay, it was evident that the leaf extract demonstrated comparatively higher levels of antioxidant activity with the IC_{50} values of 687.67 µg/mL and 128 µg/mL for SGE nanoparticles.

Phenolic compounds and tocopherol in plants are responsible for antioxidant activity. The extracts quenched DPPH free radical in a dose-dependent manner. As the concentration of the leaf extract decreased, its DPPH quenching activity also increased, as shown in **Fig. 3A** & **B**.

The presence of phenolic compound is confirmed in the FTIR analysis and ascorbic acid in HPLC analysis; thus, the presence of both flavonoids and phenols in the n-hexane extract contributed to higher antioxidant activity. Therefore, the SGE nanoparticles rendered the highest antioxidant activity when compared with individual drug concentrations of SG leaf extracts.

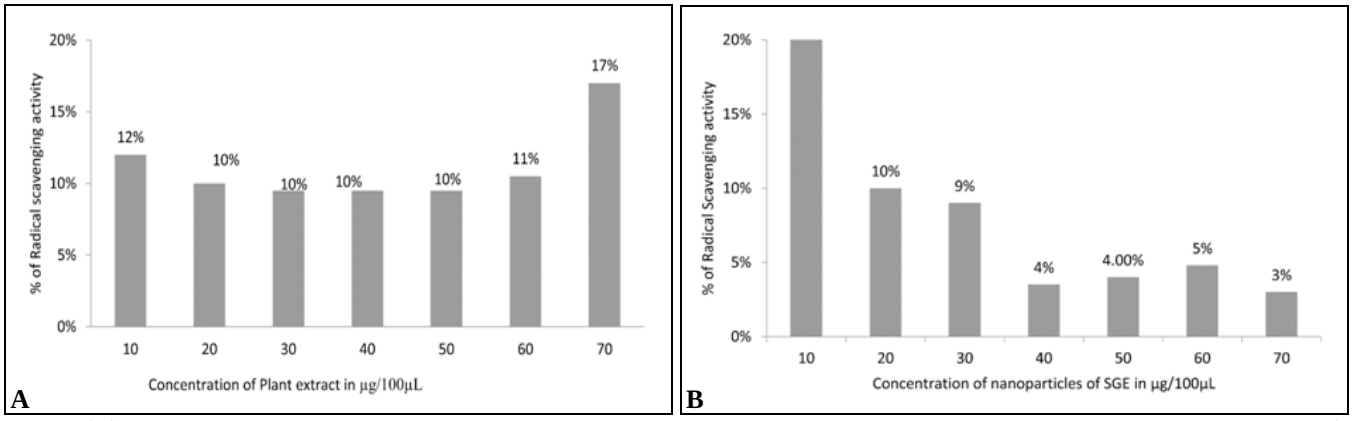

FIG. 3: (A) ANTIOXIDANT ACTIVITY OF *SESBANIA GRANDIFLORA* LEAF EXTRACTS BY DPPH METHOD. (B) ANTIOXIDANT ACTIVITY OF *SESBANIA GRANDIFLORA* NANOPARTICLES BY DPPH METHOD

Cell Viability Assay: To functionally evaluate the effect of *S. grandiflora* leaf extract and SGE nanoparticles cell viability and proliferation in Saos-2 cells MTT assay was performed. MTT is capable of detecting very small numbers of living cells (*e.g.,* 200). Only active cells will cleave MTT, which in turn forms a dark blue formazan product, while dead cells are almost completely negative. The MTT formazan reaction product was only partially soluble in the medium, and so dimethyl sulfoxide (DMSO), which is a solvent used to dissolve the formazan and produce a homogenous

solution suitable for measurement of optical density. The results in **Fig. 4A** & **B** show the percentage of cell viability in the range 10 µg-50 µg. It showed inhibition in cell viability at a higher concentration in Saos-2 cells. It was found that the % cell proliferation decreases with increasing concentration steadily up to 50 µg / 100 µL on both the extracts. The IC_{50} value of *S. grandiflora* leaf extract was 56 µg/mL and SGE nanoparticle is 72 µg/mL. In comparison between nanoparticles of SGE and leaf extract, nanoparticle shows significant difference at $p<0.05$. Cytotoxicity activity is significantly higher ($p<0.01$) in the higher concentration range 30 µg-50 µg / 100 µL when compared with the control. Overall, the study suggests that both SGE leaf extract and SGE nanoparticles have potential anti-cancerous activity on Saos-2 at higher concentration.

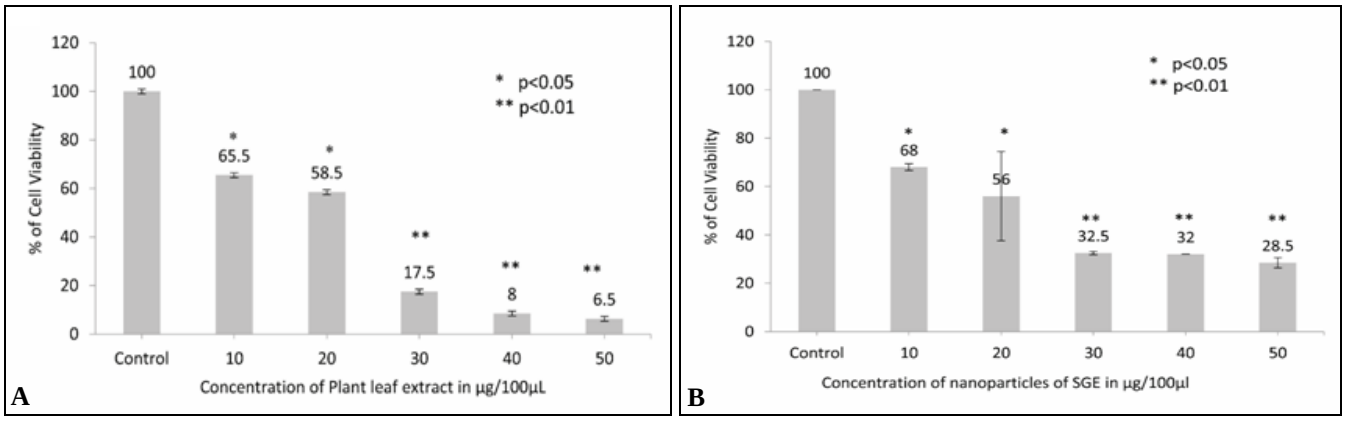

FIG. 4: (A) PERCENTAGE OF CELL VIABILITY OF *SESBANIA GRANDIFLORA* LEAF EXTRACTS ON SAOS-2 CELL LINE. (B) PERCENTAGE OF CELL VIABILITY OF *SESBANIA GRANDIFLORA* NANOPARTICLES ON SAOS-2 CELL LINE

ALP Activity: ALP activity is one of the most established markers of studying osteoblast activity. Our results indicated that the leaf extract had osteoblast stimulating potential. When compared with control, there is a significant increase in osteoblastic activity at lower doses of leaf extracts. In comparison between nanoparticle and leaf extracts of SGE there is no significant difference at $p < 0.05$, but the extracts showed a significant difference in the cell proliferation exhibited by the control and the experimental concentration ($p < 0.01$) at lower concentrations 10 & 20 µg / 100 µL in both the formulation. The intracellular enzyme is considered to mark the middle stage of bone formation. SGE nanoparticles showed increased ALP activity in Saos-2 Cells over 48H, and the maximal effect was reached when the cells were treated with 10 µg / 100 µL. The result showed a dose-dependent increase in the ALP expression as compared to control. After 30 µg / 100 µL concentration the ALP concentration started declining. The maximum activity and the proliferation were also found to be at the lower concentration in both *Sesbania grandiflora* leaf extract and SGE nanoparticles **Fig. 5A** & **B**.

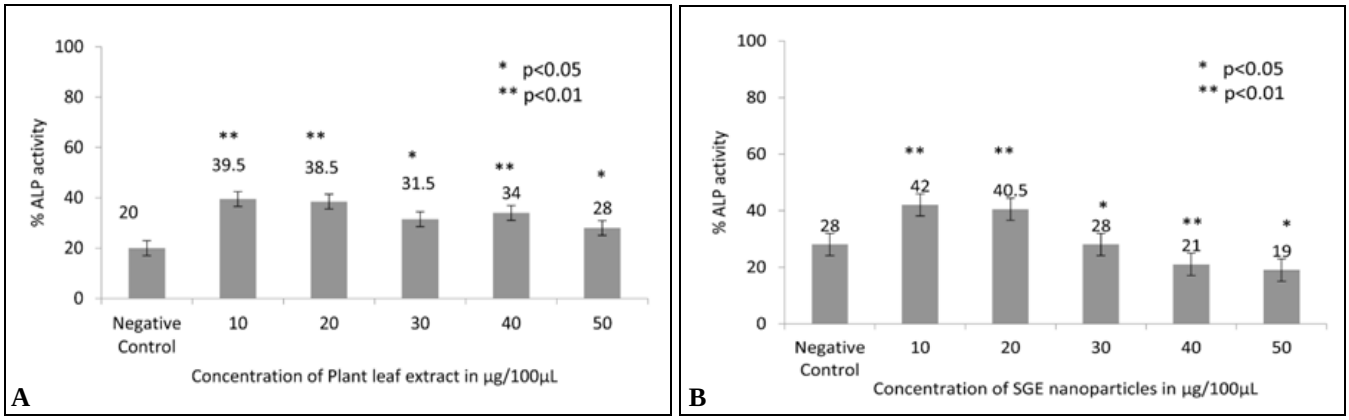

FIG. 5: (A) ALKALINE PHOSPHATASE ASSAY SHOWING PERCENTAGE OF CELL PROLIFERATION IN PLANT LEAF EXTRACT.(B) ALKALINE PHOSPHATASE ASSAY SHOWING PERCENTAGE OF CELL PROLIFERATION IN *S. GRANDIFLORA* NANOPARTICLES

Anti-hemolytic Activity: Hemolytic assay was performed because biological compounds with potent drug activity may not be useful in pharmacological preparation if they possess a toxicological effect. *In-vitro* hemolytic activity of leaf extract of SGE and nanoparticles were carried out on fresh human RBC at various concentrations. Hemolysis is the indicator of cytotoxicity towards RBC's cells. The phytochemical present in the extract can show hemolytic activity. Hemolytic activity of various crude extracts is shown in **Fig. 6A** & **B**. Each concentration showed a mean hemolysis percentage. A lower hemolytic effect on human RBC was observed by both the extracts. In general, both the extracts can be used in pharmacological preparations. A slight increase in hemolysis was observed at higher concentrations.

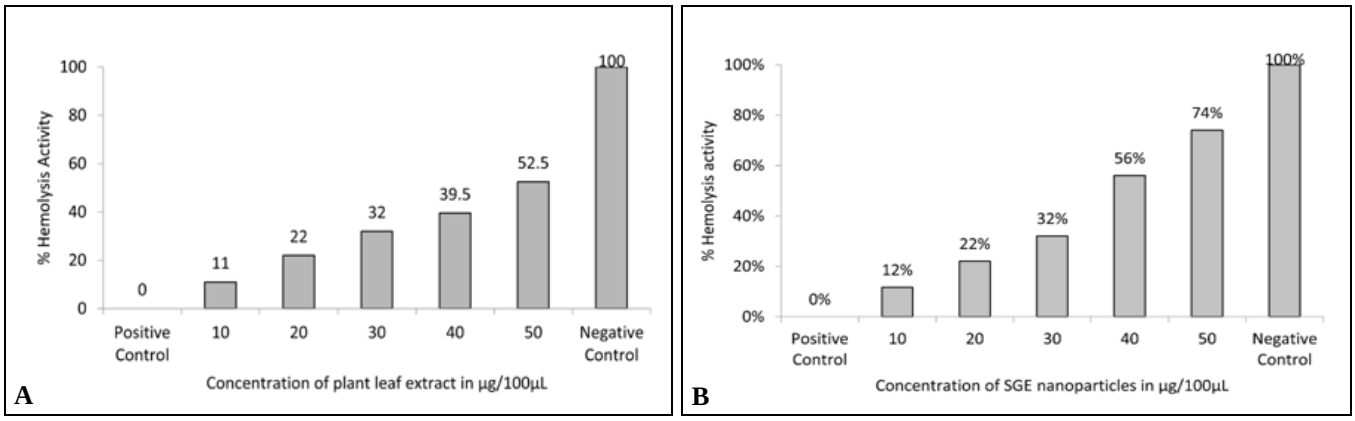

FIG. 6: (A) ANTI-HEMOLYSIS ACTIVITY OF LEAF EXTRACT OF *S. GRANDIFLORA*. (B)ANTI-HEMOLYSIS ACTIVITY OF LEAF *S. GRANDIFLORA* OF NANOPARTICLES

DISCUSSION: The impact of new, novel products from potential bioactive plant extracts for treatment and prevention of disease is still vast, despite consuming recent synthetic chemistry as a method of drug discoveries and drug productions. Plant-derived dugs like vinblastine, chymopapain, codeine, taxol had led to greatest extend in phyto-pharmacotherapy. Therefore, in this study, *S. grandiflora* leaf extract and nanoparticle was evaluated as a new anti-osteoporotic agent by using MTT and ALP assays. *Sesbania grandiflora* was chosen for this study due to its high source of calcium and potent antioxidant activity [14].

In previous studies, the Soxhlet extraction using n-hexane as the solvent is reported for its high yield of oil due to the non-polar property of n-hexane. The chosen lead compound in our study is alpha-tocopherol which is appeared to promote bone fracture healing in osteoporotic rats [21]. Since tocopherol has very low solubility in pure water owning to hydrophobic repulsion, n-hexane solvent increases the solubility mostly due to non-polar attractive interactions between non-polar solvent and vitamin molecule. Phytochemical screening revealed the presence of various bioactive compounds in the n-hexane extract, including carbohydrates, alkaloids, flavonoids, glycosides, tannins, steroids, proteins, terpenoids. Phenolic acids possess antioxidant activity and are naturally found in fruits, vegetables, and other plants; thus, they hold great potential as drug candidates to their safety, low toxicity, and wide acceptance. In order to understand the characteristics of the anti-osteoporotic activity of *S. grandiflora* leaf extract and SGE nanoparticles Saos-2 cell lines were selected. Saos-2 cell line has all the markers that are found in Osteoblast cells that are involved in bone formation; hence it is chosen as a substitute for osteoblast cells.

Recently, the synthesis of nanoparticles fascinated the field of nanotechnology because of their diverse properties like catalysis, magnetic and optical polarizability, electrical conductivity, and drug delivery activity. The SGE nanoparticles extract was successfully prepared by the nano-precipitation method. This novel attempt was taken to enhance the bioavailability of the poor water-soluble drug. Previous studies have demonstrated that the same concentration of nano-particles formulation possesses better pharmacological activity as compared with its raw drug [22]. AFM images recorded at different magnifications showed that

the nanoparticles of SGE is spherical in shape and the average particle size ranges from 60-80 nm. The interest of using alcohol and acetone as solvent essentially lies in their relatively low dielectric constant value, higher solubility, and low to no toxicity level [23, 24].

DPPH radical scavenging activity is one of the most recorded methods for evaluating the antioxidant activity of the plant extract [25]. The antioxidant properties of the leaf extracts were found to be concentration-dependent. The highest antioxidant activity was observed at 10 µg/mL in both the SGE leaf and nanoparticle extracts. Studies on medicinal plants/herbs with high phenolic contents have gained importance over the past few years due to the high antioxidant, anti-inflammatory, and anti-carcinogenic activities and are of great value in decreasing the risk of many human diseases [24, 25].

The results of the antioxidant assays indicated that all the tested compounds had certain antioxidant properties although their activity levels were significantly different. α tocopherols compounds can interrupt free radical chain reaction by capturing the free radical. The free hydroxyl group on the aromatic ring is responsible for the antioxidant properties [26]. A number of evidences showed that the natural function of α tocopherol is that of cell signaling. This property was not shared by any other antioxidant molecule [27]. Analysis of HPLC peaks showed that fatty acids or ascorbic acid or and γ tocopherols were extracted along with α-tocopherol. These results could be a useful guide in identifying lead compounds in Sesbania other than tocopherol [28]. n-hexane with 0.5% isopropyl alcohol was chosen as the optimum mobile phase composition for the separation of tocopherols standards.

Nevertheless, the separation of tocopherol in SGE leaves extracts was not satisfied with this mobile phase because α tocopherol and the co-extracted compound were eluted together by the mobile phase, and their peaks were overlapped. Even so, the experimental result showed that 0.5%isopropyl alcohol was sufficient to keep the polarity of the mobile phase in optimum conditions to separate the tocopherols. Therefore, in future optimization, work was necessary to separate the tocopherol and the co-extracted compound. FTIR measurement was carried to identify the possible biomolecules and functional groups in *Sesbania grandiflora* leaf extracts which are responsible for capping, leading to efficient stabilization of the nanoparticles and drug activity. In addition, the cell viability assay showed that it is capable of detecting very small numbers of living cells. From the results obtained the extract was found to be toxic at higher doses.

The reason for decreased cell viability may be due to the increase bioavailability of the nanoparticles of the SGE. The nano-ionization of *S. grandiflora* results in the production of numerous nanoparticles with increased surface area, due to which the dissolution rate of the nanoparticles becomes amplified, and hence its *in-vitro* absorption increases. The maximum increase in cell proliferation was observed in 10 µg/mL as compared to control. The SGE nano-particles showed a statistically significant proliferative effect at $p<0.05$. The morphological effects were more prominent in the extract-treated with higher concentration showing extensive blebbing and vacuolation, suggesting an autophagic mechanism of cell death.

The IC_{50} values for SGE nano-particle are 72 µg/mL, which is considered less cytotoxic when compared to plant leaf extract, which is 56 µg/mL. Increased cell viability at low concentration and increased ALP level in low concentration of plant extract suggests that bioactive compounds such as α tocopherol, ascorbic acid, and gallic acid in the extracts suppress ROS-induced injury, reduce the intracellular level and increase the expression of endogenous antioxidant enzymes. In addition to these *in-vitro* observations, the positive effects of Osteocare on ALP activity have also been reported during clinical trials of rickets in rats [29]. ALP is recognized as the widely accepted biochemical marker for osteoblastic activity.

Even so, the mechanism of action is poorly understood. The chosen plant extract demonstrated that SGE leaf extract and nanoparticles potentially induce osteoblastic differentiation markers such as ALP in Saos-2 cells. The erythrocytes model has been widely used in hemolysis studies as it presents a direct indication of toxicity of injectable formulations as well as general indication of membrane toxicity. Hemolysis activity is directly

related to the concentration and potency of extracts [30]. RBC's contain a high amount of polyunsaturated fatty acids; they are vulnerable to oxidative stress. Iron overload and reactive oxygen species play a major role in increasing the RBC's susceptibility to peroxidation and hemolysis. The abilities of the extract to scavenge free radicals and bind iron were confirmed by hemolysis analysis [27]. Iron overload and ROS production will be higher in patients with cardiac disease, sickle cell anemia, β thalassemia, and osteoporosis [31]. The SGE nanoparticles effectively protect RBC from hemolysis when compared with SG leaf extracts. SGE nanoparticles and leaf extracts showed reduced cell proliferation at higher dose concentration. This result is in good correlation with MTT assay showing reduced proliferation at higher concentrations. Statistically, the data suggests that SGE nanoparticle can promote matrix mineralization *in-vitro* through increased synthesis and secretion of matrix proteins than *S. grandiflora* leaf extract. In summary, the increase in ALP activity shows that the SGE leaf and nanoparticles have concentration based osteoblastogenic activity. To conclude that the chosen plant extract demonstrated that *S. grandiflora* has the potential to be selected as an alternative medicinal food plant that can be utilized in phyto-pharmacology.

CONCLUSION: In conclusion, *Sesbania grandiflora* n-hexane extract and nanoparticles of the leaf extracts increase the ALP activity of Soas-2 cells *in-vitro*, presumably indicating that it has osteoclastogenic activity. The presence of α tocopherol and ascorbic acid may is the reason for good anti-oxidant and decreased cell viability of our extract at higher concentrations. However, the above evidence is not clear as to whether a single factor is responsible for Saos-2 osteoinductivity.

SG leaf extract and nanoparticles showed that the bioactive compounds extracted have a dual function as anti-osteoporosis and anti-cancerous activity, meaning that the compounds could be developed as potent drugs, and it is purely dependent on concentration. Subsequent studies examining the efficacy of these components separately can throw more light into the anti-osteoporosis activity of SGE. Analysis involving primary cell lines and *in-vivo* studies can prove osteogenesis activity. Further research is required on the mechanism of SGE activity both *in-vitro* and *in-vivo* for safe and effective exploitation as promising anti-osteoporosis drugs.

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