RESEARCH ARTICLE ISSN 2456-0170

GODHUMA (Triticum sativum LAM.) IN ASTHIKSHAYA: AN INVITRO EVALUATION

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ABSTRACT

Asthi has prime importance in giving stability, shape, and structure to the body. Derangement in *Asthi* and its nutrients will lead to *Asthigatarogas*. *Asthikshaya* is one such condition, due to which *bhagna* etc. manifest. Around 33.6 million individuals over age 50 have low bone density of the hip and thus are at risk of osteoporosis and fractures. Hence it is one of the burning issues in the Society. Treatments available in contemporary science for Low bone density are not devoid of adverse effects. *Ayurveda* has mentioned many herbal drugs helpful in combating bone loss but are not scientifically evaluated. One such drug is *Godhuma (Triticum sativum Lam.)* which is easily available and cost effective. Hence to evaluate the effect of *Godhuma* in *Asthikshaya*, this study was taken up. Aqueous, Alcoholic and Hydroalcoholic extracts of *Godhuma* were tested for Cell proliferation, Osteoblasts mineralisation, Alkaline phosphatise and Nitric oxide scavenging assays using UMR 106 cells (osteoblasts cells) and standard as Residronate sodium. The percentage of Bone cell proliferation and scavenging of Nitric oxide was studied spectrophotometrically, production of Alkaline phosphatase by Opaque density taken at 405nm, and Osteoblasts mineralization by observing the formation of extracellular calcium deposits.All the extracts of *Godhuma* showed appreciable results in Cell proliferation, osteoblasts mineralisation and alkaline phosphatase assay, except in Nitric oxide scavenging activity. This Invitro evaluation proved that Godhuma has pivotal and potential role in treating *Asthikshaya.*

KEYWORDS: *Godhuma, Asthikshaya,* UMR 106 cells.

INTRODUCTION

"Dosha Dhatu Mala Mulam Hi Shareeram" Shareera is considered as a substratum and homeostasis of *Dosha, Dhatu* and *Mala.*Here, the function of *Dhatu* is *Dharaṇa* (maintain the structure) of the *Shareera* (body). Among them *Asthidhatu* has been given prime importance as it is responsible for giving stability, shape, and maintaining structural framework of the body.

Any derangement in *Asthidhatu* and its nutrients will lead to *Asthigata rogas. AsthiKshaya, (i.e. kshaya of Asthidhatu)* is one such condition associated with *asthisoushirya, dantanakhabhanga, asthitoda* due to which diseases like *phakkaroga, bhagna* and *asthivrana* manifest.

*Asthikshaya (*i.e. low bone density) can be compared to disease condition

Osteopenia/Osteoporosis in contemporary era. The causative factors, signs and symptoms of both are similar. Hence, *Asthikshaya* can be considered as an independent disease.

Worldwide, 1 in 3 women and 1 in 5 men over age 50 will experience Osteopenia leading to Osteoporotic fracture. Hence Low BMD is one of the burning issues in the Society.[1]

Conventional medicines like Bisphosphonates used to combat bone loss may have side effects like nausea, pain in muscle, bone and joints etc. Prolonged and excessive intake of Calcium supplements may increase the risk of developing kidney stones and cardiovascular disease. Hormone (oestrogen) therapy has an increased risk of stroke, coronary artery disease, breast cancer, and venous thrombo-embolism.[1]

Ayurveda explains various treatment aspects for treating *Asthikshaya*, among them *Shamana Chikitsa* is one under which herbal drugs mainly having *Vatahara, Tarpana, Brihmana, Balya, Sthirakara* and *Asthisandhankara* property are mentioned helpful in combating and treating *Asthikshaya* but are not scientifically evaluated.

One such drug is *Godhuma* having *gunas* as that of *Asthidhatu* and also having *Vatahara, Brimhana, Balya, Sandhanakara* and *Sthiratvakara karma* which is easily available and cost effective.

Hence to evaluate the action of *Godhuma* in *Asthikshaya* using different Invitro Assays this study was carried out.

OBJECTIVES OF THE STUDY:

1. To Carry out Analytical evaluation of *Godhuma.*
2. To Carry out Phytochemical analysis of *Godhuma.*
3. Invitro Study on UMR 106 Cells, to evaluate the efficacy of *Godhuma* in *Asthikhaya*.

MATERIAL & METHODS:

Study was designed under three headings:

1. Collection, Authentication and Preparation of the trial drug.
2. Analytical evaluation of the trial drug.
3. In-vitro Study.

The grains of *Godhuma (Triticum sativum Lam.)* were procured from Sangogi Village, Indi Taluk, Vijayapura District, Karnataka and authentified by Botanist and grains were subjected for different extractions using Standard Operative Procedures and extracts were prepared i.e. Aqueous, Alcoholic and Hydroalcoholic extract were used further for the Study.

The drug extracts were subjected for Macroscopic, Microscopic, Physicochemical, Phytochemical and HPLC studies in order to confirm the genuinity and purity of the drug.

Plate no. 1: Morphological Features of *Godhuma (Triticum sativum* Lam.)



Invitro study: The Invitro study was carried out at Skanda Life Sciences Pvt Ltd, Sunkadakatte, Bangalore-91 using UMR 106 Osteoblasts cells to evaluate the efficacy of the trail drug *Triticumsativum* in*Asthikshaya* / Low bone density.

Osteoblast like cell line 'UMR 106 Cells' was procured from Sigma Aldrich lab. 70-80% or equal to 100% Confluence UMR 106 Cells were used for different Assays.

Test Samples Preparation:

32 mg of each of Aqueous, Alcoholic and Hydro-alcoholic extract samples of *Triticumsativum* were weighed separately in digital weighing machine. The weighed samples were marked as 'Aq', 'Al', and 'Hy' for Aqueous extract, Alcoholic extract and Hydro-alcoholic extract respectively. To each Sample 1ml DMSO (Dimethyl sulfoxide) was added and mixed well to make a homogenous solution. This was used as Standard test samples preparation which were later used for all the different In-vitro assays in two-fold dilutions.

Standard Sample Preparation:

35mg of Standard Sample was weighed and was marked as 'Res.sd.' for Residronate sodium. To this 35ml of distilled water was added and mixed well to make a homogenous solution, later stored in fridge. This was used as Standard preparation of Standard drug which was later used for different In-vitro assays in two-fold dilutions.

In-vitro assays selected for the study.

Cell proliferation assay or MTT assay.[2-9]

The MTT assay is a means of measuring the activity of living cells via mitochondrial dehydrogenases. The key component is (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTT, is a water soluble tetrazolium salt. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells. This water insoluble formazan can be solubilized using DMSO (Dimethyl Sulfoxide). The resulting purple solution is spectrophotometrically measured. An increase in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of proliferation caused by the test material.

Procedure:

1. UMR 106 Cells were collected when they reach about 70-80% confluence and checked for the viability of the cells and were centrifuged.
2. 50,000 cells / well of UMR106 in a 96 well plate were seeded and incubated for 24 hrs at 37^{o}C, 5 % CO2 incubator.
3. Reagents and Solubilization solution were prepared as per assay protocol.
4. Samples to be tested i.e. Standard drug Residronate sodium, Aqueous, Alcoholic and Hydro-alcoholic extracts of *Triticum sativum* were added from 10 - 320µg/ml (2-fold variation) concentration in DMEM

(Dimethyl eagle medium) without FBS (Foetal bovine serum) & were incubated for 24 hr.

5. After incubation with Standard drug and test samples, 100µl/well (50 mg /well) of MTT (5 mg/10ml of MTT in 1X PBS) was added to the respective wells and incubated for 3 to 4 hours.

6. After incubation with MTT reagent, the MTT reagent was discarded by pipetting without disturbing cells and 100 µl of DMSO was added to rapidly solubilize the formazan.

7. Absorbance was measured at 590 nm.

Calculating proliferation:

% Proliferation = ((OD of Sample/OD of Control)×100)-100

Osteoblasts Mineralisation assay.[10-14]

1.Seeding of Osteoblasts. (UMR 106 cells): Plating of 6 x 104 UMR cells per well of a 24 well tissue culture plate (3.15 x 104 cells/cm2) using Growth Medium was done. And studied with Standard drug Residronate Sodium and different extracts of *Triticumsativum.*

2. Growing of Osteoblasts: Important: Cells to reach ≥100% confluency (24 - 72 hours).

3. Inducing of Osteoblasts: UMR 106 cells were induced with Standard drug Residronate sodium, Aqueous, Alcoholic and Hydro-alcoholic extract of *Triticum sativum* at test concentrations of 160µg/ml and 320µg/ml. UMR 106 cells growth medium was used for the remaining well as a negative control.

4. Differentiation culture of induced Osteoblasts: Incubation was done for 21 days to complete the mineralization process. And Medium was changed every third day carefully not to disturb the cell monolayer.

Detection of Calcium Deposits (Mineralization). Osteoblasts (UMR 106 cells) can **be induced** to produce **vast extracellular calcium deposits** in vitro. This process is called mineralization. Calcium deposits are an indication of successful in-vitro bone formation and can specifically be stained bright orange-red using Alizarin Red S.

1. Preparation of solutions and buffers: 2 g Alizarin Red S was dissolved in 100 ml distilled water to prepare Alizarin Red S staining solution. The dark-brown solution was filtered and stored in the dark.

2. Washing of the cells: UMR 106 cells were taken from the incubator and carefully the medium was aspirated. Later the cells were washed carefully with Dulbecco's PBS, w/o Ca++/ Mg++. Without disturbing the cell monolayer.

3. Fixation of the cells: PBS (Phosphate buffer Solution) was carefully aspirated and the flask was transferred to a fume hood. Enough neutral buffered formalin (10%) was added to cover the cellular monolayer. After at least 30 min, the formalin was carefully aspirated, and the cells were washed with distilled water.

4. Staining of the cells: The distilled water was carefully aspirated, and enough Alizarin Red S staining solution was added to cover the cellular monolayer. And Incubation was done in dark room for 45 mins at room temperature.

5. Washing of the cells: After carefully aspirating Alizarin Red S staining solution, the cell monolayer was washed four times with 1 ml distilled water. Later, the Washing

Buffer was aspirated carefully, and PBS was added.

6. Analysation of the cells: Undifferentiated Osteoblasts (UMR 106 cells), (***without extracellular calcium deposits) are slightly reddish,*** whereas ***mineralized osteoblasts (with extracellular calcium deposits) are bright orange-red.***

Alkaline phosphatase assay.[10-14]

Alkaline Phosphatase is an enzyme naturally present throughout the body. Proliferating Osteoblasts show alkaline phosphatase (AP) activity, which is greatly enhanced during in vitro bone formation. AP activity is therefore a feasible marker for Osteoblasts.

Procedure:

1. The reagents of the Assay were prepared as Standard Procedure.
2. The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using respective media containing 10% FBS.
3. To each well of 96 well microtiter plate, 100 µl of the diluted cell suspension (50,000cells/well) was added.
4. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations (10-320µg/ml) of Standard drug Residronate sodium, and Aqueous, Alcoholic and Hydro-alcoholic extract of *Triticum sativum* were added on to the partial monolayer in microtiter plates.
5. The plates were then incubated at 37^{o}C for 24hrs in 5% CO_2atmosphere. After incubation, the Standard drug and trial sample test solutions in the wells were collected. To these collected 20µl of test solutions, 1000µl working reagent is added.
6. Optical density (OD) is taken at 405nm every 1min for 3-4 times.
7. Alkaline phosphatase activity (ALP activity) is calculated later with the formula.

ALP activity (IU/L)= ΔA/min*2720

Nitric oxide scavenging assay.[15-19]

Nitric oxide is a gaseous free radical that mediates served diverse biological events. Nitric oxide can react with superoxide anion and give rise to powerful free radicals called peroxynitrite radicals (NOO).It is clearly known that exacerbated ***production of NO isinvolved in inflammation.*** NO is similar to several other inflammatory mediators, probably sub serves different functions as local conditions dictate. Due to these reasons scavengers of NO are supposed to be useful in the control of inflammation.

Procedure:

1. Preparation of working solutions along with Griess reagent and reference standard (Curcuminoids) were prepared as per SOP.
2. Nitric oxide scavenging assay was carried out as per the method of Sreejayan and Rao *et al.* (1997).
3. 200 µl of 10 mM sodium nitroprusside and 200 µl of test solutions i.e. Standard drug Residronate sodium, and Aqueous, Alcoholic and Hydro-alcoholic extracts of *Triticum sativum* of various concentrations in two-fold variations (10-320µg/ml) along with reference standard "Curcuminoids" with various test concentrations (2.5-80µg/ml) were incubated at room temperature for 150 minutes.
4. Later, 500µl ofGriess reagent was added and incubated for 10 minutes at room temperature. The absorbance was measured at 546 nm. Test samples were replaced by buffer solution for a control.

5. The percentage inhibition of Nitric Oxide is calculated as follows:

$$\%\text{inhibition} = \frac{[\text{Absorbnce(control)} - \text{Absorbance(test Sample)}] * 100}{\text{Absorbance (control)}}$$

OBSERVATIONS AND RESULTS:

Analytical Evaluation of Trial Drug:

Powder Microscopy showed the presence of Abundant **Simple Starch grains,** Lignified Xylem Parenchyma, **Cotyledons with Starch grains. Physico-chemical test** results were in accordance with the standard values. Remaining tests were done in triplicates, as no standards were available.

Phytochemical analysis revealed the presence of Flavonoids, Glycosides, Proteins, Carbohydrates, Calcium, magnesium, phosphorus, sodium, iron etc.

HPLC confirmed the presence and difference in solubility of Ferulic acid in different extracts of *Triticumsativum*.

Cell Proliferation by MTT Assay:

Treatment of UMR 106 Cells with Standard drug Residronate sodium; Aqueous, Alcoholic and Hydro-alcoholic extract of *Triticumsativum* for 24 hours incubation stimulated cell proliferation in a dose dependant manner has shown ***two-fold increase in cell proliferation.***

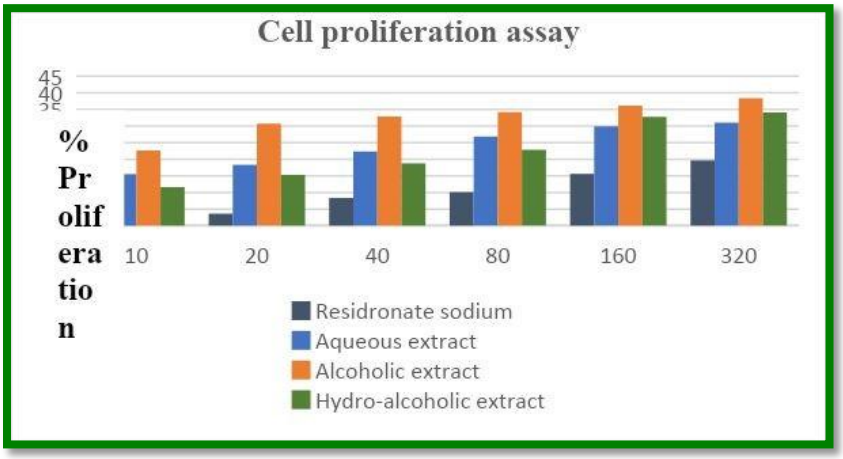
The Standard drug ***Residronate sodium*** showed only ***19.63 %, Aqueous extract*** stimulated ***31.03%***of cell proliferation, ***Alcoholic extract*** showed ***38.44 %*** and ***34.07%*** stimulation of cell proliferation with ***Hydro-alcoholic extract*** at the highest tested concentration of ***320µg/ml.***

Table no. 1: Data of Standard and Test Samples in Cell Proliferation assay.

Sample code	Conc. (µg/ml)	% proliferation	EC_{50}(µg/ml)
Standard (Residronate Sodium)	0	0	**
	10	2.043	
	20	3.609	
	40	8.356	
	80	10.092	
	160	15.599	
	320	19.639	
Aqueous Extract of *Triticum sativum*	0	8.305	**
	10	15.530	
	20	18.319	
	40	22.373	
	80	26.878	
	160	29.889	
	320	31.030	

Alcoholic Extract of *Triticum sativum*	0	19.413	**
	10	22.674	
	20	30.726	
	40	32.855	
	80	34.178	
	160	36.161	
	320	38.440	
Hydro-alcoholic Extract of *Triticum sativum*	0	5.369	**
	10	11.586	
	20	15.298	
	40	18.767	
	80	22.879	
	160	32.732	
	320	34.076	

Graph no. 1: Comparative results of Cell Proliferation activity of Standard sampleand test sample extracts of *Triticumsativum.*



Osteoblast Mineralisation Assay:

1. The UMR 106 cell line treated with Aqueous, Alcoholic and Hydro-alcoholic extract samples of *Triticum sativum* at 160 and 320 µg/ml has induced mineralisation when compared to Control and Standard drug Residronate Sodium.

2. However, compared to Aqueous and Alcoholic extract of *Triticum sativum*, ***hydro-alcoholic extract sample*** has shown ***less effect on mineralisation*** of UMR 106 Cells. These results can be observed in the PLATE NO.2 and PLATE NO.3.

3. On the 7th day of incubation of cells, the test concentration of 320 µg/ml of Standard Residronate Sodium showed less cell viability along with some cell's death. But the cell viability was maintained to some extent at 160 µg/ml of standard.

4. The cells treated at 160 and 320 µg/ml test concentration of standard drug Residronate Sodium, had lost the viability post 14 days incubation.

5. Cells without extracellular Calcium deposits are ***slightly reddish***, whereas mineralised osteoblasts with extracellular calcium deposits are ***bright orange-red.***

Plate no. 2: UMR 106 Cells after Alizarin Red S staining

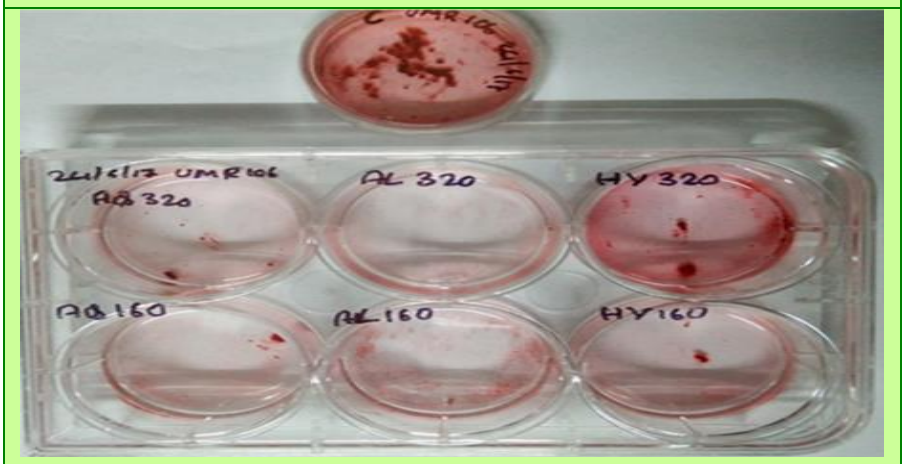
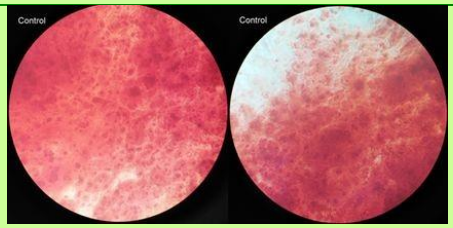
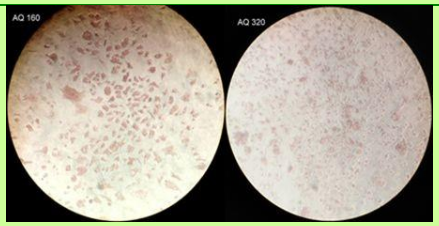
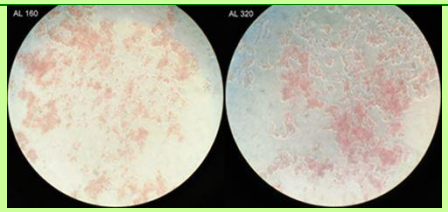
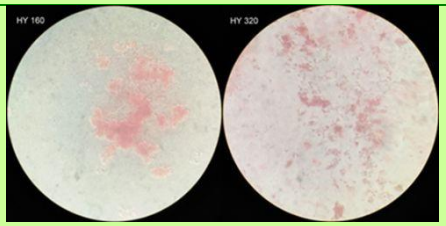


Plate no. 3: The effect of samples on mineralisation in UMR 106 cells as analysed under microscope are depicted in figures below.

Control	Aqueous Extract 160 and 320 µg/ml
	
Alcoholic Extract 160 and 320 µg/ml	**Hydroalcoholic Extract 160 and 320 µg/ml**
	

Alkaline phosphatase Assay:

1. Compared to ***Standard sample*** which showed ***38.08%*** ALP activity, the trial samples Aqueous, Alcoholic and Hydro-alcoholic extracts of *Triticum sativum* showed better ALP activity.

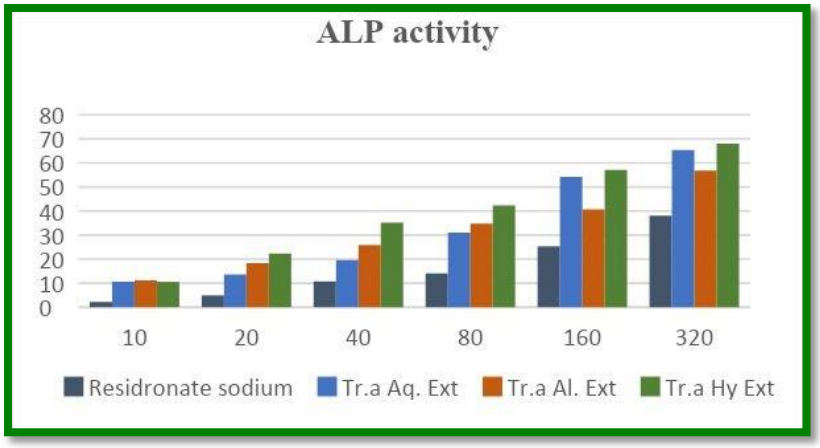
2. Hydro-alcoholic extract has shown ***68 %*** ALP activity, followed by ***Aqueous extract i.e. 65.37%*** and ***56.85%*** of ALP activity by ***Alcoholic Extract***.

Table no. 2: Alkaline Phosphatase assay of Standard and test samples.

	UMR 106	
Sample name	**Conc. µg/ml**	**ALP Activity**

Control	0	0.09
Hydro-alcoholic Extract of *Triticum sativum*	10	10.52
	20	22.30
	40	35.09
	80	42.34
	160	57.12
	320	68.00
Alcoholic Extarct of *Triticum sativum*	10	11.15
	20	18.31
	40	25.84
	80	34.73
	160	40.62
	320	56.85
Aqueous Extract of *Triticum sativum*	10	10.61
	20	13.60
	40	19.58
	80	31.01
	160	54.13
	320	65.37
Standard (Residronate Sodium)	10	2.18
	20	4.90
	40	10.70
	80	14.05
	160	25.30
	320	38.08

Graph no. 2: Comparative ALP activity results of Standard Residronate sodium and Test samples of *Triticumsativum*.



Nitric Oxide Scavenging Assay:

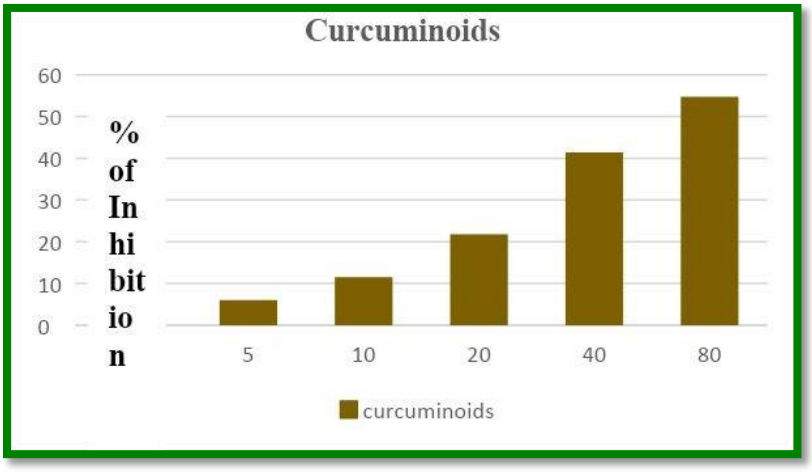
1. The Standard sample and also the test samples of *Triticumsativum* did not show any appreciable Scavenging of Nitric oxide up to the highest tested concentration of 320µg/ml.
2. The scavenging activity was observed to be ***14.85% (Standard sample)***,***Alcoholic extract*** showed ***22.35 %***, ***Aqueous extract showed 16.69%*** and ***16.64%*** was observed in ***Hydro-alcoholic extract*** at the test concentration of 320µg/ml.

Table no. 3: Nitric oxide scavenging activity of Standard sample and test samples.

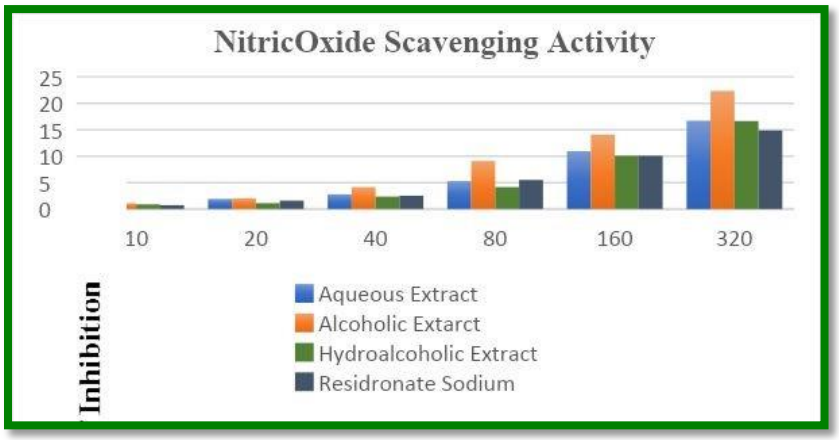
Compound name	Conc. µg/ml	% Inhibition	IC50 (µg/ml)
Control	0	0.00	56.81
Curcuminoids (Reference Standard)	2.5	2.88	
	5	6.04	
	10	11.52	
	20	21.81	
	40	41.38	
	80	54.70	
Aqueous Extract of *Triticumsativum*	10	0.98	*
	20	1.90	
	40	2.77	
	80	5.27	
	160	10.93	
	320	16.69	
Alcoholic Extract of *Triticumsativum*	10	1.09	*
	20	2.01	
	40	4.13	
	80	9.08	
	160	14.08	
	320	22.35	

Hydro-alcoholic Extract of *Triticumsativum*	10	0.87	*
	20	1.09	
	40	2.34	
	80	4.13	
	160	10.06	
	320	16.64	
Standard (Residronate sodium)	10	0.71	*
	20	1.58	
	40	2.50	
	80	5.49	
	160	10.06	
	320	14.85	

Graph no. 3: Nitric oxide scavenging activity of Reference standard curcuminoids



Graph no.4. Comparative results of Nitric oxide scavenging activity of Standard drug and test sample extracts of *Triticumsativum.*



DISCUSSION:

1. Powder microscopy, Physico-chemical and Phyto-chemical analysis, pH, HPLC confirmed the genuinity of drug.

2. The Cell line UMR 106 Cells are Osteoblastic bone cells that can be used for evaluating effectiveness of the Standard and Test samples for Low bone density.

3. In *Asthikshaya*/Low bone density, there will be Quality and Quantity wise depletion of *Asthidhatu* i.e. decrease in the bone tissue and porosity in bones. This can be studied and managed by treating with test samples which increase bone density by proliferating cells (Bone formation), and by improving bone mineral density (mineralisation) using various Invitro Assays.

Cell proliferation assay:

All the extracts of *Triticum sativum* has shown bone cells proliferation activity, thereby increasing the low bone density and helps in bone formation.

Osteoblast mineralisation assay:

1. All the three test sample extracts were effective in inducing mineralisation of osteoblastic UMR 106 Cells, when compared to Standard and Control.

2. The cells treated with Standard drug, had lost viability post 14 days of incubation. From this it can be inferred that, the standard drug may not be helpful in cell mineralisation in higher concentrations.

3. Compared to Aqueous and Alcoholic extracts, Hydroalcoholic extract sample has shown less effect on mineralisation.This may be because of Ferulic acid[68](proven to be helpful in bone formation) content observed in Aqueous and Alcoholic extract of Triticumsativum by HPLC analysis acting as supportive factor in osteoblasts mineralisation.

4. Cells with extracellular Calcium deposits were observed to be 'slightly reddish' whereas mineralised osteoblasts with extracellular calcium deposits were 'bright orange-red'.

From this assay, it was proved that *Triticum sativum* is helpful in improving bone mineral density, thereby in the mineralisation process of bone by calcium deposition.

Alkaline phosphatase assay:

ALP activity in UMR 106 Cells was increased by two-fold on treatment with trial drugs and Standard Sample. Among them, Hydro-alcoholic extract was observed to have better ALP activity when compared to other samples.

The results of this assay proved the supportive role of *Triticum sativum* in osteoblast differentiation and mineralisation.

The resukts of above three assays attributes to Sandhaneeya and Sthiratva karma of Godhuma.

Nitric oxide scavenging assay:

The Standard and test sample extracts did not show any appreciable Scavenging of Nitric oxide. Among them the scavenging effect on Nitric oxide to some extent was observed more in Alcoholic extract when compared to others.

By the results of this assay, it can be inferred that - presence of reduced number of Nitric oxide scavengers in *Godhuma.*

Probable mode of action of drug:

Based on *Rasapanchaka.* All the *Rasapanchaka* of *Godhuma* i.e *Madhura rasa, Guru, Snigdha, Sheetaguna; Sheetavirya;* and *Madhuravipaka* along with

Sandhanakara, Sthairyakara, Balya, Jeevana, and *Brumhana, Vata-pitta hara* and *Kapha-kara karma* supports its role in nourishment, Strength and Stability of the *Asthidhatu* and thus may be helpful in treating *Asthikshaya*.

Based on Phytoconstituents. The Presence of Primary metabolites like Carbohydrates, Proteins, Starch, Fats and Oils[20] etc. in *Triticum sativum* have an important role in bone remodelling, bone metabolism and maintaining its health.

Secondary metabolites like flavonoids, glycosides, phenolic compounds etc., observed in extracts of *Triticum sativum* may help in managing *Asthikshaya*. It has found that Flavanoids[21] may protect against bone loss and can improve bone mineral density. Similar is the activity of glycosides. [22] Phenolic compounds[23,24] have been proven to be effective in promoting calcification of bones and inhibition of osteoclast differentiation.

Inorganic constituents like calcium, phosphorus, sodium etc., found in *Triticumsativum* may be helpful in calcification and mineralisation of bone.

CONCLUSION:

By reviewing the role of *Rasapanchaka* andphyto-constituents present in *Godhuma* , and the results of various carried out Invitro assays, it proves that *Godhuma (Triticum sativum Lam.)*has shown good results as that of the Standard drug.

The overall results of the Invitro study done on *Godhuma* with assays like Cell proliferation Assay, Osteoblast mineralisation Assay, Alkaline Phosphatase Assay revealed that it is having active/potential role and as supportive factor in *Sthirikarana* of *Asthi* i.e. bone formation along with increasing the bone density and its minimal Nitric oxide scavenging activity, indicating that it may not be much helpful in *Asthikshaya* caused due to inflammatory condition

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Source of support: Nil,
Conflict of interest: None Declared

Cite this article as
Syeda Nikhat Nausheen: Godhuma (Triticum sativum Lam.) in Asthikshaya: an Invitro Evaluation; ayurpub; IV(6): 1388-1402