ISSN: 2278-4632
Vol-10 Issue-6 No. 15 June 2020

Elucidating the pharmacological targets of *Litsea glutinosa* in Osteoporosis: An *In-silico* and *In-vitro* approach

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Abstract

Osteoporosis is a skeletal disease which is characterised by decreased bone mineral density leading to increased bone fragility and a consequent enhanced in fracture risk. Many treatments for osteoporosis are available like bisphosphonate therapy, hormonal replacement therapy but these treatments come with serious side effects. Due to this, herbal medicines have gained attraction as an alternative to current therapies. India carries a rich legacy of many of such Herbal medicines. One such herb *Litsea glutinousa* (LG) is recognized and studied for its anti-osteoporotic effects. Moreover, methanolic extract of bark of LG have been shown to contain various bioactive compounds like, alkaloids, flavonoids etc. In present study, we have employed molecular docking approach to identify specific targets which shows higher probability of binding with LG components using SwissTargetPrediction online software. The results revealed that molecules (Androstane, Crinamine, Cinnamolaurine, Thiocoumarin and Gestonorone) illustrated an affinity in the range of 0.50 to 0.89 to different target proteins. To validate results of this *in-silico* study, subsequent *in-vitro* study was conducted in which osteoblastic cell line $SaOS_2$were treated with methanolic extract of LG. It was observed that the genes of identified proteins like Androgen receptor, dopamine, glucocorticoid receptors and cytochrome 450 are significantly altered upon LG treatment. Androgen receptor, dopamine receptor (D2) and cytochrome P450 were observed to be upregulated significantly in the range of 1.3 – 2.6 whereas glucocorticoid receptor was downregulated upto 0.6 times compared to control. In conclusion, the present study proves the possible mode of action of LG regulating the osteoblastogenesis, a positive response towards osteoporosis and the application of *In-silico* studies combined with in-vitro studies in understanding the specific mechanistic action of LG's anti-osteoporotic property.

Keywords: Osteoporosis, *Litsea glutinosa*, Herbals, *In-silico, In-vitro*

Introduction:

Osteoporosis is an age-related disease which is defined as a systemic impairment of bone bulk and altered structure that results in brittleness fractures (Pietschmann *et al.,* 2016). Primary (post-menopausal and senile) and secondary osteoporosis (caused by various drugs and pathologies) can

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be distinguished (Ciccarelli *et al.,* 2015; M. De Martinis *et al.,* 2006; Güler-Yüksel *et al.,* 2018). Osteoporosis prevalence in world is very high, it affects around 200 million people and has becomes major determinant of morbidity, mortality and disability of aged people (Massimo De Martinis *et al.,* 2019). Currently the therapies like calcitonin, estrogen and other hormonal replacement therapy (HRT), denosumab, bisphosphonate etc (Cheng *et al.,* 2020) have been proved for osteoporosis. Bisphosphonate is considered as most preferred treatment (Das & Crockett, 2013).However, prime alert is, prolong treatment of bisphosphonate results in jaw osteonecrosis and fracture of femur (Cheng *et al.,* 2020; Crandall, 2014). Further, HRT is associated with thrombosis, cardiovascular diseases and breast cancers, hence, USFDA has issued a notice not to consider HRT as first line treatment (Chen *et al.,* 2019; Nelson *et al.,* 2002; Stephenson, 2003).

Thus, due to disadvantages associated with these therapies, it has been a challenge to identify new therapy or molecule for osteoporosis treatment. Therefore, researchers have focused to develop the medicine from herbals which have unique benefit in treatment of various of diseases including osteoporosis (Yang *et al.,* 2019). *Litsea glutinosa* (LG), also known as "Maida Lakri", is known to be one of the most potent plants for treatment of osteoporosis, (Sukh *et al.,* 2006), and has been revealed to have osteoprotective properties in OVX rats (Parikh, 2009; Rangrez *et al.,* 2011), by reducing serum TRAcP (Tartrate-resistant acid phosphatase) levels, restored ALP (alkaline phosphatase) and reduced rate of Ca^{++} excretion. Parikh and Rangrez, (2012) in their studies have proved that the methanolic extract to comprise many bioactive compounds like Androstane, Gestonorone and many others (Parikh & Rangrez, 2012). Phytochemical analysis of LG encouraged us to enlighten the molecular targets and possible mechanistic action of LG. Hence, in the present study an attempt is made to understand the network pharmacology using an experimental *in-vitro* approach in which target gene expression levels were studied in osteoblastic $SaOS_2$ cell line using online software SwissTargetPrediction (Ge *et al.,* 2019).

Material & methods:

Preparation of LG extract:

Bark powder of LG were purchased from local market and 50 g was suspended in 500 mL methanol, incubated overnight at room temperature on magnetic stirrer. After incubation, solution was filtered through Whatman® filter paper to remove insoluble particles. Methanolic extract was allowed to air dry. Upon drying, remaining solid extract was collected, weighed and stored at -20°c for further experiment.

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Culturing of $SaOS_2$ cells:

$SaOS_2$ cell line was procured from NCCS, Pune, and were maintained in Maccoy's 5A + 10 % FBS media at 37°c in CO_2 humidified chamber. Cells were washed with 1x PBS, followed by trypsinization and were counted using hemocytometer, and were seeded to fresh flask at 0.4 M/mL density.

LG treatment:

A stock solution of 250 mg/ml of LG dried powder was prepared in DMSO. LG extract were diluted in cell culture media + 10% FBS to achieve 250 µg/mL, 100 µg/mL & 50 µg/mL concentrations. Cells were seeded a day before treatment in T25 flask. Tests flasks (LG treated, control (untreated cells) and vehicle control (DMSO treated) flasks were incubated for 96 hrs.

Transcript analysis:

Total RNA isolation and cDNA synthesis:

Cells from all the flasks were trypsinized and were resuspended in TRIzol® reagent for total RNA isolation. Trizol reagent manual protocol was followed to isolate and purify total RNA. First strand of cDNA was synthesized using Thermo cDNA synthesis kit, 5 µg of total RNA was used. cDNA was converted using oligoDT primers using manual protocol then used as a template for qPCR study.

Quantitative PCR:

cDNA was used as sample for qPCR to analyze expression level of target genes. Different sets of primers (Table 2) were used along with PowerUP Sybr® green master mix. Standard manual protocol was used for amplification. The experiment was performed in triplicates (N=3).

Table 1: Real time PCR conditions

Stage name	Conditions	Cycles/ramp rate
Initial denaturation	95 °C for 5 min	1 cycle
Amplification stage	95 °C for 15 sec	40 cycles
	60 °C for 30 sec	
	72 °C for 45 sec	
Melt curve generation	95 °C for 15 sec	Ramp rate 1.6°C/sec
	60 °C for 1 min	Ramp rate 1.6°C/sec
	95 °C for 15 sec	Ramp rate 0.15°C/sec

Table 1 Indicates PCR conditions used for amplifying target gene

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Table 2: Primer details

	Gene Name	RefSeq ID	TM value (°C)	Primer Type	Sequence
1	DRD2	1813	62	Fw	CAATACGCGCTACAGCTCCAAG
			62	Re	GGCAATGATGCACTCGTTCTGG
2	SC6A3	6531	61	Fw	CCTCAACGACACTTTTGGGACC
			62	Re	AGTAGAGCAGCACGATGACCAG
3	GCR	2908	60	Fw	GGAATAGGTGCCAAGGATCTGG
			61	Re	GCTTACATCTGGTCTCATGCTGG
4	ANDR	367	61	Fw	ATGGTGAGCAGAGTGCCCTATC
			63	Re	ATGGTCCCTGGCAGTCTCCAAA
5	CP19A	1588	61	Fw	GACGCAGGATTTCCACAGAAGAG
			64	Re	ATGGTGTCAGGAGCTGCGATCA
6	NR1I3	9970	62	Fw	GCAGAAGTGCTTAGATGCTGGC
			61	Re	GCTCCTTACTCAGTTGCACAGG
7	ACTB	NG_007992.1	66	Fw	5' GCAACGGAACCGCTCATT 3'
			67	Re	5' AGCTGAGAGGGAAATTGTGCG 3'

Table 2 Indicating details of primers of all the genes along with Tm value and Gene RefSeq ID of each gene from NCBI. β-Actin was taken as a endogenous control & vehicle control was taken as test control in real time PCR to calculate $\Delta\Delta C_T$ and thereby RQ $\Delta\Delta C_T$ is calculated from ΔC_T mean using following formula.

ΔC_T = (Test gene C_T - Endogenous gene C_T)
$\Delta\Delta C_T$= (ΔC_T of test sample - ΔC_T of experiment control)
Relative quantification= $2^{-\Delta\Delta C_T}$

Statistical analysis

All the data were statically analysed. Dunnett's multiple comparisons test was used for statistical analysis. The statistical analysis was performed using one-way ANOVA using GraphPad Prism 8.3.1 Software to obtain p-value each sample was compared with control. (*- $p< 0.05$; **- $p<0.01$; ***- $p<0.001$) All the data are presented as mean ±S.E. and are representative of three independent experiments (n=3).

Database Construction and target prediction:

Chemical SMILES structures of ingredients were found from online software PubChem. These formula were imported in online SwissTargetPrediction network database (http://www.swisstargetprediction.ch/) (Gan *et al.,* 2019) to identify possible target proteins. Results were received with Probability for Androstane, Cinnamolaurine, Crinamine, Gestonorone, Piperzine carobnitrile, Cinnamic Acid, Thiocoumarin and Quinoline molecules. Relevant targets with high probability were identified and selected for gene expression study.

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Results:

Extraction:

The total yield obtained from LG methanolic extraction was 4.42 gm, which corresponds to ~8.8% total yield.

Target prediction:

Upon predicting targets in SwissTargetPrediction software, it was found that molecules like Piperzine carobnitrile, Cinnamic Acid and Quinoline find no probability with any of target proteins. However, other molecules like Androstane, Crinamine, Cinnamolaurine, Thiocoumarin and Gestonorone showed probability with proteins ranging from 0.05 – 0.89 out of 1.

Table 3: Target proteins

	Compound	Target	Target Class	Probability	UniProt ID
1	Androstane	Androgen Receptor	Nuclear receptor	0.67	P10275
		Cytochrome P450 19A1	Cytochrome P450(Aromatase)	0.59	P11511
		Nuclear receptor subfamily 1 group I member 3 (by homology)	Nuclear receptor	0.34	Q14994
2	Crinamine	Acetylcholinesterase	Hydrolase	0.11	P22303
		Delta opioid receptor	Family A G protein coupled receptor	0.11	P41143
		Transient receptor potential cation channel subfamily V member 3	Voltage-gated ion channel	0.11	Q8NET8
3	Cinnamolaurine	Dopamine D2 receptor	Family A G protein coupled receptor	0.67	P14416
		Dopamine D1 receptor	Family A G protein coupled receptor	0.64	P21728
		Dopamine transporter	Electrochemmical transporter	0.63	Q01959
4	Thiocoumarin	Poly [ADP-ribose] polymerase-1	Enzyme	0.05	P09874
		Rho-associated protein kinase 2	Kinase	0.05	O75116
		Poly [ADP-ribose] polymerase-2	Enzyme	0.05	Q9UGN5
5	Gestonorone	Androgen Receptor	Nuclear Receptor	0.89	P10275

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		Glucocorticoid receptor	Nuclear Receptor	0.89	P04150
		Dopamine transporter	Electrochemical transporter	0.89	Q01959

Table 3 depicts the probability of phytochemical compound binding with respective target proteins. Details of these target protein of androstane, cinnamolaurine and Gestonoronewere extracted from UniProt online protein data base (https://www.uniprot.org/) and they were selected for gene expression studies.

qPCR:

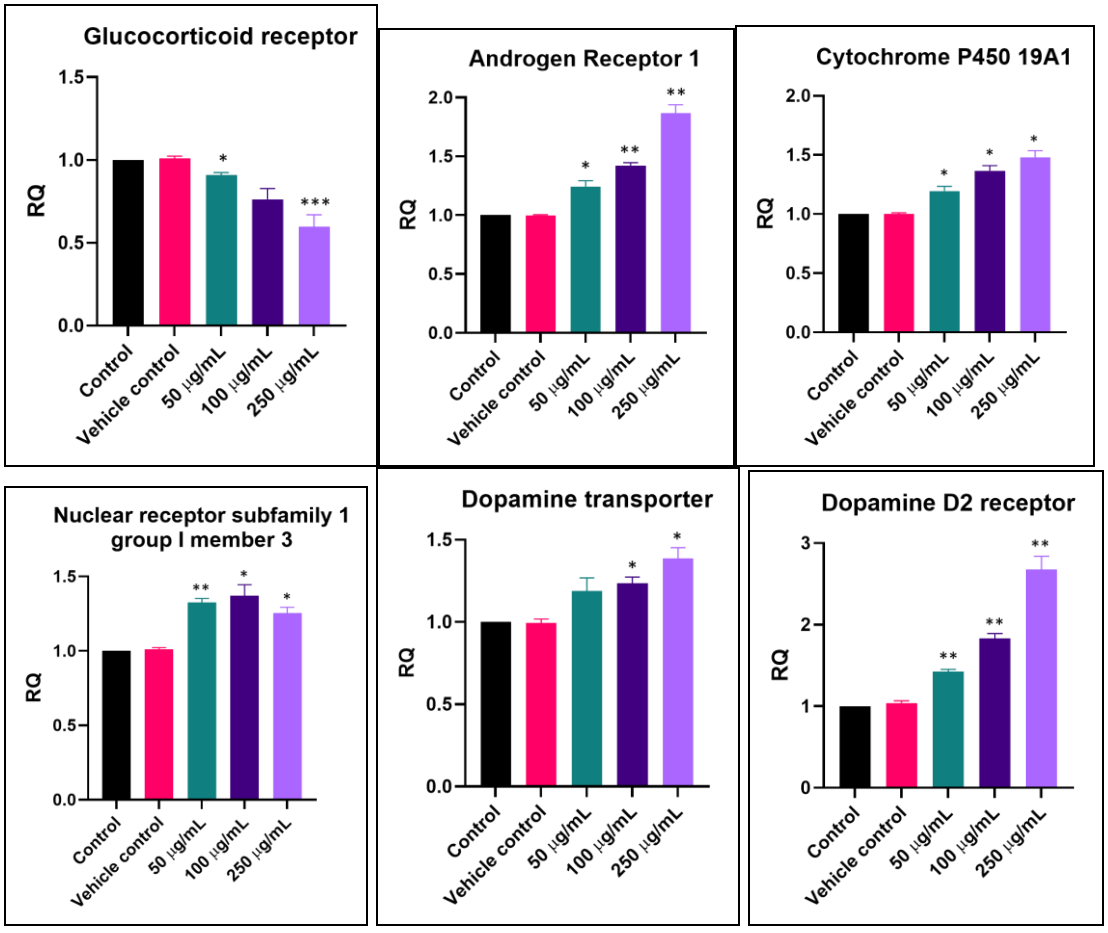

Figure 1: Gene expression profile of target genes. Each graph is showing the expression profile of Target genes.

Target Gene	Control	Vehicle control	50 µg/mL	100 µg/mL	250 µg/mL
Dopamine D2 receptor (DRD2)	1.00 ± 0.00	1.04 ± 0.02	1.43 ± 0.21**	1.83 ± 0.04**	2.67 ± 0.10**

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Dopamine transporter (SC6A3)	1.00 ± 0.00	0.99 ± 0.01	1.19 ± 0.05	1.23 ± 0.02*	1.39 ± 0.09*
Glucocorticoid receptor (GCR)	1.00 ± 0.00	1.01 ± 0.01	0.91 ± 0.11*	0.76 ± 0.04	0.60 ± 0.04***
Androgen Receptor 1 (ANDR)	1.00 ± 0.00	1.00 ± 0.00	1.24 ± 0.03**	1.42 ± 0.10**	1.87 ± 0.04**
Cytochrome P450 19A1 (CP19A)	1.00 ± 0.00	1.00 ± 0.01	1.19 ± 0.09*	1.37 ± 0.03*	1.48 ± 0.11*
Nuclear receptor subfamily 1 group I member 3 (NR1I3)	1.00 ± 0.00	1.01 ± 0.01	1.32 ± 0.05**	1.37 ± 0.04*	1.25 ± 0.02*

Table 4 Indicates relative quantification of target genes with fold change compared to control

In gene expression study, it was observed that androgen receptor, cytochrome P450, dopamine receptor D2 and dopamine transporter were getting significantly upregulated in dose dependent manner upon LG treatment in the range of 1.39 – 2.67 times compared to control when treated with 250 µg/mL dose. Whereas expression of GCR was significantly downregulated in dose dependent manner upon LG treatment. NR1I3 was also getting significantly upregulated upon LG treatment but upregulation were not consistent with dose.

Discussion:

Osteoporosis is the disease which results due to imbalance of activity of osteoblasts and osteoclasts (Ge *et al.,* 2019). Due to serious drawbacks of current available therapy, researchers are in constant efforts to discover promising herbal based medicine. With akin aim, we had conducted a study to screen all active components present in LG methanolic crude using *in-silico* approach followed by *in-vitro* verification experiments.

In present study with the help bioinformatics analysis, it was possible to study probable binding of bioactive compound of LG methanolic extract with target proteins. Androstane and Gestonorone, bioactive molecules present in LG crude, exhibited high binding probability with cytochrome P450, nuclear receptor 1I3 and dopamine transporter. Furthermore, both the molecules showed very high probability with Androgen receptor (Table 3). In our *in-vitro* experiment, a significant upregulation of cytochrome P450, which is mainly responsible for conversion of androgens to estrogen, clearly indicates that Androstane enhances the expression in a dose dependent manner probably leading to local estrogen level surge (Perez *et al.,* 2006; Shaheenah *et al.,* n.d.; Simpson, 2002). Besides this, we also have obtained upsurge in gene expression level of nuclear receptors like androgen receptor and nuclear receptor 1I3 (constitutive androstane receptor (CAR)). Studies suggest CAR and androgen receptor are closely associated with osteoblastic differentiation and bone mineralisation process (Takeuchi *et al.,* 1994; Urano *et al.,* 2009). In the present condition,

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the LG dose treatment in $SaOS_2$ cells, significantly upregulated the nuclear receptors suggesting the differentiation and growth of osteoblastic cells. Therefore it also can be stated that Androstane and Gestonorone are involved in bone formation process. The study lays a strong foundation, where the similar alteration was found in *in-vivo* assessment proposing osteoprotective effect of LG (Parikh, 2009).

In *In-silico* study, we have observed that Cinnamolaurine exhibited greater probability of binding with proteins like Dopamine receptor D1R, D2R and Dopamine transporter (a common target of Gestonorone). Dopamine which is important neurotransmitter, exerts its effect by binding with five different types of dopamine receptors of which, receptors D1R and D2R are expressed on Osteoblasts (Cheong *et al.,* 2018). Correlating the literature studies between dopamine and osteoblasts, dopamine has been shown to be involved in bone metabolism via playing positive role in proliferation of osteoblastic cells, bone mineralization and formation (Bliziotes *et al.,* 2002; Lee *et al.,* 2015). Studying *in-vitro* outcomes, dose dependent significant upregulation of dopamine receptor D2R and transporter indicates that Cinnamolaurine and Gestonorone may be involved in upregulation of these genes and hence in osteoblast proliferation. Thus, this findings elucidates the bioactive properties of Cinnamolaurine & Gestonorone found in LG extract and thus can be accounted for improving bone health.

Besides dopamine transporter and androgen receptor, Gestonorone showed a very high binding probability with Glucocorticoid receptor also. Gestonorone, (belongs to the class of steroid hormones) shows a very high binding probability with Glucocorticoid receptor which are distributed on cells of most of the tissues. However, there are controversies relating to the action of glucocorticoids and osteoblast. It has been postulated that this hormone reduces the differentiation of osteoblast thus resulting in bone loss (Gluck *et al.,* 1981; Lane & Lukert, 1998; LoCascio *et al.,* 1990; Rauch *et al.,* 2010). In the in-vitro experiment of the present study, we have reported significant down-regulation of Glucocorticoid receptor in $SaOS_2$ cell line. This finding enlightens an active involvement of Gestonorone in regulating glucocorticoid receptor and hence probably playing a crucial role in suppressing bone loss activities supports an anti-osteoporotic property of LG. Compared to similar action(s) of other bioactive compounds, Gestonorone, supports anti-osteoporotic property of LG via bone formatting and bone loss suppression.

Cumulatively, it can be concluded that all three bioactive components play an essential role in modulating the expression of various genes. Expression profile mapping of these genes draw a frame work of mechanistic action of LG by defining specific role of potent components in the direction of bone formation and preventing osteoporosis. However, this study provides only partial

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support to the inferences and seeks further detailed analysis of proteins involved in pathways via orthogonal methods and an *in-vivo* studies.

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