MATERIAL AND METHODS

2.0 PREPARATION OF LG BARK EXTRACT

Litsea Glutinosa is the herbal plant, which has been studied for its anti-osteoporotic properties *in-vivo* (Rangrez et al., 2011). As it was established that methanolic extract of LG bark is most potent with compared to aqueous and no-aqueous fraction, LG bark powder was proceed for methanolic extraction.

LG bark powder was procured from herbal shop of local market, Vadodara. Powder, which was confirmed to be from any of the bacterial or fungal contamination. 50 gm of powder was weighing balance and it was added in 400 mL of methanol in shaking condition on magnetic stirrer. After addition of all the powder, volume was made up 500 mL with methanol. Suspension was allowed to mix for 48 hrs at room temperature at highest possible shaking speed. After 48 hrs, magnetic stirrer was stopped and solution was carefully filtered to a glass bottle using Whatman® filter paper to remove undissolved particles. Multiple filter papers were used for efficient filtration. Flow-through (Methanolic extract) was collected in clean autoclaved glass bottle. After collection, extract was air dried in the waterbath (35 °C – 40 °C) to evaporate methanol 100%. Methanol evaporation was confirmed by smell the remaining paste. This paste was collected and weighed. Total weight of methanolic extract paste was 4.42 gm which corresponds to 8.8% yield. Paste was stored in 5 mL microcentrifuge tube at -20 °C temperature for further use.

Before experiment, 250 mg of methanolic extract was weighed and dissolved in 800 µL of dimethyl sulfoxide (DMSO). Solution was vortexed vigorously for 30 min for complete dissolution. After dissolving it completely, volume was made up 1 mL, resulting stock of 250 mg/mL. For the preparation of working stock, main stock was serially diluted in Maccoy's 5A media, then filtered with 0.2 µ filter before use for study.

2.1 CELL CULTURING OF SAOS-2 CELL LINE AND TREATMENT

Saos-2 cell line is one of the most chosen human cell line for the studies on bone cells. Saos-2 cell line is human sarcoma cell line, which has been characterised since very old time for displaying various properties like osteoblasts including bone mineralising ability *in-vivo* as well as *in-vitro* (Fedde, 1992; MCQUILLAN, 1995; Rodan et al., 1987).

Saos-2 cell line was procured from National Center for Cellular Sciences (NCCS), Pune, India. Cells were supplied in T25 flask in Maccoy's 5A + 10 % FBS media. Cells were sub-cultured and further propagated in same medium (Maccoy's 5A (16600082, Gibco, USA) + 10 % FBS media (RM9955-100ML, Himedia, India)). For the treatment with LG methanolic extract, it was serially diluted to achieve desired concentration. After that each solution was filtered with 0.2 µm filter and cells were treated with it.

2.2 MTT ASSAY

Rangrez et al., (2011) research group has reported all the experiments in *in-vivo* conditions in OVX rats. Till now LG treatment on Saos-2 cell line are yet to be explored. To establish suitable doses on Saos-2 cells, MTT assay was conducted. In this assay, Saos-2 cells were treated with the range of concentrations like from 500 ng/mL to 400 µg/mL of LG methanolic extract Table MM- 1.

Table MM- 1: Target concentrations for MTT assay

Test	Target Test Concentration
Vehicle control 1	DMSO 0.01%
Vehicle control 2	DMSO 0.05%
Vehicle control 3	DMSO 0.1%
Vehicle control 4	DMSO 0.2%
Test concentration 1	500 ng/mL
Test concentration 2	1 µg/mL

Test concentration 3	10 µg/mL
Test concentration 4	50 µg/mL
Test concentration 5	100 µg/mL
Test concentration 6	150 µg/mL
Test concentration 7	200 µg/mL
Test concentration 8	250 µg/mL
Test concentration 9	300 µg/mL
Test concentration 10	350 µg/mL
Test concentration 11	400 µg/mL

MTT assay was performed in 96 well cell culture plate. 15 mL sterile centrifuge tube was used for the preparation of dilutions. Sterile Maccoy's 5A + 10% FBS media was used as diluent.

Table MM- 2: Dilution flow for MTT assay

	Final conc (µg/mL)	**Stock conc (µg/mL)**	**Volume (µL)**	**Diluent(µL)**
Main Stock	NA	250000	NA	NA
Working stock - 1	2500	250000	30	2970
Working stock - 2	100	2500	200	4800
Working solution	1	100	50	4950
	20	100	1000	4000
	100	Working Stock - 2		
	200	2500	400	4600
	300		600	4400
	400		800	4200
	500		1000	4000
	600		1200	3800
	700		1400	3600
	800		1600	3400

NA: Not applicable

Table MM- 3: Plate layout for MTT assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control						300 µg/mL					
B	500 ng/mL						350 µg/mL					
C	10 µg/mL						400 µg/mL					
D	50 µg/mL						Media + 0.01% DMSO					
E	100 µg/mL						Media + 0.05% DMSO					
F	150 µg/mL						Media + 0.1% DMSO					
G	200 µg/mL						Media + 0.2% DMSO					
H	250 µg/mL						Media Blank					

Saos-2 cells were propagated upto 3 passages (P#3) during culturing. On P#4, they were seeded at 5000 cell/well/200 µL of seeding density in 96 well plate for MTT assay prior to 24 hrs of experiment. Plate was incubated at 37 °C and 5% CO_2 in static incubator with 30 – 80% humidified environment.

On the day 0, dilutions for different concentrations of LG methanolic extract were prepared as mentioned in Table MM- 2. For dilutions of vehicle controls, DMSO was diluted in same diluent media. All the dilutions of LG and vehicle were dispensed 200 µL in another fresh sterile 96 well plate as per layout mentioned in Table MM- 3. For treatment, 96 well plate with cells was removed from incubator. Each well was emptied carefully with multichannel micropipette. 100 µL of freshly prepared media (Maccoy's 5A + 10% FBS) was dispensed in each well. After that, 100 µL from dilution plate was dispensed in respective well. This will result in 1:2 final concentration in well which will result achieving target test concentration (Table MM- 1). Then after, plate was again incubated at 37 °C and 5% CO_2 in static incubator with 30 – 80 % humidified environment. Post 48 hr, spent media was removed gently with multichannel micropipette. Media change was given with exact same concentration in similar way as previous. Plates were again incubated for 48 hrs at 37 °C and 5 % CO_2 in static incubator with 30 – 80 % humidified environment.

Post total 96 hr of incubation, plate was proceeded for MTT reagent treatment. MTT assay is based on 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye (M2128, Sigma, USA) reduction in viable cells converting dye into crystal forms which are measured quantitatively at 570 nm. After removing media from each well, cells were gently washed 1x sterile PBS. Further 100 µL of cell culture media (maccoy's 5A + 10 % FBS) and 20 µL MTT solution were added to each well. Plate was then incubated at 37 °C and 5% CO_2 in static incubator with 30 – 80 % humidified environment for 4 hrs. After completion of incubation, 10 % SDS (L3771, Sigma, USA) solution was mixed in 0.01 N HCl (320331, Sigma, USA) and was added to each well and mixed thoroughly. Plate was incubated at room temperature for 10 mins in dark environment for colour development. After colour development, the absorbance of each well was measured at 570 nm using plate reader (Gen5, BioTek).

2.3 LG TREATMENT TO SAOS-2 CELL LINE

After MTT assay results interpretation, 3 doses were finalized for further experiments: 50 µg/mL, 100 µg/mL and 250 µg/mL. For further experiments like RNA isolation, protein isolation, flow cytometry analysis, higher number of test cells are required. For that purpose, LG treatment was set up in T25 cell culture flask scale. Saos-2 cells of P#5, were seeded in T25 flasks at seeding density of 0.5 million cells/5 mL/T25 flask. Total 5 such flasks were seeded and then incubated at 37 °C and 5% CO_2 in static incubator with 30 – 80 % humidified environment for 24 hrs. Next day, dilutions were prepared of LG methanolic extract in media to achieve desired concentration. Dilutions were prepared as mentioned in

Table MM- 4.

Table MM- 4: Dilution flow for treatment

Dilution for Treatment				
	Final conc (µg/mL)	**Stock conc (µg/mL)**	**Volume (µL)**	**Diluent (µL)**
Main stock	-	250000	-	-
Working stock - 1	2500	250000	30	2970
Working solution	50	2500	200	9800
	100		400	9600
	250		1000	9000

Post 24 hr incubation, spent media was removed and it was replaced with 5 mL of media + LG extract along with vehicle control (media + 0.1% DMSO). Flasks were again incubated at 37 °C and 5% CO_2 in static incubator with 30 – 80 % humidified environment for 48 hrs. On Day2, last step of replacing spent media with media + LG was repeated and flasks were incubated at same conditions for 48 hrs.

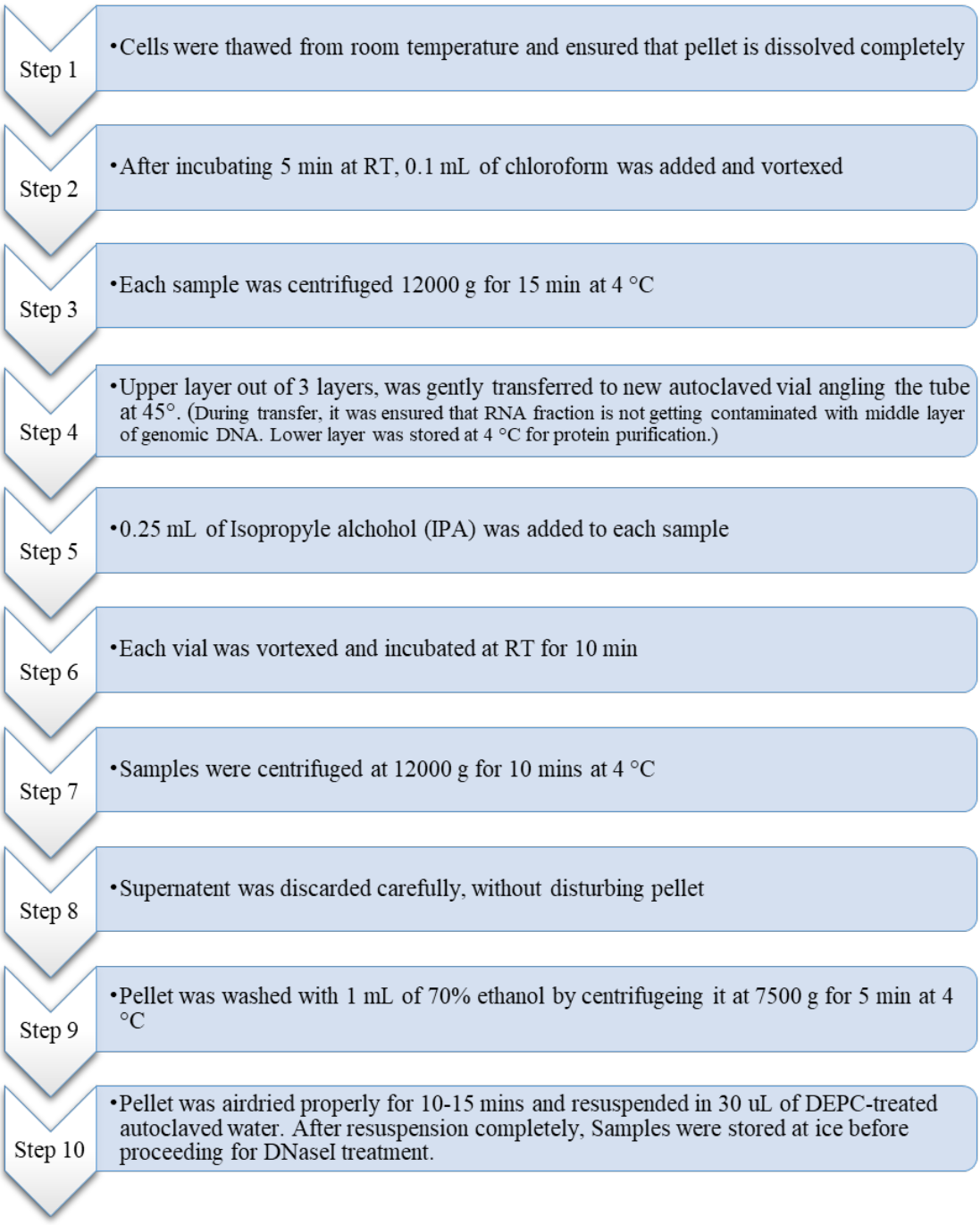
Post 96 hr incubation, cells were observed at microscope and images were recorded of all the flasks. Cells were then washed with 1x PBS. After removal of 1x PBS, 0.25 mL of trypsin was added to each flask and flasks were incubated at 37 °C for 2-5 min with microscopic observation for cell detachment. Once all the cells were detached and gained round shape, 1 mL media (Maccoy's 5A) was added to each flasks and all the cells were harvested. Cells were then centrifuged at 1000 rpm/10 min in swinging bucket rotor. Supernatant was discarded and pellet was resuspended in 0.5 mL TRIzol™ reagent (15596026, Invitrogen, USA). Tubes were then stored at -20 °C for further experimentation.

2.4 RNA ISOLATION PROTEIN ISOLATION AND CDNA CONVERSION

Cells were thawed to room temperature from -20 °C and proceeded for RNA isolation as per TRIzol manual protocol. Whole process of RNA isolation and purification was performed throughout on ice.

2.4.1 RNA ISOLATION:

2.4.1.1 PROTOCOL USED FOR RNA ISOLATION AND PURIFICATION


Step 1
•Cells were thawed from room temperature and ensured that pellet is dissolved completely
Step 2
•After incubating 5 min at RT, 0.1 mL of chloroform was added and vortexed
Step 3
•Each sample was centrifuged 12000 g for 15 min at 4 °C
Step 4
•Upper layer out of 3 layers, was gently transferred to new autoclaved vial angling the tube at 45°. (During transfer, it was ensured that RNA fraction is not getting contaminated with middle layer of genomic DNA. Lower layer was stored at 4 °C for protein purification.)
Step 5
•0.25 mL of Isopropyle alchohol (IPA) was added to each sample
Step 6
•Each vial was vortexed and incubated at RT for 10 min
Step 7
•Samples were centrifuged at 12000 g for 10 mins at 4 °C
Step 8
•Supernatent was discarded carefully, without disturbing pellet
Step 9
•Pellet was washed with 1 mL of 70% ethanol by centrifugeing it at 7500 g for 5 min at 4 °C
Step 10
•Pellet was airdried properly for 10-15 mins and resuspended in 30 uL of DEPC-treated autoclaved water. After resuspension completely, Samples were stored at ice before proceeding for DNaseI treatment.

- Once RNA was purified, DNaseI (EN0521, Thermo, USA) treatment was given to the each RNA sample to remove genomic DNA contamination as follows:

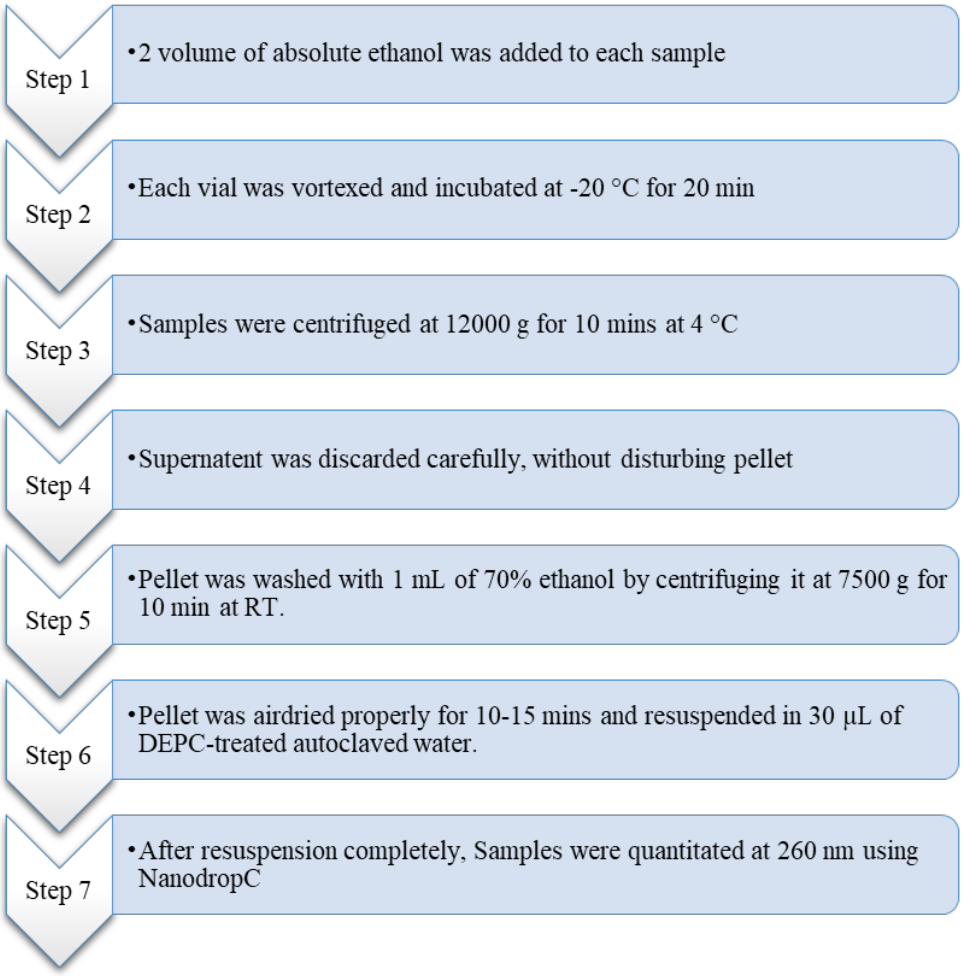
Table MM- 5: Reaction mix for DNaseI treatment

Reagent	Stock	Volume (µL)
RNA sample	NA	30
DNaseI buffer	10x	5
DNaseI Enzyme	1 U/µL	2.5
DEPC treated water (supplied with kit)	NA	12.5
Total	NA	50

After constituting all the components mentioned in Table MM- 5, each sample was incubated at 37 ℃ for 1 hr to ensure reaction completion. After that reaction was stopped by adding 2.5 µL of 50 mM EDTA (supplied with kit) and incubated at 65 ℃ for 10 min.

Post that, RNA was precipitated using following protocol:

Protocol for RNA precipitation:



RNA samples were checked for its OD_{260nm} using Nanodrop C.

2.4.2 CDNA CONVERSION (REVERSE TRANSCRIPTION (RT) PCR):

When RNA isolation and purification was completed, cDNA first strand was synthesised from each sample using First strand cDNA synthesis kit (K1216, Thermo, USA). In reaction, 5 µg RNA was used as a template for cDNA synthesis using oligo dT primers. Reaction mix is mentioned in

Table MM- ***6***. After setting up reaction mix, RNA samples were stored at -80 °C.

Table MM- 6: Reaction mix for cDNA synthesis

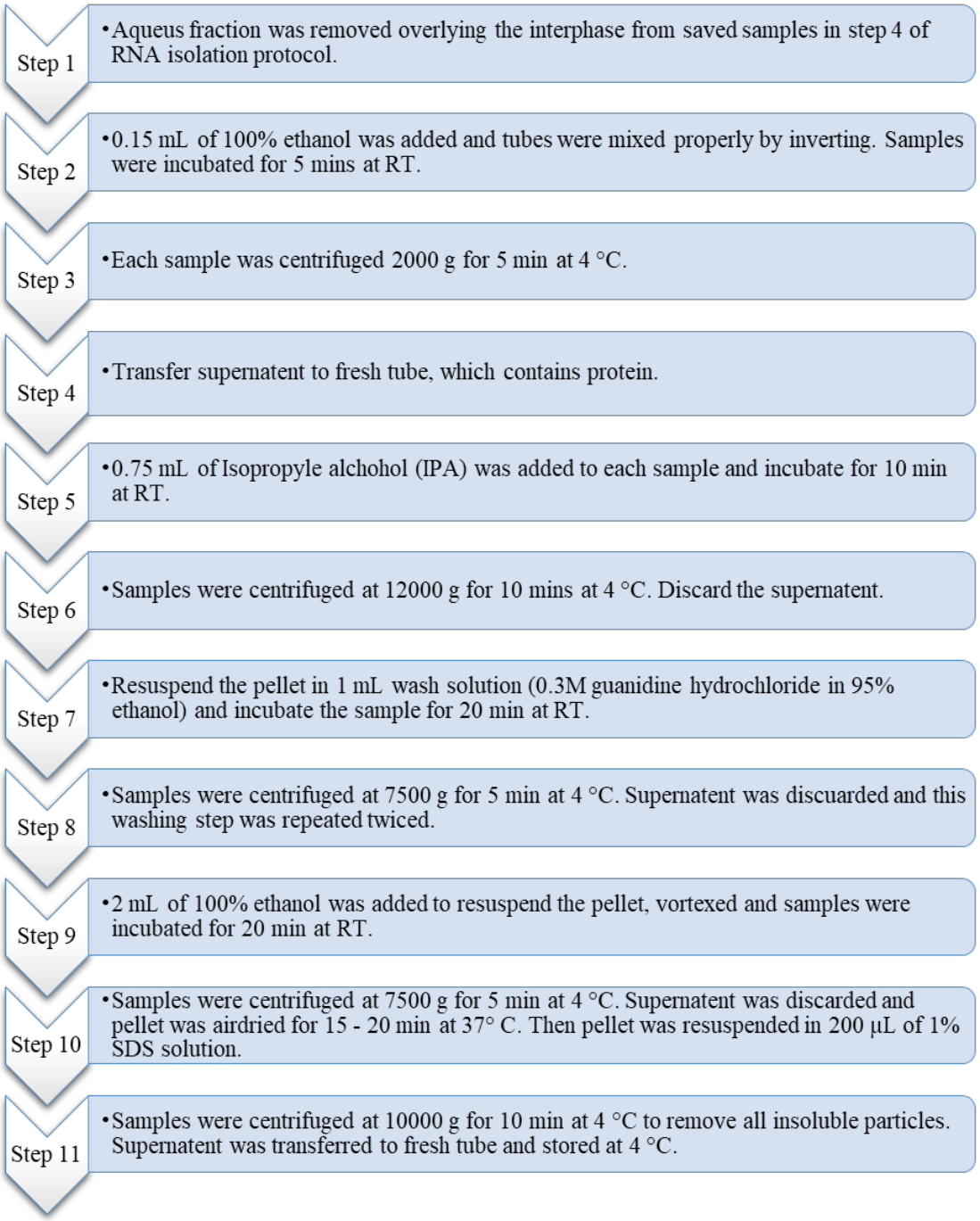
Reagent	Stock	Volume (µL)
RNA sample (5 µg)	NA	*x**
Reaction buffer	5x	10
Oligo (dT)18 primers	100 µM	2.5
dNTPs	10 mM	5
Ribolock RNase Inhibitor	20 U/µL	5
M-MuLV Reverse transcriptase	20 U/µL	2.5
Water	NA	25 – *x*
Total		50

*: *x* indicates variable volume of each RNA sample to take 5 µg of RNA quantity. For example, if RNA concentration is 300 ng/µL, then for 5 µg 16.6 µL of RNA was taken in cDNA synthesis reaction mix

Whole reaction was set up on ice. Once reaction set up was completed, each vial was mixed gently and given short spin. Samples were incubated at 37°C for 60 min, followed by terminated by incubating at 70°C for 5 min. After incubation, cDNA samples were stored at -20 °C.

2.4.3 PROTEIN ISOLATION AND PURIFICATION:

2.4.3.1 PROTOCOL USED FOR PROTEIN ISOLATION AND PURIFICATION



Isolated proteins were quantified at OD280nm using NanodropC.

2.5 QUANTITATIVE PCR:

Gene expression of desired genes was performed using quantitative PCR (QuantStudio™ 5, Applied Biosystem, USA). For quantitation, primers were designed in-house using online available primer designing tool (www.eurofinsgenomics.eu). For primer designing, DNA sequences of human designed genes were obtained from NCBI database. Total 18 genes were considered for primer designing which are listed in Table MM- 7. Post designing, primers were chemically synthesized out-sourced from Sigma Pvt ltd (Sigma, USA). Primers details are mentioned inTable MM- 8.

Table MM- 7: List of Targeted Genes

Sr No	Category	Gene Name	Gene Code
1	Protein Kinase	MAP Kinase 3	*MAPK3*
2	Nuclear receptor	Estrogen Receptor β	*ESR2*
3	ATP catalase	Adenylate cyclase	*ADCY1*
4	Transcription Factor	Runx2	*RUNX2*
5		Egr-2	*EGR-2*
6		NFATc1	*NFATC1*
7		CREB	*CREB1*
8	Proliferation marker protein	PCNA	*PCNA*
9	Globular protein (Bone marker protein)	Osteocalcin	*BGLAP*
10	TNF family member – Apoptosis marker	Fas Ligand	*FASLG*
11	Globular protein – Apoptosis marker	Cytochrome C	*CYCS*
12	Cysteine protease – Apoptosis marker	Caspase 3	*CAP3*
13	Family A G protein coupled receptor	Dopamine D2 receptor	*DRD2*
14	Electrochemical transporter	Dopamine transporter	*SC6A3*
15	Nuclear receptor	Glucocorticoid receptor	*GCR*
16		Androgen Receptor	*ANDR*
17		Nuclear receptor subfamily 1 group I member 3	*NR1I3*
18	Aromatase enzyme	Cytochrome P450 19A1	*CP19A*
19	Endogenous control	β-actin	*ACTB*

Table MM- 8: Primer Details of targeted genes

Sr No.	Gene Name	RefSeq ID	TM value (°C)	Primer Type	Sequence
1	MAPK3	NM_001109891.2	62	Forward	5' GGATGCCGATGACATTCTC 3'
			64	Reverse	5' CATCAAGAAGATCAGCCCCT 3'
2	RUNX2	NM_001015051.4	67	Forward	5' CCTAAATCACTGAGGCGGTC 3'
			64	Reverse	5' CAGTAGATGGACCTCGGGAA 3'
3	ESR2	NM_001437.3	64	Forward	5' ACCAAAGCATCGGTCACG 3'
			62	Reverse	5' CATGATCCTGCTCAATTCCA 3'
4	ADCY1	NM_001281768.2	64	Forward	5' CGACACGCAGTAGTAGCA 3'
			65	Reverse	5' ATGAGCTCTTCGGCAAGTTC 3'
5	EGR-2	NM_001136178.2	64	Forward	5' AGCAAAGCTGCTGGGATAT 3'
			64	Reverse	5' TTGACCAGATGAACGGAGTG 3'
6	NFATC1	NM_001278675.2	65	Forward	5' CACCTCAATCCGAAGCTCAT 3'
			65	Reverse	5' CCTGTCCCCTACGTCCTA 3'
7	CREB1	NM_134442.5	66	Forward	5' GCTGGGCTTGAACTGTCATT 3'
			68	Reverse	5' GTGACGGAGGAGCTTGTACC 3'
8	PCNA	NM_182649.2	65	Forward	5' AAGAGAGTGGAGTGGCTTTTG 3'
			64	Reverse	5' TGTCGATAAAGAGGAGGAAGC 3'
9	CASP3	NM_001354783.2	62	Forward	5' TCGCTTCCATGTATGATCTTTG 3'
			63	Reverse	5' CTGCCTCTTCCCCCATTCT 3'
10	FASLG	NM_000639.3	65	Forward	5' CAGAGGCATGGACCTTGAGT 3'
			65	Reverse	5' GTCTACCAGCCAGATGCACA 3'
11	BGLAP	NM_199173.6	63	Forward	5' GCCTGGGTCTCTTCACTA 3'
			65	Reverse	5' TCACACTCCTCGCCCTATT 3'
12	CYCS	NM_018947.6	65	Forward	5' GGGGTAAACAGTGATACC 3'
			68	Reverse	5' GAATGACTGACAGAATATTTTG 3'
13	DRD1	NM_000795.5	62	Forward	5’ CAATACGCGCTACAGCTCCAAG 3’
			62	Reverse	5’ GGCAATGATGCACTCGTTCTGG 3’
14	SC6A3	NM_001044.5	61	Forward	5' CCTCAACGACACTTTTGGGACC 3'
			62	Reverse	5' AGTAGAGCAGCACGATGACCAG 3'

15	GCR	NM_000176.3	60	Forward	5' GGAATAGGTGCCAAGGATCTGG 3'
			61	Reverse	5' GCTTACATCTGGTCTCATGCTGG 3'
16	ANDR	NM_000044.6	61	Forward	5' ATGGTGAGCAGAGTGCCCTATC 3'
			63	Reverse	5' ATGGTCCCTGGCAGTCTCCAAA 3'
17	CP19A	NM_000103.4	61	Forward	5' GACGCAGGATTTCCACAGAAGAG 3'
			64	Reverse	5' ATGGTGTCAGGAGCTGCGATCA 3'
18	NR1I3	NM_001077482.3	62	Forward	5' GCAGAAGTGCTTAGATGCTGGC 3'
			61	Reverse	5' GCTCCTTACTCAGTTGCACAGG 3'
19	ACTB	NM_001101.5	66	Forward	5' GCAACGGAACCGCTCATT 3'
			67	Reverse	5' AGCTGAGAGGGAAATTGTGCG 3'

Post synthesis of cDNA, reaction was set for quantitative PCR. As the expression of genes varies in their expression level, cDNA volume used for each gene was optimised. For some high expressing genes, cDNA was diluted 1:2, and for rest, neat cDNA was used in the reaction. Each primer was resuspended in respective volume (as per primer technical sheet provided by Sigma) of autoclaved distilled water to achieve 100 µM stock concentration. Each primer was diluted 1:10 in autoclaved distilled water in fresh tube, resulting 10 µM working solution.

Table MM- 9: qPCR Reaction mix for MAPK3, CYCS & CREB

Component	Stock	volume (µL)
PowerUP Sybr® green master mix	2x	10
Respective Fw primer	10 µM	0.5
Respective Re primer	10 µM	0.5
cDNA*	NA	3
Nuclease free water	NA	6
Total	--	20

* cDNA was diluted 1:2 using DEPC treated water

Table MM- 10: qPCR Reaction mix for RUNX2 & CP19A

Component	Stock	volume (µL)
PowerUP Sybr® green master mix	2x	10

Respective Fw primer	10 µM	0.5
Respective Re primer	10 µM	0.5
cDNA*	NA	2
Nuclease free water	NA	7
Total	--	20

* cDNA was diluted 1:2 using DEPC treated water

Table MM- 11: qPCR Reaction mix for ESR2, ANDR & FASL

Component	Stock	volume (µL)
PowerUP Sybr® green master mix	2x	10
Respective Fw primer	10 µM	0.5
Respective Re primer	10 µM	0.5
cDNA	NA	3
Nuclease free water	NA	6
Total	--	20

Table MM- 12: qPCR Reaction mix for ADCY1

Component	Stock	volume (µL)
PowerUP Sybr® green master mix	2x	10
ADCY1 Fw primer	10 µM	0.5
ADCY1 Re primer	10 µM	0.5
cDNA	NA	2.5
Nuclease free water	NA	6.5
Total	--	20

Table MM- 13: qPCR Reaction mix for EGR-2 & NR1I3

Component	Stock	volume (µL)
PowerUP Sybr® green master mix	2x	10
Respective Fw primer	10 µM	0.5
Respective Re primer	10 µM	0.5
cDNA	NA	1.5

Nuclease free water	NA	7.5
Total	--	20

Table MM- 14: qPCR Reaction mix for NFATC1 & PCNA

Component	Stock	volume (µL)
PowerUP Sybr® green master mix	2x	10
Respective Fw primer	10 µM	0.5
Respective Re primer	10 µM	0.5
cDNA	NA	4
Nuclease free water	NA	5
Total	--	20

Table MM- 15: qPCR Reaction mix for CASP3

Component	Stock	volume (µL)
PowerUP Sybr® green master mix	2x	10
CASP3 Fw primer	10 µM	0.5
CASP3 Re primer	10 µM	0.5
cDNA*	NA	3.5
Nuclease free water	NA	6
Total	--	20

Table MM- 16: qPCR Reaction mix for BGLAP & GCR

Component	Stock	volume (µL)
PowerUP Sybr® green master mix	2x	10
Respective Fw primer	10 µM	0.5
Respective Re primer	10 µM	0.5
cDNA*	NA	2.5
Nuclease free water	NA	6.5
Total	--	20

* cDNA was diluted 1:2 using DEPC treated water

Table MM- 17: qPCR Reaction mix for DRD1

Component	Stock	volume (µL)
PowerUP Sybr® green master mix	2x	10
DRD1 Fw primer	10 µM	0.5
DRD1 Re primer	10 µM	0.5
cDNA	NA	2
Nuclease free water	NA	7
Total	--	20

Table MM- 18: qPCR Reaction mix for SC6A3

Component	Stock	volume (µL)
PowerUP Sybr® green master mix	2x	10
SC6A3 Fw primer	10 µM	0.5
SC6A3 Re primer	10 µM	0.5
cDNA*	NA	5
Nuclease free water	NA	4
Total	--	20

* cDNA was diluted 1:2 using DEPC treated water

Table MM- 19: qPCR Reaction mix for ACTB

Component	Stock	volume (µL)
PowerUP Sybr® green master mix	2x	10
ACTB Fw primer	10 µM	0.5
ACTB Re primer	10 µM	0.5
cDNA*	NA	1.5
Nuclease free water	NA	7.5
Total	--	20

* cDNA was diluted 1:2 using DEPC treated water

Reaction mixture was prepared on ice. For each gene, total 5 vials of reaction mixture was prepared each for host, vehicle control, 50 µg/mL, 100 µg/mL and 250 µg/mL tests. For each gene, 6x master mix was prepared consists of 2x PowerUP sybr green master mix, Fw primer, Re primer and water, described in Table MM- 20. After preparation of master mix, tube was

vortexed and short spun. After that, 15 µL of master mix was distributed to each labelled well of 96 well plate. To complete the reaction mix, remaining amount of water and cDNA was added to each well. After completing the reaction in 96 well, plate was sealed with 96 well adhesive film. Plate was then short spun at 1200 rpm for 2 min in swinging bucket rotor, then run in QuantStudio real time PCR machine.

Table MM- 20: Master Mix preparation for each reaction

	1x	**6x**
PowerUP Sybr® green master mix (2x)	10 µL	60 µL
Fw primer (10 µM)	0.5 µL	3 µL
Re primer (10 µM)	0.5 µL	3 µL
cDNA	NA	NA
Nuclease free water	4 µL	24 µL
Total	**15 µL**	**90 µL**

In QuantStudio real time PCR machine, following parameters were selected.

- Experiment Type: Comparative C_T ($\Delta\Delta C_T$)
- Chemistry: SYBR® Green Reagents
- Run Mode: Standard
- Endogenous Control: β-Actin

PCR Conditions are mentioned in

Table MM- **21**.

Table MM- 21: 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

2.6 WESTERN BLOT:

2.6.1 SDS-PAGE

Protein samples were removed from 4 °C and thawed to room temperature. 30 µg of protein was transferred to fresh tube. Each sample volume was normalized with autoclaved distilled water by adding remaining volume of water along with 6x sample loading dye to make up final volume of 30 µL. All the samples were properly mixed and vortexed. Then samples were boiled for 10 min in boiling water bath. After that each sample was short spun and all the samples were loaded in well of 4 -20 % glycine gradient gel (XP04205BOX, Thermo, USA) along with pre-stained protein ladder. Samples were run at 25 mA constant current for ~45 mins.

2.6.2 TRANSFER AND BLOT DEVELOPMENT

Once run is completed, gel plates were gently opened and gel was transferred to water. Gel was washed with distilled water twice. With parallel to this, transfer buffer was diluted to 1x from 20x stock solution using distilled water and 20% methanol was added to prepare final 1x transfer solution (20x stock solution: 0.5 M Tris-Cl, 3.8 M glycine pH 7.6). Gel was washed with 1x transfer buffer and it was kept in the same. After cutting nitrocellulose membrane of approx. size of gel, it was socked in 1x transfer buffer. Then after, gel was gently positioned above the nitrocellulose membrane. It was

ensured that gel doesn't break and no air bubble gets entrapped between gel and membrane. Both, gel as well as membrane, were covered with 2 equal sized Whatman filter paper-I on each side which was covered with 2 sponges on each side. This whole set up was put inside the Biorad Mini Trans-Blot® Cell system in such a way that gel faces anode and membrane faces cathode of system. Tank was filled with 1x transfer buffer in the presence of ice pack and run was started at 200 mA of constant current for 2 hrs.

After completion of transfer, blot was carefully separated from gel and immediately dipped in blocking solution and followed below protocol.

Required buffers and solutions:

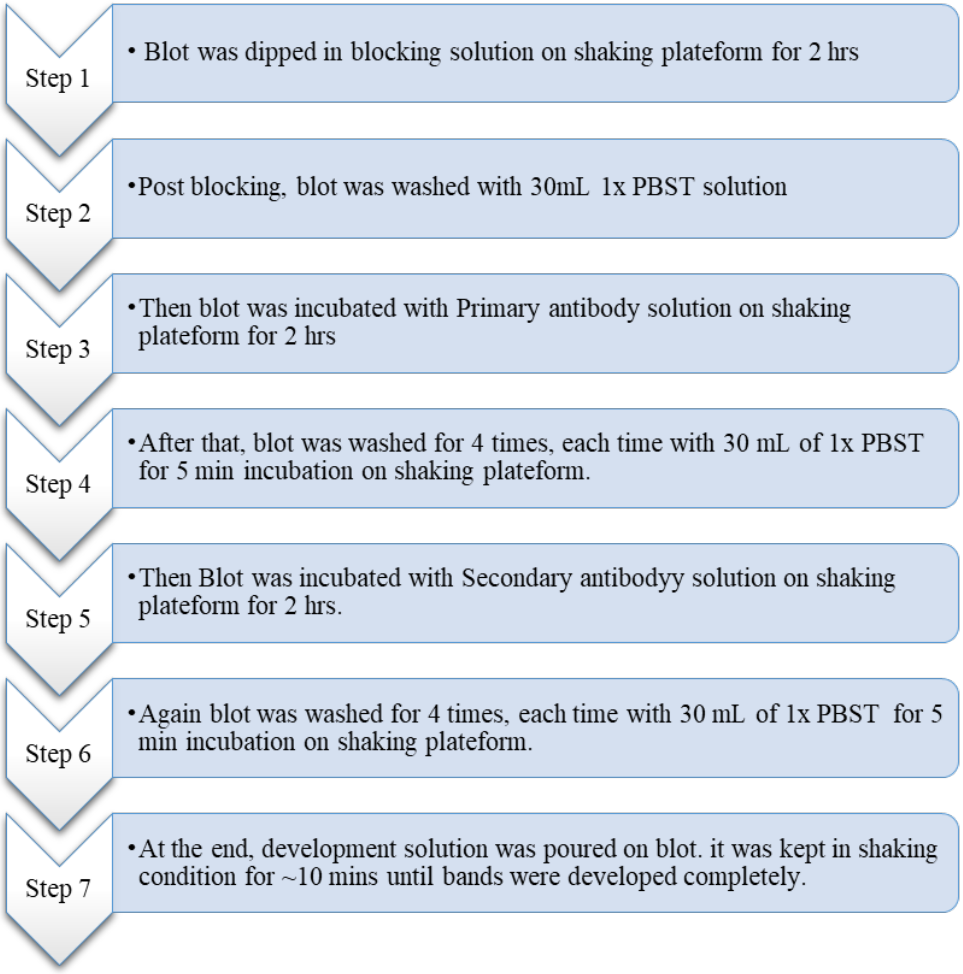
1. **1x PBS:**
   5 tablets of PBS (Sigma, USA) were completely dissolved in 950 mL of distilled water. Solution was then made up 1000 mL.
2. **1x PBST (1x PBS + 0.1% T20):**
   600 mL of PBS was aliquoted in a fresh beaker. 0.6 mL of Tween-20 (Sigma, USA) was added in aliquoted PBS, solution was mixed for 15 min to allow completer dissolution of Tween-20.
3. **Blocking solution (5% Skim milk in PBS):**
   100 mL of PBS was aliquoted in fresh beaker. 5 g of skim milk powder was added and dissolved in aliquoted PBS.
4. **Primary antibody solution:**
   15 mL of 5% skim milk was diluted 1:2 using distilled water to achieve 30 mL of 2.5% skim milk solution. 6 µL of anti-Egr-2 (AB108399, abcam, UK) antibody and 6 µL of anti-β-actin antibody (A1978, Sigma, USA) was added in to the solution to achieve 1:10000 dilution respectively. Solution was mixed gently.
5. **Secondary antibody solution:**
   15 mL of 5% skim milk was diluted 1:2 using distilled water to achieve 30 mL of 2.5% skim milk solution. 0.5 µL of anti-rabbit HRP conjugate (A0545, Sigma, USA) antibody and 1 µL of anti-mouse HRP conjugate antibody (AP127P, Sigma, USA) was added in to the

solution to achieve 1:60000 and 1:30000 dilution respectively. Solution was mixed gently.

6. **TMB development solution:**

   Development solution was prepared as per instruction in TMB development kit (SK-4400, Vector Laboratories, USA).

2.6.2.1 PROTOCOL OF WESTERN BLOT DEVELOPMENT:



2.7 FLOW CYTOMETRY

2.7.1 LG TREATMENT

LG treatment was set up in T25 cell culture flask scale. Saos-2 cells of P#5, were seeded in T25 flasks at seeding density of 0.5 million cells/5 mL/T25 flask. Total 4 such flasks were seeded and then incubated at 37 °C and 5% CO_2 in static incubator with 30 – 80 % humidified environment for 24 hrs. Next day, dilutions were prepared of LG methanolic extract in media to achieve desired concentration. Dilutions were prepared as mentioned in Table MM- 22.

Table MM- 22: Dilution flow for treatment

Dilution for Treatment				
	Final conc (µg/mL)	**Stock conc (µg/mL)**	**Volume (µL)**	**Diluent (µL)**
Main stock	-	250000	-	-
Working stock - 1	2500	250000	30	2970
Working solution	50	2500	200	9800
	100		400	9600
	250		1000	9000

Post 24 hr incubation, spent media was removed and it was replaced with 5 mL of media + LG extract. Flasks were again incubated at 37 °C and 5% CO_2 in static incubator with 30 – 80 % humidified environment for 48 hrs. On Day2, last step of replacing spent media with media + LG was repeated and flasks were incubated at same conditions for 48 hrs.

Post 96 hr incubation, cells were observed at microscope and images were recorded of all the flasks. Cells were then washed with 1x PBS. After removal of 1x PBS, 0.25 mL of trypsin was added to each flask and flasks were incubated at 37 °C for 2-5 min with microscopic observation for cell detachment. Once all the cells were detached and gained round shape, 1 mL media (Maccoy's 5A) was added to each flasks and all the cells were

harvested. Cells were then centrifuged at 1000 rpm/10 min in swinging bucket rotor. Supernatant was discarded and pellet was washed with 1x PBS solution.

For staining, Calcein AM (C3099, Thermo, USA) and PI (P3566, Thermo, USA) were used. All the reagents were thawed to room temperature. Calcein AM was diluted in DMSO to make 50 µM working solution. Live and healthy cells were taken as positive control of experiment. To generate negative control, live cells were treated with 0.1% Triton-x-100 detergent for 30 min at room temperature. After that cells were harvested by centrifugation and resuspended in 1x PBS before staining.

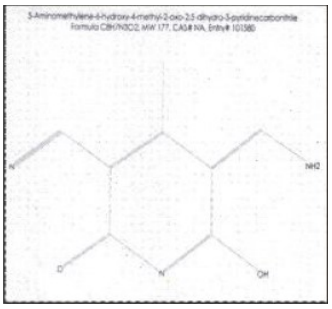
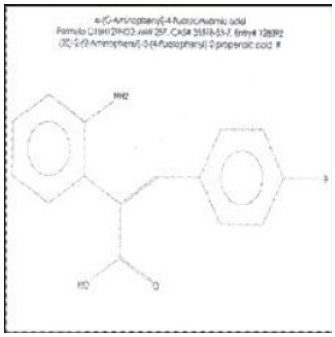
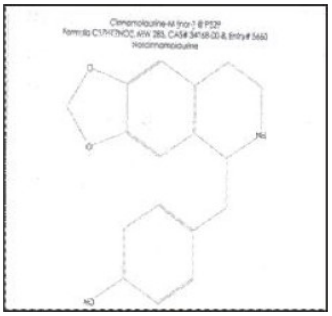
Each test solution was adjusted to have 2.5 million cells in 1 mL solution. 2 µL of calcein AM dye was added and 4 µL of 2 mM PI. Cells were incubated for 30 minutes at room temperature, protected from light. After 30 min of incubation, cells were recovered by centrifuging at 800 xg for 5 min, and resuspended in 1x PBS. After that cells were analysed by BD FACSVerse using 488 nm excitation and measuring green fluorescence emission for calcein AM (i.e., 530/30 bandpass) and red fluorescence emission for PI (i.e., 617/20 bandpass). By running positive and negative controls, gates were set to exclude debris. Total events set were 10,000.

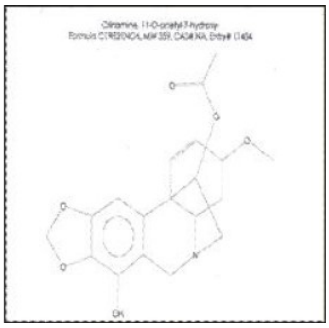
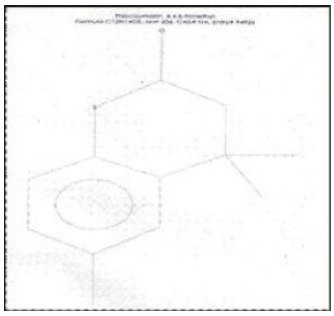
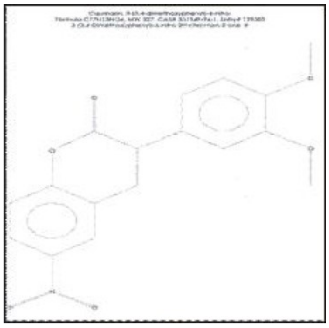
2.8 *IN-SILICO* STUDY

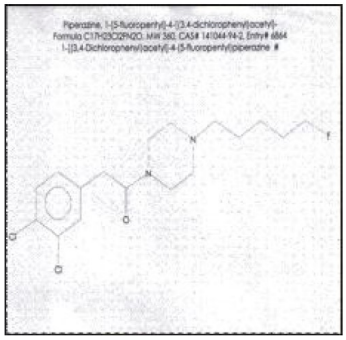
To conduct *in-silico* study for identification of probable molecular targets of phytoactive components of LG, chemical smile formulas were derived from the structure. Parikh and Rangrez 2012 research group has evaluated LG extract and\has identified major phytocomponents along with its structure

Table MM- 23.

Table MM- 23: Structures of Phytocomponents

Sr No	Phytocomponent	Structure (Parikh & Rangrez, 2012)
1	Piperzine carobnitrile	
2	Androstane	
3	Cinnamic Acid	
4	Cinnamolaurine	

5	Crinamine	
6	Gestonorone	
7	Thiocoumarin	
8	Cinnamon	

9	Piperzine	

These structures were utilized to generate respective SMILES formula, which were used to initiate target predictions. SMILES formulas were generated using pubchem NCBI online software (https://pubchem.ncbi.nlm.nih.gov/edit3/ index.html) which are listed in Table MM- 24.

Table MM- 24: SMILES formula of Phytocomponents

Sr No	Phytocomponent	SMILES Formula
1	Piperzine carobnitrile	NCC1C(O)NC(=O)C(C1C)C=N
2	Androstane	C1CC(=O)CC2CCC3C4CCC(=O)C4(C)CC(=O)C3C12C
3	Cinnamic Acid	Nc1ccccc1CC(Cc1ccccc1)C(=O)O
4	Cinnamolaurine	CN1CCc2cc3OCOc3cc2C1Cc1ccc(O)cc1
5	Crinamine	COC1C=CC23C(C1)N(CC2O)Cc1c3cc2OCOc2c1
6	Gestonorone	CC(=O)C1(O)CCC2C3CCC4=CC(=O)CCC4C3CCC12C
7	Thiocoumarin	Cc1ccc2c(c1)CC(=O)CC2(C)C
8	Cinnamon	O=C1Oc2ccc([N+](=O)[O-])cc2CC1c1ccc(O)c(O)c1
9	Piperzine	CCCCCCN1CCN(C(=O)Cc2ccc([O-])c([O-])c2)CC1

These derived SMILES formula were utilized for target prediction. These formulae were imported in the online SwissTargetPrediction network database (http://www.swisstargetprediction.ch/) to identify possible target proteins using *Homo sapiens* species. All the molecules showed binding probability with different human proteins which was represented in the range of 0 to 1.

The phytocomponents which had shown more than 0.35 probability with human target proteins, were selected for further studies. Such phytocomponents include Androstane, Gestonorone and Cinnamolaurine. Rest of 6 molecule showed very less probability with human proteins, therefore they were not considered for further studies. Target proteins of these three selected phytocomponents, were sorted as per high to low probability and top 3 targets of each were selected for in-vitro gene expression studies after LG treatment. For in-vitro gene expression studies, LG treatment to Saos-2 cell line, RNA isolation & cDNA conversion and its quantitative analysis were followed as per section 2.3, section 2.4 and section 2.5 respectively.