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ANTI-INFLAMMATORY, ANTI-CANDIDAL ACTIVITY AND *IN SILICO* PREDICTION OF PHARMACOKINETIC PROPERTIES OF *NARDOSTACHYS JATAMANSI*

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ABSTRACT: The earlier reports on *Nardostachys jatamansi* show contain a various class of chemical constituents that have influenced the anti-inflammatory and anti-candidal activities. The present study reports the evaluation of anti-inflammatory and anti-candidal activities of *N. jatamansi* and also studies pharmacokinetic properties using *in-silico* model. In ongoing research work, we used individual extracts of dichloromethane, ethyl acetate, methanol, and water were prepared. The anti-inflammatory activity of different solvent extracts was carried out using gelatin zymography through detection of MMP-2 and MMP-9 and on other side anti-candidal activity was carried out using Nitro blue tetrazolium assay (NBT). Bioactivity and pharmacokinetic parameters of all extracts were calculated using the property calculation online toolkit. From the results, it is observed that, among all the tested extracts, ethyl acetate extract was found to be more potent towards phagocytosis (more than 5 MPN) and Candida albicans (41%) at 100 µg/mL while dichloromethane extract possesses profound anti-inflammatory activity towards MMP-2 (90%) and MMP-9 (85%) as compared to standardtetracyline HCl (positive control) followed by other extracts. Desoxo-Narchinol A, Narchinol B, Selinidin/ *Jatamansin*, *Nardosinone* and Valerenal were chosen as representative compounds of *N. Jatamansi* for the prediction of pharmacokinetic parameters, *in-silico* and bioactivity scores. All the compounds were showed results in line with Lipinski Rule of 5 and best acted as enzyme inhibitors with a score greater than 0.00. In light of the above result findings, *N. jatamansi was* found to possess anti-inflammatory and anti-candidal activities. This suggests extensive investigation of chemical components present in the active extracts to establish a structure-based activity relationship.

INTRODUCTION: *Nardostachys jatamansi* (family Valerianaceae) is an important medicinal plant found in Himalayas [1, 3].

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It is a well-known Ayurvedic herb, and it has demonstrated hypotensive, hypolipidemic, anti-microbial, antiarrhythmic, hepatoprotective, *etc*. Ethanol extract of *N. Jatamansi* DC roots was studied for anticonvulsant activity [4] and it demonstrated a significant elevation in the seizure threshold against maximal electroshock seizure (MES) model [5, 7]. Chemical examination of the same plant revealed the presence of terpenoid ester, nardostachysin N, volatile, non-volatile constituents, sesquiterpenes coumarins, lignans,

neolignans and alkaloids [8, 9]. Oleanolic acid, β-sitosterol, Nardosinonediol, Acaciin, Nardosinone, Ursolic acid, Octacosanol, Kanshone A, and Aristolen-9-β-ol, are chemical constituents isolated from this plant [10, 12]. The Discovery of the angiotensin-converting enzyme (ACE) leads to the drug discovery of antihypertensive drugs. The ACE converts angiotensin decapeptide inactive form into active octapeptide angiotensin II in the kidneys, especially in the renin-angiotensin-aldosterone system [13]. Captopril, Enalapril, Zofenopril, Ramipril, Fosinopril, Lisinopril, and SQ 29852 are the well-known ACE inhibitors [14]. Generally, Captopril is used as a positive control in most of the research studies owing to its free radical scavenger activity that is highly relevant as an ACE inhibitor [13]. Considering the above facts, the ongoing research aimed to conduct phytochemical screening and to examine the anti-hypertensive constituents present in rhizomes of *N. jatamansi*.

MATERIALS AND METHODS:

Plant Material: *N. jatamansi* rhizomes were collected in the month of June 2016 from High Altitude Plant Physiology Research Centre, Srinagar [Gharwal] Uttarkhand, India. Rhizome was authenticated by comparing with the Herbarium (No. 2016/52) maintained at department of Botany J.S.S College Belagavi, Karnataka, India. The authenticated plant material was sprayed with alcohol to arrest the biochemical reactions. Further, it was shade dried to arrest any possible photochemical reactions. Then, it was finally powdered and stored for further research work [14].

Extraction: The rhizomes were coarsely powdered in a pulverize with sieve # at K.L.E Ayurveda Pharmacy college, Shahapur, Belagavi, Karnataka, India. Then, 100 g of each powdered material was subjected to Soxhlet extraction by using 400 mL of different solvents *viz*. dichloromethane, ethyl acetate, methanol, and water individually as described earlier [14]. The extracted solution was dried individually using a rotary vacuum evaporator and preserved in different amber color bottles [14, 16].

Phytochemical Screening: Phytochemical screening was carried out to investigate the various phytochemicals present in *N. jatamansi* rhizomes [14]. For instance, for the determination of alkaloids, Mayer's test, Hager's and Dragendroff's tests were performed. A Ferric chloride test was followed to identify tannins and phenolics. For the presence of glycosides, a Legal test was performed. An alkaline test was implemented for the presence of flavonoids. Ninhydrin and Biuret tests were followed to detect proteins. Steroids were analyzed through the Salkowaski test. To test the presence of carbohydrates, Biuretic and Fehling's test was employed as described in earlier literature reports [14].

Fractionation and Purification: Ethyl acetate fractional extract was purified over silica gel column chromatography (60-120 mesh) under gradient elution of hexane and ethyl acetate (500 mL each) and methanol (500 mL) to provide dried fractions: Fr.1 (10% ethyl acetate in hexane); Fr. 2 (25% ethyl acetate in hexane); Fr. 3 (50% ethyl acetate in hexane); Fr. 4 (75% ethyl acetate in hexane); Fr. 5 (100 % ethyl acetate) and Fr. 6 (100 % methanol) as described earlier [14].

Neutrophil Function Assay: Neutrophil function assay was carried out at Maratha Mandal's Central Research Laboratory, Belagavi, Karnataka, India. Blood sample was collected either with an anticoagulant (heparin or EDTA) from granulocytes or white blood cells. A 3% solution of gelatin or high molecular weight dextran in saline was recommended, which gives relatively pure population of WBCs with minimal contamination. Ficoll- Hypaque method was used to collect granulocytes. Blood sample was diluted with saline, and an equal amount of 3% gelatin or dextran was added to separate WBCs.

For settling down RBCs, tubes were kept upright for about 45 min. The supernatant was collected, centrifuged, and washed with saline. WBCs were counted in Neubauer's chamber, diluted with Hanks balanced salt. The EDTA blood was mixed with equal quantities of MEM (minimum essential medium) and 6% dextran solution of molecular weight of 150,000 D. The tube was kept upright without disturbing for about 45 min. RBCs settle down at the bottom, whereas the WBCs and the plasma would be in the upper layer. This upper layer was collected in a centrifuge tube and spun for about 10 min at 3000 rpm. The deposit containing the cells was washed thrice with

phosphate-buffered saline, and the cell concentration was adjusted to 1000000 /mL with MEM [15].

Nitroblue Tetrazolium (NBT) Assay: The nitroblue tetrazolium (NBT) test is a qualitative assay used to calculate reactive oxygen species production. The stepwise procedure involves 0.5 ml of anti-coagulated blood, 200 µl of 0.15% NBT and 0.2 ml of Hanks balanced salt solution (HBSS) were added. Another set of tubes with the above-mentioned reagents and in addition 0.05 ml of endotoxin C prepared from E. coli. was taken and incubated for 20 min at 37 ℃. A thin film of smear on glass slide stain with Giemsa was prepared and studied under an oil immersion field of the microscope. For the tube containing endotoxin, more than 80% of phagocyte cells should reduce NBT and demonstrate bluish-black granules in the cytoplasm. For the first set of tubes without endotoxin, the percentage of NBT reducing cells should not be more than 20% in a healthy individual [15].

Phagocytosis: This process involves 0.25 ml of HBSS, 0.25 ml of leukocyte suspension, and 0.25 ml of heat-killed C. albicans were added to three sets of tubes. 0.25 ml of patient's serum was added to one set of tubes. Pooled human serum from healthy individuals was taken to the second set. Serum was not added to the third set. Serum was mixed properly and incubated at 37 ℃ for 30 min. The contents were centrifuged, and smears were prepared from the deposit count at least 100 phagocytes, and the number of Candida ingested per cell were counted and expressed as mean particle number (MPN) [16].

Candidacidal Assay: Essentially, the basic procedure for anti-candidacidal activity is same as for the procedure described in phagocytosis. Only the change is, dead candida was replaced with a suspension of live candida cells, mixed the suspension thoroughly, and incubated for a period of 1 h. After the incubation period, the solution of 2.5% sodium deoxycholate was added to each tube and mixed adequately, resulting in lyses of leukocytes without damaging candida cells. Later, 4ml of 0.01% methylene blue was added carefully to each tube, mixed, and centrifuged the resultant mixture for 10 min. The deposit was resuspended in 0.5 ml of the material a film was prepared and counted the percentage of dead cells, *i.e.*, those which have taken up methylene blue and appeared blue in color [16].

Chemotaxes: For the chemotaxes analysis, 1.2% agarose gels containing minimum eagle's medium was added to pooled human serum and solution of sodium bicarbonate; after the gels were set, the series of three wells with 3 mm in diameter and 3 mm apart were cut. FMLP, a known chemoattractant, was added to the central well, whereas the test samples were added to the peripheral wells and incubated for about 2 h. The plates were fixed in methanol followed by formalin. The Agarose layer was removed and cells stained with Giemsa stain. The distance the cells have traveled from the edge of the peripheral wells towards wells containing FMLP was calculated [17].

TABLE 1(A): PREPARATION OF 10% RESOLVING GEL (10ML)

Reagents	Volume
Acryl amide – Bis-acrylamide	3.3ml
Resolving gel buffer stock	1.25ml
10% SDS	100µl
1.5% APS	500 µl
Gelatin	1ml
Water	3.8ml
TEMED	10µl

TABLE 1(B): PREPARATION OF 5% STACKING GEL (10ML)

Reagents	Volume
Acrylamide – Bisacrylamide	1.7ml
Stacking gel buffer	1.12ml
10% SDS	100µl
1.5% APS	500µl
Water	6.5ml
TEMED	10µl

Anti-Inflammatory Activity Using Gelatin Zymography (Detection of MMP-2 and MMP-9): Experiments for anti-inflammatory effects were carried out at Maratha Mandal's Central Research Laboratory, Belagavi, Karnataka, India [18]. Briefly, the electrophoresis apparatus cleaned using warm water, whereas glass plates were cleaned in methanol. Plates were set up. Large plate, then two spacers and small plate on top and were assembled into the clamp. Agarose gel was heated and poured between the two glass plates to seal the bottom surface and then left for 5, 10 min until gel cools down. Resolving gel mixed appropriately and

poured between the glass plates eradicating bubbles. Plates were filled 80% stacking gel and comb, overlaid with a small amount of water to achieve a completely flat interface between resolving gel and stacking gel and allowed to set for about 45 min [18]. Setting the resolving gel **Table 1,** the excess water was poured off.

The stacking gel was poured off and allowed to set for about 30 min. Post setting the stacking gel; wells washed and assembled the gel apparatus.

Preparation of MMP Samples: The tissue sample was chopped completely, 5 ml of Tris buffer was added and centrifuged at 3000 rpm for 15 min [18]. 50 µl of MMP samples added to 50 µl of the extract/compound (the sample given for study), incubated for 1 h. 50 µl of MMP sample for negative control; 50 µl of MMP sample + 50 µl of Tetracycline HCl (store for 1 h) for positive control. 20 µl of the sample was loaded in each well. Run was carried out at about 50V for 15 min and then 100V until the bromophenol blue reaches the bottom of the plates. After electrophoresis, the apparatus was dissembled and the gel was removed gently and kept into a plastic dish, and gel washed with zymogram renaturing buffer *i.e.*, 2.5% Triton x-100 for 1 h [18].

Staining Coomassie blue R-250 was used for staining for one hour followed by de-staining with Coomassie R-250 de-staining solution for about 2 h. After staining, the background stains blue with Coomassie stain. The lower bands were gelatinases-A (MMP-2) ~72KD, while the upper bands are gelatinases-B (MMP-9) ~ 95KD [18].

In-silico determination of pharmacokinetic properties of compounds Mol inspiration online property calculation toolkit was used to determine Polar surface area (TPSA), molecular volume, number of a hydrogen donor, milog P, number of rotatable bonds, and acceptor atoms and violations of Lipinski's rule of Five [19, 20].

RESULTS AND DISCUSSION: Preliminary phytochemical analysis showed that the occurrence of bioactive compounds such as alkaloids, flavonoids, carbohydrates, triterpenoid, glycosides, saponin and tannins in different extracts.

TABLE 2: NEUTROPHIL FUNCTION ASSAY OF EXTRACTS OF *N. JATAMANSI* RHIZOME

Extracts	NBT (%)	PHAGOCYTOSIS (MPN)	CANDIDACIDAL ASSAY (%)	CHEMOTAXIS (mm)
Dichloromethane Extract				
100 µg/mL	61%	5	40%	2.2
50 µg/mL	53%	3	40%	1.0
25 µg/mL	21%	2	32%	0.6
12.5 µg/mL	17%	2	16%	0.6
5 µg/mL	16%	2	16%	0.6
Ethyl acetate Extract				
100 µg/mL	89%	5+	40%	2.1
50 µg/mL	62%	4	37%	1.6
25 µg/mL	25%	4	24%	1.0
12.5 µg/mL	25%	4	19%	0.5
5 µg/mL	14%	2	15%	0.5
Methanol Extract				
100 µg/mL	63%	3	22%	1.4
50 µg/mL	52%	3	20%	1.0
25 µg/mL	24%	2	16%	0.6
12.5 µg/mL	19%	2	16%	0.6
5 µg/mL	17%	2	16%	0.6
Water Extract				
100 µg/mL	27%	2	24%	2.4
50 µg/mL	22%	2	21%	2.3
25 µg/mL	15%	2	19%	2.0
12.5 µg/mL	16%	2	15%	2.0
5 µg/mL	15%	2	15%	1.8
Control				
Negative Control	20%	2MPN	16%	0.5mm

It is evident from neutrophil function assay **Table 2.** That among all the tested extracts, ethyl acetate extract was found to be more potent towards phagocytosis (more than 5 MPN) and Candida albicans (41%) at 100 µg/mL **Table 3.** shows that among all the tested extracts, dichloromethane extract possesses profound anti-inflammatory activity towards MMP-2 (90 %) and MMP-9 (85%) as compared to tetracycline HCl (positive control) followed by other extracts.

TABLE 3: ANTI-INFLAMMATORY ACTIVITY OF EXTRACTS OF *N. JATAMANSI* RHIZOME

S. no	Test samples	MMP-2(%)	MMP-9(%)
1	Dichloromethane Extract	90	85
2	Ethyl acetate Extract	85	75
3	Methanol Extract	70	68
4	Water Extract	88	76
5	Tetracyline HCl	100	98
6	Negative control	10	Nil

Desoxo-Narchinol A, Narchinol B, Selinidin/ Jatamansin, Nardosinone and Valerenal were chosen as representative compounds of *N. Jatamansi* for prediction of pharmacokinetic parameters in silico and bioactivity prediction **Table 4a** & **4b.** For prediction of ADME properties, such as absorption (% ABS), polar surface area (TPSA), miLog P, number of rotatable bonds and violations of Lipinski's rule of five, Mol inspiration online property calculation toolkit was considered. Topological polar surface area (TPSA), allows prediction of transport properties of drugs in the intestines and blood-brain barrier crossing. The percentage of absorption (% ABS) was calculated by % ABS = 109-(0.345 × TPSA) [21, 24]. From all these parameters, it can be observed that all compounds exhibited a great % ABS ranging from 86.57 to 92.34%. All of the compounds generally possessed all desired parameters for good oral bioavailability and followed Lipinski Rule of five.

TABLE 4(A): PHARMACOKINETIC FILTERS FOR *N. JATAMANSI* COMPOUNDS

Compounds	miLogP[a]	TPSA[b]	*n*atoms	MW	*n*ON	*n*OHNH	*n*violation	*n*rot	volume
Desoxo-Narchinol A	1.68	37.30	14	192.26	2	1	0	0	189.67
Narchinol B	0.52	57.53	15	208.26	3	2	0	0	197.72
Selinidin/Jatamansin	4.12	65.72	24	328.36	5	0	0	3	298.49
Nardosinone	3.04	35.54	18	250.34	3	0	0	0	244.62
Valerenal	4.50	17.07	16	218.34	1	0	0	2	232.14

In general, any compound with a bioactivity score of more than 0.00 is most likely to exhibit certain pharmacological activities, while values −0.50 to 0.00 are considered as moderately active and if score is less than −0.50, totally inactive **Table 4b.** All compounds best acted as enzyme inhibitors with a score greater than 0.00. Except for desoxo-Narchinol A, the remaining compounds were found to exhibit a good bioactivity score (0.02-0.17) over nuclear ligand-receptor. However, Desoxo-Narchinol A, Narchinol B, Selinidin/ Jatamansin, Nardosinone, and Valerenal were found to be moderately active over another ligand-receptor. It can be postulated that all these compounds have drug-likeness features.

TABLE 4(B): BIOACTIVITY SCORE FOR DRUG-LIKENESS FOR *N. JATAMANSI* COMPOUNDS

Compounds	GPCR Ligand	Ion channel Modulator	Kinase inhibitor	Nuclear receptor Ligand	Protease inhibitor	Enzyme inhibitor
Desoxo-Narchinol A	-0.32	-0.20	-1.18	-0.01	-0.55	0.36
Narchinol B	-0.10	0.11	-1.06	0.17	-0.33	0.60
Selinidin/Jatamansin	-0.27	-0.28	-0.71	0.36	-0.39	0.25
Nardosinone	-0.19	-0.18	-0.85	0.04	-0.24	0.25
Valerenal	-0.40	-0.23	-0.85	0.02	-0.28	0.08

CONCLUSION: In light of the above result findings, *N. jatamansi* was found to exhibit promising anti-inflammatory and anti-candidal activities. This shows the extensive investigation of chemical components presents in the active extracts to establish a structure-based activity relationship.

The present study indicates the usage of this plant for inflammatory-related disorders. Further, ethyl acetate extract can be further investigated for the identification of potent molecules that work in a synergistic combination.

CONFLICTS OF INTEREST: The authors declare no competing financial interest exists.

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