

Evaluation of Anti Arthritic Potential of *Gloriosa superba* (L.) Elite Germplasm Collected from Eastern Himalayas, India

Ankita Misra^{1,2}, Akanksha Srivastava¹, Mohammad Khalid², Poonam Kushwaha², Sharad Srivastava^{1*}

Ankita Misra^{1,2}, Akanksha Srivastava¹, Mohammad Khalid², Poonam Kushwaha², Sharad Srivastava^{1*}

¹Pharmacognosy and Ethnopharmacology Division, CSIR-National Botanical Research Institute, Lucknow, Uttar Pradesh 226001, INDIA.

²Faculty of Pharmacy, Integral University, Lucknow, Uttar Pradesh 226001, INDIA.

Correspondence

Dr. Sharad Srivastava

Pharmacognosy and Ethnopharmacology Division, CSIR-National Botanical Research Institute, Lucknow, Uttar Pradesh 226001, INDIA.

Phone No: 91-522-2297818

E-mail: sharad_ks2003@yahoo.com

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ABSTRACT

Introduction: *Gloriosa superba* (L.) is a traditionally known medicinal plant for its potential anti-gout property. The species is rich source of colchicine alkaloid and is commercially exploited in the international market for the same. **Method:** In the present study, elite chemotype of *G. superba* was identified from natural population in Eastern Himalayas based on their colchicine content through HPTLC calibrated method. The selected elite chemotypes were further evaluated for *in vitro* anti-arthritic potential via inhibition of protein denaturation along with hydroxyl radical scavenging potential. **Result:** The HPTLC quantification data reveals that the content of colchicine varies from 0.044 to 0.184% having maximum content in NBG-128 from Jorhat, Assam. The results of bioassay reflect a potentiating anti-arthritic and hydroxyl radical scavenging with statistically insignificant difference within the elite germplasms. **Conclusion:** The presence of bioactive polyphenolics with significant hydroxyl radical scavenging will further suggest that inhibition of inflammatory mediator cells by extract is superimposed action of colchicine and other chemical inhibitors like polyphenolics. The study will aid in site specific exploration of high metabolite yielding chemotype(s) with validated pharmacological action for commercial cultivation to meet out the industrial demand of colchicine and herbal product development.

Key words: *G. superba*, Colchicine, Elite chemotype, HPTLC, Anti arthritic.

INTRODUCTION

Gloriosa superba L. (Colchicaceae) is a tuberous climbing herb found throughout tropical parts of India up to an altitude of 1800 m and also distributed in other tropical parts of the World. Indigenously *Gloriosa* is known as 'kalihari' in Hindi, 'Agnishikha' in Sanskrit and its trade name is "Glory lily".¹ The species exhibits various medicinal properties *viz.* analgesic, anti-inflammatory, antimicrobial, larvicidal, antipoxviral, antithrombotic, antitumor and, also used in treatment of snake bite, gout, respiratory disorders.^{2,3,4}

Gloriosa superba is a potential source of phenethylisoquinoline category of alkaloids, among which colchicine is the major metabolite for which *Gloriosa* is industrially exploited from time long and hence became a threatened species. Other chemical constituents in tubers are lumicolchicine, 2-demethylcolchicine, 3-demethylcolchicine, N-formyl-N-deacetyl-colchicine, benzoic acid, salicylic acid, sterols, resinous substances, colchicoside, gloriosine, tannins and superbine.^{5,6}

The chemotaxonomic studies of *G. superba* were scarcely documented.^{7,8,9} We had reported the variations of colchicine content in *G. superba* L., germplasms collected from Central India using OPLC technique.¹⁰ The use of colchicine in gout was well established by several biochemical and clinical studies,^{11,12,13,14,15} however

scientific validation of *G. superba* for its traditional claim in gouty arthritis is still very less.¹⁶ Thus, looking towards the commercial relevance of colchicine, the study was aimed to record the intra-specie variation in colchicine content of *G. superba* population from Eastern Himalayas. This was further extended to validate the pharmacological potential *i.e.* anti-arthritic activity of elite germplasm selected from the population. In addition, antioxidant activity of elite germplasms was also established. This will aid in site specific exploration of high colchicine yielding chemotype(s) with validated pharmacological action to meet out the medicinal and commercial demands.

MATERIALS AND METHODS

Chemicals and reagents

Colchicine (99.8% w/w), Bovine Serum Albumin (BSA flakes, >99.8 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents, *viz.* chloroform, acetone, and diethyl amine of analytical grade, are obtained from Merck, Mumbai (India). Solvents were filtered (0.45 mm filter, Millipore, Bedford, MA, USA) and sonicated for 15 min before use. HPTLC (20 cm × 20 cm), precoated silica gel alumi-

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num plates 60 F₂₅₄ (0.25 mm) were purchased from Merck (Darmstadt, Germany).

Plant material and extraction protocol

The tubers were collected during the month of September from North-Eastern Himalayan region covering Sikkim and the adjoining area of West Bengal. Five samples were collected from varied condition(s); specimens were authenticated and deposited in repository of CSIR-NBRI with individual voucher number. GPS information of the collected samples is recorded and summarized in Table 1.

The samples were washed with water, chopped, and shade dried. The dried tubers were coarsely powdered by passing through 40 mesh sieves (up to 500 mm), and the powdered sample (about 5 g) is defatted using petroleum ether by maceration method (cold) to remove the fatty materials/impurity. The defatted material was then macerated (cold method) with methanol (25 ml) for 24 h at room temperature (25°C ± 2°C) for 3 consecutive days. Extraction was repeated thrice, filtered (Whatman no. 4), and the pooled filtrate was dried in a rotatory evaporator (Buchi, USA) under standard conditions of temperature (55°C ± 2°C) and pressure (40 mbar). The concentrated extract was finally lyophilized to solid residue (Labconco, USA).

HPTLC quantification of bioactive metabolite colchicine

The collected samples of *G. superba* were quantified for bioactive alkaloid(s), colchicine through HPTLC calibrated method as per ICH guidelines (2005).¹⁷ Quantified markers are expressed as % dry weight of crude samples.

In vitro anti-oxidant activity

Estimation of total phenolics

Assay of total phenolic¹⁸ (TPC) and total flavonoid¹⁹ (TFC) content was quantified by spectroscopic method as per standard protocol based on regression curve of reference standard. Total phenolic and flavonoid content was expressed in mg/g of gallic acid equivalent (GAE) and quercetin equivalent (QE).

Hydroxyl radical scavenging activity (HRSA)

Hydroxyl radical scavenging activity of samples was determined according to the standard method²⁰ with slight modification. The reactive mixture, containing methanolic extract (0.2 mg/ml) in variable concentration (20–100 µl) was incubated with 0.1 ml EDTA (0.1 mM), 0.01 ml of ferric chloride (10 mM), 0.36 ml of deoxy ribose (10 mM), 0.33 ml of phosphate buffer (pH 7.9, 50 mM) and 0.1 ml ascorbic acid (1 mM). The reaction was initiated by addition of 0.1 ml of H₂O₂ (10 mM), followed by incubation at 37°C for 1h. An aliquot of 1.0 ml of the incubated mixture was mixed with 1.0 ml of 10 % trichloro acetic acid and 1.0 ml of 0.5 % thio barbituric acid (in 0.025 M NaOH) to develop the pink chromogen measured at 532 nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxy ribose degradation.

DPPH radical scavenging activity

The radical scavenging activity of extract was measured using DPPH stable radical.²¹ As per standards, the reaction mixture contains methanol extract of sample (0.1 ml) and 2.9 ml of freshly prepared DPPH solution (6 × 10⁻⁵ M). The mixture was then shaken vigorously before incubation for a period of 30 min in dark at 27°C. The decrease in absorbance at 517nm was recorded and the results were expressed in terms of concentration required for 50% inhibition of DPPH radical by test sample as IC₅₀ (mg/ml). Quercetin is used as positive control having similar treatment protocol as mentioned above.

In vitro anti arthritic activity

Inhibition of protein denaturation

Inhibition of protein denaturation by test extract was evaluated by the method with slight modification.^{22,23} 450 µL of Bovine Serum Albumin (5% aq. Solution) was added to 50 µL of test solution (methanol extract, 0.05 mg/ml). The reaction mixture was kept at 37°C for 30 min, followed by heating at 50°C for 10 min. In the resulting solution 2.5 ml of phosphate buffer (pH 6.5) was added and allowed to cool down at room temperature, absorbance was than recorded at 660 nm. Acetyl salicylic acid was taken as a positive control. The experiment was carried out in triplicates and percent inhibition for protein denaturation was calculated using, % Inhibition=100-((A_i-A_c)/A_i)*100. Where A_i is the absorbance of the sample, A_c is the absorbance of the product control.

Statistical analysis

Results were reported as means ± standard deviation (S.D) of three replicates of the same extract. Data were subjected to one-way analysis of variance (ANOVA) to test the level of significance (XLSTAT, 2010, Microsoft Corporation, USA). The cluster analysis was done by PAST 2.15 for the selection of elite chemotypes and clustering of samples.

RESULTS

HPTLC quantification of colchicine

Seven germplasm of *G. superba* (tuber) were collected from the phyto-geographical region of Eastern Himalayas of India, exhibiting the altitudinal variation from 94.48 to 1162.812 meter Table 1. Chemical profiling and method optimization for quantification of colchicine were carried out on 20 cm × 10 cm TLC aluminum precoated plates with 200 nm layer thickness of silica gel 60 F₂₅₄ (SD. Fine-Chem Ltd., Mumbai, India). Tracks (standard and sample) were applied as 6 mm bandwidth using Camag 100 µl sample syringes (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland) under a flow of N₂ gas. The linear ascending development was carried out with chloroform: acetone: diethylamine (5:4:1 v/v/v) as a mobile phase in a Camag glass twin trough chamber.²⁴

After development, the plates were air dried for 30 min, and scanning was performed using Camag TLC Scanner 3 at λ_{max} of 350 nm in UV absorbance-reflectance mode operated by win CATS software (version 3.2.1) for the colchicines content. Quantification was performed using regression analysis of peak area versus concentration of standard marker in the range of 100–400 ng/band having statistically, acceptable regression coefficient (r²) of 0.9987. The slope and intercept of linear line was 14.837 and 417.63 with statistically acceptable LOD and LOQ Table 2. Quantification data (% dry wt. of sample) reveal that the content of colchicine varies from 0.044 to 0.184 % Figure 1, maximum content was observed in NBG-128 from Jorhat, Assam. While minimum content of colchicine was observed in NBG-24 and NBG-25 from 6th mile, Kalimpong, Darjeeling and Kalimpong, West Bengal.

Identification of elite chemotype

Hierarchical cluster analysis was performed to group the samples on basis of colchicine content and altitude in seven samples (Hammer *et al.* 2001) as other biotic and abiotic factors are considered salient within the same phyto-geography of Eastern Himalayas. A UPGMA dendrogram Figure 2 reveals that the whole population was divided into two branches, clustering NBG-23, NBG-24 and NBG-25 into one branch (I), whereas NBG-26, NBG-27, NBG-52 and NBG-128 into other one (II). Branch II was further bifurcated and separating the one germplasm, NBG-128 as elite chemotype having highest content of colchicine. Beside this, NBG-23 and NBG-24 having lowest content of metabolite was also selected with an approach that the pharmacological response may be due

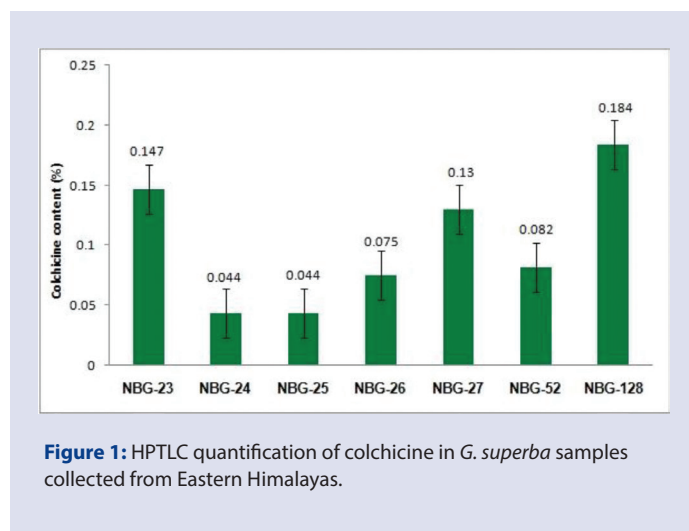
Table 1: GPS information of collected samples of *Gloriosa superba*.

Collection code	Voucher No.	Places/location/ District	Height (feet)	Latitude (N)	Longitude (E)	Soil type
NBG-23	305323	3rd mile Kalimpong/ Darjeeling/W.B	1162.812	27°03'28.72"	88°27'35.47"	Red gravel hilly soil
NBG-24	305324	6th mile, Kalimpong/ Darjeeling/W.B	1162.812	27°03'28.72"	88°27'35.47"	Red gravel hilly soil
NBG-25	305325	Kalimpong/W.B	1162.812	27°03'36.00"	88°28'12.00"	Red gravel hilly soil
NBG-26	305326	Sumbuk/ W. Sikkim	359.664	27°05'54.93"	88°22' 51.81"	Red gravel hilly soil
NBG-27	305327	Jorethang/W. Sikkim	321.25	27°07'55.88"	88°16'53.02"	Red gravel hilly soil
NBG-52	305352	Itanagar, Arunanchal Pradesh	296.87	27°05'12.84"	93°36'31.00"	Red soil
NBG-128	307928	Jorhat, Assam	94.48	26°44'47.47"	94°12'09.31"	Red gravel hilly soil

W.B: West Bengal, AAU: Assam Agriculture University

Table 2: Statistical parameter for linearity calibration of standard colchicine

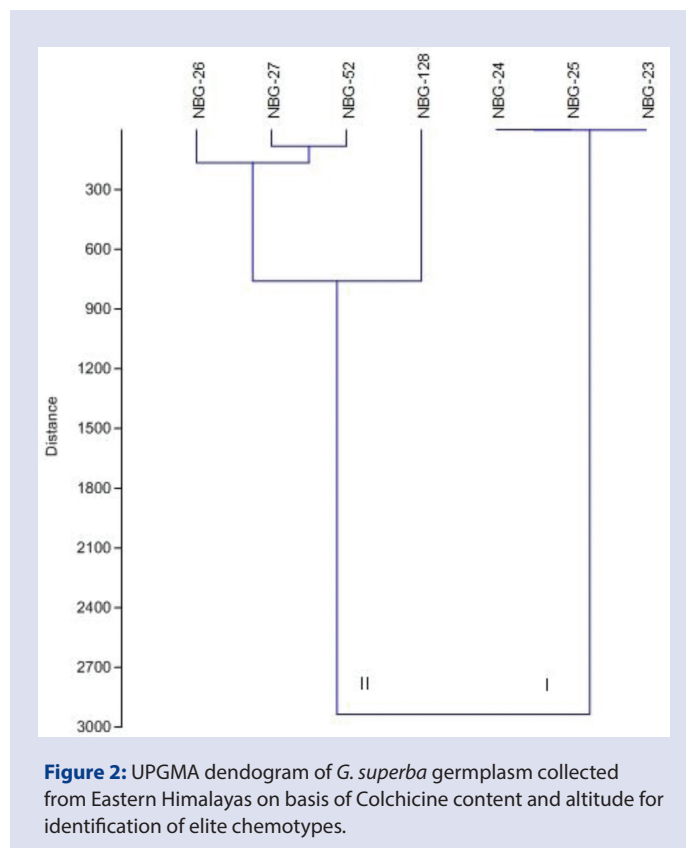
Statistical parameters*	Colchicine
R_f	0.71
r^2	0.9987
Slope	14.837
Intercept	417.63
LOD (ng)	6.245
LOQ (ng)	18.926
RSD (%)	0.715

* n=3, r^2 : regression coefficient**Figure 1:** HPTLC quantification of colchicine in *G. superba* samples collected from Eastern Himalayas.

to synergistic effect and not exclusively due to colchicine content. Thus, three germplasm NBG-24, NBG-25 and NBG-128 are selected for *in vitro* anti-arthritic activity.

Total polyphenolic content

All the seven samples were quantitatively analysed through spectrophotometric method for the estimation total phenolic (TPC) and flavonoid content (TFC) in extract and results reveal that there is significant difference within the samples. The total phenolic content in extract(s) was estimated against calibration curve of standard Gallic acid (0.1 mg/ml) at concentration range of 20 – 100 μ l using regression equation; $y = 95.54x + 0.384$ and coefficient of 0.989. Whereas, total flavonoid content was calculated against calibration curve of quercetin (0.01 mg/ml) at variable dilutions of 0.2 - 0.5 μ l, using regression equation; $y = 74.61x$

**Figure 2:** UPGMA dendrogram of *G. superba* germplasm collected from Eastern Himalayas on basis of Colchicine content and altitude for identification of elite chemotypes.

+ 0.058 and $r^2=0.998$. The TPC ranges from 0.3 to 2.95%, maximum content was in NBG- 25 (Kalimpong, W.B) and minimum was in NBG-52 (Itanagar, Arunanchal Pradesh). The TFC varies from 0.0074 to 0.0335%, maximum was in NBG- 27 (Jorethang, West Sikkim) and minimum in NBG-24 from 6th mile Kalimpong, Darjeeling, West Bengal Figure 3.

A cluster analysis Figure 4 was performed to segregate the population based on TPC and TFC for identification of elite germplasm, UPGMA dendrogram was constructed and it was observed that the population was divided into two branches (I and II). Branch one was further divided to separate NBG-23, NBG-24 and NBG-52 into one group. The other arm (II) of dendrogram contains four germplasm among which NBG-25 was distinctively separated out as elite germplasm with considerably higher content of both TPC and TFC.

Variability in anti-arthritic and antioxidant activity

The selected elite germplasm (based on colchicine and polyphenolic content) *viz.* NBG-24, NBG-25 and NBG-128 were evaluated for anti-

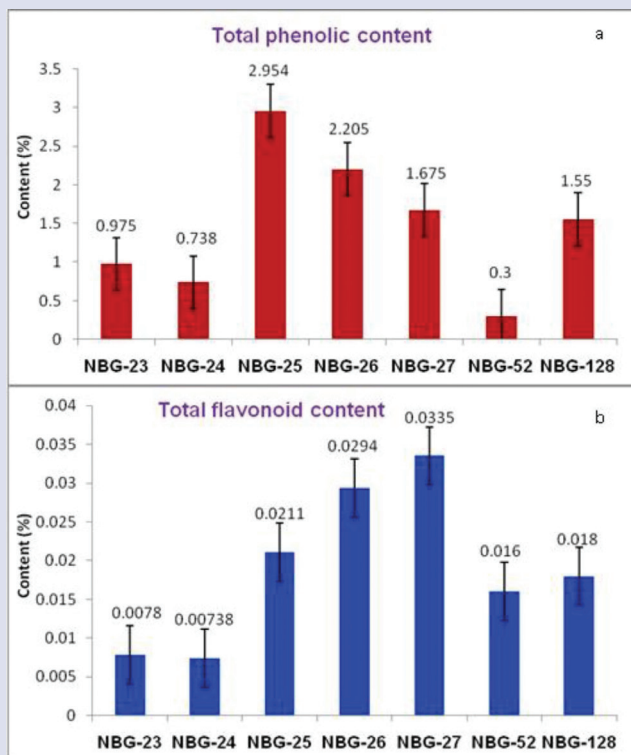


Figure 3: Total phenolic (TPC) and Flavonoid content (TFC) of *G. superba* germplasm.

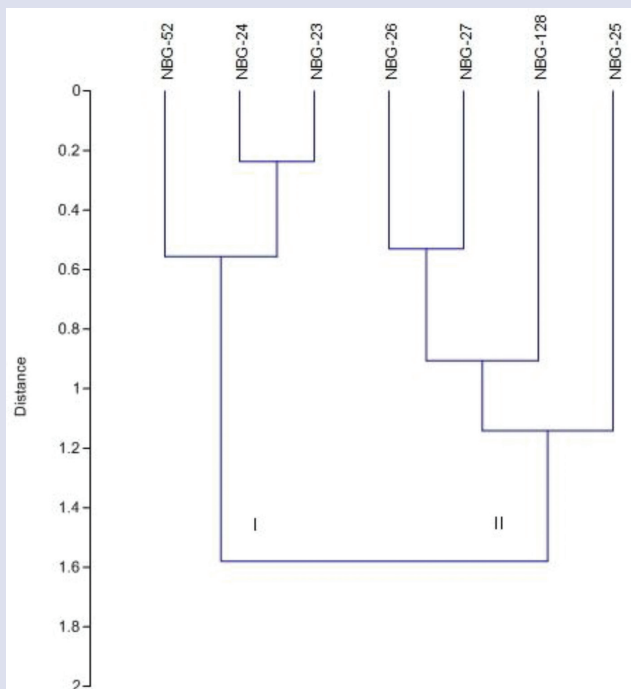


Figure 4: UPGMA dendrogram of *G. superba* germplasm collected from Eastern Himalayas on basis of TPC and TFC.

arthritic potential. In addition to this, radical scavenging potential via DPPH radical scavenging and hydroxyl radical scavenging assay was also checked. The anti-arthritic potential of elite germplasm was analyzed by inhibition of protein denaturation of Bovine serum albumin, with increase in concentration of test extract the denaturation of albumin protein decreases characteristically. At a concentration range of 0.001 to 0.005 mg/ml, the inhibition (%) in NBG-24, NBG-25 and NBG-128 varies from 25.81 to 67.66 %, 23.21 to 60.18 % and 28.33 to 64.46 % respectively with IC_{50} at 0.0031, 0.004, 0.0032 mg/ml respectively. Pure colchicine under the identical *in vitro* experimental condition exhibit an IC_{50} at 0.0048 mg/ml, at a concentration range of 0.001 to 0.005 mg/ml. Aspirin, the positive control of study exhibit the maximum inhibition of 74.64 % at concentration of 200 μ g/ml with regression coefficient of 0.989.

The elite germplasm was found to have promising radical (Hydroxyl) scavenging activity when tested under standardized protocol. The DPPH radical scavenging assay was performed under the mentioned protocol at stock concentration of 2 mg/ml, the IC_{50} of elite germplasm NBG-24, NBG-25 and NBG-128 was at 2.18, 1.62 and 0.531 mg/ml respectively. The inhibition (%) of DPPH free radical by test extract of elite germplasms varies from 1.77 to 8.6 %, 4.08 to 20.18 % and 10.53 to 44.52 % for NBG-24, NBG-25 and NBG-128 respectively. The calibration curve of elite sample was documented in supplementary Figure 1. The Inhibition (%) of quercetin ranges from 12.77 to 72.43 % at a concentration range of 0.002 to 0.026 mg/ml. IC_{50} of quercetin was recorded at 5.95 ± 0.115 μ g/ml. In the other assay, the hydroxyl radical scavenging activity of elite germplasm were evaluated at stock concentration of 0.05 mg/ml, exhibit a statistically significant regression coefficient Supplementary Figure 2. The inhibition (%) of hydroxyl radical in elite sample varies from 25.81 to 67.66%, 23.21 to 60.18 % and 28.33 to 64.46 % for NBG-24, NBG-25 and NBG-128 having IC_{50} at 0.0029, 0.0048 and 0.003 mg/ml respectively. The scavenging activity of quercetin standard (positive control) at a concentration range of 20 - 100 μ g/ml varies from 10.03 to 82.15% with an IC_{50} value of 41.05 μ g/ml.

DISCUSSION

It is well evident that the use of *Gloriosa superba* in gout is due to action of colchicine on several biochemical reactions, majorly on inflammatory mediators. The effect is multifacet and involves at molecular level underlying the cascade of inflammatory action. Specifically, it is attributed due to various proposed actions as disruption of microtubule in neutrophils and inhibiting their migration towards the chemotactic factors, alteration in distribution of adhesion molecules on the surface of both neutrophils and endothelial cells, which significantly inhibit the interaction between WBC and endothelial cells, inhibition of monosodium urate crystal activation of the NLRP3 inflammasome, inhibit the release of IL- 1β and suppression of gene expression.^{12,13,14,15} This however is dependent upon the dose of colchicine and duration of treatment; the effect is at both molecular and genetic level.²⁵ The competitive binding of colchicine to serum albumin was well established²⁶ and thus supports the selection of appropriate method in study to check the existing variability in targeted pharmacological activity.

Quantification data reveals significant variation in the colchicine content of collected samples, among which NBG-128 was statistically selected as the elite chemotype with high colchicine content. However pharmacological response of anti arthritic activity was not significantly varied in selected three elite chemotypes viz. NBG-24, NBG-25 and NBG-128. Moreover, the elite germplasm(s) exhibit more prominent anti-arthritic activity than pure colchicine and thus we presumed that this may be due to synergistic action of other unknown metabolites along with colchicine in extract for such potentiating response towards the inhibition of pro-

tein denaturation. The results of radical scavenging assays were variable but pronounced in elite germplasm. Thus, there may be a possibility that the inhibition of inflammatory mediators will be more accentuated when whole extract of *G. superba* is used in the form of herbal medicine/formulation than pure colchicine, especially in cases of long term treatment considering the toxicity factor of species. As in such cases anti-inflammatory action of colchicine is super imposed with the anti oxidant action of other polyphenolics present in extract.

CONCLUSION

Seven germplasm were collected from different location of Eastern Himalayas up to an altitude of 1162.812 meters. A calibrated HPTLC method was used to quantify colchicine in collected samples, ranging from 0.044 to 0.184% having maximum content in NBG-128 from Jorhat, Assam. While minimum content of colchicine of observed in NBG-24 and NBG-25 from 6th mile, Darjeeling, W.B. and Kalimpong, W.B. Germplasm from Jorhat, Assam (NBG-128) was statistically screened as elite chemotype with highest colchicine content. Three germplasm viz. NBG-24, NBG-25 and NBG-128 with highest and lowest colchicine content was pharmacologically validated. The test extracts reflect potential anti arthritic activity with promising hydroxyl radical scavenging activity in addition with no statistically significant difference within the selected germplasm. The results, thus suggested that the effect of *Gloriosa superba* whole extract on curbing the inflammation causing cells is more pronounced than pure colchicine and which may due to superimposed action of colchicine and, other chemical inhibitors like polyphenolics. Thus, it will aid in site specific exploration and commercial cultivation of high metabolite yielding chemotype(s) with validated pharmacological action to meet out the medicinal and commercial demands of colchicine. This will also promote the use of species in herbal product development as an adjuvant therapy in acute gouty flare for inhibition of inflammation.

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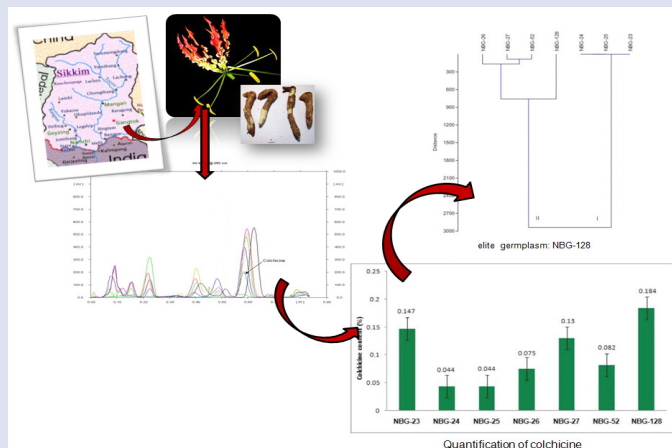
CONFLICT OF INTEREST

Authors do not have any conflict of Interest.

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GRAPHICAL ABSTRACT



ABOUT AUTHORS



Ms. Ankita Misra: Is working as a Research Associate in Pharmacognosy Division at CSIR-National Botanical Research Institute; Lucknow, INDIA. She is working in the area of analytical chemistry on medicinal plants, chemotaxonomy, bio-prospection and natural product development, handling major analytical instruments including HPLC, HPTLC, OPLC and column chromatography for quality control of herbal drugs. She has 23 publications in peer reviewed journals and 1 patent is.



Dr. Sharad Srivastava: Is Principal Scientist in Pharmacognosy Division at CSIR-National Botanical Research Institute; Lucknow, INDIA. He has made significant contributions to quality control of crude drugs/products, chemotaxonomy, bio-prospection and natural product development and have developed quality parameters of single crude drugs (more than 70 medicinal plants) and also identified biomarkers for their quality control. He has contributed 30 monographs of single herbal drugs in Ayurvedic Pharmacopoeia of India. He has 135 publications in peer reviewed journals, 17 patents and developed some technologies/formulations, few has already been transferred to industry.

SUMMARY

- Seven germplasm were collected from different location of Eastern Himalayas up to an altitude of 1162.812 meters.
- A calibrated HPTLC method was used to quantify colchicine content in samples, ranging from 0.044 to 0.184 % having maximum content in NBG-128 from Jorhat, Assam.
- The test extracts reflect potential anti arthritic activity in addition with promising hydroxyl radical scavenging activity.
- The results, thus suggested that the effect of *Gloriosa superba* whole extract on curbing the inflammation causing cells is more pronounced than pure colchicine and which may due to superimposed action of colchicine and, other chemical inhibitors like polyphenolics.
- This will aid in site specific exploration and commercial cultivation of high metabolite yielding chemotype(s) with validated pharmacological action to meet out the medicinal and commercial demands of colchicine.

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