Current Botany 2024, 15: 41-46
doi: 10.25081/cb.2024.v15.8785
https://updatepublishing.com/journal/index.php/cb

Research Article

ISSN: 2220-4822

Occurrence of guggulsterone content based chemotypes in *Commiphora wightii*

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ABSTRACT

Commiphora wightii is a critically endangered plant endemic in the arid and semi-arid regions of India. Oleogum is produced by the plant schizogenously. Upon making an incision, this gum exudes from the wound and solidifies in the arid environment. This solidfied gum-resin called 'gum guggul or guggal' is mentioned in Ayurvedic, Unani and Siddha literature for the treatment of many ailments. The steroidal ketones, *E*- and Z- guggulsterone are believed to be the active principles responsible for the medicinal properties. These resin samples were collected from plants in a few regions of Rajasthan, India, for quantitation of guggulsterone content using HPLC. Based on the quantitation of the guggulsterone content, we were able to identify three chemotypes. The first and the most common chemotype showed relatively much higher Z-guggulsterone than *E*-guggulsterone, the second type showed the absence of *E*-guggulsterone, the third and rare type showed the presence of equal amounts *E*- and Z-guggulsterone.

Received: January 15, 2024
Revised: March 28, 2024
Accepted: March 29, 2024
Published: April 20, 2024

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KEYWORDS: Guggul, Guggulsterone, Chemotypes, Endemic, Critically endangered

INTRODUCTION

Commiphora wightii is a critically endangered plant (Ved *et al.*, 2014) that belongs to the family Burseraceae. It is also identified as the incense tree, frankincense and myrrh family. Vernacularly, it is also known as Indian bdellium, Indian myrrh, Guggul, etc., and in Ayurvedic literature as Guggulu. Fragrant resins like frankincense, copal and myrrh which are of cultural, economic and medical use are produced by this incense tree family. *Commiphora* spp. is normally found in semi-arid to arid regions and is mostly common in Pakistan and the Indian region of the Thar Desert. In India, the plant is present predominantly in the state of Rajasthan, a few plant populations are also found in Maharashtra and Gujarat.

Oleogum-resin produced by *C. wightii* is present in the balsam canals in the phloem of the base of the stem and larger veins in the leaves. These canals develop in the young stem of the plant schizogenously. Tapping the plant for its gum is done in winter, a circular incision is made on the main branch (or base of the stem), and from this wound yellowish white latex is slowly exuded. The arid environment helps in the solidification of the latex to form the guggul resin. The resin looks like golden brown tears or pieces of a stalactite with a distinct balsamic aroma. The oleo-resin, guggul, is made up of a variety of resins, gums, volatile oils, minerals, inorganic foreign materials, and contaminants. From the separation of different components of the complex guggul resin mixture, ketonic compounds were seen to be present in the neutral fraction. The neutral ketonic fraction was found to include the active principles of C_{21} or C_{27} steroids and some defence related secretory ketones. The two closely related steroidal ketones, *E*-guggulsterone and Z-guggulsterone (Figure 1), {4,17(20)-pregnadiene-3,16-dione}, are said to be responsible for the therapeutic effectiveness of guggul resin extracts. *C. wightii* is differentiated from other species of the genus because of the presence of both the steroidal ketones i.e. Z- and *E*- guggulsterone. Guggul and its purified steroidal extracts acted as a helpful hypolipidemic principle in patients who were affected with ischemic heart disease, hyperlipidemia, obesity and hypercholesterolemia (Kunnumakkara *et al.*, 2018). The ability of guggul to act as an antioxidant also promotes cardiovascular health (Mester *et al.*, 1979). Guggulsterones are proposed to have similar activity like propranolol and nifedipine (cardio-protective drugs), in reversing the elevation of both lipid peroxidases and xanthine oxidase, while decreasing superoxide dismutase activity (Kaul & Kapoor, 1989). Guggulsterones are beneficial against multiple myeloma and breast cancer in humans (Ichikawa & Aggarwal, 2006), causing apoptosis in human

prostate cancer cells (Singh *et al.*, 2007), inhibiting angiogenesis (Xiao & Singh, 2008) and formation of skin tumours in SENCAR mouse (Sarfaraz *et al.*, 2008). However, recent reports do suggest that guggul has no statistically significant hypolipidemic activity (Szapary *et al.*, 2003; Donato *et al.*, 2021).

A major portion of rural the population in India depends on medicine from natural resources for treatment against various diseases and ailments. This dependence on herbal medicine is primarily due to Ayurveda. As *C. wightii* is well documented in Ayurvedic texts and is used to cure a variety of ailments. 'Guggulu', the dried gummy exudate, is sold in the open market at a high price (~800 INR Kg^{-1}). The worldwide belief in the natural and herbal remedies is continuously increasing. Industries involved in the production of natural healthcare remedies, supplements and cosmetic products are on the rise, as well as the driving force behind this is the increased demand for natural resources.

Chemotypes are defined as chemically distinct entities in plants or microorganisms, with differences in the composition of the secondary metabolites. Minor genetic changes bring about a marked difference in the secondary metabolite makeup of the organism. Since ancient times, knowledge about chemotypes has been used to propagate and cultivate plants producing high amounts of a favoured metabolite. Chemical variations between populations can be attributed to environmental or genetic factors, but field studies have demonstrated that the majority of chemotypes are not affected by environmental factors and are controlled genetically (Bolchi *et al.*, 2021). Therefore, it is imperative to analyse and identify plants producing high amounts of desired active principle, and use this information to develop cultivation strategies to augment income as well as develop germplasm resources. Policy formulation and development will help to ease the foraging pressure and dependence on natural populations, which has pushed this species to be critically endangered and on the brink of extinction (Cunningham *et al.*, 2018).

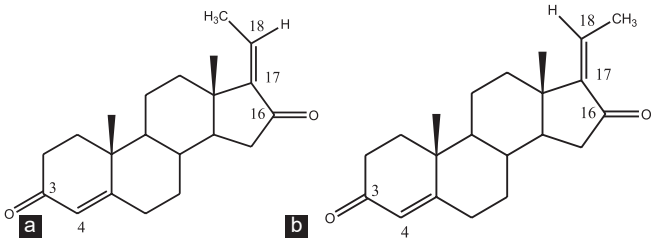

Figure 1: Chemical structures of the guggulsterones present in *C. wightii* resin. (a) *E*-guggulsterone and (b) *Z*-guggulsterone

MATERIAL AND METHODS

Sample Collection

The plant remains leafless throughout the year except for the short rainy season between July to October. Field surveys were carried out in the large districts of Jaipur and Ajmer. Sampling was done in seven sites of the two districts. Dr. M.L. Sharma of SKN Agriculture University, Jobner, Rajasthan accompanied them during field surveys and helped in the identification of the plants. Resin samples were collected during the winter season, from 64 mature plants from 7 populations (Table 1). Under the supervision of Dr. Sharma, a number 11 sterile (scalpel) blade was used to make a small and shallow incision (slanted and linear) on the main stem to allow the gum from the stem to exude. After a week, the sites were visited again to collect the solidified resin which had exuded from the incision made earlier, the resin samples were carefully collected in sterile and labelled plastic tubes. Dr. M.L. Sharma deposited the resins and voucher specimens of plant material at SKN University College Herbarium in Rajasthan, the details of voucher numbers are mentioned in Table 1.

Preparation of Reference Standards, Resin Extracts

Primary grade reference standards of both the guggulsterones i.e. *E*- and Z- were purchased from ChromaDex Inc. (Irvine, CA). Mobile phase and solvents (ethyl acetate, methanol and acetonitrile) were of HPLC grade and purchased from Merck (Whitehouse Station, NJ). Milli Q water was used for all purposes. Guggulsterone (both *E* & Z) standards for HPLC were prepared in a volumetric flask by adding 1 mg of respective guggulsterones in 3 mL of ethyl acetate, and then adding 7 mL of methanol to make the final volume 10 mL. Resin samples collected from plants (50 mg) were crushed in 2 mL of ethyl acetate, with the help of a micro-pestle, and the whole extracts were added to 8 mL of methanol. The standards and the prepared sample solutions were filtered through Acrodisc 4CR PTFE filters (Pall Life Sciences, NY) to remove particulate debris, and were kept at 4 °C for storage.

Separation and Quantitative Estimation of *E*- and *Z*-guggulsterone by HPLC

Separations of guggulsterone (*E* & Z) were done in an HPLC (Waters, Allianz, Milford, MA) which had a 717plus auto-sampler, Spherisorb C_{18} Reverse Phase Column (2.1 mm ID × 250 mm, with particle size 5 µm), 1525 Binary HPLC pump, and a PDA

Table 1: Location of the sampled populations of *C. wightii*

Locality	Terrain	Acronym	Sample size	Latitude (°N)	Longitude (°E)	Elevation (ft asl)	Vouchers
Mangliyawas	Hilly	M	23	26° 16′ 59.88″	75° 30′ 0″	1433	SKNCWM-125001-125023
Ajmer	Hilly	A	5	26°26′44″	74°38′49″	1548	SKNCWA-125024-125028
Kishangarh	Hilly	KG	4	26° 34′ 0.12″	74° 52′ 0.12″	1420	SKNCWK- 125029-125032
Jobner	Plains	J	15	26° 58′ 0.12″	75° 22′ 59.88″	1243	SKNCWJ-125033-125047
Bobas	Plains	B	9	26°54′21″	75°29′54″	1220	SKNCWB-125048-125056
Hirnoda	Plains	H	5	26°53′8″	75°19′41″	1116	SKNCWH-125057-125061
Galta Hills	Hilly	GH	3	26°55′1″	75°51′28″	1698	SKNCWG-125062-125064

detector. The software used for the analysis of chromatograms was EmPower (build 1154, Waters, Milford, MA) which was provided by the vendor. Solvents used for the mobile phase of the gradient method were Acetonitrile (A) and water (B). The solvent program was optimized following Mesrob *et al.* (1998) as follows: 0-3 min: 35% A and 65% B; 3-70 min: A= 35 to 100% and B= 65 to 0% (convex, 5); 70-82 min: 100% A; 82-83 min: A= 100 to 35% and B= 0 to 65% (linear, 6); 83-87 min: 35% A and 65% B. The injection volume was 20 µL, and the flow rate was set to 0.5 mL min^{-1}. The process was similar to that of Mesrob *et al.* (1998) except that the flow rate was reduced from 1 mL min^{-1} to 0.5 mL min^{-1} for better separation. Guggulsterone content was estimated by the EmPower software by measurement of the ratio of peak area to the areas of guggulsterone standards. The software used for statistical analysis was Unscrambler ver 9.8.

RESULTS AND DISCUSSION

The retention time for *E*-guggulsterone was 32.739 minutes and for Z-guggulsterone was 45.218 minutes (Figure 2a). Typical HPL chromatograms obtained for samples are depicted in Figures 2b to 2d. Peak assignments from the chromatograms were made by comparing individual peak retention times with that of guggulsterone standards by the EmPower software. An unknown peak having a retention time of 43.5 min was always found to co-elute with Z-guggulsterone.

Guggulsterone content varied largely among the samples included in the study (Table 2). The highest total guggulsterone content was measured in voucher SKNCWH-125061 from the Hirnoda population (5.027%), while the lowest content was detected in voucher SKNCWK-125032 from the Kishangarh population (0.346%). Relevant data on the amount of total guggulsterone in the methanolic extract of resins from the seven populations of *C. wightii* has been summarized in Table 3.

Important insights were gained upon analyses of the HPL chromatograms. Earlier reports indicate that in total guggulsterone content, Z-guggulsterone was present in much higher amounts than *E*-guggulsterone, both in natural resin (Patil *et al.*, 1972; Mesrob *et al.*, 1998) and drug formulations (Agrawal *et al.*, 2004). In our study, it was observed that in most of the samples the amount of Z-guggulsterone was much higher than *E*-guggulsterone (Table 2). This ratio was found in all populations (Table 3) and on average was highest in the Galta Hills population (more than 50 times) and lowest in the Mangliyawas population (less than 6 times). Also, some variations were observed. In some samples *E*-guggulsterone was found to be absent (Table 2 & Figure 2d), these vouchers were found in populations of Mangliyawas (SKNCWM-125005, 125020 and 125022), Ajmer (SKNCWA-125028), Kishangarh (SKNCWK-125032) and Jobner (SKNCWJ-125035, 125038, 125043 and 125045) but not in the populations Hirnoda, Bobas and Galta Hills. Only in one voucher (SKNCWM-125001) belonging to Mangliyawas population proportionate amounts of guggulsterone (both *E*- and Z-) were found (Figure 2c).

The occurrence of samples that lack *E*-guggulsterone has not pertained to a particular population but is distributed among a majority of them. The possibility that such a character could have arisen purely due to edaphic or environmental factors is

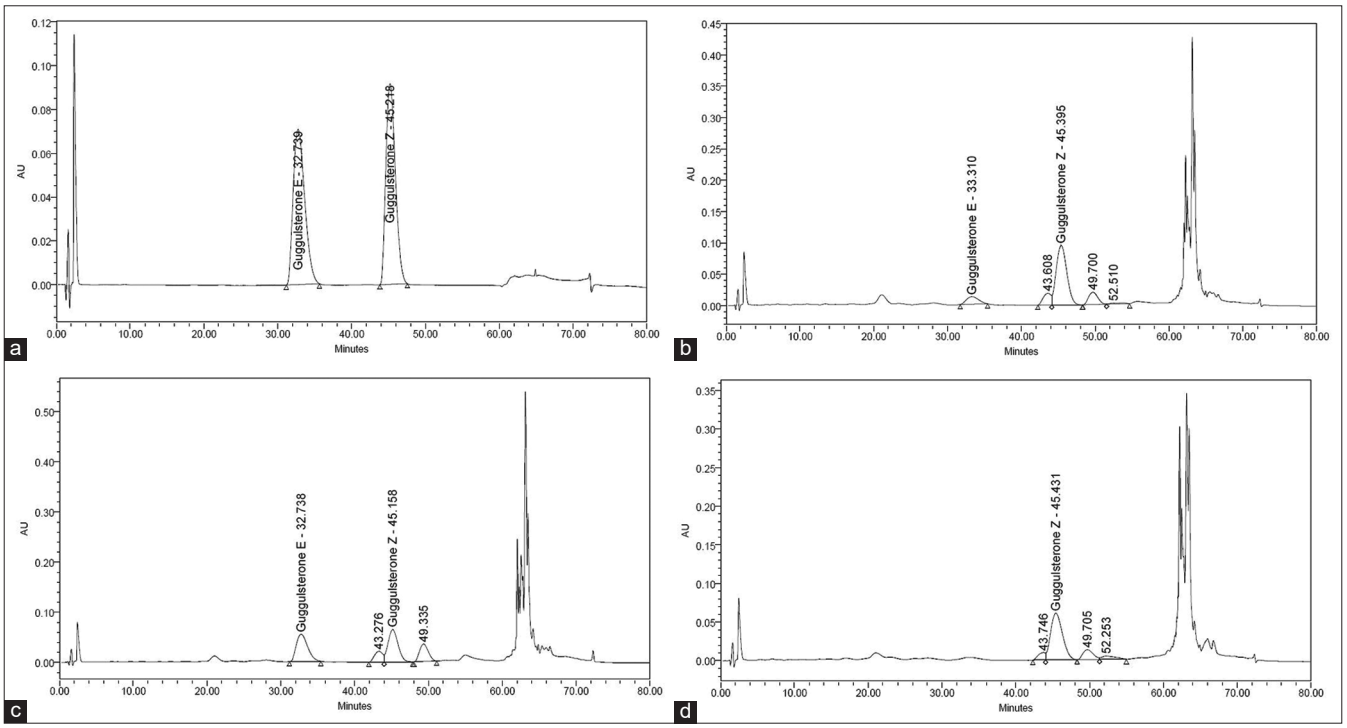

Figure 2: a) HPL chromatograms of standards of *E*- and *Z*-guggulsteron, b) chromatograms showing higher *Z*-guggulsterone than *E*-guggulsterone, c) almost equal amounts of *Z*- and *E*-guggulsterone and d) complete absence of *E*-guggulsterone

Table 2: Guggulsterone content of samples

Accession No.	Sample Code	*E*-guggulsterone (µg)	*Z*-guggulsterone (µg)	% Guggulsterones in resin
SKNCWM-125001	M-1	0.752	0.737	1.489
SKNCWM-125002	M-2	0.141	1.211	1.352
SKNCWM-125003	M-3	0.024	0.530	0.554
SKNCWM-125004	M-4	0.028	0.528	0.556
SKNCWM-125005	M-5	0	0.477	0.477
SKNCWM-125006	M-6	0.288	0.602	0.890
SKNCWM-125007	M-7	0.179	1.148	1.327
SKNCWM-125008	M-8	0.169	0.636	0.805
SKNCWM-125009	M-9	0.124	0.661	0.785
SKNCWM-125010	M-10	0.124	1.085	1.209
SKNCWM-125011	M-11	0.219	1.100	1.319
SKNCWM-125012	M-12	0.163	1.235	1.398
SKNCWM-125013	M-13	0.181	1.097	1.278
SKNCWM-125014	M-14	0.237	1.268	1.505
SKNCWM-125015	M-15	0.099	0.485	0.584
SKNCWM-125016	M-16	0.228	0.986	1.214
SKNCWM-125017	M-17	0.198	0.893	1.091
SKNCWM-125018	M-18	0.089	0.948	1.037
SKNCWM-125019	M-19	0.062	0.796	0.858
SKNCWM-125020	M-20	0	0.595	0.595
SKNCWM-125021	M-21	0.151	1.194	1.345
SKNCWM-125022	M-22	0	0.820	0.820
SKNCWM-125023	M-23	0.021	0.527	0.548
SKNCWK-125029	KG-1	0.001	0.951	0.952
SKNCWK-125030	KG-2	0.189	2.023	2.212
SKNCWK-125031	KG-3	0.116	0.778	0.894
SKNCWK-125032	KG-4	0	0.346	0.346
SKNCWA-125024	A-1	0.191	1.328	1.519
SKNCWA-125025	A-2	0.103	0.797	0.900
SKNCWA-125026	A-3	0.025	1.087	1.112
SKNCWA-125027	A-4	0.024	0.588	0.612
SKNCWA-125028	A-5	0	0.781	0.781
SKNCWJ-125033	J-1	0.165	3.600	3.765
SKNCWJ-125034	J-2	0.068	1.7984	1.866
SKNCWJ-125035	J-3	0	0.873	0.873
SKNCWJ-125036	J-4	0.011	0.790	0.801
SKNCWJ-125037	J-5	0.084	1.249	1.333
SKNCWJ-125038	J-6	0	0.967	0.967
SKNCWJ-125039	J-7	0.152	2.285	2.437
SKNCWJ-125040	J-8	0.212	2.328	2.540
SKNCWJ-125041	J-9	0.150	3.269	3.419
SKNCWJ-125042	J10	0.016	1.520	1.536
SKNCWJ-125043	J-11	0	1.162	1.162
SKNCWJ-125044	J-12	0.007	1.127	1.134
SKNCWJ-125045	J-13	0	0.832	0.832
SKNCWJ-125046	J-14	0.126	2.965	3.091
SKNCWJ-125047	J-15	0.032	2.213	2.245
SKNCWH-125057	H-1	0.162	2.805	2.967
SKNCWH-125058	H-2	0.242	4.225	4.467
SKNCWH-125059	H-3	0.166	3.212	3.378
SKNCWH-125060	H-4	0.108	2.076	2.184
SKNCWH-125061	H-5	0.272	4.755	5.027
SKNCWB-125048	B-1	0.106	1.882	1.988
SKNCWB-125049	B2	0.053	1.014	1.067
SKNCWB-125050	B-3	0.125	1.245	1.37
SKNCWB-125051	B-4	0.021	1.600	1.621
SKNCWB-125052	B-5	0.058	1.465	1.523
SKNCWB-125053	B-6	0.040	1.024	1.064
SKNCWB-125054	B-7	0.057	1.015	1.072
SKNCWB-125055	B-8	0.063	1.923	1.986
SKNCWB-125056	B-9	0.023	1.240	1.263
SKNCWG-125062	GH-1	0.026	1.224	1.250
SKNCWG-125063	GH-2	0.023	1.242	1.265
SKNCWG-125064	GH-3	0.027	1.039	1.066

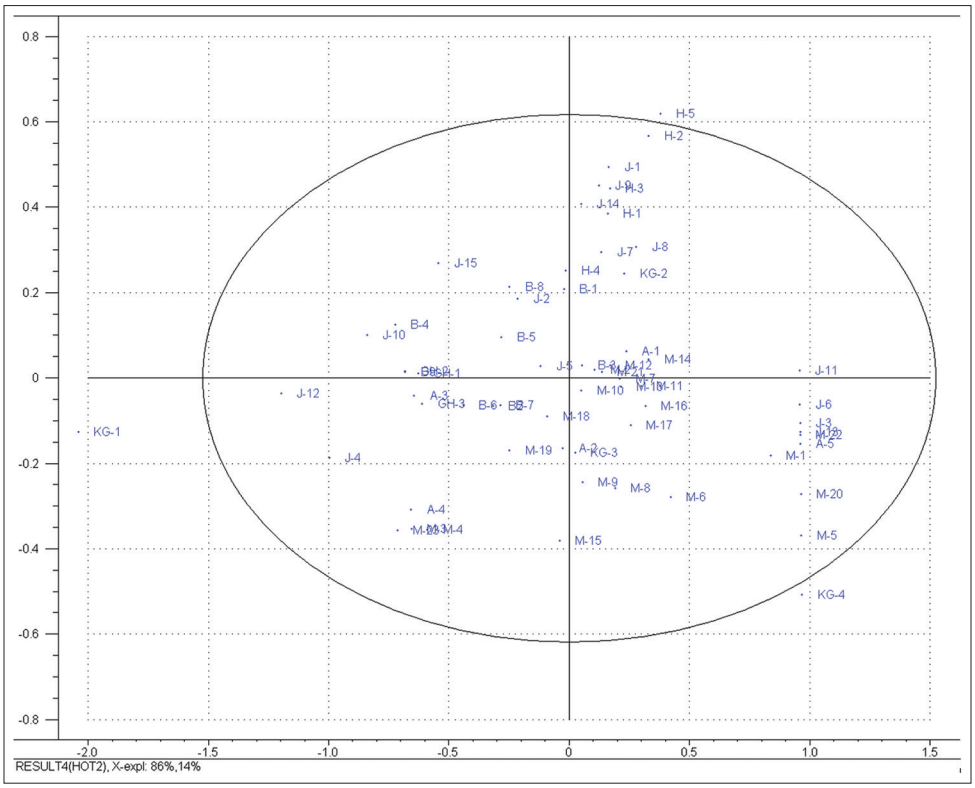

Figure 3: Score plot of PCA of the samples based on individual guggulsterone content. The ellipse represents the Hotelling's T^2 with 95% confidence in score plots

Table 3: Guggulsterone content of populations

Population	% *E*-guggulsterone in resin	% *Z*-guggulsterone in resin	% Total guggulsterone in resin
Jobner	0.068 (112.83)	1.799 (53.84)	1.867 (55.32)
Hirnoda	0.190 (61.03)	3.415 (55.52)	3.605 (55.80)
Bobas	0.061 (69.28)	1.379 (32.66)	1.440 (33.69)
Mangliyawas	0.152 (103.14)	0.850 (32.46)	1.002 (34.50)
Ajmer	0.069 (114.69)	0.916 (31.77)	0.985 (35.51)
Kishangarh	0.077 (121.15)	1.025 (69.56)	1.102 (71.70)
Galta Hills	0.025 (7.96)	1.168 (9.62)	1.193 (9.20)

Figures in parenthesis denote %RSD

miniscule. Many populations studied showed the occurrence of vouchers of the abovementioned character both in the hilly terrain (Mangliyawas, Ajmer and Kishangarh) and in the plains (Jobner). Although only a single voucher showed the presence of proportionate amounts of guggulsterone, its occurrence due to non-genetic characters is very little as none of the other vouchers collected from the same site showed this character. Thus genetic factors may be responsible for such changes in guggulsterone content (both *E*- and Z-) and the presence of three chemotypes is proposed.

Chemotype 1: The predominant one, showing very high levels of Z-guggulsterone when compared to *E*-guggulsterone.

Chemotype 2: Showing absence of *E*-guggulsterone but the presence of Z-guggulsterone.

Chemotype 3: The very rare type, showing proportionate amounts of *E*- and Z-guggulsterone.

To test for statistical significance of the guggulsterone concentration, principal component analysis (PCA) was performed, after log transformation of the guggulsterone data, followed by Hotelling's T^2. PCA reduces the dimensionality of the multivariate data while maintaining the majority of the data inside it since it is an unsupervised clustering approach that requires no prior knowledge of the data set. It is possible to visualise the principal component as a score plot graphically, which helps in identifying any groups that are present within the data set. Hotelling's T^2 is a generalization of the square of the T-statistic, the ellipse is generally constructed with $\alpha = 0.05$ (95% confidence), thus the scores bound by it are statistically significant. Hotelling's ellipses are free from unknown theoretical parameters and are applicable to both, dependent as well as independent series of measurements. As seen in the PCA (Figure 3), all the samples are distributed in two major groups. The most prevalent chemotype-1 is distributed throughout and present in all the four quadrants whereas chemotype-2 is seen to form a small cluster at the third quadrant completely separated from chemotype-1. The only sample that showed equal amounts of guggulsterone (M-1) was also present in the third quadrant.

CONCLUSION

C. wightii is a critically endangered plant, and it is well known that unsustainable utilization of any species can cause a very

severe decrease in the number of plants of that species, leading to the disappearance of entire populations and severely affecting genetic diversity (Haque *et al.*, 2010). The primary threat to the species, aside from demographic and genetic limitations, is the overexploitation of the species for its medicinally and economically significant resin (Cunningham *et al.*, 2018). It is generally known that *in-situ* or on-site conservation, in which a wild species or stock of a biological community is safeguarded and conserved in its native environment, is the most successful and economical method of preserving the current genetic diversity. *Ex-situ* conservation or conservation of the plant outside its natural habitat can be done by maintenance and cultivation in a suitable site or botanic gardens, but this methodology is very labour intensive and has a high economic burden. Conservation strategies should include genotypes having high guggulsterone content. Sampling intensively from different sites can fulfill the objective of capturing most of the genetic variability.

ACKNOWLEDGMENTS

We thank late Dr. M. L. Sharma and Dr. M. Krishnamohan for helping to locate and identify *C. wightii* plants. The authors are thankful to BTISNet SubDIC (BT/BI/04/065/04) for providing facilities for statistical analyses and the Government of Jharkhand, Dept. of Agriculture for providing infrastructure development fund. This study was financially supported by the Department of Biotechnology, Govt. of India (NBDB Grant 102/IFD/SAN/1192/2004-05). Fellowships provided [9/554 (13) 2007-EMR-I].

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