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RESEARCH ARTICLE

Quantitative determination of guggulsterone in existing natural populations of *Commiphora wightii* (Arn.) Bhandari for identification of germplasm having higher guggulsterone content

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Abstract Guggulsterone is an aromatic steroidal ketonic compound obtained from vertical rein ducts and canals of bark of *Commiphora wightii* (Arn.) Bhandari (Family - Burseraceae). Owing to its multifarious medicinal and therapeutic values as well as its various other significant bioactivities, guggulsterone has high demand in pharmaceutical, perfumery and incense industries. More and more pharmaceutical and perfumery industries are showing interest in guggulsterone, therefore, there is a need for its quantitative determination in existing natural populations of *C. wightii.* Identification of elite germplasm having higher guggulsterone content can be multiplied through conventional or biotechnological means. In the present study an effort was made to estimate two isoforms of guggulsterone i.e. E and Z guggulsterone in raw exudates of 75 accessions of *C. wightii* collected from three states of North-western India viz. Rajasthan (19 districts), Haryana (4 districts) and Gujarat (3 districts). Extracted steroid rich fraction from stem samples was fractionated using reverse-phase preparative High Performance Liquid Chromatography (HPLC) coupled with UV/VIS detector operating at wavelength of 250 nm. HPLC analysis of stem samples of wild as well as cultivated plants showed that the concentration of E and Z isomers as well as total guggulsterone was highest in Rajasthan, as compared to Haryana and Gujarat states. Highest concentration of E guggulsterone (487.45 μg/g) and Z guggulsterone (487.68 μg/g) was found in samples collected from Devikot (Jaisalmer) and Palana (Bikaner) respectively, the two hyper-arid regions of Rajasthan, India. Quantitative assay was presented on the basis of calibration curve obtained from a mixture of standard E and Z guggulsterones with different validatory parameters including linearity, selectivity and specificity, accuracy, auto-injector, flow-rate, recoveries, limit of detection and limit of quantification (as per norms of International conference of Hormonization). Present findings revealed the role of environmental factors on biosynthesis of guggulsterone isomers under natural conditions.

Keywords Biochemical characterization · Burseraceae · Endangered species · Guggulsterone · Oleo-gum resin

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Introduction

Commiphora wightii (Arn.) Bhandari (Burseraceae) is a gum bearing (Greek word *kommi* means 'gum' and *pheros* means 'to bear') plant species indigenous to the Indian subcontinent and growing in rocky tracts of arid and semi-arid lands of India, Bangladesh, Pakistan, China, Ethiopia, Arabia, Tropical and Northern Africa, and many other countries (Deng 2007; Kant et al. 2010). It is commonly known as Guggulu in Sanskrit and guggul in Hindi due to the presence of E and Z isomers of aromatic steroidal ketonic compound guggulsterone in vertical rein ducts and canals of bark. Oleo-gum resin of *C. wightii* is widely used in traditional medicines to treat various afflictions

including rheumatism, arthritis, arteriosclerosis, obesity, hypercholestermia, inflammation and cancer (Samudio et al. 2005; Kulhari et al. 2012; Harish et al. 2014). Various successful clinical studies on effectiveness of herbal formulations containing guggulsterone made it popular in the pharmacy world. Destructive and unscientific harvesting for economic benefits with negligible conservation efforts led to dwindling of natural population of this species making it endangered and led to its categorization in 'data deficient' category ver. 2.3 (1994) of the Red Data Book of IUCN (International Union for the Conservation of Nature) assemblage. However, the Government of India has included it under RET (Rare, Endangered, Threatened) category and now only few wild populations exists in the states of Rajasthan and Gujarat (Haque et al. 2007; Samantaray et al. 2011; Kulloli and Kumar 2014). Lack of organized cultivation strategies, over exploitation, increasing desertification and environmental vulnerabilities, slow growth, poor seed setting (16 %), very poor seed germination (5 %), and long dormant phase has hampered its natural regeneration and led to scarcity of raw as well as finished products of this valuable drug in the country. According to an estimate, the demand of gum guggul is 1000 MT but India produces only 100 MT against its requirement (Maheshwari 2010). Even the price of gum guggul has increased manifold (from Rs. 100–600 kg^{-1}) in the last 10–years and the deficiency is being met through imports from Pakistan and Afghanistan. India spends approximately Rs 45 crores on its import mainly from Afghanistan indicating many fold increase in its demand with decreasing availability of natural sources in the country.

Guggul is the dried form of oleo-gum resin obtained primarily from the bark of the guggul plant. It is a complex mixture of resin (61 %), gum (29.3 %) and other chemicals (6.1 %) including several plant sterols, steroids, esters, diterpenes and higher alcohols. However, its main active compounds are inter-convertible isomeric forms (E and Z) of guggulsterone which are steroidal in nature (Agrawal et al. 2004a). Two different arrangements of CH_3 at C_{20} in three-dimensional space and the hindered rotation about the carbon–carbon double bond at C_{17} and C_{20} classifies guggulsterone into Z-{4,17(20)-*cis*-pregnadiene-3,16-dione} and E-{4,17(20)-*trans*-pregnadiene-3,16-dione} isomers.

Genetic variability of this species is facing a great threat since more and more pharmaceutical and perfumery industries and showing interest in this wonder plant, thereby severely increasing pressure on natural wild populations. Therefore, there is an urgent need for reliable and consistent quantitative determination of bioactive ingredients in existing natural populations of *C. wightii* for identification of elite germplasm having higher oleo-gum resin yielding ability which can be multiplied and mass propagated through tissue culture (Kumar and Nadgauda 2014) and vegetative propagation (Tripathi et al. 2014) methods for afforestation purposes. Kulhari et al. (2013) recently determined guggulsterone content in 11 samples of *C. wightii* using High Performance Thin Layer Chromatography (HPTLC). Mesorb et al. (1998) validated gradient HPLC method for quantification of E and Z stereoisomers in oleo-gum resin exudates of *C. mukul* Engl. Soni et al. (2010) estimated the content of guggulsterone isomers in resin using HPLC while Dass and Ramawat (2009) used reverse phase column HPLC to determine guggulsterone content in cell and callus cultures of *C. wightii*. Verma et al. (1998) and Singh et al. (1995) quantified the two isomeric forms of guggulsterone, simultaneously by HPLC in rat serum after administration of a single dose (50 mg/kg). Agrawal et al. (2004a) determined the concentration of E (R_f 0.38) and Z (R_f 0.46) guggulsterone in pharmaceutical dosage forms while Agrawal et al. (2004b) carried out stress degradation studies on guggulsterone using HPTLC. Recently, Kulhari et al. (2012) elaborately and exhaustively reviewed the status of pharmacological, biochemical and biotechnological progress made in the genus *Commiphora* with emphasis on *C. wightii*.

Keeping in view the endangered status and importance of *C. wightii*, the present investigation was designed to develop a HPLC based quick and validated procedure, conforming to ICH recommendations, for simultaneous estimation of bioactive constituents, E and Z guggulsterone, in wild as well as cultivated *C. wightii* samples collected from diverse geographical regions (75 locations from 26 districts of 3 states namely Rajasthan, Gujarat and Haryana) of North-western India for identification of elite genotypes. A total of 75 samples from eleven agro-climatic regions of India were screened to determine the nature and extent of variability in these two steroidal components among *C. wightii* accessions.

Materials and methods

Procurement of samples

Stem samples of wild as well as cultivated *C. wightii* plants were collected from Rajasthan (19 districts), Haryana (4 districts) and Gujarat (3 districts), the three hot spot states for guggul occurrence in India.

Preparation of raw material

Collected plant material was washed with running tap water followed by deionized autoclaved water to remove the dust particles and possible parasites. Stem samples (10 g) were shade dried, pulverized and finally coarsely powdered before subjecting to extraction with petroleum ether (60–80 °C) in Soxhlet apparatus for 8–10 h. Extracted samples were concentrated under vacuum using rotary evaporator at high temperature to near dryness. These concentrated sticky samples were reconstituted quantitatively in acetone, filtered through syringe filters (size 0.45 μ, Axiva) and the final volume was made to

Table 1 Validatory parameters for E and Z guggulsterone - Linear regression equation, R^2, LOD and LOQ values

Compound	Wavelength	Regression	R^2	Retention time	LOD (3.3*SD)/S (μg ml^{-1})	LOQ (10*SD)/S (μg ml^{-1})
E guggulsterone	250 nm	Y=14296x−69744	0.994	15.009 min	0.214	0.649
Z guggulsterone	250 nm	Y=11278x+43499	0.993	17.809 min	2.789	8.454

5 ml in volumetric flasks. Purified samples were stored at 4 °C and subjected for sonication prior to HPLC analysis.

Chemicals and reagents

For quantitative estimation of guggulsterone, reference compounds (isomeric form of E and Z guggulsterone) were procured from Chromadex, USA. HPLC grade solvents (methanol, petroleum ether, acetone, water, acetonitrile and trifluroacetic acid) were obtained from Sigma, Hi- Media, Fisher Scientific and Qualigens.

Preparation of stock solution

Accurately weighed (5 mg) standards of E and Z guggulsterone were transferred to 5 ml volumetric flask and volume was made using methanol. This stock standard solution was further diluted to obtain working standard solution of different concentrations ranging from 20 to 100 μg ml^{-1} and stored at 4 °C.

HPLC instrumentation and chromatographic conditions

HPLC analysis was performed on Rapid Separation LC (RSLC) system (Shimadzu, Japan), equipped with auto sampler, LC-2010 pump (low pressure gradient mode), with a Sentry C_{18} guard column, degasser, column oven and a UV/VIS detector. Chromatographic separation of analytes was carried out using a reverse phase Nucleosil C_{18} column (5 μm, 4.6×250 mm). Mobile phase (pH 3.0+/−0.2), a binary gradient system consisting of eluent A (0.05 % trifluroacetic acid in water) and eluent B (0.03 % trifluroacetic acid in acetonitrile), was properly filtered and degassed in ultrasonic bath for 20 min prior to use. Injected volume (20 μl) was maintained at a constant flow rate (0.6 ml/min) and column temperature (35 °C). The spectral data was collected at 250 nm detection wavelength (LC- 2010 UV detector with Duterium D2 lamp) and data acquisition was performed by LC- Solution software version 1.25. All the samples were analyzed in triplicate and data was subjected to standard error calculation using SAS 9.3 software.

Method validation

The described method was validated according to the ICH guidelines (ICH 1993) including following validation characteristics: linearity, selectivity and specificity, accuracy, repeatability, precision (Intraday and Interday), limit of detection and quantification (LOD and LOQ).

Linearity

The calibration graphs were obtained for each individual compound (E and Z) by plotting the peak area versus the

Table 2 Validatory parameters for E and Z guggulsterone - Accuracy and recovery for quality consistency evaluation

Conc. (μg ml^{-1})	20		60		100	
Component	E	Z	E	Z	E	Z
Conc. Determined 1	19.43	19.73	59.59	59.89	100.12	100.02
Conc. Determined 2	19.79	19.99	59.68	59.68	99.98	99.92
Conc. Determined 3	19.96	19.96	60.49	60.29	99.92	99.87
Conc. Determined Mean	19.72	19.89	59.92	59.95	100.00	99.93
Std. deviation (SD)	0.27	0.14	0.49	0.30	0.10	0.076
RSD	1.37	0.71	0.82	0.519	0.105	0.07
Recovery 1 (%)	97.15	98.65	99.32	99.82	100.12	100.02
Recovery 2 (%)	98.95	99.95	99.47	99.47	99.98	99.92
Recovery 3 (%)	99.8	99.80	100.82	100.48	99.92	99.87
Recovery mean (%)	98.63	99.46	99.86	99.92	100.00	99.93
Std. deviation (SD)	1.35	0.71	0.82	0.51	0.10	0.076
RSD	1.371	0.71	0.82	0.51	0.10	0.076
% **Bias**	**−1.366**	**−0.533**	**−0.133**	**−0.077**	**0.006**	**−0.063**

Table 3 Method precision for E and Z guggulsterones – (A) Intra-day precision

Session	1						2					
Conc.	20		60		100		20		60		100	
Component	E	Z	E	Z	E	Z	E	Z	E	Z	E	Z
Conc. Determined 1	19.63	19.43	59.89	59.59	100.12	100.08	19.33	19.93	59.99	59.89	100.62	100.22
Conc. Determined 2	19.89	19.79	59.67	59.68	99.98	99.88	19.89	19.79	59.58	59.88	99.48	99.98
Conc. Determined 3	19.76	19.96	60.19	60.49	99.92	99.97	19.86	19.66	60.19	60.09	99.62	99.54
Mean	19.76	19.73	59.92	59.92	100.01	99.98	19.69	19.79	59.92	59.95	99.91	99.9
Standard deviation	0.13	0.27	0.26	0.50	0.08	0.10	0.30	0.14	0.26	0.12	0.51	0.34
RSD	0.65	1.37	0.44	0.83	0.08	0.10	1.52	0.68	0.43	0.20	0.51	0.34

concentration. Regression analysis calibration curves demonstrated linearity in the range of 20–100 μg ml^{-1} after evaluating five concentrations of standards. Linearity of the developed method was presented in terms of regression coefficient (R^2) and it was>0.99 in the two reference compounds, E (0.994) and Z (0.993) guggulsterone, at 250 nm wavelength.

Selectivity and specificity

The selectivity of the method was determined by comparing the retention time of representative chromatogram of ultraviolet–visible (UV/Vis) spectra of sample extracts with the reference compounds of E and Z guggulsterone. E and Z guggulsterone reference compounds were eluted at 15.009 and 17.809 min, respectively.

Accuracy

Three standard concentrations (20, 60 and 100 μg ml^{-1}) were used. The negative values of % bias in the recovery of standard samples verified the accuracy of the analytical method.

Table 4 Method precision for E and Z guggulsterones – (B) Inter-day precision

Conc.	20		60		100	
Component	E	Z	E	Z	E	Z
Day 1						
Conc. Determined 1	19.79	19.79	59.68	59.68	99.98	99.88
Conc. Determined 2	19.96	19.96	60.49	60.49	99.92	100.08
Conc. Determined 3	19.93	19.88	59.89	59.89	100.12	99.88
Mean	19.89	19.87	60.02	60.02	100.01	99.94
Standard deviation	0.09	0.09	0.42	0.42	0.10	0.11
RSD	0.46	0.43	0.70	0.70	0.10	0.11
Day 2						
Conc. Determined 1	19.71	19.33	59.78	59.67	99.78	99.67
Conc. Determined 2	19.69	19.89	60.69	60.19	99.97	99.44
Conc. Determined 3	19.70	19.61	60.24	59.93	99.88	98.97
Mean	19.7	19.61	60.235	59.93	99.875	99.36
Standard deviation	0.01	0.28	0.46	0.26	0.09	0.36
RSD	0.05	1.43	0.76	0.43	0.10	0.36
Day 3						
Conc. Determined 1	19.33	19.93	59.99	59.89	100.62	100.22
Conc. Determined 2	19.89	19.79	59.58	59.88	99.48	99.98
Conc. Determined 3	19.86	19.66	60.19	60.09	99.62	99.54
Mean	19.69	19.79	59.92	59.95	99.91	99.91333
Standard deviation	0.30	0.14	0.26	0.12	0.51	0.344867
RSD	1.52	0.68	0.43	0.20	0.51	0.345166

Table 5 Analysis of E and Z guggulsterones in oleo-gum resin exudates of different accessions of *Commiphora wightii*

Code	District	Specific location	Agroecological region	Latitude	Longitude	Status	Rainfall (mm/yr)	Height (m)	Girth (cm)	Canopy E-W (cm)	Canopy N-S (cm)	E Guggulsterone (μg g^{-1})	Z Guggulsterone (μg g^{-1})	Total guggulsterone (μg g^{-1})
Haryana														
H1	Fatehabad	Herbal farm	Trans-Gangetic Plains Region	29º31 N	75º27 E	C	260.45	1.63	6.2	48	53	143.68±0.35	155.37±0.32	299.05
H2	Hisar	HAU Campus	Trans-Gangetic Plains Region	28º84 N	75º54 E	C	325.85	1.9	6.4	52	57	148.23±0.34	163.26±0.24	311.49
H3	Rewari	Mandola	Trans-Gangetic Plains Region	28º24 N	76º58 E	W	474.45	3.39	8.4	67	61	153.10±0.36	163.10±0.25	316.20
H4	Rewari	Nursery	Trans-Gangetic Plains Region	28º24 N	76º58 E	C	474.45	1.10	4.7	49	32	145.14±0.39	169.98±0.26	315.12
H5	Mahendergarh	Narnaul	Trans-Gangetic Plains Region	28º09 N	76º07 E	W	416.95	3.26	6.7	70	62	195.36±0.07	190.08±0.33	385.44
H6	Mahendergarh	Khudana	Trans-Gangetic Plains Region	28º02 N	76º07 E	W	416.95	3.19	6.2	58	43	190.92±0.32	187.80±0.29	378.72
H7	Mahendergarh	Kultajpur	Trans-Gangetic Plains Region	28º02 N	76º07 E	W	416.95	2.89	5.1	61	47	188.11±0.47	183.69±0.46	371.80
Gujarat														
G8	Aanand	GAU	Plains & Hills Region	22º57 N	72º93 E	C	610.30	2.04	4.6	54	42	138.49±0.07	142.44±0.45	280.93
G9	Jamnagar	Nursery	Plains & Hills Region	22º47 N	70º07 E	C	516.00	1.93	4.8	57	49	160.00±0.44	161.83±0.06	321.83
G10	Kachchh	Nakhatrana	Plains & Hills Region	23º62 N	71º20 E	C	350.00	1.93	4.8	57	49	145.86±0.34	147.55±0.30	293.41
Rajasthan														
R11	Udaipur	MLSU Campus	Sub-humid	24º62 N	73º68 E	C	724.45	2.78	5.4	46	49	178.21±0.13	173.87±0.44	352.08
R12	Udaipur	Neemach Mata	Sub-humid	24º62 N	73º68 E	W	724.45	2.78	5.4	46	49	182.50±0.10	176.21±0.32	358.71
R13	Udaipur	Moti Magri	Sub-humid	24º62 N	73º68 E	W	724.45	3.2	6.7	69	52	182.73±0.14	174.42±0.57	357.15
R14	Udaipur	Kaler ka jungle	Sub-humid	24º62 N	73º68 E	W	724.45	3.39	6.7	58	56	188.65±0.18	164.24±0.31	352.89
R15	Udaipur	Kaler ka jungle	Sub-humid	24º62 N	73º68 E	W	724.45	3.40	6.7	78	69	187.47±0.18	170.85±0.52	358.32
R16	Udaipur	Sajangarh forest	Sub-humid	24º62 N	73º68 E	W	724.45	3.38	7.9	54	56	186.22±0.09	192.32±0.17	378.54
R17	Udaipur	Brahmino Ki Hunder	Sub-humid	24º62 N	73º68 E	W	724.45	3.318	8.7	69	62	183.43±0.03	188.15±0.09	371.58
R18	Udaipur	Bagdhara Nature park	Sub-humid	24º62 N	73º68 E	W	724.45	4.11	7.6	56	55	181.12±0.35	186.50±0.07	367.62
R19	Udaipur	Bagdhara Nature park	Sub-humid	24º62 N	73º68 E	W	724.45	3.80	6.4	52	50	202.47±0.12	205.68±0.54	408.15
R20	Udaipur	Bagdhara Nature park	Sub-humid	24º62 N	73º68 E	W	724.45	3.49	8.6	61	56	208.80±0.28	212.08±0.34	420.88
R21	Udaipur	Baanki forest	Sub-humid	24º62 N	73º68 E	W	724.45	3.48	6.5	67	63	200.81±0.31	205.97±0.30	406.78
R22	Udaipur	Baanki forest	Sub-humid	24º62 N	73º68 E	W	724.45	3.31	5.8	69	64	207.19±0.67	211.78±0.32	418.97
R23	Udaipur	Sisaram	Sub-humid	24º62 N	73º68 E	W	724.45	3.19	5.4	70	62	206.39±0.67	212.48±0.13	418.87
R24	Udaipur	Gogunda	Sub-humid	24º62 N	73º68 E	W	724.45	3.19	8.2	58	49	183.84±0.44	191.84±0.32	375.68
R25	Udaipur	Chirwaghata forest	Sub-humid	24º62 N	73º68 E	W	724.45	2.89	8.1	61	55	188.98±0.50	192.34±0.28	381.32
R26	Udaipur	Chirwaghata forest	Sub-humid	24º62 N	73º68 E	W	724.45	2.16	8.8	72	67	186.98±0.17	195.83±0.17	382.81
R27	Udaipur	Balicha	Sub-humid	24º62 N	73º68 E	W	724.45	2.28	8.1	75	68	185.11±0.48	191.14±0.49	376.25
R28	Udaipur	Adamagra	Sub-humid	24º62 N	73º68 E	W	724.45	2.39	8.4	67	61	182.74±0.23	188.06±0.32	370.80
R29	Udaipur	Macchlamgra	Sub-humid	24º62 N	73º68 E	W	724.45	2.78	8.9	65	64	183.89±0.33	189.14±0.32	373.03
R30	Rajsamand	Dhermata mines	Semi-arid, Rocky tract	25º17 N	73º51 E	W	540	3.18	8.7	78	69	220.46±0.37	202.12±0.41	422.58
R31	Rajsamand	National Highway-8	Semi-arid, Rocky tract	25º17 N	73º51 E	W	540	3.22	8.7	70	72	238.12±0.27	219.51±0.35	457.63
R32	Rajsamand	Haldighati Area	Semi-arid, Rocky tract	25º17 N	73º51 E	W	540	3.61	8.9	84	82	252.94±0.37	257.03±0.37	509.97
R33	Rajsamand	Haldighati Area	Semi-arid, Rocky tract	25º17 N	73º51 E	W	540	3.65	8.8	81	82	252.61±0.28	259.62±0.32	512.23
R34	Rajsamand	Piplantri green belt	Semi-arid, Rocky tract	25º06 N	73º88 E	W	540	3.51	8.9	65	64	265.28±0.37	272.15±0.32	537.43
R35	Rajsamand	Puthol village	Semi-arid, Rocky tract	25º06 N	73º88 E	W	540	3.54	8.4	70	74	269.23±0.40	276.97±0.31	546.20

Table 5 (continued)

Code	District	Specific location	Agroecological region	Latitude	Longitude	Status	Rainfall (mm/yr)	Height (m)	Girth (cm)	Canopy E-W (cm)	Canopy N-S (cm)	E Guggulsterone (μg g^{-1})	Z Guggulsterone (μg g^{-1})	Total guggulsterone (μg g^{-1})
R36	Chittorgarh	Bhadesar	Sub-humid	24º88 N	76º63 E	W	708	3.78	7.4	73	66	201.80±0.40	246.82±0.24	448.62
R37	Chittorgarh	Bhadsoda	Sub-humid	24º88 N	76º63 E	W	708	3.73	7.6	77	69	215.04±0.37	281.33±0.02	496.37
R38	Chittorgarh	Chittorgarh fort	Sub-humid	24º88 N	76º63 E	W	708	3.82	7.3	73	65	274.41±0.33	247.02±0.13	521.43
R39	Chittorgarh	Chhoti sadadi	Sub-humid	24º88 N	76º63 E	W	708	3.86	7.6	77	69	294.82±0.59	229.16±0.44	523.98
R40	Chittorgarh	Nimbahera	Sub-humid	25º75 N	71º38 E	W	708	3.71	7.4	72	65	217.92±0.35	268.05±0.03	485.97
R41	Bhilwara	Bhillo ki jhopadi	Sub-humid	25º35 N	74º63 E	W	633.90	3.22	7.6	77	76	164.24±0.48	176.54±0.41	340.78
R42	Bhilwara	Mandavgarh	Sub-humid	25º35 N	74º63 E	W	633.90	3.02	7.3	73	70	173.14±0.23	178.50±0.09	351.64
R43	Bhilwara	Det village	Sub-humid	25º35 N	74º63 E	W	633.90	3.13	7.4	75	74	170.53±0.24	176.08±0.44	346.61
R44	Ajmer	Mangliawas herbal farm	Semi-arid, Rocky tract	26º25 N	74º51 E	W	550	3.9	8.9	77	79	259.07±0.46	209.56±0.32	468.63
R45	Ajmer	Pitamber-ki-gal	Semi-arid, Rocky tract	26º25 N	74º51 E	W	550	3.81	8.7	89	86	270.43±0.33	211.27±0.55	481.70
R46	Ajmer	Nasirabad valley	Semi-arid, Rocky tract	26º25 N	74º51 E	W	550	3.82	8.7	779	82	267.52±0.33	208.46±0.33	475.98
R47	Ajmer	Nagpahar	Semi-arid, Rocky tract	26º25 N	74º51 E	W	550	3.88	8.6	76	56	260.88±0.28	209.92±0.48	470.80
R48	Jaipur	Neem Ka Thana	Semi-arid	27º73 N	75º78 E	W	565	2.78	6.1	72	65	244.09±0.16	285.22±0.21	529.31
R49	Jaipur	Jhalana	Semi-arid	27º73 N	75º78 E	W	565	2.82	6.4	65	62	236.22±0.30	240.03±0.23	476.25
R50	Jodhpur	CAZRI	Arid	26º11 N	73º42 E	C	301.5	1.44	5.9	43	47	162.96±0.34	179.26±0.34	342.22
R51	Jodhpur	Kailana lake	Arid	26º11 N	73º42 E	W	301.5	2.88	6.7	64	58	343.10±0.40	173.11±0.40	516.21
R52	Barmer	Mungeria hills	Arid, Desert	25º75 N	71º38 E	W	277	3.44	7.6	78	73	382.02±0.41	324.26±0.04	706.28
R53	Jaislmer	Dabra	Hyper- arid	26º92 N	70º90 E	W	209	3.56	7.8	65	63	455.21±0.30	450.15±0.41	905.36
R54	Jaislmer	Devikot	Hyper- arid	26º92 N	70º90 E	W	209	3.49	7.9	76	74	487.45±0.19	472.88±0.19	960.33
R55	Jaislmer	Akal Wood fossil forest	Hyper- arid	26º92 N	70º90 E	W	209	3.40	7.4	67	65	480.85±0.37	447.22±0.18	928.07
R56	Jaislmer	Pithla	Hyper- arid	26º92 N	70º90 E	W	209	3.48	7.6	69	67	475.82±0.29	470.02±0.21	945.84
R57	Bikaner	Palana	Hyper- arid	28º01 N	73º18 E	W	266	3.12	6.7	74	69	467.65±0.19	487.68±0.18	955.33
R58	Churu	Rajgarh	Hyper- arid	28º38 N	72º02 E	W	331.5	3.00	6.6	71	74	358.74±0.52	364.69±0.27	723.43
R59	Jhunjhunu	Khetri	Rocky tract, Inland drainage	27°52 N	75º46 E	W	500	3.56	6.5	67	63	373.98±0.35	388.62±0.31	762.6
R60	Jhunjhunu	Khetri	Rocky tract, Inland drainage	27°52 N	75º46 E	W	500	3.82	5.8	69	64	364.90±0.36	351.91±0.05	716.81
R61	Jhunjhunu	Khetri	Rocky tract, Inland drainage	27º52 N	75º46 E	W	500	3.90	6.1	78	64	328.88±0.39	318.78±0.06	647.66
R62	Jalore	Govindgarh	Arid, Desert	27º35 N	72º62 E	W	420	3.49	7.8	82	80	427.14±0.42	447.24±0.40	874.38
R63	Jalore	Jaswantpura	Arid, Desert	27º35 N	72º62 E	W	420	3.80	8.1	87	83	422.80±0.19	424.12±0.36	846.92
R64	Alwar	Prithripura	Flood prone	27º57 N	76º60 E	W	724	3.42	6.4	55	56	181.13±0.49	185.67±0.08	366.80
R65	Dungarpur	Baroda	Humid	28º83 N	73º72 E	W	701	3.12	6.4	56	59	171.23±0.36	182.99±0.26	354.22
R66	Dungarpur	Mandli	Humid	28º83 N	73º72 E	W	701	3.00	6.6	58	56	172.61±0.15	181.24±0.42	353.85
R67	Dungarpur	Balota	Humid	28º83 N	73º72 E	W	701	3.29	6.9	62	64	166.82±0.08	179.17±0.17	345.99
R68	Banswara	Bagidora	Humid	23º55 N	74º45 E	W	781	3.22	6.5	55	57	160.35±0.16	172.44±0.19	332.79
R69	Banswara	Kushalgarh	Humid	23º55 N	74º45 E	W	781	3.12	6.6	57	58	128.19±0.22	125.23±0.19	253.42
R70	Banswara	Dungra	Humid	23º55 N	74º45 E	W	781	3.04	6.4	43	48	120.82±0.44	116.99±0.19	237.81
R71	Banswara	Sarwa Kalan	Humid	23º55 N	74º45 E	W	781	3.11	6.3	42	43	118.35±0.28	111.74±0.28	230.09
R72	Sawai Madhopur	Ranthambor	Humid	25º98 N	76º36 E	W	800	3.44	7.5	76	74	347.10±0.32	282.60±0.34	629.70
R73	Hanumangarh	Kohla farm house	Trans-Gangetic Plains Region	29º58 N	74º32 E	C	225	2.28	6.3	43	39	132.51±0.21	140.55±0.19	273.06

Table 5 (continued)

Code	District	Specific location	Agroecological region	Latitude	Longitude	Status	Rainfall (mm/yr)	Height (m)	Girth (cm)	Canopy E-W (cm)	Canopy N-S (cm)	E Guggulsterone (μg g^{-1})	Z Guggulsterone (μg g^{-1})	Total guggulsterone (μg g^{-1})
R74	Hanumangarh	Nursery	Trans-Gangetic Plains Region	29°58 N	74°32 E	C	225	1.98	6.1	40	37	135.02±0.29	143.89±0.28	278.91
R75	Ganganagar	Nursery	Trans-Gangetic Plains Region	29°92 N	73°88 E	C	200	1.90	6.2	42	41	121.19±0.27	128.73±0.07	249.92

C – Cultivated; W - Wild

Repeatability

Accuracy data was used to determine the repeatability of the method. For total 9 determinations for each component (E and Z isomers) the values of mean % recovery and mean relative standard deviation (% RSD) were 99.6387 and 0.601 respectively. The value of mean recovery as well as mean and individual RSD (<2.0) verified the repeatability of the method.

Precision

The intraday and interday precision was determined in terms of % RSD (n=2). The 3 standard concentration samples were analyzed in triplicate on 2 different sessions to determine the intraday precision. The data obtained, indicated that % RSD for any sample was not more than 2 % and thus verified the precision.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were calculated on the basis of slope of the calibration curve and standard deviation of the response using the following formula;

$$\mathrm{LOD} = (3.3 * \text{Standard deviation for lowest concentration})/\text{Slope}$$
$$\mathrm{LOQ} = (10 * \text{Standard deviation for lowest concentration})/\text{Slope}$$

Results and discussion

Various chemical fingerprinting means are employed for quantification and also for quality control of herbal remedies including chromatographic, spectroscopic, thermogravimetric analysis, capillary electrophoresis and polarography techniques (Choudhary and Sekhon 2011). Among them HPLC is a highly efficient, robust and quick analytical method for quantitative estimation of desirable components with optimum resolution. It can also be exploited as a regular investigative method for testing purity of drugs in marketed products as well as for detection of resinous adulterants from related plants like *Mangifera indica* L., *Acacia nilotica* (L) Willd. Ex Delile, *Ficus religiosa* L., while trading pharmacologically important oleo-gum resins. No reports are available regarding quantitative estimation of guggulsterone isomers in wild collections of *C. wightii* using HPLC. However, the technique has been used for estimation of E and Z isomers of guggulsterone in gum resin exudates of *C. wightii* and *C. mukul*, and dietary supplements containing *C. mukul* guggulipids (Soni et al. 2010; Nagarajan et al. 2001; Musharraf et al. 2011). Preliminary results indicated that negligible amount of guggulsterone isomers were detectable in the leaf and root samples of *C. wightii*, therefore, only the

stem samples showing presence of substantial quantity of guggulsterone were taken up for further studies. The oleo-gum resin is mainly found in the stem only therefore, selective distribution of the resin and its components is expected (Kulhari et al. 2013).

The E and Z isomers of guggulsterone, individual as well as mixture, depicted a clear peak during separation at a retention time of 15.009 and 17.809 min., respectively. A calibration plot was obtained by plotting peak area against concentration of guggulsterone. A linear straight line was observed for guggulsterone E and Z standards using the regression equation Y=14296x–69744 and Y=11278x+43499 respectively. LOD and LOQ values for E isomer were 0.214 μg ml^{-1} and 0.649 μg ml^{-1} respectively, while for Z isomer they were 2.789 μg ml^{-1} and 8.454 μg ml^{-1}. The correlation coefficients for E and Z guggulsterones were 0.994 and 0.993 with a linear calibration graph (linearity range of 20–100 μg/ml) (Table 1). The value of mean recovery as well as mean and individual RSD (<2.0) verified the repeatability, accuracy and precision of the method (Tables 2, 3 and 4).Chemical profiling of all the 75 samples collected from various locations could elucidate the difference in guggulsterone content without interference of any other constituents at 250 nm wavelength (Table 5). Figure 1 depicts a representative HPLC chromatogram showing separation of guggulsterone isomers as well as other constituents in stem extracts of *C. wightii* samples. Concentration of guggulsterone E varied from 120.82 μg g^{-1} to 487.45 μg g^{-1} while that of guggulsterone Z varied from 111.74 μg g^{-1} to 487.68 μg g^{-1}. Concentration of guggulsterone was highest in Rajasthan, as compared to Haryana and Gujarat, due to water deficiency and adverse climatic conditions. Among different geographical locations highest concentration of E guggulsterone (487.45 μg g^{-1}) and Z guggulsterone (487.68 μg g^{-1}) was found in the samples collected from Devikot (Jaisalmer - Western Rajasthan) and Palana (Bikaner - North-Western Rajasthan) respectively, both belonging to hyper-arid agro-climatic region (Fig. 1). Least concentration of guggulsterone isomers viz. 118.35±0.28 μg g^{-1} (E isomer) and 111.74±0.28 μg g^{-1} (Z isomer) was found in wild accessions collected from Banswara (Southern Rajasthan). Central part of Rajasthan, such as Ajmer district, also reflected a significantly higher amount of E (270.43±0.33 μg g^{-1}) and Z (211.27±0.55 μg g^{-1}) guggulsterone. Total guggulsterone was also highest in sample collected from Devikot, Jaisalmer (960.33 μg g^{-1}) followed by that collected from Palana, Bikaner (955.33 μg g^{-1}), the two hyper-arid districts of Rajasthan. Major difference in concentration of the two isomeric forms was found in the sample collected from Jodhpur {E guggulsterone (343.10 μg g^{-1}); Z guggulsterone (173.11 μg g^{-1})} followed by the samples collected from Swai Madhopur, Ajmer and Chittorgarh districts of Rajasthan. While minor variation was observed in concentration of both the isomers in the samples gathered from Nakhatrana, Gujarat {E guggulsterone (160.00 μg g^{-1}); Z guggulsterone (161.83 μg g^{-1})} followed by Jawantpura (Jalore), (Kushalgarh, Dungra and Sarwa Kalan), Banswara, Rajasthan. Among the 26 districts having diverse (eleven) agro-climatic conditions, collections made from Trans-Gangetic plains (Mahendergarh), semi-arid-rocky tracts (Rajsamand and Ajmer), sub-humid (Chittorgarh, Bhilwara and Udaipur), humid (Swai Madhopur) and hyper-arid regions (Jaisalmer) were found to contain higher concentration of E isomer compared to Z guggulsterone. During quantification both the isomers exhibited a wide disparity which can be discerned on the basis of geographical inequality as well as on environmental factors, unambiguously. The recoveries of both E and Z isomers from different samples were found greater than 98 % in the present study, whereas it was >96 % in serum samples (Verma et al. 1998) and >90 % in *C. mukul* extract and dietary supplements (Nagarajan et al. 2001) while Akhade et al. (2013) reported 103.84 % recovery of Z guggulsterone in tablet formulations.

Both the isomeric forms of guggulsterone are interconvertible as was reported in callus and cell cultures of guggul (Ramawat et al. 2008). Agrawal et al. (2004a) also

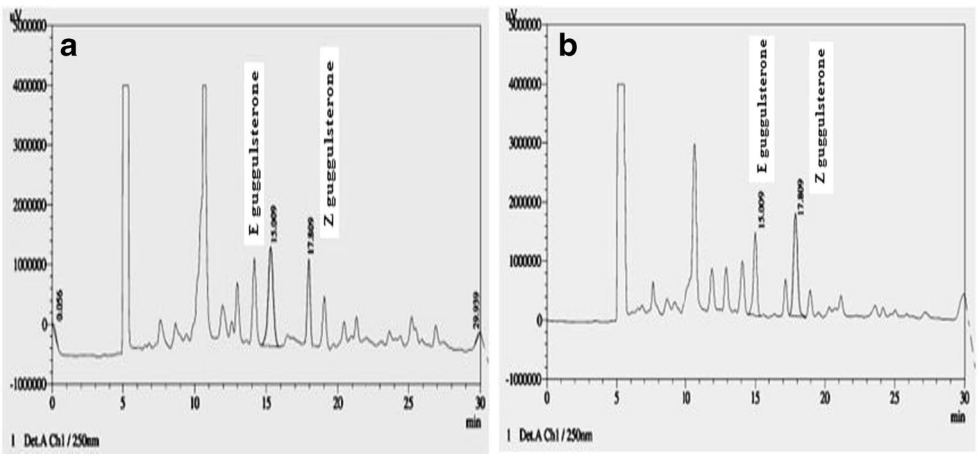

Fig. 1 Chromatogram of oleo-gum resin of *C. wightii* collected from (**a**) Devikot (Jaisalmer) and (**b**) Palana (Bikaner); two hyper-arid regions showing separation of various components including guggulsterone E (R_t : 15.009) and Z (R_t : 17.809)

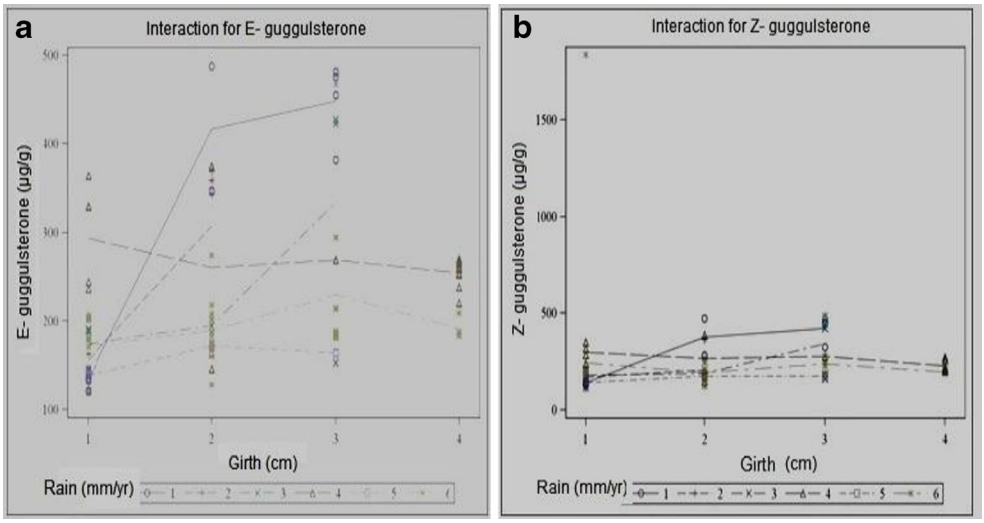

Fig. 2 Correlation between rainfall and concentration of guggulsterone (**a**) E isomer and (**b**) Z isomer

reported that significant variations are likely to occur in the component content of guggul oleo-gum resin depending upon the climatic conditions under which the plants are grown and the resin is harvested. Concentration of the bio-active agents has been found to be influenced by atmospheric factors (seasonal variation, geographical variation, average rainfall, and temperature), agriculture practices (planting strength, genotype of plant, time of sowing and harvesting period) and laboratory factors (chromatographic conditions, mobile phase composition, extraction solvents). Along with these factors guggul gum yield also depend on the age of the plant (Jain and Nadgauda 2013). Influence of these factors has also been monitored in other medicinal plants like *Lepidium sativum* L. (Nayak et al. 2009, 2012), *Plantago ovata* Forsk (Mann and Vyas 1996) and *Andrographis paniculata* (Burm. f.) Wall. ex Nees (Saxena et al. 2000). Quantitative estimation of methanolic seed extract of *L. sativum* through HPTLC exhibited an apparent variation in sinapic acid concentration owing to difference in date of sowing and harvesting period (Nayak et al. 2009). Highest efficiency of extraction of *andrographolide* derivatives was gained in methanol as compared to chloroform, ethyl acetate and ethanol extracts of *A. paniculata* (Saxena et al. 2000). Maceration under sonication was the most effective extraction method compared to maceration alone and its infusion with supercritical fluid extraction (after consideration of extraction yield/extraction time ratio) in HPLC analysis of coumarin in hydroalcoholic extracts of *Mikania glomerata* Spreng (Celeghini et al. 2001).

Production of oleo-gum resin is a stress induced phenomenon. Both biotic and abiotic factors including ecological (geographical and seasonal), individual plant performance (genotypes and morphotypes), pathogens and elicitors (methyl jasmonate, ethrel and salicyclic acid) as well as cultivation practices affect the production of secondary metabolite guggulsterone in intact plants as well as in tissue culture (see Kulhari et al. 2012; Suthar and Ramawat 2010). In the present investigation, a strong correlation was seen between average rainfall of the area and guggulsterone content; regions with lower rainfall exhibited higher amount of guggulsterone (Fig. 2). Duncan's multiple range test revealed a relationship

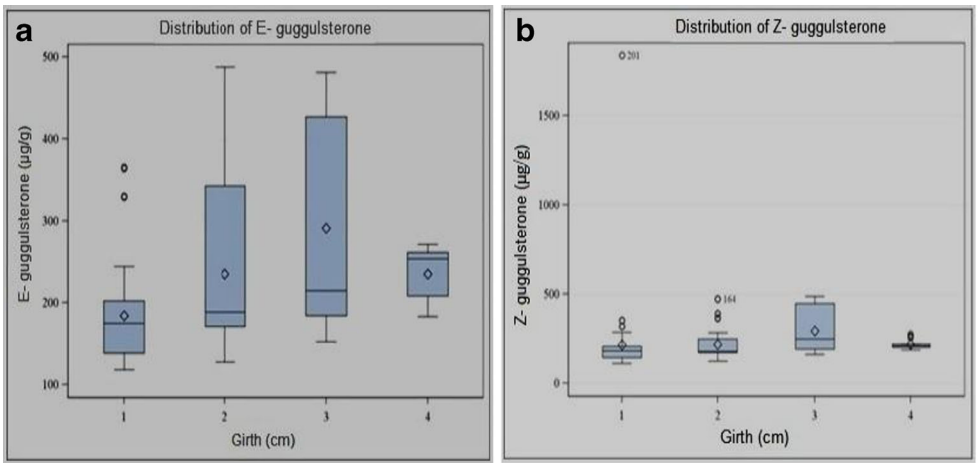

Fig. 3 Correlation between girth and distribution of guggulsterone (**a**) E isomer, and (**b**) Z isomer

between developmental stage of the plant and guggulsterone content. Mature plants with thick trunk produced higher guggulsterone content against nursery raised smaller plants (Fig. 3). These outcomes depicted that wild mature guggul plants (more than eight years of age) growing in adverse conditions or in rocky tracts had more guggulsterone content. Variation in guggulsterone content among accessions has also been reported by Kulhari et al. (2013); Soni et al. (2010) and Yadav et al. (1999). Soni et al. (2010) attributed the discrepancy in guggulsterone content to environmental factors like temperature and rainfall of the concerned geographical region; higher guggulsterone content was obtained during summer (May-July, highest in May) which gradually decreased in the rainy season (Aug-Oct) and was lowest in winter (Nov-March). In case of geographical locations northern, western and central part of Rajasthan showed maximum amount of guggulsterone whereas southern part of the state produced lower amount. They also reported that Z isomeric form was dominating over E guggulsterone however the same trend was not found in the present study. E and Z isomers were found in an approximate constant ration of 4:1 by Mathur and Ramawat (2007) while Kumar et al. (2006) reported 46.3 μg g^{-1} and 104.3 μg g^{-1} respectively of E and Z isomers in stem samples of *C. wightii*, and Musharraf et al. (2011) reported their concentration as 51.042 ng μL^{-1} and 28.399 ng μL^{-1} respectively in *C. mukul* extracts. Total guggulsterone yield was variable in different regions of same agroclimatic provinces (hyper-arid) viz. 2.291 w/w in Churu; 2.088 w/w in Bikaner and 1.871 w/w in Jaiselmer (Soni et al. 2010). Agrawal et al. (2004a) also reported that the content of guggulsterone produced in winters was very limited due to dormant phase of the plant while its production enhanced in summers.

The developed technique is a precise, specific and accurate method for estimation of guggulsterone content within a 17-min run using single reverse phase HPLC in *C. wightii* extracts. Present finding revealed the role of environmental factors on biosynthesis of guggulsterone isomers under natural conditions. By HPLC based fingerprinting the botanical identity of the plant can be linked with its biochemical profile as well as it allows for the determination of variations in the guggul resin component's content in different collected accessions grown and harvested at different climatic conditions. These methods would also be useful for comparing the resinous analysis in related plant species. The present work is unique in terms of wide collection from three hot spot biodiversity rich Indian states for quantification of these bioactive agents in stem samples. The given method is gainful as it showed good reproducibility with higher resolution, competence as well as separation of marker compounds. Additionally, no peaks of other constituents present in the extracts were found to interfere with that of the marker compounds, indicating no hindrance.

Identification of high guggulsterone producing lines will play an important role in designing mass propagation as well as conservation strategies. Reverse phase HPLC analysis evaluated that embryogenic callus is the best alternate for guggulsterone production in vitro (Kumar et al. 2006; Dass and Ramawat 2009). Tissue culture is the only available method for production of secondary metabolites via cell, callus culture and cloning of selected high yielding guggul varieties. However, cytodifferentiation in callus and cell cultures is a prerequisite for the production of secondary metabolites, which are produced in complex tissue systems like laticifers and resin canals (Ramawat et al. 2008). The superior genotypes identified can also be used in forward genetics for isolation of gene(s) responsible for guggulsterone production which can be integrated in to other plants or micro organisms for production of higher quality and quantity of guggulsterone. This in turn would reduce the pressure to undertaken commercial cultivation of *Commiphora wightii*, and will also reduce over-exploitation of this plant in the wild and will thereby complement the conservation process.

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