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High-performance liquid chromatographic method for fingerprinting and quantitative determination of *E*- and *Z*-guggulsterones in *Commiphora mukul* resin and its products

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Abstract

A high-performance liquid chromatographic method has been developed and validated for the fingerprinting (profiling) and quantitative determination of *E*- and *Z*-guggulsterones, the hypolipidemic agents in the gum-resin exudate of *Commiphora mukul*, currently marketed worldwide as hypocholesterolemic. The method involves extraction of the guggul-resin from either the raw exudate or compounded tablets (or capsules) with ethyl acetate, concentration of the combined extracts and chromatography on a reversed-phase C_{18} column using an acetonitrile–water gradient. The method has a validated quantitation range of 15–85 μg/ml for *E*-guggulsterone and 25–130 μg/ml for *Z*-guggulsterone with a precision of ±2% S.D. and a recovery of >99.5%. Standard curve correlation coefficients of 0.992 or greater were obtained during validation experiments. The method was applied to six commercial (OTC) products, all of which were found to contain significantly less (in most cases very little or none) of the claimed guggulsterones.

Keywords: Guggulsterones; Guggul-resin; Commiphora mukul

1. Introduction

Commiphora mukul (C. mukul) is a small thorny plant indigenous to the Indian subcontinent and parts of the Near East [1–3]. It produces a dense, oily resin identified in Sanskrit as guggulu, but in western texts is generally referred to by the somewhat shortened appellation of guggul [1,2].

The traditional uses of guggul-resin extract are well documented in the Ayurveda – the ancient system of traditional Indian medicine [1,4–6] – where it is prescribed to treat a variety of ailments including lipid-related disorders such as obesity and arteriosclerosis. In recent years, studies designed to elicit the scientific basis of these folk uses have demonstrated that guggul-resin extract possesses clinically verifiable hypolipidemic activity [4–6].

The reported distribution of hypocholesterolemic activity for various guggul-resin extracts as a function of resin refinement is summarized in Table 1. The cholesterol-reducing activity of the guggul-resin extracts can be traced to the two closely related steroidal ketones, *E*-guggulsterone and *Z*-guggulsterone (Fig. 1). However, related studies have demonstrated that other extract components which, either individually or collectively do not exhibit any

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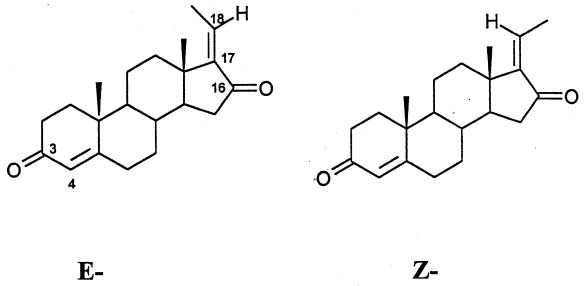
Table 1
Hypocholesterolemic activity of guggul-resin extracts as a function of resin refinement [1].

Fraction	Guggulsterone Content	Hypocholesterolemic Activity
Guggul-resin (crude)	~2%	+
↓ EtOAc Extract	4 - 4.5%	+
↓ Neutral Compounds in EtOAc Extract	4.2 - 4.7%	+
↓ Ketonic Compounds	35 - 40%	+
↘ Refined *E*-and Z-guggulsterone	100%	+
↓ Other Ketones	0%	-

hypocholesterolemic activity, but synergistically enhance the extracts' collective hypocholesterolemic activity beyond that observed for isolated *E*- and *Z*-guggulsterones [4].

Our investigations of the pharmacokinetic properties of these necessitated a broadly applicable method for analyzing these complex mixtures and, in particular, for quantifying the pharmacologically active components *E*- and *Z*-guggulsterones. All previously reported procedures [1,7] afforded incomplete resolution of the major components (guggulsterones and guggulsterols) even after multiple combined preparative TLC isolations and HPLC determinations.



E- **Z-**

Fig. 1. Structures of *E*- and *Z*- guggulsterones according to Sukh Dev [1].

The HPLC-method reported by Singh et al. [8] is oriented only to blood plasma analysis of guggulsterones. The method is developed and tested on guggulsterones alone, using hexane as a means of extraction. The use of PDA is advantageous, since the UV-spectra of guggulsterones are specific and can easily be differentiated from the other plasma components. The high concentration of acetonitrile (MeCN) in water as a mobile phase (65:35, v/v) in Singh's method is useful only for single steroid compounds as an analyte. The method however is not applicable to the complicated mixture of the Guggul resin ethyl acetate extract (GREAE) used to produce guggul-resin based drugs and nutritional supplements, because of the complexity of steroid type compounds with HPLC behavior and UV spectra similar to the guggulsterones in the GREAE.

Hung et al. [9] reported a qualitative procedure for profiling the constituents of several resin-based extracts, including that of *C. mukul*. This method, however, does not afford sufficient component resolution to provide the precision and accuracy required for reliable quantitation studies.

Numerous suppliers of guggul-resin extract or of formulated guggul-resin extract used in nutraceutical supplements utilize various unreported procedures to assay the guggulsterones content in their product. These methods include utilization of HPLC methods [10] or a spectrophotometric assay (λ=327 nm) [11]. Our studies indicated that none of these methods provide reliable, accurate assays of the levels *E*- and *Z*-guggulsterones in guggul-resin or its products. Accordingly, we developed a broadly applicable, high-sensitivity method for the quantitative analysis of guggul-resin extract and, in particular, of its principal bioactive components, *E*- and *Z*-guggulsterones.

2. Experimental

2.1. Materials

Crystalline reference samples of *E*- and *Z*-guggulsterones and authentic *Commiphora mukul* (guggul) resin were provided by Professor Sukh Dev, Delhi University, India. Several commercial suppliers pro-

vided botanically authenticated raw guggul resin. HPLC analysis of *E*- and *Z*-guggulsterones revealed assays of 97.0 and 96.4%, respectively. Seven over-the-counter brand-name nutraceutical supplements, claiming to contain guggul-resin, were purchased at retail outlets within the US. Ethyl acetate (HPLC grade) was purchased from Spectrum Quality Products (New Brunswick, NJ, USA). Methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Fisher (Fair Lawn, NJ, USA).

2.2. Instruments

HPLC experiments were conducted on two different instruments to check the ruggedness of the method: a Perkin-Elmer Integral 4000 and a Waters Alliance. The first series of experiments were carried out on a Perkin-Elmer Integral 4000 (Perkin-Elmer, Beaconsfield, UK) equipped with a photo-diode array (PDA) detector. The system included a Legend-4 PC (Packard Bell, Westlake Village, CA, USA) for system control, data collection, and analysis. Data were recorded on an Oki Model GE5253A printer (Oki America, Mount Laurel, NJ, USA). A second series of HPLC experiments were performed on a Waters Alliance (Waters, Milford, MA, USA.) equipped with PDA detector and a MILLENIUM 2010 Workstation.

LC–MS determinations were performed on two different instruments. One series of analyses was carried out on an Alliance LC–MS (Waters) equipped with tandem 3-D PDA and 3-D TM electron impact (EI) detectors. A MILLENIUM 2010 Workstation was used for system control, data collection, and analysis. Electron impact spectra were obtained using an ion-spray tip (70 eV). The effluent stream was not split.

Another series of analyses was performed on a Varian 9012 LC–MS (Varian, Chicago, IL, USA) equipped with a variable wavelength UV detector in tandem (no effluent splitting) with a Micromass Platform (Micromass, Altricham, UK) atmospheric pressure CI detector.

Full-scan mass spectra were obtained on both instruments while continuously scanning from $m/z=$ 100 to $m/z=600$ in increments of 0.4, using a dwell time of 1.0 ms. All analyses were automated by integration of the HPLC with the MS control computer.

2.3. Procedures

Standard solutions of guggulsterones were prepared by accurately weighing quantities of *E*- and *Z*-guggulsterones (~1 mg ±1 μg) into separate 10-ml volumetric flasks, dissolving each sample in 3 ml of ethyl acetate (EtOAc) and diluting to volume with methanol.

Resinous extract (25–30±0.1 mg) was accurately weighed and placed in a 10-ml volumetric flask, dissolved with 2 ml of ethyl acetate, and the volume adjusted with methanol.

Commercial tablets, or the contents of a commercial capsules, or commercial powdered extract were finely ground in an agate mortar, after which a weighed quantity (2–3 g) was transferred to a 30-ml centrifuge tube equipped with a PTFE-coated magnetic stirrer bar. Ethyl acetate (20 ml) was added and the mixture stirred for 30 min., then centrifuged (10 min, 2200 rpm). The clear supernatant solution was transferred to a tared 100-ml RB flask and the contents of the flask concentrated to dryness on a rotary evaporator at 40°C. A second and a third extraction yielded small additional amounts of resin; however, further extraction failed to provide any additional resin.

The combined residue was then dried in a vacuum oven (40°C/1 mm Hg). A portion of the dried extract (25~30 mg accurately weighed) was transferred to a 10-ml volumetric flask, dissolved in 2 ml of EtOAc, and adjusted to volume with methanol.

Two different HPLC methods were used to accommodate differing analytical requirements. For qualitative identification of the resinous components in commercial products, a procedure (Method I) was developed which allows fingerprinting for comparison with authentic *C. mukul* resin extract. For quantitative determination of the bioactive components *E*- and *Z*-guggulsterones, a second procedure (Method II) was used. General parameters that apply throughout in both the methodologies include: an Adsorbophere™ HS-C_{18} reversed-phase column (150×4.6 mm; 5 μm) (Alltech, Deerfield, IL, USA); 20 μl/injection sample size and UV monitoring of effluent at 245 nm and 327 nm (Method I). For

reasons discussed below, Method II assays were performed only at 245 nm. An acetonitrile (A) and water (B) gradient was the mobile phase for both methods.

Method I – Solvent program (PE 4000 System): 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. Flow-rate=1.0 ml/min.

Method II – Solvent program (Waters' Alliance): 0–30 min: 36% A and 64% B; 30–50 min: A=36 to 45% and B=64 to 55% (linear, 6); 50–56 min: 45% A and 55% B; 56–66 min: 100% A (curve, 1); 66–67 min: A=100 to 36 and B=0 to 64% (linear, 6); 67–76 min: 36% A and 64% B. Flow-rate=1.2 ml/min.

The use of other HPLC system is possible if the system is capable of reproducing the gradient slope discussed here. A validation procedure for Method II is described below for quantitation work.

Procedure validation (Method II):

(i) System suitability was established by calculating and tabulating the resolution and tailing factors for *E*- and *Z*-guggulsterones. These factors, obtained from the HPLC profiles resulting from three sequential injections of a resolution solution consisting of 42 μg of *E*- and 63 μg of *Z*- guggulsterone per milliliter, were calculated as follows:

$$R_{(\text{resolution})} = \frac{2(t_2 - t_1)}{w_1 + w_2},$$

where t_1 is the average retention time of component 1, t_2 is the average retention time of component 2, w_1 is the average width at baseline of component 1 and w_2 is the average width at base line of component 2.

$$T_{(\text{tailing})} = \frac{w_{0.05}}{2f}$$

where $w_{0.05}$ is the width of the peak at 5% of its height, f is the distance between the peak maximum and its leading edge, measured at 5% of the peak height.

(ii) Method linearity was demonstrated by determining a calibration curve and calculating the regression coefficient (r^2) and intercept (y) based on data obtained from three sequential injections of each of the following five standard solutions containing, respectively, the following concentrations of *E*- and *Z*-guggulsterones: (1) 84+126 μg/ml, (2) 67.2+100.8 μg/ml, (3) 50.4+75.6 μg/ml, (4) 33.6+50.4 μg/ml and (5) 16.8+25.2 μg/ml.

(iii) Method specificity was demonstrated by establishing the homogeneity of the *E*- and *Z*-guggulsterones peaks by mass spectrometry over the entire profile of each peak.

(iv) Method accuracy was determined by calculating the mean percentage recovery [average of the percentage recoveries for solutions (2), (3), and (5)] for each guggulsterone, determined from the calibration curve [obtained in (ii)] using the mean peak areas for *E*- and *Z*-guggulsterones resulting from three sequential injections of solutions (2), (3), and (5), according to the equation [12]:

$$\%\ \text{Recovery}_{\text{Sol.i}} = \frac{\text{Conc}_{\text{Sol.i}}\ (\text{calculated from the calibration curve}) \times 100}{\text{Concentration as prepared}}$$

(v) Method ruggedness was established by repeating the entire validation procedure by a second analyst. The robustness was proved by using a second HPLC system.

3. Results and discussion

The HPLC profile provided by Method I (Fig. 2) affords a valuable fingerprint for identifying authentic *C. mukul* (guggul) extract, which is noticeably different from those observed for the resinous extracts of various related plant species [9]. As such, Method I affords a procedure for screening both raw materials and formulated products for the authenticity of the used resin. Selected peak assignments, specifically that of *E*- and *Z*-guggulsterones were made by: (1) comparison of individual peak retention times with those observed for authentic *E*- and *Z*-guggulsterones and (2) by comparing the UV and mass spectra of individual components to that of authentic *E*- and *Z*-guggulsterones.

The UV profile of the components that elute during HPLC analysis of *C. mukul* (guggul) resin extract (Method I, Fig. 2) with the following retention times: 27.4 min (*E*- guggulsterone), 30.6 min

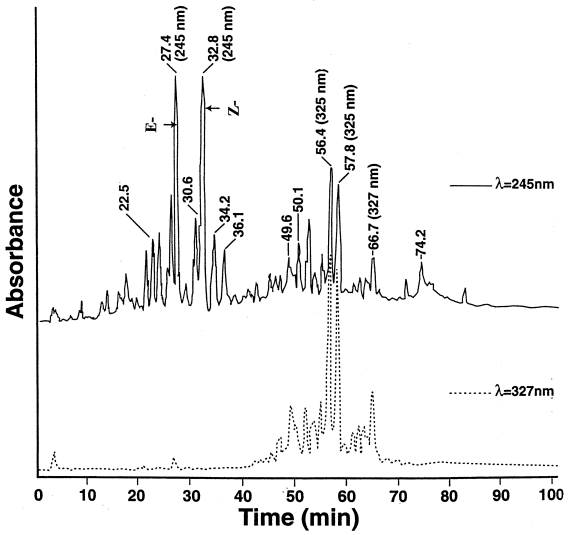


Fig. 2. HPLC profile of *C. mukul* (guggul) resin extract (Method I) showing the retention time and λ_{max} of the major components simultaneously recorded at 245 nm (———) and 327 nm (. . . ....) using a PDA detector. Chromatography conditions: See text.

(unknown), and 32.8 min (*Z*-guggulsterone) are shown in Fig. 3a. The UV profiles of both guggulsterones are equivalent ($\lambda_{max}=245\pm1$ nm). For comparison, Fig. 3b shows the UV profile of some of the major slower-eluting components, i.e. those with a retention time between 49 min and 65 min (Fig. 2). These latter spectra exhibit strong similarities including a $\lambda_{max}=327$ nm. Collectively, these data demonstrate the importance of using multiple monitoring wavelengths for determining system as related to reliable quantitation of *E*- and *Z*-guggulsterones in *C. mukul* (guggul) resin extract.

The HPLC profile provided by Method II (Fig. 4) affords the best separation of the active guggulsterones and makes the method applicable for their quantitative determination. Fig. 5 shows the mass spectra of the two guggulsterones and their vicinal compounds.

As is evident from Fig. 5, the separated peaks are free of any contaminant, since only one pseudomolecular ion $[M+H]^+$ at $m/z=313.3$ is present in both the HPLC-separated guggulsterone peaks ($M_R=312.3$). The ion at $m/z=354.3$ is assigned to $[M+MeCN]^+$ formed with acetonitrile used in the mobile phase. The HPLC-assay method described here is

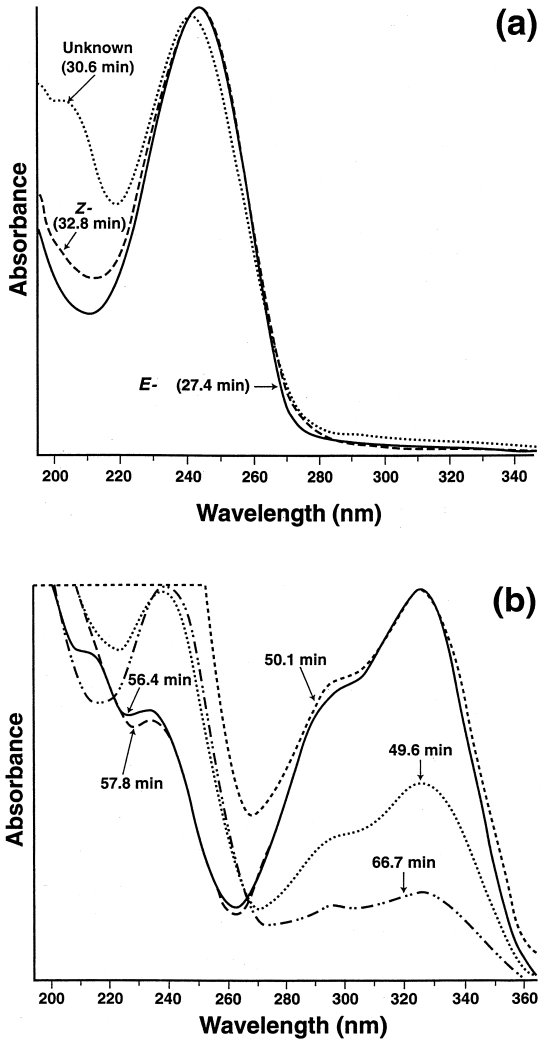


Fig. 3. UV Profiles of (a) *E*-guggulsterone ($t_R=27.4$ min) and *Z*-guggulsterone ($t_R=32.8$ min) and an unknown ($t_R=30.6$ min) and (b) several of the slower moving components ($t_R=49.6$, 50.1, 56.4, 57.8 and 66.7 min) recorded during the analysis of *C. mukul* (guggul) resin extract (Method I, Fig. 2) using a PDA detector.

selective for *E*- and *Z*-guggulsterones determination in a natural mixture of the plant exudate and its different extracts.

The method is applicable for guggulsterones determination in resin or powder with ~1% *E*- and *Z*-content as well as for ~1 mg per tablet *E*- and *Z*-content.

The absolute recovery for four extractions of *C. mukul* (guggul) resin averaged is ≥99.5% The lower

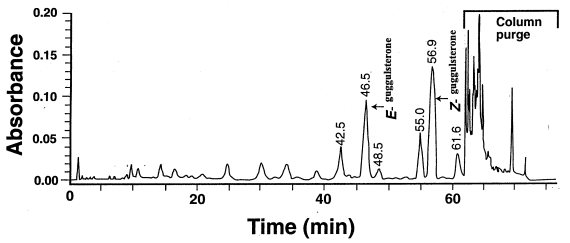


Fig. 4. HPLC profile of *C. mukul* (guggul) resin extract (Method II). Peak assignment is based on a comparison of component retention time and UV and LC-mass spectra with those of authentic *E*-1 and *Z*-1. (PDA detector, $\lambda = 245$ nm). Chromatography conditions: see text.

limit of guggulsterone quantitation is established as 4 μg/ml.

The assay for *E*-guggulsterone [Method II (Fig. 4)] is linear over the concentration range 15–85 μg/ml while the corresponding assay for *Z*-guggulsterone is linear over the concentration range 25–130 μg/ml. Correlation coefficients of 0.992 or better were obtained throughout the validation. The linearity and accuracy parameters for Method II are summarized in Table 2. Each five-point calibration curve was performed in triplicate. Resolution and tailing performance parameters associated with validation of Method II are summarized in Table 3.

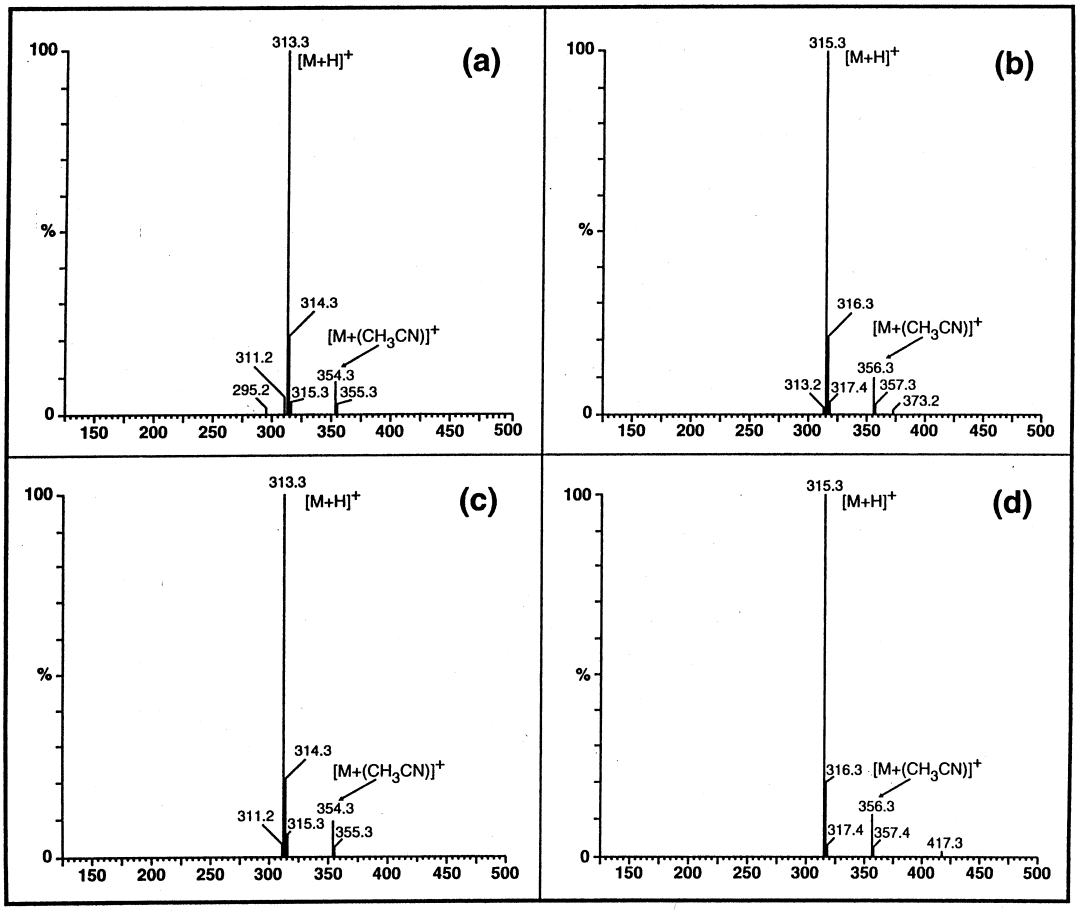


Fig. 5. Liquid chromatography–mass spectra (AP–CI) of selected peaks of *C. mukul* (guggul) resin extract recorded during analysis as described in Fig. 4. Spectra shown correspond to components with retention times of (a) 46.5 (*E*-guggulsterone); (b) 55.0 (unknown); (c) 56.9 (*Z*-guggulsterone) and (d) 61.6 (unknown) min.

Table 2
Summary of linearity and accuracy parameters for Method II

System	Guggulsterone	Linearity					Accuracy	
		r^2	*y*-Intercept		Range (μg/ml)	%SD	%Recovery	%SD
			Area	%				
Waters	*E*-	0.9997	24719	1.9	15–85	0.39	102.7	0.13
	Z-	0.9995	79294	2.1	25–130	0.24	103.9	0.48
PE	*E*-	0.9971	676.9	1.8	15–85	0.55	100.8	1.85
	Z-	0.9917	1157	1.9	25–130	0.27	100.0	2.26

Table 3
Summary of resolution and tailing performance

Parameter	HPLC system	
	Waters	Perkin-Elmer
Resolution	$R_{(mean)}$	$R_{(mean)}$
E-Guggulsterones	1.50	1.36
Z-Guggulsterones	1.36	1.21
Tailing Factor	$T_{(mean)}$	*T*(mean)
E-Guggulsterones	1.02	1.502
Z-Guggulsterones	1.04	1.356
%S.D. = R.S.D.		
E-Guggulsterones	0.28[a]	1.34[a]
Z-Guggulsterones	0.99[a]	0.53[a]

[a] Average of three values.
Parameters associated with validation of Method II.

The HPLC methods described above were used to separate, identify (Fig. 4) and assay the bioactive components, *E*- and *Z*-guggulsterones, present in commercial guggul-resin extract (i.e. the resin extract prior to its formulation and tableting or encapsulating) as well as in various OTC tablets/capsules containing formulated guggul-resin extract. The quantitative studies of various *C. mukul* based resin extracts and formulated products as summarized in Table 4 show that the content of *E*- and *Z*-guggulsterone in these materials varies widely, and is always significantly less than claimed for both of the guggulsterones.

The data obtained from the raw materials and the tablets/capsules used as nutraceuticals clearly show

Table 4
Quantitative studies of various *C. mukul*-based resin extracts and formulated products

Sample type and No.	Quantity of extracted resin (mg)		Guggulsterones content (*E*- + *Z*-)	
	Claimed	Found	Claimed	Found
Raw resins				
1. XC-008	–[a]	–	20.0%	2.8%
2. XC-264	–	–	5.9%	3.8%
3. XC-272	–	–	3.0%	0.57%
4. XC-333	–	–	3.2%	2.1%
5. XC-334	–	–	3.2%	2.2%
6. XC-343	–	–	10–12%	0.92%
Formulated products				
1. XC-89-09	250	125	25 mg	Negligible
2. XC-89-11	250	147	25 mg[b]	7.8 mg
3. XC-89-13	–	150	25 mg[b]	7.4 mg
4. XC-89-66	–	–	25 mg	7.8 mg
5. XC-159	340	117	Unreported	None
6. XC-187	–	–	25 mg	5.5 mg
7. XC-320	–	–	25 mg	0.91 mg

[a] Not available.
[b] Supplier reported an ‘equivalent’ rather than an exact guggulsterone content.

the lack of quantitative analysis at all (no certificate of analysis) or the use of incorrect methods of analysis. The methods used (when supplied) are non specific for *E*- and *Z*-guggulsterones, even when their presence and quantity is claimed. For example when UV-spectrophotometry at 327 is used [11], it detects only the most nonpolar components of the resin (Fig. 3b), not the active guggulsterones which do not absorb in this wavelength region (Fig. 3a). HPLC methods reported earlier [10] leads to poorly separated, contaminated guggulsterone peaks or a contaminated peak where the guggulsterones are only a small part of the peak.

Some of the results of the tablets/capsules analysis show incorrectly prepared formulations since the extracted resin is much less than its claimed content. In the case of XC-320 the claim is based on an incorrect certificate of analysis of the raw material used (XC-343) which is quantified by UV absorption at 327 nm.

The sample XC-89-09 and XC-159 are examples where the method is used as identity criteria. The HPLC profile of XC-89-09 shows negligible guggulsterone peaks and profile intensities. This product is probably based on a mixture primarily of other resins used with a small amount of guggul resin extract. The sample XC-159 does not contain any guggul resin extract!

4. Conclusion

Two HPLC procedures are developed for the fingerprinting and quantitation with high sensitivity. The methods are selective, accurate and reproducible for the two biologically active components *E*- and *Z*-guggulsterones present in *Commiphora mukul* crude resin extracts and final products (tablets, capsules). The extraction procedure used ensures the quantitative isolation of the guggul resin components from its raw material intermediates as well as from its tablets and capsules. HPLC-identification method (fingerprinting) is described which allows for the determination of variations in the guggul resin component's content in products grown and harvested at different climatic conditions. These methods would also be useful for comparing the resinous extracts of related plant species.

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