ORIGINAL RESEARCH PAPER

# Combination of high-performance thin-layer chromatography and liquid chromatography–quadrupole time-of-flight–tandem mass spectrometry analysis: a promising analytical tool for discrimination between oleo-gum resin of raw and purified *Commiphora wightii*

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## Abstract

Guggulu (*Commiphora wightii* oleo-gum resin) is exported in more than 42 countries including developed countries like the United Kingdom and the United States of America. The demand for guggulu is more than its production. Various purification processes have been mentioned in Ayurvedic classical literature which are not explored well yet, so it is impossible to discriminate between purified and raw guggulu. In the present study, an effort was made where guggulu was purified by using cow urine and changes were analyzed by using high-performance thin-layer chromatography (HPTLC) and liquid chromatography–quadrupole time-of-flight–tandem mass spectrometry (LC–QTOF–MS/MS). In HPTLC analysis, three new bands ($R_F$ 0.31, 0.68, 0.74) and twelve other bands were observed including the bands of marker compounds guggulsterone E ($R_F$ 0.81) and Z ($R_F$ 0.85) at 254 nm. In LC–QTOF–MS/MS analysis, two new peaks at Rt 5.00 and 16.21 min were observed in cow urine-purified guggulu. After purification, all the peaks were resolute well which shows the effects of purification. Overall, in LC–MS/MS study, metabolites, viz*.,* 5-(13′Z-nonadecenyl)resorcinol (Rt 8.31), 8*β*-hydroxy-3,20-dioxopregn-4,6-diene (Rt 10.33), guggulsterone E (Rt 12.99), guggulsterone Z (Rt 13.28), guggulsterol I (Rt = 15.19), mangiferolic acid (Rt = 15.27), 20(*S*),21-epoxy-3-oxocholest-4-ene (Rt = 15.75), and guggulsterol II (Rt 16.23) were identified. The present study reports the first time a comparative analysis of raw guggulu and cow urine-purified guggulu by using advanced analytical tools like HPTLC and LC–QTOF–MS/MS. This study may be helpful in the standardization of and quality control of raw guggulu and cow urine-purified guggulu used in various formulations of guggulu.

## Abbreviations

| | |
|---|---|
| ICH | International Council for Harmonisation |
| HPTLC | High-performance thin-layer chromatography |
| RA | Rheumatoid arthritis |
| $R_F$ | Retention factor |
| UV | Ultraviolet |
| SD | Standard deviation |
| RSD | Relative standard deviation |
| rpm | Revolution per minute |
| GC | Gas chromatography |
| LC–MS | Liquid chromatography–mass spectrometry |
| CCRAS | Central Council for Research in Ayurvedic Sciences |
| Shodhana | Purification |
| Gowmutra | Cow urine |

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# 1 Introduction

It is a well-known fact that many plants are the basis of modern medicine. Well-known drug molecules like taxol, aspirin, and morphine originate from plants or the natural system [1–9]. As per the World Health Organization (WHO) reports, for primary healthcare, more than 70% of the world population relies on natural resources [10, 11]. Recent studies report the important role of traditional medicines in combating coronavirus disease 2019 (COVID–19) [1]. To certify the safety of consumers and to gain their faith, all the drugs, either herbal or modern, need standardization [8, 10]. Recently, for the global acceptance of herbal drugs, WHO and European Agency for the Evaluation of Medicinal Products (EMEA) have released guidelines for the standardization of herbal drugs [12–15].

Guggulu, an important Ayurvedic drug used in various herbal formulations, is an oily resin produced from *Commiphora mukul* (Arn.) Bhandari [5, 16, 17]. The versatile applications of guggulu and their polyherbal formulations have been mentioned in various Ayurvedic pharmacopoeias including the Ayurvedic Pharmacopoeia of India (API) [16–20]. It is used to treat a variety of disorders or ailments like obesity, COVID-based obesity [21], hyperlipidemia [22–24], inflammation [5], cardiovascular diseases [25], rheumatoid arthritis [23], cancer [26, 27], and arteriosclerosis [5]. The major biological active compounds of guggulu are two ketosteroids, viz*.,* guggulsterone E and guggulsterone Z [5, 16]. In the Indian medicine system, guggulu is recommended to be used after being processed or purified [5, 16]. The recommended media for the guggulu shodhana are gomutra (cow urine), triphala kashaya (decoction of triphala), vasapatra kasaya (decoction of *Adhatoda vasica* leaves), vasapatra savrasa (aqueous extract of *Adhatoda vasica* leaves), Godugdha (cow milk), and water [15]. Shodhana or purification is used to remove the foreign matters and to reduce toxicity [5, 16, 28].

Various chromatographic analytical methods like high-performance liquid chromatography (HPLC) [29–32], liquid chromatography−mass spectrometry (LC−MS) [33–35], high-performance thin-layer chromatography (HPTLC) [36, 37], and gas chromatography−mass spectrometry (GC−MS) [5] have been reported by various research groups for the quantification of guggulsterone E and Z. Moreover, there is no HPTLC cum LC−MS analytical method to identify the effect of purification on guggulu.

The aim of this work is to optimize the HPTLC method for the identification of guggulsterone E and Z and confirmation and identification of major phytochemicals and effect of shodhana by LC−MS analysis. Moreover, quality control parameters like loss on drying, pH, total ash, acid-insoluble ash, water-extractive value and ethanol-extractive value were performed as per the guidelines of API [18–20].

# 2 Experimental

## 2.1 Materials

Marker compounds guggulsterone E and guggulsterone Z (Natural Remedies Ltd., Bangalore, India; purity: 98%) were used. Solvents like toluene, ethyl acetate, and methanol were of analytical reagent (AR) grade. TLC plates $60F_{254}$ (Merck Ltd., Bangalore, India; purity: 98% or AR grade) were used in the present study. The authentic materials of guggulu were collected from Rajasthan (longitude of 76.41 and latitude of 27.25), India, by the Botanist of the Regional Ayurveda Research Institute, Jaipur, Rajasthan, in May 2021. Fresh gowmutra (cow urine) was collected from local gowshala (cow diary farm), Jhansi, India.

## 2.2 Guggulu purification (shodhana)

The raw sample of guggulu was checked for the foreign matters which were removed by physical observation-based method. To purify guggulu, an amount of 3 kg guggulu was made into small pieces and kept in a vessel, then 10 L gomutra was added. The vessel was kept over medium flame and stirred continuously to dissolve the guggulu content. The dissolved guggulu was filtered through muslin cloth. The filtrate was kept in a vessel and heated on low flame (60 °C) to get semisolid mixture of shodhita (purified) guggulu.

## 2.3 Analysis of physicochemical parameters

The quality control parameters of guggulu and shodhita guggulu were analyzed as per the Ayurvedic Pharmacopoeia of India [18–20]. Quality control parameters like loss on drying, pH, total ash content, acid-insoluble ash content, water-extractive value, and ethanol-extractive value were analyzed. The ultraviolet−visible (UV−VIS) spectra of methanolic extracts of guggulu and shodhita guggulu were recorded by using methanol as blank.

## 2.4 HPTLC instrumentation and chromatographic conditions

CAMAG (Muttenz, Switzerland) HPTLC instrument, syringe (100 μL; Hamilton, Bonaduz, Switzerland), and glass twin-trough chamber were used for the HPTLC profiling of guggulu, shodhita guggulu, and marker compounds. Methanolic extracts (10 μL) of guggulu and shodhita

guggulu and methanolic standard solutions (5 μL) were applied on aluminum plate pre-coated with silica gel 60 F$_{254}$ of 0.2 mm thickness (Merck) using an applicator CAMAG Linomat 5 and software visionCATS 2.5. The TLC plate track number details are track 9: guggulu (G), track 10: shodhita guggulu (SG), track 11: guggulsterone E (GE), and track 12: guggulsterone Z (GZ).

The plate was developed in glass twin-trough chamber pre-saturated (for 15 min) with the mobile phase chloroform−ethyl acetate−formic acid−acetic acid (7.5:2.25:0.5:0.5, V/V). The developed plate was visualized at 254 nm and 366 nm by using CAMAG visualizer. The plate was derivatized with anisaldehyde−sulphuric acid reagent and heated at 105 °C till the development of visible spots. The visible spots were capture by using CAMAG visualizer. The developed visible spots of the derivatized plate were scanned using CAMAG Scanner 4 at 514 nm. The $R_F$ values and densitograms were recorded by using the CAMAG visionCATS 2.5 software and final report was generated. Images were captured by keeping plates in photo-documentation chamber.

*Stationary phase* TLC silica gel 60 F$_{254}$ (Merck).

*Developing solvent* toluene−ethyl acetate−acetone−formic acid: (8.5:0.5:1:1, V/V).

*Saturation time* 15 min at $25 \pm 2$ °C.

*Test solutions* 100 mg/mL methanolic solutions of guggulu and shodhita guggulu were prepared. To prepare the methanolic solutions, an aliquot of 100 mg sample was dissolved in 100 mL methanol, sonicated for 10 min at 25 °C, centrifuged at 10,000 rpm for 5 min, and supernatant kept in glass vials for HPTLC analysis.

*Standard solution* the standard solutions of guggulsterone E and Z of concentration 1 mg/mL were prepared in methanol.

## 2.5 LC−QTOF−MS/MS instrumentation and chromatographic conditions

### 2.5.1 Sample preparation

To prepare the extracts of samples, all the samples were grounded by using mixture grinder. All the samples were passed through a sieve of mesh size 80 mm. To prepare the sample extracts, 100 mg of sample and 100 mL methanol were taken in beaker, sonicated for 10 min at 25 °C, and filtered by using HPLC syringe filters (size 45 micron).

### 2.5.2 Instrumentation and chromatographic conditions

Metabolite profiling was done by using liquid chromatography–quadrupole time-of-flight–tandem mass spectrometry (LC−QTOF−MS/MS) in positive ion mode (electrospray ionization, ESI$^+$). The 5-μL solution of each sample extract

was injected at a flow rate of 0.300 mL/min to 1290 Infinity ultra-high-performance liquid chromatography (UHPLC) system (Agilent Technologies, Santa Clara, CA, USA) coupled to a QTOF/LC−MS (Agilent Technologies). A Hypersil column Gold C$_{18}$, 100 × 2.1 mm, 3 micron, was used. The solvent composition was $A = 100\%$ water with 0.1% formic acid and $B = 100\%$ acetonitrile with 90% acetonitrile + 10% water with 0.1% formic acid. The run time of experiment was 20 min with the following steps: 1 to 20 min, solvent $A = 95\%$ and $B = 5\%$; 21 to 25 min, solvent $B = 100\%$; 26 to 30 min, solvent $A = 95\%$ and $B = 5\%$. The compound databases, namely PubChem, KEGG Compound and ChemSpider, were used to search the metabolites.

## 2.6 Statistical analysis

All the results were performed in triplicate and expressed as mean. The Student's *t* test and one-way ANOVA (analysis of variance) tests were applied to check the significant difference between control *versus* sample or treated *versus* untreated group at $p < 0.001$.

## 3 Results and discussion

### 3.1 Guggulu purification (shodhana)

During the shodhana process of guggulu, the following facts were observed: (i) initially the liquid was light brown in color and subsequently turned into dark brown; (ii) evaporation was started at 70 °C, which was aggravated on stirring; and (iii) the maximum temperature was recorded up to 90−100 °C. On average, semisolid guggulu was prepared after 3 h (avg. for three batches). The final yield of guggulu (semisolid) obtained was 680 g (avg. for three batches). The final quantity of purified guggulu residue obtained was 320 g (avg. for three batches). During the purification, it was mandatory that all vessels were non-reactive and cleaned properly before use. During boiling, the liquid should be stirred continuously. Care should be taken to avoid direct product contact.

### 3.2 Analysis of physicochemical parameters

Table 1 shows that foreign matter, loss on drying or moisture content, pH, ethanol-extractive value, total ash, and acid-insoluble ash of guggulu and shodhita guggulu were significantly different (at $p < 0.001$). This significant variation in the moisture content was due to the interaction of guggulu with water molecules during its shodhana process. This significant variation in pH might be due to the reduction in the acidic metabolites of guggulu during its shodhana process. It is documented that the shodhana process helps

**Table 1** Physicochemical parameters and HPTLC analysis of guggulu and shodhita guggulu

| Test(S) | Results ± SD<br>Guggulu | Results ± SD<br>Shodhita guggulu |
|---|---|---|
| Foreign matter (% *w/w*)** | 0.10 ± 0.014 | 0.00 ± 0.000 |
| Loss on drying (% *w/w*)** | 5.08 ± 0.818 | 9.55 ± 1.027 |
| pH (10% solution)** | 5.10 ± 0.623 | 7.44 ± 0.879 |
| Water-extractive value (% *w/w*)* | 61.08 ± 3.434 | 63.56 ± 4.026 |
| Alcohol-extractive value (% *w/w*)** | 50.73 ± 2.237 | 26.50 ± 1.957 |
| Total ash value (% *w/w*)** | 2.00 ± 0.177 | 16.08 ± 1.012 |
| Acid-insoluble ash value (% *w/w*)** | 0.16 ± 0.072 | 3.38 ± 0.443 |
| UV−visible ($\lambda_{max}$ in methanol)* | 324 ± 2 nm | 324 ± 2 nm |

HPTLC analysis

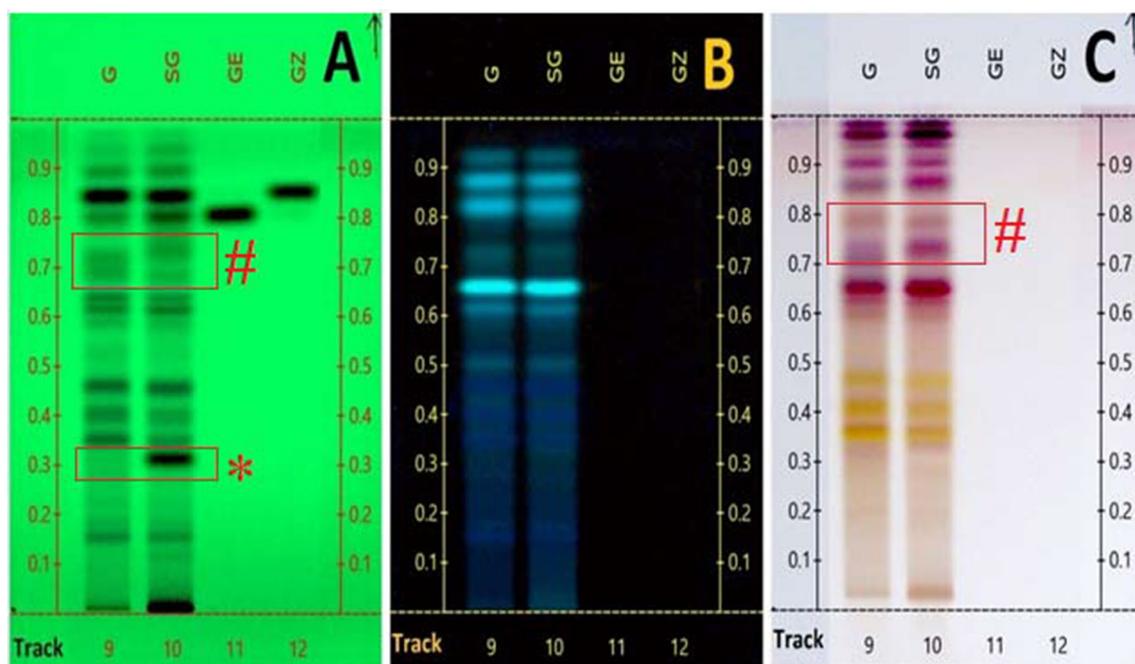| | Guggulu | Shodhita guggulu | Markers |
|---|---|---|---|
| 254 nm | Twelve bands<br>0.008, 0.153, 0.350, 0.409, 0.459, 0.615, 0.637, 0.849, 0.855, 0.891, 0.935 | Fourteen bands<br>0.011, 0.152, 0.311, 0.346, 0.407, 0.456, 0.611, 0.636, 0.683, 0.735, 0.851, 0.857, 0.894, 0.938 | Two bands<br>Guggulsterone E 0.807<br>Guggulsterone Z 0.853 |
| 366 nm | Seven bands<br>0.503 (blue), 0.613 (cyan), 0.658 (cyan), 0.727 (cyan), 0.821 (cyan), 0.872 (cyan), 0.919 (cyan) | Seven bands<br>0.502 (blue), 0.610 (cyan), 0.654 (cyan), 0.730 (cyan), 0.821(cyan), 0.872 (cyan)<br>0.922 (cyan) | No band |
| After derivatization | Eight bands<br>0.364 (Yellow), 0.420 (Yellow), 0.472 (yellow), 0.654 (pink), 0.734 (brown), 0.794 (brown), 0.864 (pink), 0.913 (pink) | Eight bands<br>0.366 (yellow), 0.414 (yellow), 0.469 (yellow). 0.647 (pink), 0.729 (brown), 0.789 (brown), 0.864 (pink), 0.908 (pink) | No band |

*Non-significantly differ at $p < 0.001$

**All groups/formulations significantly differ at $p < 0.001$

to reduce the gastric irritation effects of guggulu [28, 38]. The water-extractive values of guggulu (61.08% *w/w*) and shodhita guggulu (63.56% *w/w*) were non-significantly different (at $p < 0.001$) which means gowmutra shodhana has non-significant effects on the polar molecules of guggulu. The ethanol-extractive value of guggulu and shodhita guggulu were significantly different which means gowmutra shodhana has significant effects on the polar as well as non-polar molecules of guggulu as ethanol is highly miscible in non-polar solvents like hexane. The increase in total ash and acid-insoluble ash after shodhana confirms the facts and trends observed in water and ethanol-extractive values. As non-significant changes in water-extractive value means the polar molecules including metal ions are remains after shodhana, similarly, the ethanol-extractive value after shodhana was decreased almost to 50% which means that the ratio of polar molecules including metal ions has increased and the ratio of non-polar molecules has decreased. The observed values of $\lambda_{max}$ of guggulu and shodhita guggulu were 324 nm which was non-significant, meaning that the major molecules of guggulu are not depleted after shodhana (Table 1).

## 3.3 HPTLC analysis of guggulu and shodhita guggulu

In a HPTLC study, mobile phase optimization is the main phase. Better mobile phase means good resolution and better separation of compounds. In the present study, the mobile phase consisting of toluene−ethyl acetate−acetone−formic acid (8.5:0.5:1:1, *V/V*) at room temperature (25 ± 2 °C) was optimized for the HPTLC analysis. The observed results of HPTLC analysis for guggulu, shodhita guggulu, and marker compounds (guggulsterone E and guggulsterone Z) are given in Table 1. In the HPTLC analysis, at 254 nm, twelve bands were observed including the bands of the two marker compounds guggulsterone E ($R_F$ 0.81) and Z ($R_F$ 0.85) (Fig. 1). Marker compounds were not detected at 366 nm and after derivatization of TLC plate (Table 1 and Fig. 1). In the HPTLC analysis at 254 nm, a new band at $R_F$ 0.31 was observed after the shodhana of guggulu sample. After shodhana, the intensity as well as the resolution of bands also increased which means fatty organic matter with volatile nature was removed. Further, the pH study results

**Fig. 1** Comparative HPTLC analysis of guggulu (G) and shodhita guggulu (SG) at 254 nm (**A**), 366 nm (**B**), and 514 nm (**C**) w.r.t. their marker compounds. Track 9: guggulu (G), track 10: shodhita guggulu (SG), track 11: guggulsterone E (GE), and track 12: guggulsterone Z (GZ). *New peaks after shodhana. #Intensity or resolution increased after shodhana

revealed that the removal of organic matters having acidic nature occurred during the shodhana process.

There are various studies on the quantification guggulsterone E and Z alone. The developed method is better than recently reported methods for the profiling of guggulsterone E and Z alone. *Musharraf* et al. (2011) have developed simultaneous quantification of guggulsterone E and guggulsterone Z, where the mobile phase hexane−ethyl acetate−formic acid (55:40:5, *V/V*) was used to quantify guggulsterone E and Z [36]. The basic difference between the present study and the study reported by *Musharraf* et al. (2011) is the resolution and the HPTLC plate used. In the present study, simple TLC plates were used, whereas *Musharraf* et al. (2011) used glass HPTLC plates which are costly and not every laboratory can afford it. In terms of resolution, Fig. 1 shows the bands of high resolution that is blade-like sharpness, which is very rare and needs more skills to develop such plates. In another study, the mobile phase toluene−acetone (9.3:0.7, *V/V*) was used to quantify guggulsterone E and Z in herbal extract where simple TLC plates were used, but resolution was less than in the present study [37].

### 3.4 LC−MS analysis of guggulu and shodhita guggulu

To verify and validate the HPTLC analysis results, LC−QTOF−MS/MS analysis of guggulu and shodhita guggulu was performed. To attain the significant results, the same concentration of guggulu and shodhita guggulu and the same method conditions were used. The LC−QTOF−MS/MS analysis of the methanolic extract of guggulu and shodhita guggulu revealed the presence of 45 compounds (in ESI$^+$) including the presence of the marker compounds, i.e., guggulsterone E and Z were confirmed in the HPTLC analysis (Fig. 2). A detailed literature and library search has confirmed the presence of eight major phytochemicals as indicated by green lines in the chromatograms of guggulu and shodhita guggulu in Fig. 2. Figures 2 and 3 show the presence and fragmentation pattern of 5-(13′Z-nonadecenyl)resorcinol (Rt 8.31), 8β-hydroxy-3,20-dioxopregn-4,6-diene (Rt 10.33), guggulsterone E (Rt 12.99), guggulsterone Z (Rt 13.28), guggulsterol I (Rt = 15.19), mangiferolic acid (Rt = 15.27), 20(S),21-epoxy-3-oxocholest-4-ene (Rt = 15.75), and guggulsterol II (Rt 16.23). Figure 2 shows that between Rt from 4 to 21 min, maximum peaks became sharp and intense in shodhita guggulu sample. Various humps and doublets have been eliminated after the shodhana process.

The compound databases, namely PubChem, KEGG Compound and ChemSpider were used to search the metabolites. Moreover, detailed literature search was executed to confirm the phytochemicals of guggulu and shodhita guggulu as shown in Table 2. The patterns of the elution of phytochemicals were confirmed as almost the same conditions were used by *Haque* et al. (2009) and *Ahmed* et al. (2016) [29, 32]. In HPLC study, they
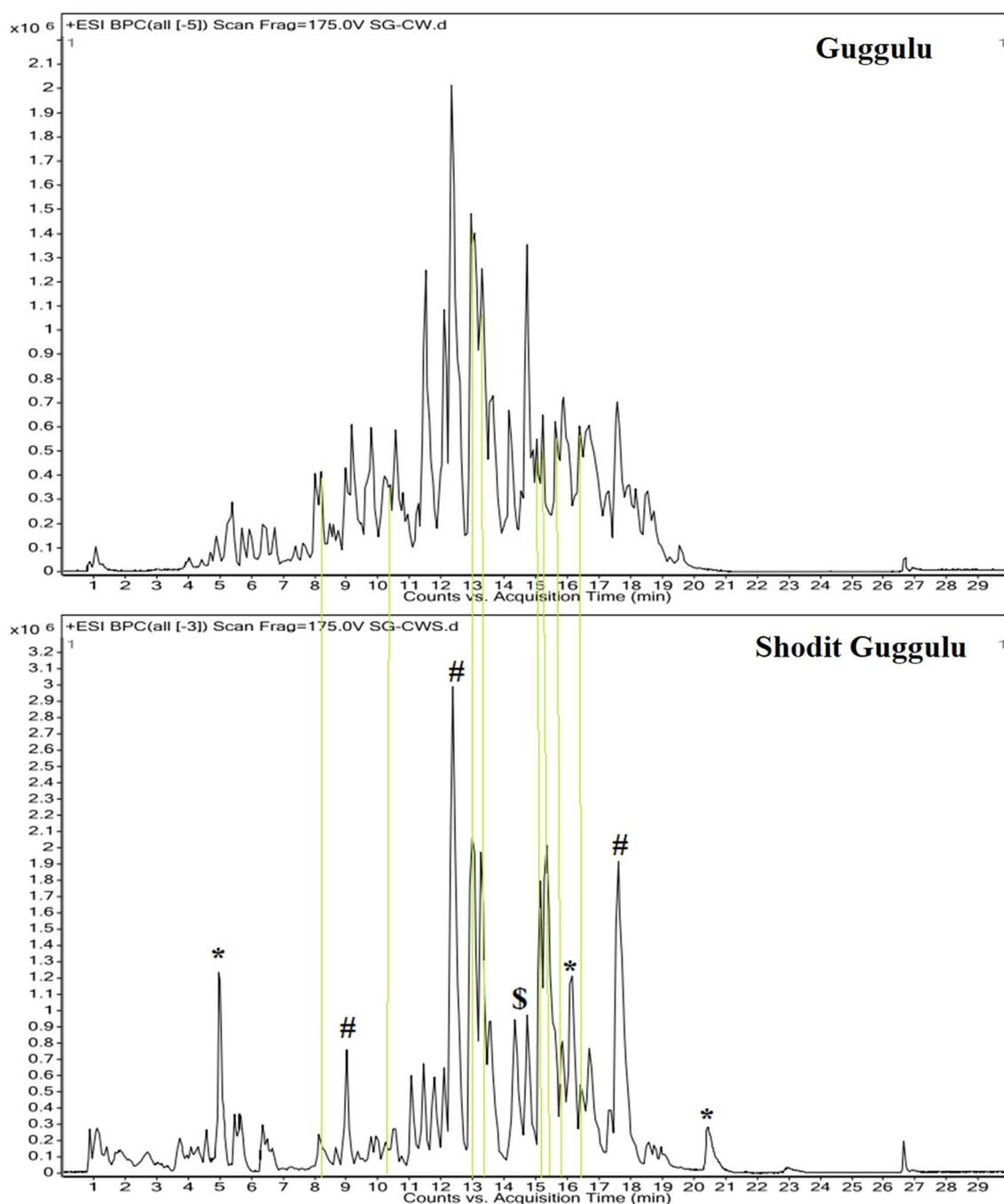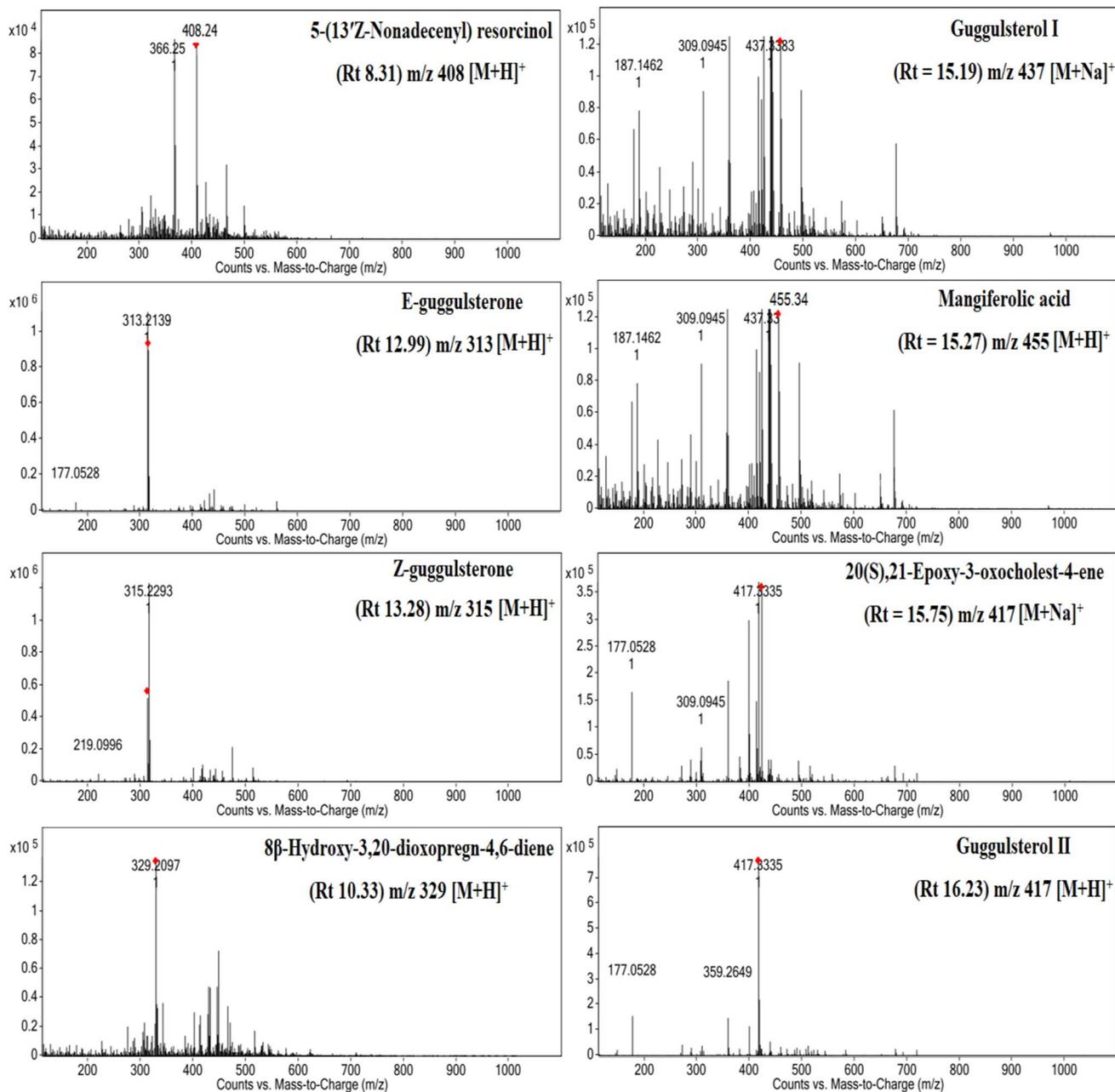
**Fig. 2** Comparative LC−MS chromatograms of guggulu and shodhita guggulu ESI (+ve) mode; 5-(13′Z-nonadecenyl)resorcinol (Rt 8.31); 8β-hydroxy-3,20-dioxopregn-4,6-diene (Rt 10.33); guggulsterone E (Rt 12.99); guggulsterone Z (Rt 13.28); guggulsterol I (Rt = 15.19); mangiferolic acid (Rt = 15.27); 20(S),21-epoxy-3-oxocholest-4-ene (Rt = 15.75); and guggulsterol II (Rt 16.23). *New peaks after shodhana. #Intensity increased after shodhana. $Intensity decreased after shodhana

confirmed the elution pattern as 5-(13′Z-nonadecenyl)resorcinol, 8β-hydroxy-3,20-dioxopregn-4,6-diene, guggulsterone E, guggulsterone Z, guggulsterol I, mangiferolic acid, 20(S),21-epoxy-3-oxocholest-4-ene, and guggulsterol II; 5-(13′Z-nonadecenyl)resorcinol with $m/z$ 408 $[M+H]^+$ having the fragmentations at 366, 321, and 242. *Chhonker*

et al. (2018) confirmed that guggulsterone E and Z can be fragmented into various $m/z$ ratios after multimechanisms [33]. The main fragmentations of guggulsterone E and Z were $m/z$ 295, 285, 277, 255, 230, 201, 162, 109, and 97 [26, 34]. Hydroxylation process can convert guggulsterone E and Z into molecule having molar mass $m/z$ 329 with

**Fig. 3** Major fragmentations pattern of guggulu and shodhita guggulu observed in LC−MS ESI (+ ve) mode study; 5-(13′-nonadecenyl) resorcinol (Rt 8.31); 8β-hydroxy-3,20-dioxopregn-4,6-diene (Rt 10.33); guggulsterone E (Rt 12.99); guggulsterone Z (Rt 13.28); guggulsterol I (Rt = 15.19); mangiferolic acid (Rt = 15.27); 20(S),21-epoxy-3-oxocholest-4-ene (Rt = 15.75); and guggulsterol II (Rt 16.23)

main fragmentations 293, 269, and 215. Dihydroxylation process can convert guggulsterone E and Z into molecule having molar mass $m/z$ 345 with main fragmentations 327, 309, 267, and 263. Trihydroxylation process can convert guggulsterone E and Z into molecule having molar mass $m/z$ 361 with main fragmentations 325, 311, and 283. Quaternary hydroxylation process can convert guggulsterone E and Z into molecule having molar mass $m/z$ 377 with main fragmentations 359, 317, 198. Acetylation process can

convert guggulsterone E and Z into molecule having molar mass $m/z$ 355 with main fragmentations 337, 327 [29–33]; guggulsterol I with $m/z$ 437 [M + Na]$^+$ having the fragmentations at 423, 413, 359, 309, and 227; mangiferolic acid with $m/z$ 455 [M + H]$^+$ having the fragmentations at 437, 423, 413, 359, 309, 227, 187, and 115; 20(S),21-epoxy-3-oxocholest-4-ene with $m/z$ 417 [M + Na]$^+$ having the fragmentations at 399, 359, 309, and 177; guggulsterol II with $m/z$ 417 [M + H]$^+$ having the fragmentations at

**Table 2** LC−MS data of phytochemicals detected in guggulu and shodhita guggulu

| Phytochemical | Rt (min) | Mass (m/z) | Fragmentation (m/z) | References |
|---|---|---|---|---|
| 5-(13′Z-Nonadecenyl) resorcinol | 8.31 | 408 [M+H]$^+$ | 366, 321, and 242 | [32–39] |
| Guggulsterone E | 12.99 | 313 [M+H]$^+$ | 295, 285, 277, 255, 230, 201, 162, 109, and 97 | |
| Guggulsterone Z | 13.28 | 315 [M+H]$^+$ | 295, 285, 277, 255, 230, 201, 162, 109, and 97 | |
| Guggulsterol I | 15.19 | 437 [M+Na]$^+$ | 423, 413, 359, 309 and 227 | |
| Mangiferolic acid | 15.27 | 455 [M+H]$^+$ | 437, 423, 413, 359, 309, 227, 187 and 115 | |
| 20(S),21-Epoxy-3-oxocholest-4-ene | 15.75 | 417 [M+Na]$^+$ | 399, 359, 309 and 177 | |
| Guggulsterol II | 16.23 | 417 [M+H]$^+$ | 399, 359, 309, 227 and 177 | |

399, 359, 309, 227, and 177 [29–33]. *Bhatia* et al. (2016) [34] and *Kumar* et al. (2020) [5] revealed guggulu as a potential source for pharmaceuticals, nutraceuticals, and a wide class of phytochemicals, so no surprise for the presence and identification of biological potent compounds in guggulu.

Guggulu has shown a broad range of activities, but the main activities are anti-hyperlipidemic, anti-inflammation, and anti-arthritic activities [5, 16, 17]. A study has revealed that guggulu helps to reduce the COVID-19-based obesity [21]. Guggulu is exported in more than 42 countries including developed countries like United Kingdom and United State America. The demand for guggulu is more than its production [39]. It has been reported that older guggulu is more potent than fresh guggulu [40, 41]. Further, purification process (shodhana) makes it more potent [39–41]. There is a lack of scientific reports where detailed studies on purified and non-purified guggulu are missing. In the present study, cow urine-processed guggulu and raw guggulu were studied by using advanced tools of HPTLC and LC−MS techniques including the basic parameters as mentioned in API. Under HPTLC study, band resolution was increased with purification and new bands were also noticed, e.g., a sharp and new band at $R_F$ 0.31 was noticed in the purified guggulu. Similarly, in LC−MS/MS study, peak resolution was increased and new peak at Rt 5 min was observed. In Fig. 2, the intensity of peaks was increased and broad peaks of raw guggulu turned into sharp peaks after purification process. There are various guggulu formulations and other formulations are mentioned in the Ayurvedic Formulary of India (AFI) where guggulu is the main ingredient [40]. There are various polyherbal drugs already available in the national and international market [5, 16, 17]. Shodhana of guggulu is the mandatory part of each formulation as mentioned in AFI [5, 39]. There are various methods of shodhana prescribed in AFI [40]. Purification may enhance the activity of guggulu by removing its toxicity. There is no report on gowmutra shodhana (cow urine-based purification) yet. This is the first study where purified and non-purified guggulu were compared by using advanced tools, and major phytochemicals like guggulsterone E, guggulsterone Z, and guggulsterols were identified.

## 4 Conclusion

The present study revealed that after the shodhana process of guggulu, parameters like pH, ethanol-extractive value, and ash (total and acid-insoluble) changed significantly. The comparative HPTLC analysis at 254 nm with the mobile phase consisting of toluene−ethyl acetate−acetone−formic acid (8.5:0.5:1:1, *V/V*) revealed the presence of one extra band ($R_F$ 0.31) in shodhita guggulu, the bands of the two marker compounds guggulsterone E ($R_F$ 0.81) and Z (0.85). The comparative LC−QTOF−MS/MS analysis of guggulu and shodhita guggulu revealed the presence of new peaks in shodhita guggulu at Rt 5 min. A total of eight major metabolites of guggulu were confirmed including the presence of the marker compounds guggulsterone E and Z; those were confirmed in HPTLC analysis. This study may be used as replica or blueprint for quality control in various formulations of guggulu.

## Declarations

# References

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