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Exploring Guggulsterone Isomer Diversity in Commiphora Mukul: A TLC and FTIR Investigation

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Abstract:
The resinous extract derived from *Commiphora mukul*, known as gum-resin, possesses significant therapeutic properties. This gum-resin has been widely recognized for its potential in treating various ailments including obesity, liver disorders, ulcers, inflammation, and even cancer. By employing diverse solvents like ethyl acetate, methanol, acetone, and hexane, the crude extracts obtained from *Commiphora mukul* resin were subjected to qualitative analysis to detect the presence of E- and Z-guggulsterone compounds. This analysis involved employing both Thin Layer Chromatography (TLC) and Fourier Transform Infrared Spectrometry (FTIR). The separation of E- and Z-guggulsterone through Thin Layer Chromatography was executed using different solvent systems, namely ethyl acetate, methanol, acetone, and hexane. This separation was carried out through two distinct extraction methods: method-1 (Soxhlet) and method-2 (Cold extraction). The outcomes of these analyses were particularly informative. The results obtained through TLC and FTIR underscored the presence of pure compound secondary metabolites, specifically the steroids E-Z guggulsterone. Remarkably, the active compounds of E and Z guggulsterone were successfully detected within all four diverse extracts derived from *Commiphora mukul*.

Keywords: *Commiphora mukul*, *g*uggulsterone, FTIR, TLC, Soxhlet extraction

Introduction

Commiphora mukul, a member of the Burseraceae family, is a slow-growing, intricately branched shrub native to the arid rocky terrains of Rajasthan, Gujarat, Madhya Pradesh, and Karnataka states in India, as well as the Sind and Baluchistan states in Pakistan. Notably, it predominantly thrives in the arid landscapes of Rajasthan and Gujarat within India (T. Janakiram 2019). The Kutch region, a district in Gujarat, houses over 70% of the guggul plants, showcasing wide distribution. Despite its abundant presence in Gujarat, the species has encountered endangered status in Rajasthan due to its sluggish growth, unsustainable tapping methods for guggul gum-resin extraction, limited seed production, and poor seed germination rates – a scenario that underscores the urgent need for conservation measures (Akhter and Javed 2023). This, however, offers an advantageous opportunity for the exploration of secondary metabolite extraction purposes in Gujarat, as noted by Gantait et al. (2021).

Commiphora mukul, commonly known as guggul, is a resin-producing tree native to India and surrounding regions. The resin has been used for centuries in traditional medicine due to its numerous pharmacological properties (Kumar et al. 2005). One of the major bioactive components of guggul resin is guggulsterone, a steroid with two major isomers: E and Z. These guggulsterone isomers are known for their potential anti-inflammatory, anti-oxidant, anti-hyperlipidemic, and anti-cancer effects (Akhter and Javed 2023). Moreover, E and Z-guggulsterones have been associated with hypoglycemic activity attributed to their role as farnesoid X receptor (FXR) antagonists, ultimately contributing to reduced cholesterol levels in the liver (Shah et al. 2012). Across India and internationally, various forms of guggul powder and capsules are available in the market for the treatment of diverse ailments.

The Central Drug Research Institute (CDRI) in Lucknow has successfully harnessed the therapeutic potential of guggul through the creation of a drug utilizing ethyl extracts containing two vital bioactive compounds – E and Z guggulsterones – collectively referred to as guggulipid. Guggulsterones, secreted through specialized ducts, are particularly abundant in the stem-bark of these plants (Cunningham et al. 2018). The composition of guggul gum-resin stands at 6.9% moisture, 0.6% volatile oil, 61% resin, 29.6% gum, and 3.2% insoluble substances (Joshi et al. 2018). The synthesis and stereochemistry of these compounds were elucidated prior to their actual isolation. In fact, compounds extracted from guggul gum were confirmed to be identical to their synthesized counterparts in all aspects. The establishment of the crystal structures of these steroids required the use of chemical synthesis methods, as highlighted by Han et al. (2020). Beyond guggulsterones, sterols have also been isolated from guggul and evaluated for their pharmacological properties (Shah et al. 2012).

To harness the full therapeutic potential of guggulsterones, it is imperative to accurately identify and quantify these isomers in *Commiphora mukul* extracts (Kulhari et al. 2013). Thin Layer Chromatography (TLC) and Fourier-Transform Infrared Spectroscopy (FTIR) have emerged as invaluable analytical techniques for such purposes (Mohd et al. 2014). TLC provides an efficient means of separating and visualizing guggulsterone isomers based on their migration distances on a thin layer of stationary phase (Kalász et al. 2020), while FTIR offers insights into the chemical composition and functional groups present in the samples (Cuello et al. 2020). Thin Layer Chromatography (TLC) is a widely utilized separation technique in the field of phytochemical analysis. It involves the separation of compounds in a sample based on their differential migration on a stationary phase (the TLC plate) due to differences in polarity and interactions with a mobile phase (solvent system). TLC is known for its simplicity, speed, and cost-effectiveness, making it suitable for qualitative analysis of complex mixtures like plant extracts (Mohd et al. 2014).

The study aims to develop a TLC method for the qualitative analysis of E and Z guggulsterones from *C. mukul* extract. The method's effectiveness lies in its ability to identify the presence of these bioactive compounds, which can contribute to the quality control of guggul-based herbal products and further our understanding of their therapeutic potential. This research endeavors to explore the diversity of guggulsterone isomers within *Commiphora mukul* through the combined use of TLC and FTIR analyses. By employing these complementary techniques, we aim to differentiate and quantify the E- and Z-guggulsterone isomers, shedding light on their relative concentrations and distribution within *Commiphora mukul* resin. This investigation is critical not only for enhancing our understanding of the chemical constituents of guggul but also for facilitating informed decisions regarding its utilization in traditional and modern healthcare practices.

Materials and Method

In the course of this study, plant samples were meticulously collected from the pristine Balamar Forest in the Ambaji Arrival Mountains, located in the state of Gujarat, India, during the concluding days of December.

Collection and Preparation of Plant Materials: Initially, the resin sourced from *Commiphora mukul* was carefully gathered, subsequently sliced into smaller pieces, and then allowed to desiccate naturally in the shade. The dried resin was then stored at an ambient room temperature of approximately 30°C ±2 until it was ready for use. To ascertain the purity of the resin, a qualitative test was conducted. A 2.5-gram portion of the dried resin was immersed in 25 milliliters of 95% methanol and subjected to boiling for a duration of 20 minutes. The presence of resin was confirmed by the formation of precipitates in the filtrate following the addition of 2.5 milliliters of distilled water. In addition, two sorts of methods were used for extraction of guggulsterone form plant.

Method 1: For the preparation of extracts, the plant materials underwent sequential extraction, following the methodology established by Murugan and Saranraj in 2011. The sequence of solvents employed for extraction, in order of increasing polarity, included hexane (69°C), ethyl acetate (77°C), acetone (56°C), and methanol (64°C). Fifteen grams (15g) of the plant resin were submerged in 100 milliliters of the respective extraction solvent, and extraction was meticulously performed within a Soxhlet apparatus until the solvents became colorless. The resulting extracts were then placed in petri plates and allowed to undergo solvent evaporation at room temperature (30°C ±2) for a period of 48 hours. The desiccated extracts were subsequently stored in sealed screw cap tubes within a refrigerated environment, maintained at a temperature of 7-8°C.

Method 2: In an alternative approach to extract preparation, the plant materials were extracted, following the procedure described by Rajaselvam et al. in 2012. Again, sequential extraction was conducted using hexane (69°C), ethyl acetate (77°C), acetone (56°C), and methanol (64°C) as solvents, progressing in polarity order. Fifteen grams (15g) of the plant resin were soaked in 100 milliliters of the respective extraction solvent, and the extraction was carried out at an ambient room temperature of approximately 30°C ±2 for a duration of 92 hours, utilizing a rotary shaker set at 150 rpm. The remaining plant materials were separated by filtration through Whatman filter paper-1. Each solvent extraction was executed once, and the resulting extracts were subsequently filtered and placed in petri plates for 48 hours to facilitate solvent evaporation at room temperature (30°C ±2). The dried extracts were carefully stored in sealed screw cap tubes within a refrigerated environment, maintained at a temperature of 7-8°C.

Extraction efficiency was calculated for each solvent obtained from method 1 and method 2 extractions by following formula.

%Yield efficiency= [Weight of extracted sample/Weight of sample] ×100.

Qualitative detection of E- and Z-Guggulsterone by TLC

In their study, Musharraf et al. developed a robust qualitative analysis method for the detection of E- and Z-Guggulsterone in *Commiphora mukul*. This analysis was extended to include Guggulipid and its pharmaceutical formulation. Their technique utilized Thin-Layer Chromatography (TLC) on

silica gel 60F-254-coated glass plates as the stationary phase.To optimize the separation of these compounds, various solvent combinations, such as methanol, toluene, ethyl acetate, chloroform, and acetone, were tested. While these combinations successfully resolved the E and Z Guggulsterone isomers, they struggled with separating samples containing 17, 20-dihydroguggulsterone. Calculate the R_f value following by this formula,

$$R_f = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent front}}$$

Fourier Transform Infrared Spectrometry (FTIR)

Fourier Transform Infrared Spectrometry (FTIR) stands as a robust analytical method employed for the identification and characterization of chemical compounds, primarily relying on their interaction with infrared radiation. In this study, a thin film of an active eluted fraction of Guggul gum dissolved in ethyl acetate was uniformly applied to a glass substrate. Subsequently, infrared (IR) spectra were meticulously recorded using a state-of-the-art FTIR spectrophotometer, specifically the IR affinity UV-visible and non-visible spectrum instrument Paragon 1000 PC, located at the sophisticated research center of St. Xavier's Foundation for Research at Ahmedabad, Gujarat, India.

Results

Extraction efficiency

During the extraction process, certain plant-derived natural products were effectively extracted. In this study, Method 1 involved Soxhlet extraction, a heated extraction technique, while Method 2 utilized room temperature extraction, a cold extraction method. It's noteworthy that the utilization of various solvent systems in our investigation consistently yielded higher extraction yields when employing Method 1 as compared to the yields achieved through Method 2. Data depicted in **Table 1 and Figure1**.

Table 1 Showing the extraction efficiency from crude samples

Method	Ethyl Acetate	Methanol	Acetone	Hexane
Method -1	44.56%	44.24%	51.28%	38.41%
Method -2	37.24%	40.25%	40.25%	11.8%

Note: Method -1= Hot extraction, Method-2=cold extraction.

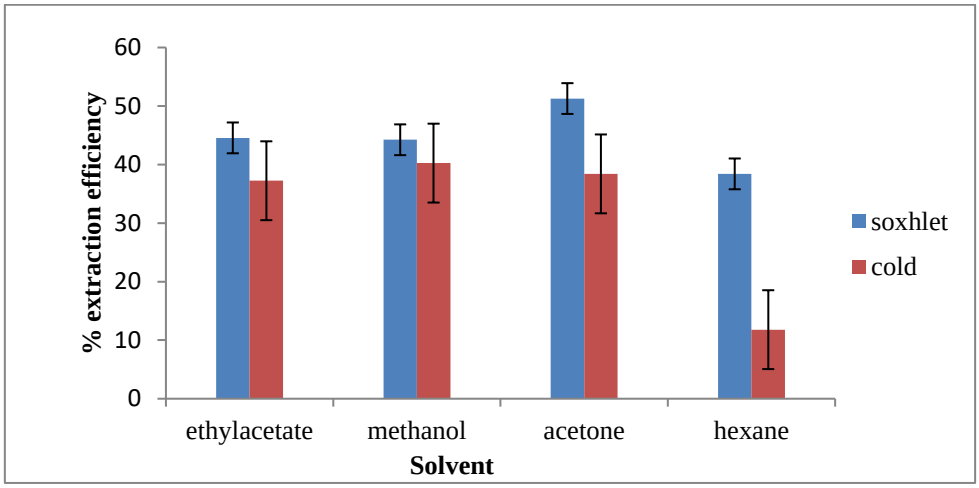

Figure 1. Extraction efficiency of Soxhlet and Cold method for different solvent

Thin Layer Chromatography

TLC profiling of four extracts of method-1 and method-2 gave promising results that directs towards the presence of number of phytochemicals present in the extracts. Various phytochemicals giving different RF values in solvent system provide a very important clue in understanding of their polarity and also help in selection of appropriate solvent system for separation of pure compounds. Compound showing high RF value in less polar solvent system have low polarity and whereas compounds with less RF value have high polarity. However, the use of a toluene-acetone solvent system (9.3:0.7 v/v) yielded superior results, with a resolution of E and Z Guggulsterones and 17, 20-dihydroguggulsterone at RF values of 0.52±0.01, 0.67±0.01, and 0.60±0.01, respectively **Figure.2**. Notably, Guggul resin and Guggulipid samples displayed excellent resolution after a single TLC run and even better results upon a second run. Furthermore, the E and Z Guggulsterone isomers, along with 17, 20-hydroguggulsterone, exhibited distinctive staining colors on the TLC plates when sprayed with vanillin as the agent. This method proves to be a valuable tool for the qualitative analysis of these compounds in *Commiphora mukul* and related formulations.

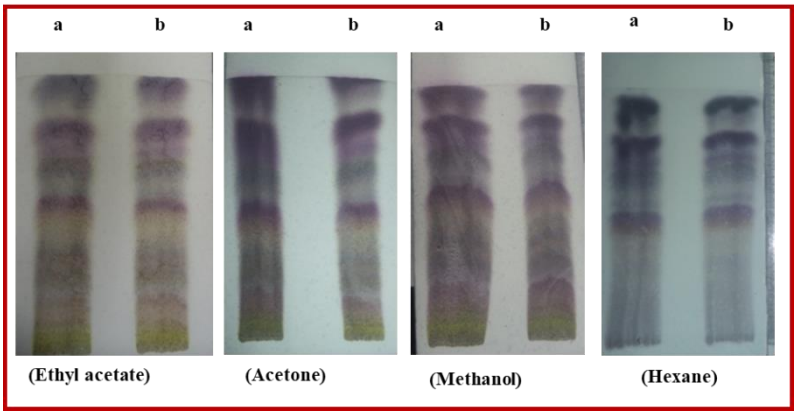

Figure.2 TLC plates showing different band with vanillin sprayed, using toluene-acetone solvent system(9.3:0.7v/v)
Note: A=Method 1, B=Method2.

Table 2 Separation of E-and Z- Guggulsterone from various extracts employing TLC

Extract	**Solvent system**	**Visualizing agent**	**Detection color**	**Rf value**	**Components**
Ethyl acetate	Toluene-acetone (9.3:0.7, v/v)	Vanillin, sulphuric acid reagent	Blue	0.52±0.1 0.67±0.1 0.60±0.1	E-Guguulsterone
			Brown yellow		Z-Guguulsterone
			Blue		(17,20-dihydroguggulsterone)
Methanol	Toluene-acetone (9.3:0.7, v/v)	Vanillin, sulphuric acid reagent	Brown	0.52±0.1 0.67±0.1 0.60±0.1	E-Guggulsterone
			Blue		Z-Guggulsterone

			Brown		(17,20-dihydroguggulsterone)
Acetone	Toluene-acetone (9.3:0.7, v/v)	Vanillin, sulphuric acid reagent	Purple	0.52±0.1 0.67±0.1 0.60±0.1	E-Guggulsterone
			Black Purple		Z-Guggulsterone (17,20-dihydro guggulsterone)
Hexane	Toluene-acetone (9.3:0.7, v/v)	Vanillin, sulphuric acid reagent	Purple	0.52±0.1 0.67±0.1 0.60±0.1	E-Guggulsterone
			Blue		Z-Guggulsterone
			Brown		(17,20 dihydroguggulsterone)

FTIR

The study of infrared spectra revealed the presence of amino, alkenes and phenol as major functional group. In both methods were soxhlet contain more functional group compared to cold extraction.

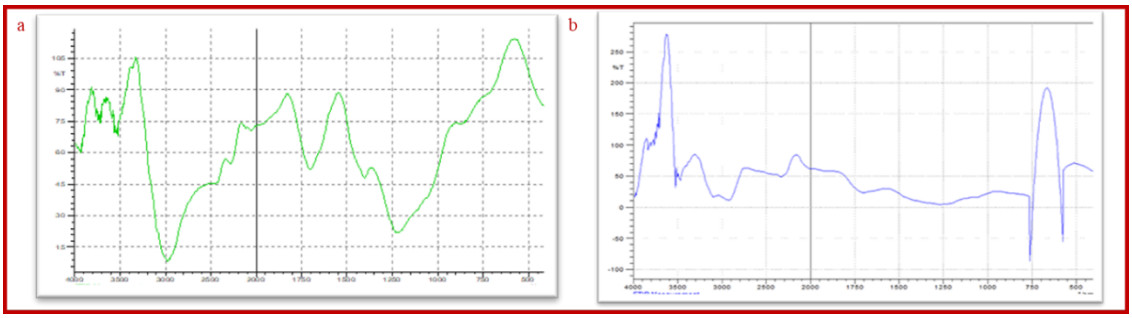

Figure 3. Display of (a) IR spectra of ethyl acetate Soxhlet extract and (b) Cold extract of *Commiphora mukul*

Table 3 IR spectra of *Commiphora mukul* from ethyl acetate Soxhlet extract

Absorption(cm^{-1})	Absorption(cm^{-1})	Functional group
3600	3640-3160	O-H stretching-Alcohol, Phenol
3400	3600-3200	O-Stretching- Alcohol, Phenol
35100	3500-3300	N-H stretching- Amines
2940	2960-2850	C-H stretching- Alkenes
2250	2260-2220	C≡N stretching-Nitriles
1620	1660-1500	NO2 asymmetrical stretching-Nitro
1430	1470-1350	C-H scissoring and bending-Alkenes
1220	1260-1000	C-O stretching-Ether, Carboxylic acid, Ester, Alcohols.

Table 4 IR spectra of *Commiphora mukul* from ethyl acetate Cold extract

Absorption (cm^{-1})	Absorption range (cm^{-1})	Functional group
3600	3640-3160	O-H stretching-Alcohol, Phenol
35100	3500-300	N-H stretching- Amines
34300	3500-3300	N-H stretching- Amines
3100	3100-3000	C-H stretching-Aromatic Ring
3059	3080-3020	C-H stretching-Alkenes
730	870-675	C-H bending- Phenyl Ring
1600	1680-1640	C=C stretching-Alkenes

Discussion

Commiphora mukul, commonly known as Guggul, has been used for centuries in traditional medicine due to its potential health benefits (Shishodia et al. 2008). Guggulsterones, including E and Z isomers, have garnered attention for their therapeutic properties, including anti-inflammatory and cholesterol-lowering effects. Understanding the diversity of Guggulsterone isomers and their extraction methods is crucial for pharmaceutical and nutraceutical industries (Mukherjee et al. 2015). These compounds have the potential for use in drugs and dietary supplements. The study delves into the choice of extraction methods, which is a fundamental consideration in natural product research. It provides insights into the effectiveness of these methods and their impact on yield and compound diversity.

The study focused on the extraction of bioactive compounds, specifically E and Z Guggulsterone, from *Commiphora mukul* using two distinct extraction methods. One of the primary findings of the study is the remarkable consistency in the yield of bioactive compounds obtained using two different extraction methods. This is a noteworthy discovery as it suggests that the choice of extraction method does not significantly affect the overall yield. Consequently, researchers can opt for the cold extraction method (method 2) with confidence, knowing that it minimizes the risk of compromising valuable compounds during the extraction process. The choice of solvent used in the Soxhlet extraction method was found to have a significant impact on the yield of bioactive compounds (Kothari et al. 2012). Specifically, acetone was identified as the superior solvent for this method, resulting in higher yields when compared to alternative solvents. This information is vital for researchers and professionals seeking to optimize their extraction procedures, as it underscores the importance of solvent selection. The study also revealed that both cold and Soxhlet extraction methods, when employing ethyl acetate as the solvent, led to superior separation on TLC plates. This separation was particularly pronounced when using a toluene:acetone solvent system. The enhanced separation led to a more pronounced presence of bioactive compounds, specifically E and Z Guggulsterone. This finding is significant, as it points to the importance of the solvent system in obtaining purer and more concentrated extracts. The FTIR analysis provided insights into the functional groups present in the crude extracts. It was found that the Soxhlet ethyl acetate method (method 1) produced extracts with a greater variety of functional groups, closely followed by the cold ethyl acetate method (method 2). This diversity of functional groups suggests that these methods are capable of extracting a broader range of compounds from the source material.

Conclusion

The experiments conducted in this study have unveiled some intriguing findings. First and foremost, the yield per extraction was found to be remarkably consistent between two distinct extraction methods. This suggests that opting for the cold extraction method (method 2) could be a prudent choice, as it minimizes the risk of compromising valuable compounds during the extraction process. Moreover, it's noteworthy that higher yields were achieved when utilizing the solvent of acetone for Soxhlet method in comparison to alternative solvents. Additionally, the extracts obtained through cold and Soxhlet extraction using ethyl acetate exhibited superior separation on TLC plates with a toluene:acetone solvent system (9.3:0.7 v/v). Notably, this resulted in a more pronounced presence of bioactive compounds, specifically E and Z Guggulsterone, as compared to other solvents such as acetone, methanol, and hexane. Furthermore, the FTIR analysis indicated that the crude extract obtained through the Soxhlet ethyl acetate method (method 1) contained a greater variety of functional groups, followed closely by the crude extract from the cold ethyl acetate method (method 2). These findings collectively suggest that the cold extraction method (method 2) using ethyl acetate may be an optimal approach for preserving valuable compounds and obtaining higher yields.

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Conflict of interest: The authors declare no competing interests.

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