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Indu M Menon
PG Scholar, Department of Dravyaguna Vijnanam, Govt. Ayurveda College, Thiruvananthapuram, Kerala, India

Jollykutty Eapen
Director of Ayurveda medical Education (Rtd), Kerala, India

MS Deepa
Associate Professor, Department of Dravyaguna Vijnanam, Govt. Ayurveda College, Thiruvananthapuram, Kerala, India

Corresponding Author:
Indu M Menon
PG Scholar, Department of Dravyaguna Vijnanam, Govt. Ayurveda College, Thiruvananthapuram, Kerala, India

In vitro antibacterial evaluation and HPTLC fingerprint profile of purified *Guggulu* (*Commiphora wightii*. Arn. Bhand) by ayurvedic method

Indu M Menon, Jollykutty Eapen and MS Deepa

Abstract
Guggulu, oleo gum resin from the plant *Commiphora wightii*, is a renowned and potent drug used in *Ayurvedic* formulations. Before incorporating in formulations, *Guggulu* has to be purified as per *Ayurvedic* texts. The purification method adopted in the present study was *swedana* (boiling) in *Triphala-Guduchi kwatha (*decoction of *T. chebula, T. bellerica, E. officinalis* and *T. cordifolia*). There are no studies performed to evaluate the antibacterial activity of purified *Guggulu*. Therefore, an attempt to find out the anti-bacterial activity of purified *Guggulu*was done. Phytochemical screening and quantification of Guggulsterone E and Z were also evaluated and compared the values with that of raw *Guggulu*. It was observedthat the purified *Guggulu* hadmore antibacterial activity against *Pseudomonas aeruginosa* and *Proteus vulgaris*. There were differences in the phytochemical parameters including the mean quantities of Guggulsterones E and Z before and after purification.

Keywords: *Guggulu, Shodhana*, purification, antibacterial activity, Guggulsterones E& Z, HPTLC

1. Introduction
Guggulu, oleogum resin from the plant Commiphora wightii. Arn. Bhand, is a highly beneficial and popular drugusedin *Ayurvedic* medications since time immemorial. As per *Ayurvedic* classical texts, *Guggulu* is indicated in many disease conditions like *Sandhigatavata* (Osteo arthritis), *medoroga* (Hyper Lipidemia), *Krimi* (Heliminthic infestation), *Bhuta* (Microbial infection), *Galaganda* (Thyroid disorder), *Hridroga* (Cardiac disorder), *Prameha* (Diabetic mellitus), *Kushta* (Skin diseases*), Vrana* (Wounds) etc [1]. All these indications were validated through various studies and found out that the major active constituents responsible for such pharmacological actions are Guggulsterone E & Z. Guggulsterones E& Z are responsible for the wide range of pharmacological actions such as Anti-microbial, Anti-inflammatory, anti hyperlipidemic, anti-oxidant, thyroid stimulatory, cardioprotective, anti-diabetic [2] etc. The *Bhutaghna* property is closely related with the anti microbialaction. It was proved that unpurified *Guggulu* possess significant antibacterial activity against Gram-positive bacterialike B. cereus, B. subtilis, S.aureus and moderate activity against Gram-negative bacterialike E. Coli, K. pneumonia, P. aeruginosa and S. typhi [3].
There are a large number of polyherbal formulations which contain *Guggulu* as chief ingredient. It is recommended to purify *Guggulu*before including in the formulations. Ayurveda emphasizes different processing methods of drugs which are capable of modifying the quality and efficacy. Any process that is adopted to alleviate impurities and to improve efficacy of the drugs is called *Shodhana*, which literally means purification.It is expected that different methods of purification will have a profound effect on the therapeutic safety and efficacy. Many *Shodhana* processes using different media are described for *Guggulu* invarious *Ayurvedic* classical texts. However no studies have been conducted to evaluate the antibacterial potential of purified *Guggulu*. Thereare only few studies which explore the possible changes after the *Shodhana* (purification) process of *Guggulu* and its probable effect on the quantity of Guggulsterones E&Z.
In this study, *Shodhana* (purification) was performed as mentioned in the *Ayurvedic* text, Bhaishajyaratnavali [4]. Purification was done using*swedana* method (Boiling and straining) in the *Triphala Guduchi kwatha (TGK)* - Decoctionprepared with *Guduchi* (fresh stem of *Tinospora cordifolia*) and *Triphala* (dried fruit rind of *Terminalia chebula*, *Terminalia bellerica*, *Emblica officinalis)*.The bacterial strains selected were Pseudomonas aeruginosa (Gram negative), Proteus vulgaris(Gram negative) and Klebsiellapneumonia (Gram negative), which are common causative microorganisms responsible for wound infections and urinary tract infections.

The present study intends to evaluate the antibacterial activityagainst selected bacterial strains and also analyse the changesin phytochemical screening and quantitative estimation of Guggulsterones E&Z using HPTLC before and after *Shodhana* (purification) of *Guggulu*.

2. Materials and Methods

2.1 Collection of Plant Materials

Oleo gum resin of *Guggulu*, collected from its natural habitat (Near Rajasthan) through a dealer and genuinity assessed as per API standards. Fresh stem of Guduchi (*Tinospora cordifolia*) and dried fruit rind of Triphala (*Terminalia chebula, Terminalia bellerica, Emblica officinalis)* were collected and authenticated by a Botanist

2.2 Method of purification of *Guggulu*

2.2.1 Preparation of *TGK*

Fresh stem of *Guduchi* (*Tinospora cordifolia*), dried fruit rinds of *Triphala* [*Hareetaki* (*Terminalia chebula*), *Vibhitaki* (*Terminalia bellerica*), *Amalaki* (*Emblica officinalis*)] (125g each) were taken, washed, moderately crushed and put in a clean stainless steel vessel. Four times of water was added (2 L). Boiled the contents and reduced to one fourth of its original volume (500 ml). The decoction thus got was strained into a clean dry vessel. 500 ml of decoction was obtained.

2.2.2 Procedure of Purification by *swedana* (boiling) method

125 g raw *Guggulu* was boiled in TGK (500 ml) until whole *Guggulu* dissolved in the decoction (approx. 45 minutes) and filtered. This filtrate was again boiled till the water evaporated completely to obtain a semisolid consistency. This semisolid *Guggulu* was transferred to a stainless steel tray smeared with ghee. It was kept in sunlight and dried. 103 g of *Guggulu* was obtained after purification with variation in texture, colour, odour, taste etc.as from raw *Guggulu*. The samples (Raw and purified *Guggulu*) werestored in air tight glass containers. Figure. 1 demonstrates the method of *Shodhana* (purification) of *Guggulu*.

Fig 1: Method of *Shodhana* (purification) of *Guggulu*

2.3 Physicochemical and Phytochemical Screening

Preliminary Physicochemical analysis and qualitative phytochemical screening were evaluated as per the standard procedures mentioned in Ayurveda pharmacopeia of India [5]

2.4 HPTLC Fingerprinting Profile

2.4.1 Chemicals and Reagents Used

Standard Guggulsterone E and Guggulsterone Z (Sigma Aldrich), HPLC grade solvents (Merck, Germany) were used

2.4.2 Preparation of Extracts

The raw and purified *Guggulu* were weighed (2 g) separately, refluxed with methanol for 20 minutes in water bath. Then filtered through whatmann filter paper no. 1 and volume was made to 10 ml in standard flask.

2.4.3 Preparation of standard Guggulsterone E solution

A stock solution of Guggulsterone E was prepared by dissolving 5 mg of Guggulsterone E standard in 100 ml Methanol (0.05 mg/ml)

2.4.5 Preparation of standard Guggulsterone Z solution

A stock solution of Guggulsterone Z was prepared by dissolving 5 mg of Guggulsterone Z standard in 100 ml Methanol (0.05 mg/ml)

2.4.6 Chromatographic conditions

Chromatography was performed on 20 cm×20 cm precoated silica gel aluminium 60 F_{254} plates (Merck, Germany). The solutions were applied to pre coated, prewashed (methanol) and activated Silica gel plates as 6 mm wide bands, 15 mm from the bottom edge, 15 mm from side edge and 17 mm apart using a CAMAG Linomat 5 applicator. The Application Volume of Standards were 3µl,5 µl,7 µl,9 µl,11 µl,13 µl and Samples were 6 µl. The applied plates were run to solvent front of 70mm using Petroleum ether: Ethyl acetate in the

ratio 7:2 as mobile phase by ascending development in twin trough chamber at room temperature (23±2 ^{0}C). The plate was observed under UV (254nm and 366nm) for detection of standard, followed by densitometric scanning. For obtaining calibration curve, densitometric scanning was performed at 251 nm for Guggulsterone E and 256 nm for Guggulsterone Z using CAMAG TLC Scanner 3 equipped with WIN CATS software version 1.3.4. Quantitative data was obtained from the software by fixing % deviation to get an appropriate regression line having a desirable standard deviation (<3) and regression coefficient (<1), while including maximum sample spots in the regression line. Percentage weight by weight (%w/w) of Guggulsterone E and Z in two samples were calculated with respect to the % weight of raw and purified *Guggulu*.

2.4.7 Method Validation
Validation of method was done by assessing linearity, specificity, precision, accuracy, Limit of Detection (LOD) and Limit of Quantitation (LOQ).

2.4.8 Statistical Analysis
The mean quantities of Guggulsterone E and Z of *Guggulu* before and after purification were tested statistically using Paired t test using SPSS Software.

2.5 *In vitro* Anti bacterial Evaluation
2.5.1 Materials Used
Glass ware, Mueller Hinton Agar(MHA)(Merck, Germany), Micropipettes, Petriplates, Pipette tips, Conical flasks, cotton swab, Ciprofloxacin, DMSO (dimethyl sulpho oxide)solvent

2.5.2 Collection of test microorganisms
Microbial strains of Pseudomonas aeruginosa, Proteus vulgaris and Klebsiella pneumonia were procured from Public health laboratory, Thiruvananthapuram, Kerala.

2.5.3 Preparation of extracts
50g of purified *Guggulu* was mixed with 100mlmethanol, reluxed for 30 minutes in a soxhlet apparatus and flitered using a filter paper. The filterate was then distilled out using a simple distillation apparatus and the concentrated extractive was transferred into a dry beaker. It was evaporated and allowed to dry. This extract (around 5g) was scraped out and kept in a beaker. 5 mg from this extract was added with 1 ml of DMSO and formed a stock solution of 5mg/ml. I mg of the standard (Ciprofloxacin) was suspended in 1 ml of DMSO and formed a stock solution of 1 mg/ml.

2.5.4 Anti-bacterial Evaluation (Agar well diffusion method)
All the test organisms were inoculated into the Mueller Hinton Broth (MHB) and incubated at 37 ^{0}C for 3-4 hours. Selected microorganisms were seeded on Mueller Hinton Agar (MHA) plates with the help of Sterile cotton swabs. It was streaked back and forth from edge to edge. This was done to ensure that the inoculums are evenly distributed and incubated at 37 ^{0}C for overnight. Wells of uniform size (approximately 10 mm) were made in the sterilized media. 10 µlfrom 5mg/ml of sample was pipetted into the well made in agar plates. 5 µl of ciprofloxacin (1mg/ml) and DMSO as negative control are also placed in the Agar plate. 3wells were made in such a way that the antibacterial zones of the drug added were easily visible. The petri dishes were incubated at 37 ^{0}C for one night. The incubated culture plates were checked for zone of inhibition. The zone that inhibits the growth of bacteria around the well is known as Zone of inhibition. The diameter of zone of inhibition was measured using a geometric scale.

3. Results and Discussion
3.1 Preliminary Physicochemical and Phytochemical Screening
It was observed that purified *Guggulu* had variations in texture, colour, odour, taste, etc. from raw *Guggulu* Variations in the organoleptic features of purified *Guggulu* are shown in Table 1. Physicochemical evaluation of raw and purified *Guggulu* showed variations. Results of physico chemical evaluation are tabulated in Table 2. Adherence of foreign matter like sand, dried leaves etc can be take place while collecting raw *Guggulu* from the Plant. Manual removing of such physical impurities is a difficult task. It was observed that after *Shodhana* (purification), no foreign matter content was detected. Thus, *Shodhana* (purification) is beneficial in removing physical impurities. Total sugar and reducing sugar were estimated using Fehling's solution test. It was found that percentage of Total and reducing sugars increased in purified *Guggulu*. It was observed that purified *Guggulu* (3.87) had pH less than that of raw *Guggulu* (5.52). Lower the pH value indicates more acidic nature, which is more capable to inhibit microbes [6]. Table 3 shows the results of qualitative screening of phytoconstituents. Raw *Guggulu* had only the presence of steroids, alkaloids and flavonoids. On evaluation, tannins, steroids, phenols, alkaloids, saponin and flavonoids were present after *Shodhana* (purification). As purified *Guggulu* had all the phytochemicals, it is expected to possess immense anti-microbial action.

Table 1: Organoleptic characters of raw and purified *Guggulu*

Characters	Raw *Guggulu*	Purified *Guggulu*
Form	Agglomerated, varying sizes	Agglomerated
Surface conditions	Rough, waxy	Smooth
Fracture and hardness	Granular	Elastic
Colour	Yellowish- reddish brown	Black
Odour	Aromatic	Aromatic
Taste	Bitter, Astringent	Bitter

Table 2: Preliminary physicochemical evaluation of *Guggulu*

Physicochemical parameters	Raw *Guggulu*	Purified *Guggulu*
Foreign matter (%)	1.27	0
Moisture content (%)	13.03	11.29
Volatile oil (%)	2.8	0
Total ash (%)	3.9	4.3
Acid insoluble ash (%)	0.8	1
Water soluble extractive (hot) (%)	32.15	25.26
Alcohol soluble extractive	50.07	31.11
Total sugar	19.76	28.61
Reducing sugar	2.98	11.5
pH(at 36 ^{o}C)	5.52	3.87

Table 3: Qualitative screening of Phytochemicals

Phytoconstituents	Test	Raw *Guggulu*	Purified *Guggulu*
Tannin	Lead acetate test	-	++
Steroids	Salkowaski test	+	+
Phenols	Ferric chloride test	-	+++
Alkaloids	Dragendorff's test	++	++
Saponins	Foam test	-	+
Flavonoids	Shinoda test	+	+

3.2 HPTLC Fingerprint Profile

Results of validation methods of HPTLC for estimation of Guggulsterone E (GE) and Guggulsterone Z (GZ) are given in Table 4. The mean concentrations of Guggulsterone E and Z of *Guggulu* before and after purification are represented in Table 5. Figure. 2 shows the spectral comparison of standard and samples (raw and purified *Guggulu*). There was statistically significant increase in the quantity of Guggulsterone E after purification ($p< 0.01$). But, the quantity of Guggulsterone Z has been reduced after purification. However, on qualitative evaluation, presence of all phytochemicals was noticed. The antimicrobial action of Guggulsterones E and Z were proven and thus the antimicrobial activity of purified *Guggulu* can be inferred.

Table 4: Results of validation studies of HPTLC for estimation of standards

Validation parameters	Guggulsterone E	Guggulsterone Z
Mobile phase	Petroleum ether: Ethyl acetate (7:2)	
Linearity range(ng/spot)	150-550 ng	150-650 ng
Detection wavelength	251 nm	256 nm
Rf value	0.3766±0.0206	0.811±0.0222
Slope mean±SD	10.575±2.72	12.256±2.74
Y intercept±SD	095.646±2.72	469.115±2.74
Regression coefficient(r^2) ±SD	0.997441±2.72	0.99662±2.74
Limit of detection	0.8487µg	0.7377µg
Limit of Quantitation	2.5721µg	2.2356µg
Linearity	Linear	Linear
Specificity	Specific	Specific
Instrumental precision	Precise	Precise
Accuracy	Accurate	Accurate

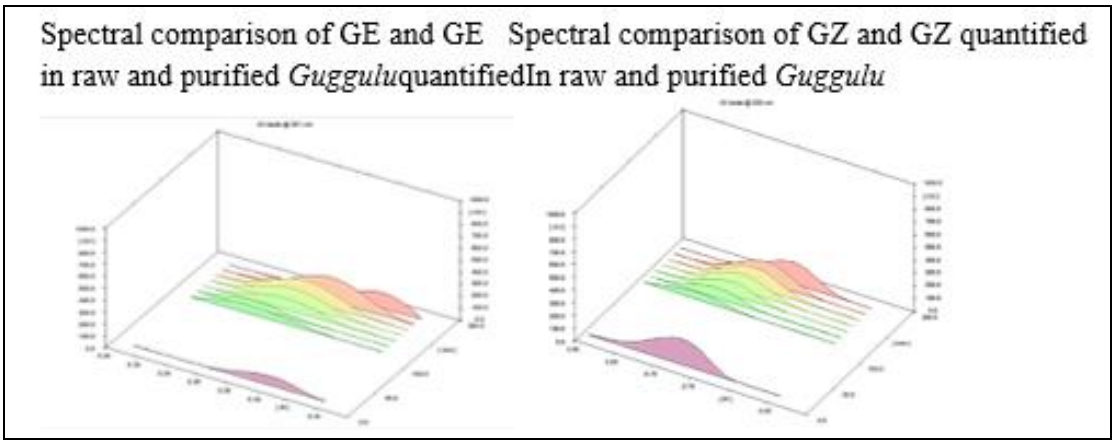

Fig 2: 3D display of chromatogram - Spectral comparison of standard and samples

Table 5: Mean quantities of Guggulsterone E &Z

Sample	%w/w of Guggulsterone E (Mean±SD)	%w/w of Guggulsterone Z (Mean±SD)
Raw *Guggulu*	13.5941±0.0004	44.3698±0.0002
Purified *Guggulu*	16.8801±0.0005	13.2843±0.001

3.3 *In vitro* Anti-bacterial Evaluation

Diameter of zone of inhibition of extract of purified *Guggulu*, Negative control and Ciprofloxacin against tested bacterial strains are given in Table 6. Figure 3 shows the antibacterial study report of extracts of purified *Guggulu* against Pseudomonas aeruginosa, Proteus vulgaris and Klebsiella pneumonia. The data indicates that the purified *Guggulu* extract has significant antibacterial activity against Pseudomonas aeruginosa and Proteus vulgaris, when compared with the standard, Ciprofloxacin.

Many researches have brought out the antibacterial potential of *Guggulu* without purification. It was established that extract of *Guggulu* possess moderate activity against Klebsiella pneumonia. Raw *Guggulu* exhibited very less activity against Pseudomonas aeruginosa (4 mm for 10mg/ml of *Guggulu* extract) [3]. But in the present study, significant activity with increased zone of inhibition was noticed for purified *Guggulu* against Pseudomonas aeruginosa.

No studies were carried out for evaluating the activity of *Guggulu* against Proteus vulgaris. In this study, Maximum zone of inhibition was observed against Proteus vulgaris. The anti-bacterial activity of raw *Guggulu* against Klebsiella pneumonia was already established [3]. But, the extract of purified *Guggulu* is resistant to Klebsiella pneumonia

The organisms like Pseudomonas aeruginosa and Proteus vulgaris causes Urinary tract infections (UTI), wound infections [7, 8] etc. So, in *Guggulu* containing formulations which are beneficial in treating such diseases, *Guggulu* purified in the decoction of Triphala and Guduchi can be used for getting better results

Table 6: Diameter of Zone of inhibition of purified *Guggulu* against bacterial strains

Organisms	Samples (in mm)		
	Purified *Guggulu* (1mg/ml)	Ciprofloxacin (5mg/ml)	DMSO
Pseudomonas aeruginosa	15	14	0
Proteus vulgaris	18	20	0
Klebsiella pneumonia	0	19	0

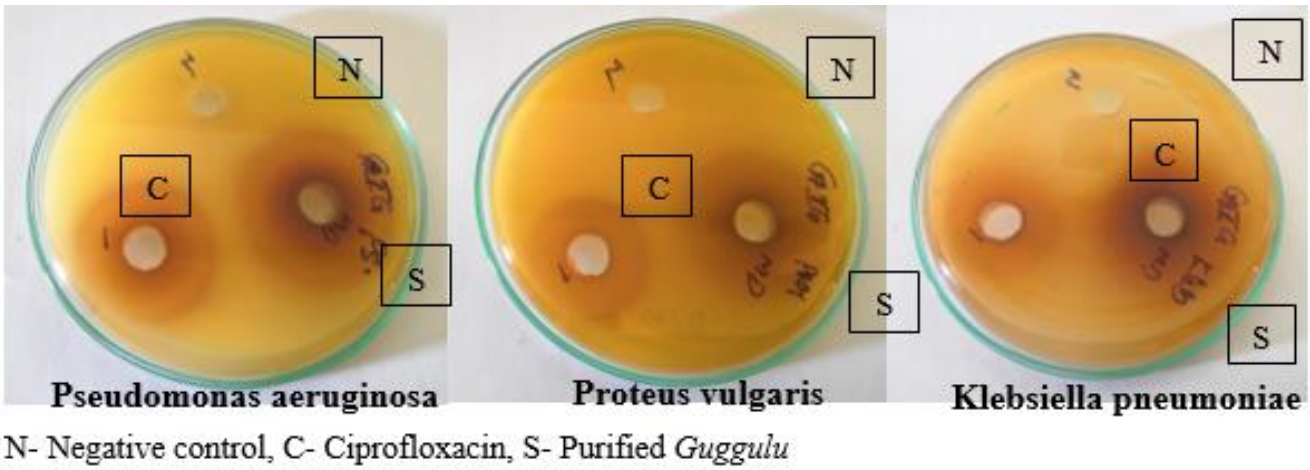

Fig 3: Antibacterial study Results of purified *Guggulu* against various bacterial strains

Along with other pharmacological actions like anti-oxidant anti-inflammatory, anti-arthritic, hypolipidemic, wound healing, cardio protective and thyroid stimulatory, anti-microbial activity of Guggulsterones E and Z has also been established [9]. Though slightly lesser quantity of Total Guggulsterones (E+Z) present in purified *Guggulu*, the improved antibacterial action can be due to the presence of active phytoconstituents like alkaloids, flavonoids, phenols, steroids, Tannins, Total sugars, reducing sugars and also due to acidic pH.

4. Conclusion

The study proved the antibacterial potential of purified *Guggulu* with significant activity against Pseudomonas aeruginosa and Proteus vulgaris. Presence of various phytoconstituents like tannins, phenols, saponins, alkaloids, steroids and flavonoids, also suggestive of the improved antibacterial activity of *Guggulu* after purification. On HPTLC Fingerprinting, it was observed that the quantity of Guggulsterone E has been increased and thus has an impact on the anti-microbial potency of *Guggulu*.

Due to the emergence of antibacterial resistance as well as the evolution of new strains of bacteria, there is a need of natural pharmacotherapeutic agents. Hence the purified *Guggulu* can be explored to discover new bioactive compounds that may serve new pharmaceutical agents. Further, antimicrobial studies of purified *Guggulu*can be performed on different other microbial strains to get a braoder view of the activity.

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6. Conflict of Interest

No conflict of interest declared

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