Research Article

OPTIMIZED SEPARATION AND QUANTIFICATION OF EUGENOL FROM A TRADITIONAL UNANI MEDICINE *JAWARISH-E-BISBASA* USING HPTLC

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Accepted on: 08-04-2011; **Finalized on:** 11-07-2011.

ABSTRACT

Jawarish-e-Bisbasa (*JEB*) is a traditional Unani medicine commonly used for clinical treatment of stomach ulcers, weakness of digestion, piles, flatulence, nausea, gastritis and as energy enhancer for stomach. It is a polyherbal preparation containing 10 ingredients which are mainly used as a remedy for many gastric disorders. Though this Unani semi solid formulation enjoys great reputation, its standardization and quality control parameters are not well defined. In this paper an attempt has been made to standardize *JEB* using modern analytical methods. In-house preparation of *JEB* was carried out as per traditional Unani Materia Medica. Preliminary phytochemical evaluation of *JEB* revealed the presence of flavonoids, essential oils, tannins, glycosides, alkaloids and resins. *JEB* was evaluated for its crude fiber and reducing sugar content. Eugenol is one of the active components found in plant ingredients added during preparation of *JEB*. A simple, rapid and accurate HPTLC method was developed for quantification of Eugenol from *JEB* and its ingredients. Method was validated as per ICH guideline and applied for stability studies of *JEB* stored at different storage periods. A comparative evaluation of *JEB* prepared in-house was carried out with three available marketed samples in terms of Eugenol content. Evaluation of *JEB* by these scientific methods may be used as Quality control methods for the Standardization of *JEB*.

Keywords: Standardization, *Jawarish-e-Bisbasa*, HPTLC, Eugenol, Stability study.

INTRODUCTION

In the past decade, there has been renewed attention and interest in the use of traditional medicine (Ayurveda, Naturopathy, Unani, Siddha, and Homeopathy) and Yoga globally. Under the parasol of traditional medicine systems, the Unani system of medicine is also gaining global acceptance due to the amazing clinical efficiency of the formulations[1].

Tibb-e-Unani (Unani medicine) claims to possess many safe and effective single drugs and compound formulations of herbal, animal and metal origin which are used to cure a wide range of diseases[2]. Unani compound preparations are commonly used in four forms viz. solid (*Habb, Qurs, Safoof, Kushta*), semi solid (*Majoon, Laooq, Marham, Zimaad*), liquid (*Sheera, Rooh, Sharbat, Tila*) and gaseous (*Bakhoor, Inkibaab, Ghalia*) etc[3].

Although such Unani medicines have been used since ancient times, there is negligible documented evidence regarding their standardization and quality control[1]. Quality control and quality assurance of such compound traditional formulations relies upon good manufacturing practices with adequate batch to batch analysis and standardized method of preparation. These traditional formulations must confirm test for identity, potency, purity, safety and efficacy as per WHO guidelines[5-7].

Majoon is a type of semi solid medicinal preparation obtained by mixing powdered drugs in a base of simple syrup (*Qiwam*) made of purified honey, sugar or jaggery. *Jawarish* is a type of *Majoon* prepared by mixing coarse powder of drugs to the *Quiwam*[4].

Jawarish-e-Bisbasa (*JEB*) is a polyherbal preparation used as a remedy for many gastric disorders. Formula composition of *JEB* includes ten medicinal herbs namely *Bisbasa* (Aril of *Myristica fragrans* Houtt.), *Taj-Qualmi* (Stem bark of *Cinnamomum tamala* Nees & Eberm), *Heel-e-Khurd* (Seeds of *Elletaria cardamomum* Maton), *Zanjabeel* (Rhizomes of *Zingiber officinale* Rosc.), *Filfil Daraz* (Fruits of *Piper longum* Linn), *Darchini* (Stem bark of *Cinnamomum zeylanicum* Blume.), *Asaroon* (Roots of *Asarum europaem* Linn), *Qaranfal* (Fruits of *Syzygium aromaticum* (L.) Merr. & Perry), *Filfil Siyah* (Fruits of *Piper nigrum* Linn) and *Heel-e-Kalan* (Seeds of *Ammomum subulatum* Roxb.)[4, 8].

Due to lack of modern pharmacopoeial standards laid down and followed for processing of *JEB*, the medicine prepared using the traditional methods may not have desired quality and batch to batch consistency. Hence there is a need for standardization of *JEB* using modern scientific procedures[9].

In the present work, quality of raw materials used for preparation of *JEB*, was assessed using parameters of proximate analysis such as ash values, loss on drying and foreign matter as per standard pharmacopoeial methods[10]. In–house *JEB* was prepared in the Herbal Research Laboratory of Ramnarain Ruia College, Mumbai as per the classical reference.

Preliminary phytochemical evaluation, reducing sugar content and crude fiber content of *JEB* was evaluated as per standard methods[11-13].

Many ingredients of *JEB* are reported to possess antibacterial, antioxidant and gastroprotective activities[14-

[21]. Eugenol is the active constituent present in all the ten ingredients of the formulation[22-24] and reported to possess antioxidant[25], antibacterial[26-27], antifungal[28], anthelmintic,[29] gastroprotective and hepatoprotective activities[30].

A precise, accurate and reproducible HPTLC densitometric method was developed and validated for quantification of Eugenol from the complex matrix of *JEB* and its ingredients.

Although, quantification of Eugenol from some of the plants and compound preparations using TLC technique have been reported, viz. *Aegle marmelos*, *Trachyspermum ammi*, *Foeniculam vulgare*[31], *Taj-Qualmi*[22], *Qaranfal*[23, 32], *Filfil Siyah*[24], *Amukkara Churnam*[33], *Lavangadi Vati*[24] etc. but eugenol has not yet been quantified from *Bisbasa*, *Heel-e-Khurd*, *Zanjabeel*, *Filfil Daraz* , *Asaroon*, *Heel-e-Kalan* and *JEB*. This is the first attempt for quantification of Eugenol from the same.

Stability testing of herbal drug preparations and finished products and its comparison with available marketed formulations is necessary to ensure the quality of the formulation. Stability tests on herbal drug preparations and finished products are a rather new application of HPTLC[9]. Hence the developed HPTLC method was further applied to study the effect of different storage periods on Eugenol content of *JEB*. A comparative evaluation of three marketed brands of *JEB* was carried out with in-house *JEB* on the basis of respective Eugenol content.

MATERIALS AND METHODS

Plant Materials: Raw materials used for the preparation of *JEB* and three different marketed brands of *JEB* (M-01, M-02, M-03) were procured from Ratan Gandhi Shop, Pydhonie, Mumbai and authenticated by Herbal Research Lab, Ramnarain Ruia College. Collected materials were dried in oven at 45^{0}C, powdered and sieved through an 85-mesh (BSS) sieve. All the raw materials were stored in an air tight container at ambient temperature.

Standard and Reagents: The organic solvents and chemicals used for extraction under study were of analytical grade and procured from Qualigens Fine Chemicals, Mumbai, India. Standard Eugenol (≥ 98 % purity, **Figure 1**) was procured from Sigma Aldrich Chemical Company, Germany. Derivatizing reagent i.e. Anisaldehyde Suphuric acid was prepared as per the standard procedure[34].

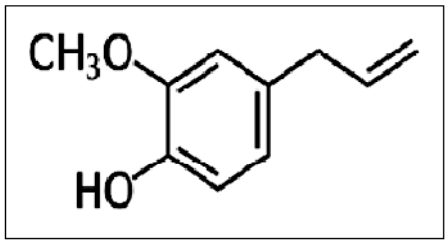


Figure 1: Structure of Eugenol

Proximate analysis of raw materials: To assess the quality of raw materials, proximate parameters like ash values (total ash, acid insoluble ash and water soluble ash), loss on drying and foreign matter were determined using standard pharmacopoeial methods as per WHO guidelines[10].

Preparation of *JEB*: *JEB* was prepared as per classical reference using traditional formula composition **(Table 1)**. *Qiwam* (base for *Jawarish*) of one *Tar* consistency was prepared using *Aab* (Water), *Qand Safaid* (Sugar) and *Asal* (Honey). As per the formula composition, all the ingredients of pharmacopoeial quality and quantity except *Qand Safaid* and *Asal* were taken and mixed thoroughly. The mixture was then added into the prepared *Qiwam* (when hot), homogenized, cooled to room temperature and packed in air tight container for further analysis.

Table 1: Formula composition for *JEB*

Sr. No.	Ingredients		Quantity
	Unani Name	Botanical Identity	
1	*Bisbasa*	Aril of *Myristica fragrans* Houtt.	1 part
2	*Taj-Qualmi*	Stem bark of *Cinnamomum tamala* Nees & Eberm	1 part
3	*Heel-e-Khurd*	Seeds of *Elletaria cardamomum* Maton	1 part
4	*Zanjabeel*	Rhizomes of *Zingiber officinale* Rosc.	1 part
5	*Filfil Daraz*	Fruits of *Piper longum* Linn	1 part
6	*Darchini*	Stem bark of *Cinnamomum zeylanicum* Blume.	1 part
7	*Asaroon*	Roots of *Asarum europaem* Linn	1.5 part
8	*Qaranfal*	Fruits of *Syzygium aromaticum* (L.) Merr. & Perry	2 part
9	*Filfil Siyah*	Fruits of *Piper nigrum* Linn	5 part
10	*Heel-e-Kalan*	Seeds of *Ammomum subulatum* Roxb.	25 part
11	*Qand Safaid*	Sugar	25 part
12	*Asal*	Honey	1 part

Preliminary phytochemical evaluation: Phytoconstituents in *JEB* were evaluated by performing preliminary phytochemical tests for flavonoids, essential oils, tannins, glycosides, alkaloids and resins as per standard methods[11].

Physicochemical evaluation: *JEB* was also subjected to physicochemical evaluation. Crude fiber and reducing sugar content of *JEB* was determined using Dutch method[12] and DNSA method[13] respectively.

Chromatographic evaluation

HPTLC conditions: Chromatographic separation was achieved on HPTLC plates (20 X 20 cm) precoated with silica gel 60 F_{254} (E. Merck) of 0.2 mm thickness with aluminum sheet support. Samples were spotted using CAMAG Linomat IV Automatic Sample Spotter (Camag Muttenz, Switzerland) equipped with syringe (Hamilton, 100 µL). Plates were developed in a glass twin trough chamber (CAMAG) pre-saturated with mobile phase. Scanning device used was CAMAG TLC Scanner 2 equipped with winCATS software. The experimental condition was maintained at 20 ± 2^{0}C.

Preparation of Standard solutions of Eugenol: Stock solution of Eugenol (1000 µg mL^{-1}) was prepared by dissolving 10 mg of accurately weighed standard in methanol and making up the volume to 10 mL in standard volumetric flask. Aliquot of 40-180 µg mL^{-1} was prepared from this stock solution for calibration curve of Eugenol. Further three quality control samples (LQC: MQC: HQC) of Eugenol (50, 85 and 145 µg mL^{-1}), were prepared for precision, accuracy and ruggedness studies.

Extraction of Phytoconstituents from *JEB*, its ingredients and marketed samples: Extraction of phytocostituents from *JEB* was optimized to achieve good fingerprinting and also to resolve the marker compound Eugenol efficiently. To the accurately weighed *JEB* (1 g), 10 mL of methanol was added, vortexed for 1-2 minutes and kept standing overnight at room temperature. Next day it was filtered through Whatmann filter paper No. 41(E. Merck, Mumbai, India) and the filtrate was further used for HPTLC analysis. Similar extraction procedure was followed for ingredients of *JEB* and its marketed samples.

Solvent system: Solvent system consisting of Toluene: Ethyl Acetate: Glacial Acetic Acid (8: 2: 0.1, v/v/v) was used to resolve and quantify Eugenol from the matrix of *JEB*, its ingredients and available marketed samples.

Method Validation: ICH guidelines were followed for the validation of the developed analytical method (CPMP/ICH/281/95 and CPMP/ICH/381/95).

Specificity: Specificity was ascertained by analyzing standard compound with sample. The band of Eugenol from sample solution was confirmed by comparing its R_f and spectra with that from standard. The peak purity of all the compounds was analyzed by comparing the spectra at three different levels, i.e. start, middle, and end positions of the bands.

Instrumental Precision and Repeatability: Instrumental precision was checked by repeated scanning (n = 7) of the same spot of Eugenol (50 µg mL^{-1}) and further expressed as relative standard deviation (% RSD). The repeatability of the method was affirmed by analyzing 50 µg mL^{-1} of Eugenol on a HPTLC plate (n = 5) and expressed as % RSD.

Inter-Day and Intra-Day Precision: Variability of the method was studied by analyzing Quality control samples of Eugenol (50, 85 and 145 µg mL^{-1}) on the same day (intra-day precision, n = 3) and on different days (interday precision, n = 3). The results were expressed as % RSD.

Limit of Detection and Limit of Quantitation: Limit of detection (LOD) and limit of quantitation (LOQ) of the developed method was affirmed by analyzing progressively low concentrations of Eugenol along with methanol as blank. Limit of detection (LOD) and limit of quantitation (LOQ) were established at a signal to noise ratio of 3:1 and 10:1 respectively.

Assay of Eugenol from *JEB*, ingredients of *JEB* and marketed samples: Sample solution (10 µL) of each was applied in triplicate to a pre-coated silica gel 60 F_{254} HPTLC plate (E. Merck) with the Camag Linomat 3 sample spotter. The plate was developed in above mentioned mobile phase, air dried and derivatized with Anisaldehyde Sulphuric acid reagent. Scanning of the plate was done at 550 nm.

Recovery: The accuracy of the method was assessed by performing recovery studies at three different levels (25, 50 and 100 %). Appropriate concentrations of Eugenol were spiked into *JEB* matrix and then each was analyzed as per the developed method. The percent recoveries at each level were calculated to deduce average percent recovery.

Ruggedness: Ruggedness of the method was assessed by deliberately incorporating the small variations in the optimized chromatographic condition. Effect of change in analyst, change in mobile phase composition [Toluene: Ethyl Acetate: Glacial Acetic Acid (8.1: 1.9: 0.1, v/v/v) and Toluene: Ethyl Acetate: Glacial Acetic Acid (7.9: 2.1: 0.1, v/v/v)] and change in spotting volume (9 µL and 11 µL) on the response and R_f of quality control samples was observed.

Method application: The developed HPTLC method was applied further to study the stability of *JEB* samples stored at different storage periods. Comparative evaluation of in-house *JEB* was carried out with three marketed brands (M-01, M-02 and M-03) in terms of their respective Eugenol content using developed method.

RESULTS AND DISCUSSION

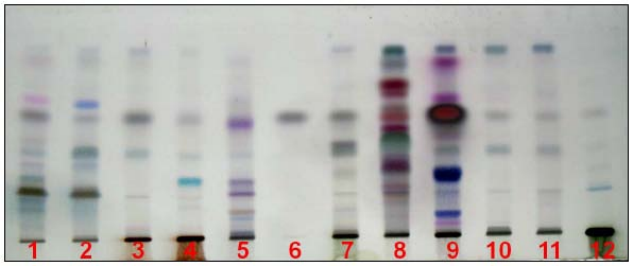
Quality assurance is an integral part of all systems of medicine to ensure quality medicament. Thus, there is an urgent need to evaluate such parameters which can be adopted by the pharmaceutical industries. There are reports on standardization of some popular Unani medicines[2, 3, 35].

In the communication an attempt has been made to standardize the raw materials as well as finish product of *JEB* using modern scientific procedures. The results for proximate analysis of raw materials for parameters like ash values (total ash, acid insoluble ash and water soluble ash), loss on drying and foreign matter were found in compliance with pharmacopoeial limits (data not shown).

Preliminary phytochemical evaluation of *JEB* revealed presence of all the phytoconstituents studied. Crude fiber and reducing sugar content of *JEB* was determined using standard methods and it was found to be 0.707 ± 0.003 % and 73.717 ± 0.6809 % respectively.

Of the various solvent systems tried, mixture containing Toluene: Ethyl Acetate: Glacial Acetic Acid (8: 2: 0.1, v/v/v) gave the best resolution for Eugenol (R_f = 0.56) from the plant matrix which enabled its quantification as well as phytochemical fingerprint **(Figure 2)**. The identity of the band of Eugenol in *JEB* and its ingredients was confirmed by their UV absorption spectra with that of the standard **(Figure 3)**.

Figure 2: Detection of Eugenol from *JEB* and its ingredients at 550 nm using HPTLC



Track 1: *Filfil Siyah*, Track 2: *Filfil Daraz*, Track 3: *Darchini*, Track 4: *Taj-Qualmi*, Track 5: *Zanjabeel*, Track 6: Eugenol Standard, Track 7: *Asaroon*, Track 8: *Bisbasa*, Track 9: *Qaranfal*, Track 10: *Heel-e-Khurd*, Track 11: *Heel-e-Kalan*, Track 12: *JEB*

Figure 3: Specificity of the method showing absorption spectra of Eugenol from *JEB* and its ingredients

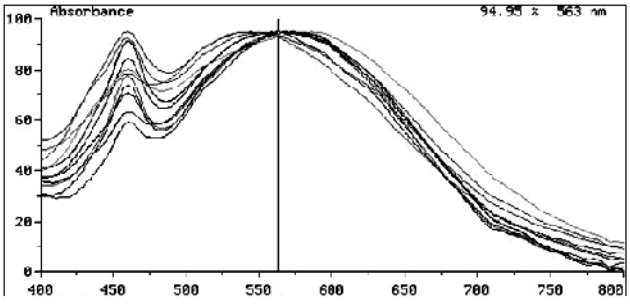
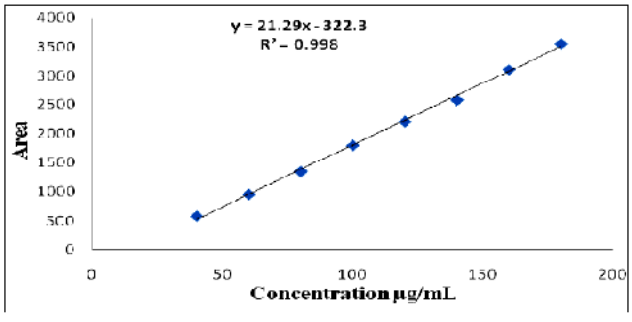


Figure 4: Linearity of Eugenol



Pharmacologically active phytoconstituent Eugenol was quantified from *JEB* by HPTLC densitometric method and it was validated in terms of precision, repeatability and accuracy as per ICH guidelines **(Table 2)**. Response for Eugenol was found to be linear in the range of 40–180 µg mL^{-1} with a correlation coefficient (r^2 value) of 0.998 **(Figure 4)** which resulted in a regression equation y = 21.29x - 322.3. This regression equation was then used to determine respective Eugenol content of *JEB*, its ingredients and, marketed samples. The assay results of the same are represented in **Table 3**, **4, 5**. Developed TLC densitometric method was found to be precise with % RSD in the range of 0.65-0.91% and 1.31-1.57% for intra-day precision and inter-day precision respectively for studied Quality control samples of Eugenol **(Table 6)**. This indicates that the method is precise.

Table 2: Method Validation parameters for Eugenol

Parameters	Results
Instrumental precision (% RSD, n = 7)	0.81
Repeatability (% RSD, n = 5)	0.75
Linear Working Range (µg mL^{-1})	40 to 180
Regression equation	y = 21.29x - 322.3
Correlation coefficient (r^2)	0.998
LOD (µg mL^{-1})	20
LOQ (µg mL^{-1})	40
Specificity	Specific
Ruggedness	Rugged

Table 3: Eugenol content in ingredients of *JEB*

Ingredients	Eugenol content (mg/g) [Mean (n=3) ± S. D.]
Bisbasa	0.7755 ± 0.0151
Taj-Qualmi	0.9784 ± 0.0132
Heel-e-Khurd	1.4402 ± 0.0062
Zanjabeel	1.9666 ± 0.0044
Filfil Daraz	0.4509 ± 0.0033
Darchini	1.7229 ± 0.0024
Asaroon	1.7433 ± 0.0087
Qaranfal	5.4002 ± 0.0026
Filfil Siyah	0.6826 ± 0.0021
Heel-e-Kalan	0.9375 ± 0.0026

Table 4: Stability study of *JEB*

Storage period (Months)	Eugenol content (mg/g) [Mean (n=3) ± S. D.]
0	0.4689 ± 0.0137
0.5	0.8730 ± 0.0059
1	1.7632 ± 0.0097
2	2.7600 ± 0.0038
3	2.8736 ± 0.0026
4	2.9447 ± 0.0048
5	3.1567 ± 0.0052
6	3.8637 ± 0.0031

Table 5: Comparative evaluation of In-House *JEB* with Marketed Samples

Samples (stored for 6 months)	Eugenol content (mg/g) [Mean (n=3) ± S. D.]
In-House	3.8637 ± 0.0031
M-01	0.6244 ± 0.0094
M-02	0.4791 ± 0.0071
M-03	0.5027 ± 0.0111

Table 6: Precision studies for Eugenol from *JEB*

Concentration (µg mL^{-1})	Intra-day (% RSD)	Inter-day (% RSD)
50	0.85	1.42
85	0.91	1.31
145	0.65	1.57

LOD and LOQ value for Eugenol was found to be 20 and 40 µg mL^{-1} respectively **(Table 3)**. Average recovery at three different levels of Eugenol was found to be 99.45 % **(Table 7)**. Ruggedness of the method for change in analyst and change in mobile phase composition showed variations within acceptable limits. Change in spotting volume at 9 and 11 µL did not affect the R_f of Eugenol but change in response was observed which was within acceptable limits.

Increase in Eugenol content was observed for the stability samples stored at different storage periods **(Table 4)**. The results of stability studies are supported by the frequent references in Unani Pharmaceutical Methods regarding use of *Jawarish*. Eugenol content was maximum in the in-house *JEB* sample when compared with other marketed formulations **(Table 5)**.

Such reproducible modern techniques can make the traditional medicines more acceptable in the local and global market. Thus rationally designed, carefully standardized, synergistic traditional herbal formulations and botanical drug products with robust scientific evidence can also be alternative to modern medicine.

Table 7: Recovery studies for Eugenol from *JEB*

Amount of Eugenol			Recovery (%)	Accuracy (average % recovery)
Present in *JEB* (µg mL^{-1})	Added in *JEB* (µg mL^{-1})	Amount found (µg mL^{-1})		
46.89	0	46.56	99.30	99.45 ± 1.053
46.89	11.72	59.12	100.87	
46.89	23.45	71.25	101.29	
46.89	46.89	90.35	96.34	

CONCLUSION

Present results can be used as a quality control method for characterization of Unani compound samples in industry to check their uniformity. The obtained values of physical and chemical parameters for the finished product can be adopted to lay down new pharmacopoeial standards to be followed in the traditional preparation of *JEB* with batch to batch consistency. The developed HPTLC method for the quantification of Eugenol can be applied to various polyherbal formulations containing Eugenol. A routine use of such scientific techniques will lead to standardization of the Unani medicine to a certain extent and would definitely help in building confidence in use of these products.

Acknowledgement: Authors are thankful to Dr. Sharique Zafar for his help and support.

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