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Standardization of Some Commercial Anti-diabetic Herbal Products containing Syzygiumcumini

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Abstract An isocratic HPLC method was developed for standardization of five commercially available products containing *S. cumini* seeds (DIABECON, MADHULENE, HYPONIDD, D-FIT and DIABEGON)using methylxanthoxylin (MXX)as marker. The method was validated in accordance with ICH Q2(R1) guidelines. The chromatographic separation of MXX was achieved on a C_{18} column by mobile phase composed of methanol and water (60:40%*v/v*) at a flow rate 0.5 mL/min. The eluent was detected at 280 nm. The method was linear over concentration of 1–200 μg/mL with correlation coefficient of 0.9999. The LOD and LOQ were 0.175 and 0.530 μg/mL, respectively. The method was precise (%RSD <0.31), accurate and robust for determination of MXX in herbal extracts. The content of MXX in the seed extract was found to be 0.0433%*w/w* while it was ranging from 0.026–0.041%*w/w* in the products. The content of MXX was found to be equivalent to the pure seed extract only in DIABECON tablets and D-FIT soft gelatine capsules while it was found to be significantly less in the other formulations.

Keywords: Methylxanthoxylin, *Syzygiumcumini*, HPLC, stability testing, anti-diabetic.

1. INTRODUCTION

Syzygiumcumini(*Eugenia jambolana or Syzygiumjambolanum* DC; commonly known as Black Plum or Java Plum), family Myrtaceae is commonly employed in Ayurveda for its antidiabetic properties (Gupta, R. *et al*, 2011; Shinde, J. *et al*, 2008). In addition to this, it also possesses anti-diarrheal (Shamkuwar, P.B. *et al*, 2012), antioxidant (Kaneria, M. *et al*,

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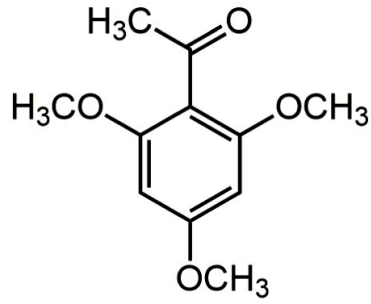

Figure 1: Structure of methylxanthoxylin (MXX).

2013), gastro-protective (Chaturvedi, A. *et al*, 2007), anti-allergic (Brito, F.A. *et al*,2007), anti-inflammatory (Kumar, A. *et al*, 2008), antimicrobial (Pareek, A. *et al*, 2015) and anti-hyperlipidemic (Ravi, K. *et al*, 2005) activities. The major classes of phytoconstituents present in *S. cumini*are phenols, tannins, alkaloids, triterpenoids and volatile oils (Kavitha, K. *et al*, 2011; Murti, K. *et al*, 2012). Methylxanthoxylin(MXX), chemically known as 1−(2,4,6−trimethoxyphenyl)ethanone (Figure 1), is the principal chemical constituent in *S. cumini*seeds. The later is employed as one of major constituents in all anti-diabetic herbal products, which are used rampantly in developing countries to treat diabetes mellitus. However, no method is reported for standardization of *S. cumini* seeds. Moreover, the chemical constituent responsible for anti-diabetic activity of *S. cumini*seeds is also not known so far. Hence, the present study is designed to develop and validate a HPLC method to standardize herbal formulations containing *S. cumini* seed taking MXX as analytical marker.

2. MATERIAL AND METHODS

2.1 Plant Materials and Chemicals

Fruits of S. *cumini* were purchased from local market of Patiala (India). MXX (Sigma-Aldrich, Bangalore, India) and methanol (HPLC grade, Qualigen, Mumbai, India) were procured commercially. The solutions were prepared using Milli-Q water obtained from Milli-Q Water purification System (Synergy, Millipore, USA) in the laboratory. Herbal formulations containing seeds of *S. cumini* (DIABECON, Himalaya Drug Company, Bangalore, India; MADHULENE, TrigunaAyurvedic Research Laboratory, New Delhi, India; HYPONIDD, CharakPharmaPvt. Ltd. Mumbai, India; D-FIT, Shridhanvantri Herbals, Amritsar, India and DIABEGON, Him Aushadh Pharmacy, Jallandar, India) were purchased from local pharmacy store.

2.2 Instruments

The Waters HPLC system comprising 2487 UV/VIS dual wavelength detector, 515 binary pumps andRheodyne manual injector was employed for chromatographic analysis (Waters, Milford, USA). The chromatographic separation was achieved on Inertsil® ODS-3V (4.6×100 mm; 3μ) column (GL Sciences Inc., Japan). Data was acquired and processed in Waters Empower 2 software. Rotary vacuum evaporator (Perfit, Ambala, India) was employed for concentration of the extracts. Freeze drier (Allied Frost®) from Macflow Engineering Pvt. Ltd., New Delhi, India) was employed for freeze drying the extracts.

2.3 Preparation of Extract and Solutions

Pulp of the fruits of *S.cumini* was removed. Seeds were dried in shade and ground to powder. For preparation of aqueous extract,100 g of the seed powder was soaked in 100 mLof water for 24 h with occasional shaking. The extract was filtered and extraction of the marc was repeated. The two aqueous extracts were combined and concentrated on rotary vacuum evaporator to about 20 mL. For preparation of extracts of herbal products, contents of 10 units of each formulation were extracted with water (10 mL) separately by the same method as for *S. cumini*seeds. The final extracts of the seeds as well as formulations were freeze dried and used as and when required as 1 mg/ml solution in water. A stock solution of MXX (1 mg/mL) in water was serially diluted with mobile phase (used as diluent) to obtain standard solutions of MXX in concentration range of 1−500 μg/mL.

2.4 Chromatographic Separation Method

The chromatographic separation of MXX from the other constituents of the seeds was achieved on a C_{18} column (4.6×100 mm; 3 μ, Inertsil®) by mobile phase composed of methanol and water (60:40%*v/v*) flowing at a rate 0.5 mL/min at ambient temperature (25−30°C). The eluent was detected at 280 nm and injection volume was 20 μL.

2.5 Method Validation

The method was validated by evaluating linearity, precision, accuracy, robustness, specificity, selectivity, limit of detection (LOD) and limit of quantification (LOQ) in accordance with ICH guidelines Q2(R1) (ICH, 2005). Linearity was evaluated by analyzing standard solutions of MXX having concentrations in range of 1−500 μg/mL. The standard solutions were prepared in triplicate to generate one calibration curve from each set

of standard solutions and subjected to linear regression analysis to calculate slope, intercept and correlation coefficient (r^2). The precision was determined by analyzing each of three concentrations (5, 50 and 200μ*g/mL*) of MXX on the same day (intra-day) as well as on three different days (inter-day). Each concentration was analyzed six times consecutively and precision was expressed as %RSD of each calculated concentration. Accuracy of the method was expressed as percent recovery of MXX from the aqueous extract fortified with known concentration of MXX. An aqueous solution of the extract was diluted with equal volume of water and analyzed for the concentration of MXX. The resultant dilute solution served as unfortified solution. The same aqueous extract solution was also mixed separately with equal volume of MXX standard solutions of concentrations 10, 100 and 500μ*g/mL* in order to produce the solutions having concentrations of MXX in the dilute solutions fortified by 5, 50 and 125μ*g/mL*, respectively. The unfortified and fortified solutions were prepared in triplicate and recovery of MXX from fortified solutions with respect to unfortified solution was calculated. LOD and LOQ were determined through the method of calibration curves using the formulae LOD = 3.3σ/S and LOQ = 10σ/S (σ = SD of slope and S = mean intercept). Subsequently, solutions of MXX corresponding to the calculated LOD and LOQ were prepared and analyzed (n=10) on the same day to determine the %RSD of the peak areasat each level. The method was also evaluated for its robustness by making deliberate but small changes in various chromatographic parameters of the optimized method such as composition of mobile phase, detection wavelength (λ_{max}), flow rate and column brand. Standard solution of MXX (50 μg/mL) was analyzed (n=3) at each varied chromatographic condition and recovery of MXX was calculated. The percentchange in content as well as change in retention time (RT) of MXX at each varied condition was noted vis-à-vis the optimized chromatographic conditions.

2.6 Standardizationof extract and products

The solutions of freeze dried extract of the seed powder and each formulation in water (1 mg/mL) wereanalyzed for the content of MXX (as %*w/w* of freeze dried extract) using the validated HPLC method.

3. RESULTS AND DISCUSSION

3.1 HPLC Method Development

There is no report on HPLC-UV method for determination of MXX available in the literature. Hence, initially a mixture of methanol and water (50:50%*v/v*) was run as mobile phase at a flow rate of 0.5 mL/min over a C_{18} column

(4.6×100 mm; 3 μm, Inertsil®) using 280 nm detection wavelength (l_{max} of MXX). The MXX was eluted as asymmetrical sharp peak with significant tailing and fronting. Increase in content of methanol to 60% with concomitant decrease in water content eluted the MXX as symmetrical peak at 7.1 min with no tailing and fronting (Figure 2). The HPLC chromatogram of aqueous extract of *S. cumini* showed a peak at 7.4 min almost overlapping with peak of MXX which indicated that MXX may be present in the extract. In order to verify this, UV scans of the standard MXX peak and the similar peak in the extract were obtained through Photo Diode Array (PDA) detector. The two scans were found very similar (Figure 3) which suggested that peak in aqueous extract at 7.4 min was due to MXX only. Further the purity angle and purity threshold of the peak in extract was 22.39 and 7.34, respectively which indicated that peak was pure. The minor deviation in retention time (0.2 min) was attributed to manual injection system or inherent manual inter-injection variability.

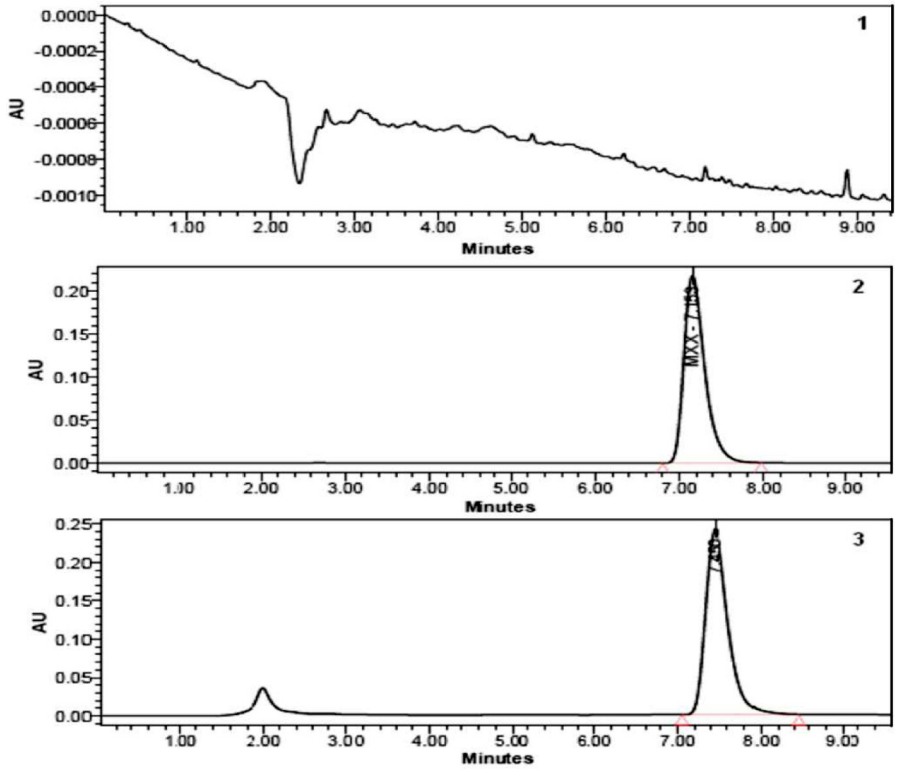

Figure 2: HPLC−UV chromatograms of Blank (1), MXX standard (2) and the extract (3).

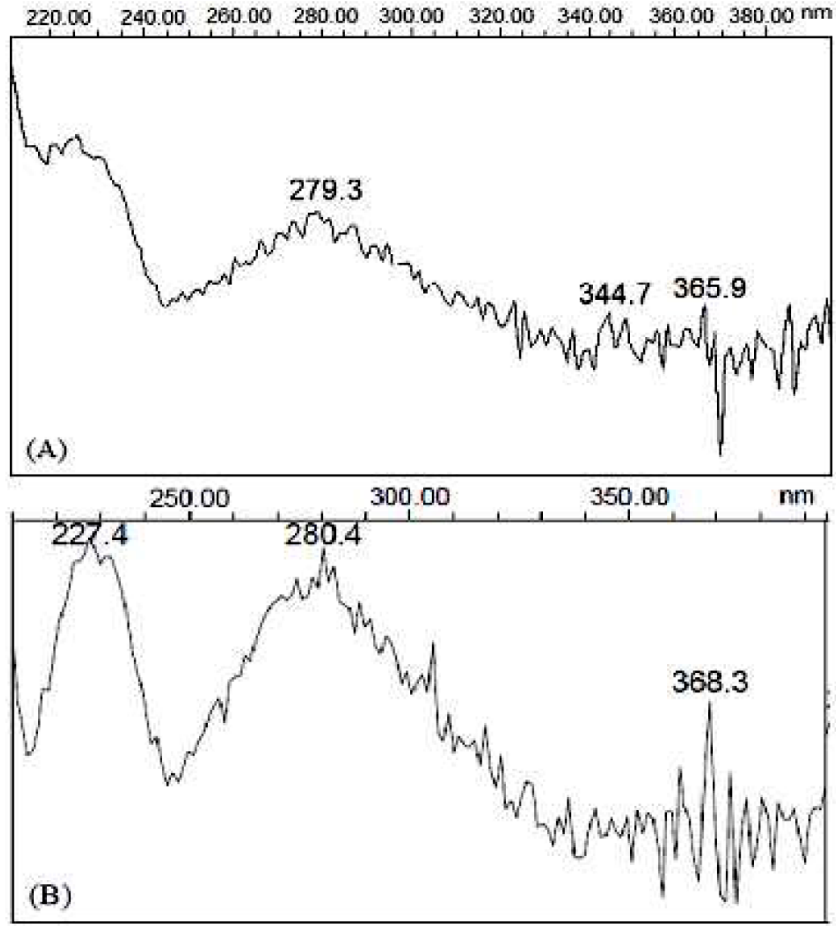

Figure 3: HPLC–PDA scans of peak in MXX solution (A) and the extract (B).

3.2 Method Validation

The method was found linear for determination of MXX in the concentration range of 1–200 μg/ml. The mean (±SD) slope, intercept and r^2 were 72934 (577), –19600 (6655) and 0.99995 (0.00004), respectively. Excellent recoveries (97.18–100.30%) of each fortified MXX concentration were achieved with RSD less than 0.66% (Table 1) which suggested the method to be sufficiently accurate for quantification of MXX in the extract. The method was sufficiently repeatable (intra-day) and reproducible (inter-day) as indicated by %RSD for inter-day and intra-day precision studies being less than 1 (Table 1). The LOD and LOQ were calculated to be 0.175 and 0.530 μg/ml, respectively. The RSD values of 10 injections of each of the LOD and LOQ concentration was found

Table 1: Inter-day and intra-day precision and accuracy studies.

Precision Study		
Actual concentration (µg/mL)	**Calculated concentration(µg/ml) Mean ± SD, %RSD (n = 6)**	
	Inter-day	**Intra-day**
5	4.99 ± 0.01, 0.29	5.00±0.01, 0.27
50	49.96 ± 0.15, 0.29	49.99±0.15, 0.31
200	200.01 ± 0.40, 0.20	200.05±0.38, 0.19
Accuracy Study		
Concentration fortified (µg/mL)	**Mean concentration found (µg/mL)**	**Mean ± SD; %RSD (n = 3)**
5	4.89	97.86 ± 0.64; 0.65
50	48.71	97.42 ± 0.21; 0.22
125	124.94	99.90 ± 0.33; 0.33

to be 3.10% and 1.86%, respectively. The percent change in calculated MXX content and change in R_T of MXX was found insignificant after deliberate changes in method variables including $\lambda_{max,}$ composition of mobile phase, pH of mobile phase, flow rate and column (Table 2). It established the method to be sufficiently robust for the purpose. The method was found specific and

Table 2: Robustness Study.

Chromatographic Parameter	**RT of MXX (min)**	**DRT w.r.t optimized conditions**	**MXX content (µg/mL)**	**% Difference in MXX content**
Optimized condition	7.15	––––– –––––	50.74	––––––– ––
Flow rate *(0.45mL/min)*	7.91	0.76	50.13	1.18
Flow rate *(0.55mL/min)*	6.56	0.59	49.23	2.87
Mobile phase Water:Methanol *(38:62)*	7.04	0.11	48.62	4.17
Mobile phase Water:Methanol *(42:58)*	7.27	0.12	50.52	0.43
Detection wavelength (*275 nm*)	7.19	0.04	49.38	2.68
Detection wavelength (*285 nm*)	7.17	0.02	48.62	4.17
Column (*Spherisorb*®)	4.34	2.81	49.18	3.07

selective to MXX as indicated by its good resolution from the other constituents in the extract as well as purity of the peak through PDA analysis.

3.3 Standardization of Seed Extract and Herbal Products

The content of MXX in freeze dried aqueous extract of *S. cumini* seeds was found to be 0.0433%*w/w* of the extract. MXX was found to be present and resolved from the other constituents present in extracts of all the five formulations (figure 4). The content of *S. cumini* seed powder in the selected formulations varied from 20 mg to 150 mg per tablet. The content of MXX was found to be equivalent to the pure seed extract only in DIABECON tablets and D-FIT soft gelatine capsules while it was found to be significantly less in the other formulations (Table 3).

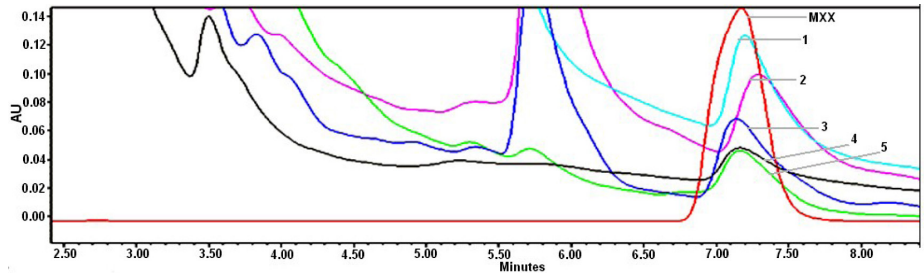

Figure 4: Overlaid chromatograms showing MXX in standard solution and in different anti-diabetic herbal formulations (1: Diabecon; 2: D-fit; 3: Diabegon; 4: Madhulene and 5: Hyponidd).

Table 3. Amount of MXX in different herbal formulations.

Herbal formulation	S. cumini content (mg)	Content of MXX (% w/w)
Diabecon	20	0.041
D−fit	50	0.039
Diabegon	25	0.029
Madhulene	72	0.024
Hyponidd	150	0.026

4. CONCLUSION

An HPLC-UV method for determination of MXX has been developed for standardization and stability testing of herbal formulations containing *S. cumini* seeds. The method has been validated and found linear in the concentration

range of 1–200 mg/mL with a correlation coefficient of 0.9999, precise, accurate and sensitive for MXX. The method has also been found robust as no significant difference (less than 4.2%) in the calculated amount of MXX is noted with deliberate changes in various chromatographic conditions including composition of mobile phase, flow rate, column and detection wavelength. Additionally, the PDA analysis revealed that no peak in the extracts co-elute with peak of MXX. The contents of MXX in only two formulations were found equivalent to that in the pure seeds.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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