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Method Development and Validation of *Aegle marmeleous*

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ABSTRACT

Aegle marmelous is one of the important plant with several medicinal & nutraceutical properties. It is commonly known as woody apple plant which belongs to rutaceace family. It is known for various medicinal properties in traditional medicinal system & use to cure various diseases. The quantitation and method development of 6-methylchromate hydrate from fruit pulp of bael using HPLC technique has not been reported so far this contains different class of compounds of alkaloids, coumarin, terpenoids, fatty acids and amino acids. 6-methylchromate hydrate is one of coumarin of *Aegle marmeleous* with its potential pharmacological activities such as hypoglycemic, anti-inflammatory, anticancer, antidiabetic, antioxidants. The aim of the present study is to screen different parts of bael for the estimation of 6-methyl chromate hydrate and to quantify 6-methyl chromate hydrate from bael fruit powder collected from standard compound and its formulation using HPLC techniques. The developed method was validated as per ICH guidelines.

KEY WORDS: 6-methyl Chromone hydrate, HPLC, Method development, Validation, Biological activities.

1. INTRODUCTION

Aegle marmeleos is commonly known as Bael, belonging to the family rutaceae, is a slender, is considered as, manufacturing process is also important. Identification of marker compounds in herbs is considered as one of major step in development of analytical methodologies in marker –based standardization. HPLC has recently emerged as one important tool for development and validation of marker compounds in drugs because of its simplicity, specificity, accuracy, precision, for their identification. In the present work, bael fruit was screened for the estimation of 6 methyl chromone hydrate. So far no attempt has been done for the quantification of the bael fruit from different areas and its formulation is done by using HPLC.

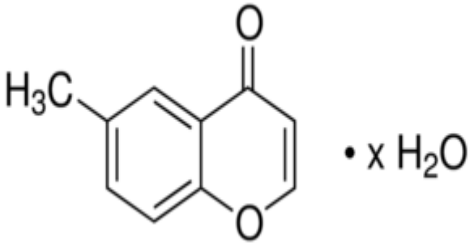

Figure.1. 6-methyl Chromone hydrate

Figure.2. *Aegle marmeleous*

2. MATERIALS AND METHODS

Materials:

Plant materials: *Aegle marmeleos* fruit pulp was collected from various regions around Andhra Pradesh. The above mentioned plant materials are tested in research Centre in Bangalore. The fruit pulp of *Aegle marmelos* was dried and pulverized into powder. About100gm of the powdered sample of each plant material were weighed in 1000ml of ethanol and methyl acetate extract in soxhlet apparatus separately and the process is carried out for 7days at 40^0c-50^0c. The filtrate was evaporated to dryness in desiccator and the process was repeated in several times, until the required amount of extract is obtained.

Chemicals & Solvents: All the chemicals used in this experiment were analyzed in analytical grade. Ammonium acetate of HPLC grade was obtained from Rankem (India). HPLC water is purchased from Merck pvt. Ltd (India). The working standard of 6 methyl chromone hydrate (purity>95%) was purchased from Sigma Arch, Bangalore baseline chromatography, data system.

Syringe: 5µl Hamilton (Switzerland).

Chromatographic condition: The elution was isocratic and the mobile phase is consisting of mixture 40% of buffer and 60% of Methanol. The mobile phase was filtered through 0.45 micron Millipore filter paper. The column Phenomenex Gemini-NX-5 µm C18 (2) 110 Å, LC Column (250 x 4.6 mm), Ea was used for determination. The flow rate was 1mL/min the column was operated at ambient temperature. The volume of sample is injected 20µl. After injecting solutions, column was calibrated for at least 20 minutes with mobile phase flowing through the system. The UV detector was set at a wavelength 254 nm.

Preparation of mobile phase: 0.005mM solution of citric acid buffer solution of 40% and methanol 60% and degassed in ultrasonic water bath for 5min. Filter through 0.45 micron Millipore filter paper under vacuum filtration.

Method:

Preparation of standard solution: Weigh and transfer accurately 50mg of 6-methylcromone hydrate was taken in 50ml volumetric flask and make up the volume to 50 ml with methanol (the concentration of this solution is 1000 µg/ml). Dissolve the substance by using sonicator. Further the volume was made up to the mark.

Preparation of sample solution: Weigh and transfer accurately 50mg of 6-methylcromone hydrate was taken in 50ml volumetric flask and make up the volume to 50 ml with methanol (the concentration of this solution is 1000 µg/ml). Dissolve the substance by using sonicator. Further the volume was made up to the mark.

Method Development: To establish and validate an efficient method for analysis of the drugs in formulation many preliminary tests are conducted. Different chromatographic techniques were employed for the analysis of 6 methyl chromone hydrate in pure dosage form. The pure drug of 6 methyl chromone hydrate was injected into the HPLC system and run by using standard solvents. Water, Acetonitrile, Methanol, Ammonium Acetate were tested to find the best conditions for the separation of 6 methyl chromone hydrate on focus to the develop good symmetrical peak. Water was replaced by citric acid buffer and it is observed that buffer. Methanol gave us a satisfactory results. This mobile phase was tested at different proportion

Finally the optimum condition is chosen as citric acid buffer of pH 3.0: methanol of (40:60) V/V.

This composition of drug is resolved well results. All the measurements were carried out in ambient temperature of the column. To optimize the flow rate, various flow rates are tested. At finally the flow rate 1mL/min for the present work.

Validation of HPLC Method: Validation method was carried out in various as per ICH guidelines. The parameters were assessed are specificity, accuracy, precision, linearity, stability, limit of detection (LOD), Limit of Quantification (LOQ).

Specificity: Specificity is an ability to measure accurately and specifically the concentration of analyte in the sample solution without any interference from other diluents. Solvents of standard and sample solutions were injected in liquid chromatography technique.

Linearity: It is an ability of an assay to obtain test results that are directly proportion to the concentration of analyte in the sample. For the establishment of linearity level different concentrations of 6 methyl chromone hydrate into the HPLC system. Plotting the graph area of peak response against the concentration and to determine

The correlation coefficient (r^2), Y-intercept & % RSD of response factor was tabulated in table.1.

Accuracy: Accuracy is an analytical procedure in which the closeness agreement between the convectional true values was found. Percentage of accuracy was 50%, 100%, 150% of each level was injected three times. The data was shown in table.2.

Precision: It express closeness of agreement between the series of measurement to obtain multiple sampling of same homogenous sample under prescribed conditions.

It is determined in both terms of repeatability (injection & analysis) and intermediate precision. It shows the degree of reproducibility of test result obtained by analyzing the sample under various test conditions such as analyst instruments.

System precision: 10µl of standard solution was injected for 5times and measured peak area for all five injection in HPLC. The % RSD for five replicate injections were calculated and shown in table.3.

Method precision: 10µl of sample solution was injected six times and peak area of chromatogram was used for the calculation of standard deviation and relative were shown in table.3.

Intermediate precision: The ruggedness of an analytical method is the degree of reproducibility of test results obtained by same sample under different conditions. The standard injections were
Injected two times in a day. And calculated the mean &%RSD were tabulated in table.3.

LOD & LOQ: The detection and quantification of 6 methyl chromone hydrate was performed and calculated by S/N (signal to noise ratio) method. The values were tabulated in table.4.

Robustness: It is an analytical method to measure its capacity to remain unaffected small but deliberate variations in the method parameter and provide an indication of its reability during normal usage. Robustness measures the lack of internal influences at test conditions. As a part of robustness and change in mobile phase change in pH was made to evaluate the test result. The method was robust by change in the mobile phase and change of pH.

System suitability: System suitability was carried in freshly prepared standard stock solutions of 6 methyl chromone hydrate was injected to HPLC system for five times and its values are recorded.

3. RESULTS AND DISCUSSION

6 methyl chromone hydrate can be effectively analyzed by the RP-HPLC method with citric acid buffer of pH 3.0: methanol of (40:60) v/v at wavelength of 254nm. The retention time of drug was found to be 2.195. The total time of analysis will be less than 15 min.

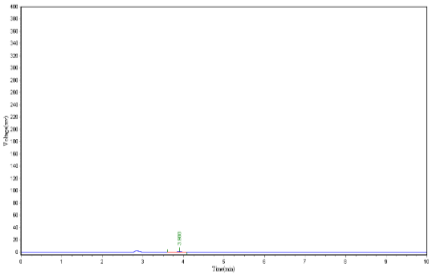

Figure.3. Chromatogram showing blank

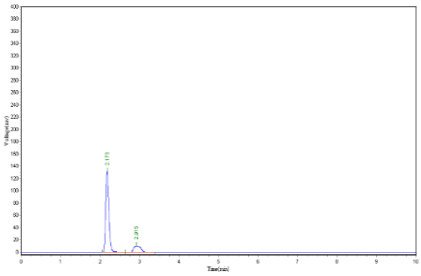

Figure.4. Chromatogram standard solution

Table. Chromatogram of Standard solution

S.no	Peak name	R_t	Area	Height	USP Plate Count	USP Tailing
1	6 methyl chromone	2.173	725665.563	131308.125	3483.841	1.354

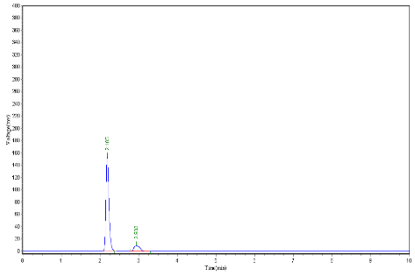

Figure.5. Chromatogram of sample injection

Table. Chromatogram of Sample

s.no	Peak name	R_t	Area	Height	USP Plate Count	USP Tailing
1	6 MCH	2.185	122685.797	10181.948	1145.115	1.247

The specificity of the method whether there is no interferences of other substances in the retention time of the analytical peak. The system suitability of develop, where theoretical plates were 2.915. The tailing factor was 1.3 within the limit.

The linearity study was performed for the concentration range of 0-0.8mg of 6 methyl chromone hydrate and the correlation coefficient was found to be 1.000.

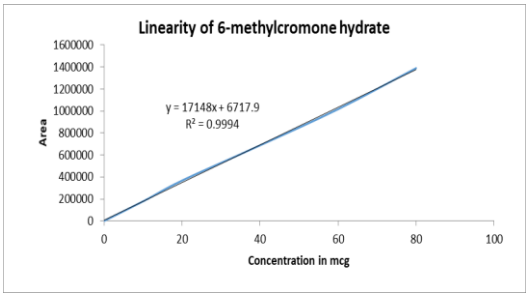

Figure.6. Linearity of 6 MCH

Table.1. Correlation coefficient of 6MCH

Conc. in mcg	Area
0	0
10	178482.052
20	367491.0213
40	688039.2917
60	1017213.188
80	1390144.292
Correlation coefficient value	1.000

The accuracy study was performed for % recovery of 6 methyl chromone hydrate at 80%, 100%, 120%. The recovery of 6 methyl chromone hydrate is 102.3%. The recovery at each level &mean recovery should be 99-102.3%. The accuracy results were tabulated in the table.2.

Table.2. Accuracy of 6 MCH

Accuracy Data sheet		**Electronic file Name: 6-methylcromone hydrate**		
sample	Percentage nominal (mean of three injections)	Amount of standard (µg)		Recovery (%)
		spike	Found	
1	80	32	31.70	99.09
2	100	40	40.95	102.3
3	120	48	47.77	99.52
Percentage	80	100	120	

The precision study %RSD was found to be less than 1% for 6 methyl chromone hydrate, the system precision indicates that the system has good reproductability in the method precision of %RSD was found to be 0.1% which indicates good repeatability in the intermediate system precision study, %RSD, was found to be 0.6% it shown on table.3.

Table.3. Intermediate precision

Intermediate Precision - Datasheet	**Electronic file Name: 6-methylcromone hydrate**		
	HPLC		
concentration	**30µg/ml**		
	Day-1	**Day-2**	**Day-3**
1	663376.375	669249.875	657264.313
2	669806.375	663376.375	661216.688
3	658817.313	669806.375	652732.563
Mean	664000.021	667477.5417	657071.188
St Dev.	5521.011833	3562.597238	4245.358313
%RSD	0.831477659	0.533740391	0.646103252

The result of LOD was found to be 0.01 for 6 methyl chromone hydrate and LOQ was found to be 0.18

6 methyl chromone hydrate	LOD	0.018938	LOQ	0.189382

Robustness of the sample was prepared and run by changing the variations in the percentage of mobile phase is affected significantly. The pH composition were done at ambient temp, the method was robust in less concentration on mobile phase.

Table.4. Robustness studies for change of mobile phase

Mobile phase	**USP Plate count**	**USP Tailing**
10% less	3811	1.32
actual	3649	1.34
10% more	3511	1.35

Similarly, by changing the pH concentration from 4.2 to 4.4 as per method was robust in less pH concentration.

Table.5. Robustness studies for change of pH

Change in pH Concentration	**USP Plate count**	**USP Tailing**
Below 4 pH	3649	1.3
actual	3330	1.4
Above 4 pH	3380.	1.4

The system suitability parameter like theoretical plates, Tailing factor were calculated and were found to be more than 2000. And the proposed RP-HPLC method was accurate & precise in the below table.

Table.6. System suitability parameters

S.No.	**Parameter**	**6 methyl chromone hydrate**
1	Retention time	2.915
2	Theoretical plates	3162.096
3	Tailing factor	1.1

4. SUMMARY AND CONCLUSION

The proposed method was found to be specific, accurate, precise, rapid and economical for the estimation of 6-methyl chromone hydrate in pure dosage form. This method was validated as per ICHs guidelines. The sample recoveries were good.

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