Simultaneous Estimation of Gallic acid and Embelin by Validated HPTLC Method in Three Marketed Formulations and In-house Formulated Manibhadra Yoga: A Polyherbal Ayurvedic Formulation

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ABSTRACT

Manibhadra Yoga Polyherbal Formulation is an official classical polyherbal formulation, mentioned in Ayurvedic Formulary of India and commonly indicated in skin's diseases, abdomen's problems, splenic disease, worm's infestation and respiratory diseases. The two biomarker gallic acid and embelin are simultaneously estimated in in-house formulated MYPF by validated high performance thin layer chromatography (HPTLC) method. The developed HPTLC method was validated on the basis of precision, linearity, specificity, LOD, LOQ, accuracy and robustness. Aluminum backed TLC of silica gel ⁶⁰ ²⁵₄ plate is use stationary phase while for mobile phase toluene: ethyl acetate: methanol: formic acid (5: 4: 0.5: 0.5 v/v) is used. LOD for gallic acid was found to be 75.24 ng/spot and for embelin was 81.60 ng/spot while LOQ for gallic acid was found 227.99 ng/spot and for embelin 247.28 ng/spot. Percentage recovery was found to be in range of 99.59-100.71 for gallic acid and 99.29-98.96 for embelin confirmed that the method was accurate. The content of gallic acid and embelin in in-house formulated MYPF were found to be 46.57 ± 0.814 & 16.15 ± 0.180 mg/mg of formulation respectively which showed highest among all tested marketed formulations (MYPF-M1, MYPF-M2 & MYPF-M3).

The proposed validated HPTLC method provides a new way for standardization and quantitative estimation of gallic acid and embelin in Manibhadra Yoga Polyherbal Formulation.

Keywords: Manibhadra Yoga Polyherbal Formulation, Standardization, Gallic acid, Embelin, Validation, HPTLC.

INTRODUCTION

Ayurveda is a time-tested, trusted worldwide plant based oldest system of medicine which is gradually developed with the time through daily life experiences with the mutual relationship between mankind and nature¹ and its main objective are preventing and curing the disease. Major formulations used in Ayurveda are based on herbs. Some time herbs are also combined with mineral preparations².

Manibhadra Yoga Polyherbal Formulation is an official classical polyherbal formulation,
mentioned in _AFI_. It is commonly indicated in skin’s
diseases, abdomen’s problems, splenic disease,
worms’ infestation and respiratory diseases. _MYPF_
consists of official traditional four medicinal herbs
like Vidanga-fruit, (_Embelia ribes_ Burm. f. 48 g),
Amalaki-fruit precarp, (_Emblica officinalis_ Geartn.
48 g), Haritaki- fruit precarp, (_Terminalia chebula_
Retz. 48 g), Nishotha or Trivrt- Root, (_Operculina_
_turpethum_ Linn. 144 g) and Guda (Jeggary, 576 g).
_MYPF_ is an example of _Avaleha kalpana_ in _Ayurvedic_
formulation because of their easy acceptance.

Chemical constituents present in plants
are very complex in nature and quantitatively differ
when they are cultivated at different places so it is
difficult to establish quality control parameter for plant
based _Ayurvedic_ formulations. Thus quality control
of such _Ayurvedic_ formulations is a challenge for
herbal drug industry and other drug development
organization and is an important task to developed
reliable method for the same. Standardization is
stepwise method to confirm a consistent biological
activity and a consistent chemical profile of _Ayurvedic_
formulation. Standardization is the process of
developing and agreeing upon technical standards.
Hence standardization is a tool in the quality control
process. HPTLC is time tested method for marker
based analysis, both qualitative and quantitative,
for herbal raw materials and finished _Ayurvedic_
products. HPTLC is most appropriate method
provides fingerprint profile and marker-based
standardization of _Ayurvedic_ drugs/formulations.

Gallic acid and embelin are common
phytoconstituents present in _MYPF_ and quantitative
estimation of both phytochemical may be used quality
control of _MYPF_ on regular basis. Therefore the present
study was directed towards validation of developed
method of HPTLC and quantitative determination of
biomarkers, gallic acid and embelin in _MYPF_ by the
HPTLC chromatographic method to ensure the quality
standard of polyherbal _Ayurvedic_ formulation.

**EXPERIMENTAL**

**Plant materials**

All four dried crude drugs, used in
formulation, were purchased from local ayurvedic
crude drug seller, Banaras Hindu University
(BHU), Varanasi (U.P.), India. The crude drugs
were authenticated by Emeritus Scientist of CSIR-
NISCAIR, New Delhi, India. Specimens of all four
crude drugs (NISCAIR/RHMD/Consult/2017/3070-
19-1, 2, 3 & 4) have been submitted in CSIR-
NISCAIR Museum, for future reference.

**Reagents**

The biomarkers Gallic acid (Hi media,
Mumbai, India) and Embelin (Natural Remedies,
Bangalore, India) were used as working standards.
Methanol, toluene, formic acid and ethyl acetate were
purchased from SDF Chem. Ltd. (Mumbai, India).

**Preparation of _MYPF_**

Manibhadra Yoga Polyherbal Formulation
was prepared in Advanced Natural Product
Laboratory, KIPM, GIDA, Gorakhpur, (U.P.), India,
as per the method described in _AFI_. The prepared
avaleha was stored in amber colored container for
a few days in cool place for maturation.

**Preparation of gallic acid (std) and embelin (std)
solution**

Weighed quantity of gallic acid (10 mg) and
embelin (10 mg) were taken in 10 mL volumetric
flask separately and dissolved in HPLC grade water
and make up volume upto mark. Thus stock solution
(1 mg/mL) of gallic acid and embelin were prepared
and from this, working standard (std) solutions
(200 μg/mL to 1200 μg/mL) were prepared.

**Preparation of _MYPF_ solution**

Dried 100 mg of in-house formulated _MYPF_
and its market formulations (_MYPF-M1_, _MYPF-M2_
& _MYPF-M3_) were extracted by maceration process
with 100 mL HPLC grade methanol for three days.
After three days, the extracts were collected and the
marc was further extracted by maceration process
with 100 mL HPLC grade methanol for three days
for each formulations. This process was repeated
one more time with the marc. Extraction efficiency
test was carried out to check the complete extraction
of marker compounds from the sample. All extracts
were combined and concentrated under reduced
temperature. After complete drying of extract, a solution
of 1 mg/mL (stock solution) was prepared in HPLC
grade methanol. Diluted extract was filtered through
filter paper and again through 0.2 μm syringe filters
for further chromatographic studies. Further required
dilutions for the study were made in mobile phase.
**Procedure and analytical conditions**

Aluminum backed TLC of silica gel 60F<sub>254</sub> plates (10x10 cm, 0.2mm thickness) previously dried at 110°C for 30 m in oven and placed in a desiccator, are used for analysis. Application of standard and sample solutions was done as 8mm wide band width by Hamilton micorsyringe with the help of Linomat V applicator. Spotted TLC plate was developed ascending pattern in a saturated CAMAG twin trough glass chamber, using toluene:ethyl acetate:methanol:formic acid (5: 4: 0.5: 0.5 v/v) as a mobile phase, at 25 ± 2°C, migration distance 80 mm for both gallic acid and embelin. The developed plates were dried at 60°C. Developed plate was kept in CAMAG TLC Scanner 3 densitometer for scanning at λ<sub>max</sub> = 254 nm for gallic acid and embelin using UV light. The HPTLC system equipped with winCATS Software.

**Limit of Detection (LOD) and Limit of Quantitation (LOQ)**

LOD was calculated at a signal-to-noise ratio of 3:1 and LOQ at signal-to-noise ratio of 10:1. Both were calculated from the equations LOD= 3 x δ/s and LOQ= 10 x δ/s (δ = SD of the peak area and s = slope of plot).

**Accuracy**

Accuracy was estimated through the % recoveries of known quantities of the combination of gallic acid and embelin added to solution with formulation. The specificity of the method was established by comparing of overlain spectra and R<sub>f</sub> values gallic acid, embelin and samples. The peak purity of gallic acid and embelin were determined by comparing the spectra.

**Robustness study**

The robustness was determined by varying the chamber saturation time by ± 10 m, changing detecting wavelength by ± 2 nm and varying the composition of major solvent (toluene and ethyl acetate) by ± 0.5 mL.

**Simultaneous determination of gallic acid and embelin**

10 μl (10 μg) of MYPF and its market formulations (MYPF-M1, MYPF-M2 & MYPF-M3) were spotted in previously dried silica gel 60F<sub>254</sub> plate. Developed plate was scanned as per the method described earlier. The data fro peak areas were noted. The quantity of gallic acid and embelin in formulations (MYPF-M1, MYPF-M2 & MYPF-M3) were calculated from equation of linear regression line obtained from curves of standard gallic acid and standard embelin. The results of triplicate analysis were expressed as average amount of gallic acid and embelin in μg/mg of formulation.

**RESULT AND DISCUSSION**

**Selection of mobile phase**

Selection of mobile phase is an art for development of successful HPTLC method. It involves a number of trials with different solvent system in different ratio. For this HPTLC method development, solvent system of toluene: methanol: formic acid: chloroform, toluene: formic acid: ethyl acetate and toluene: methanol: ethyl acetate:formic acid in different proportion was studied. The solvent
system toluene: ethyl acetate: methanol: formic acid in ratio of 5:4:0.5:0.5 showed clear and sharp peaks with significant area of gallic acid and embelin in formulation and selected.

**Validation of developed HPTLC method**

The linearity for gallic acid and embelin were determined by plotting a graph between peak area and concentration. Correlation coefficient was used to established linearity. Linearity for both the marker were linear over range 200-1200 ng/spot showing the calibration curve equation and correlation coefficient ($r^2$) were $y = 0.833x + 369.30$, $r^2 = 0.9992$ and $y = 18.35x + 2611.00$, $r^2 = 0.9991$ for gallic acid and embelin respectively (Fig. 1 and Fig. 2). The correlation coefficient ($r^2$) value confirms linear relationship between the concentration and peak area (Table 1).

Table 1: Linearity (calibration curve) data for standard gallic acid and embelin (Concentration Vs Peak area) (mean ± SD, n=3)

| S. No | Conc. of gallic acid (ng/spot) | AUC      | % RSD | Conc. of embelin (ng/spot) | AUC      | % RSD |
|-------|-------------------------------|----------|-------|---------------------------|----------|-------|
| 1     | 200                           | 535.67 ± 8.51 | 1.59  | 200                       | 6215.33 ± 4.51 | 0.07  |
| 2     | 400                           | 692.67 ± 5.03 | 0.73  | 400                       | 9984.67 ± 2.52 | 0.03  |
| 3     | 600                           | 874.33 ± 5.51 | 0.63  | 600                       | 13789.33 ± 5.13 | 0.04  |
| 4     | 800                           | 1045.33 ± 3.51 | 0.34  | 800                       | 17058.67 ± 4.51 | 0.03  |
| 5     | 1000                          | 1210.33 ± 5.51 | 0.46  | 1000                      | 21514.67 ± 4.04 | 0.02  |
| 6     | 1200                          | 1357.33 ± 3.51 | 0.26  | 1200                      | 24556.67 ± 6.03 | 0.03  |

**Percent relative standard deviation (%RSD)** is a measure of precision of the method. The % RSD values for both intraday and interday precision were found to be <2 in each ease. Method was found to be well precise as the result showed no significant variation in the intraday and interday precision study (Table 2).

Table 2: Intraday and Interday precision of Gallic acid & Embelin (mean ± SD, n=3)

| Conc. of gallic acid (ng/spot) | Peak area     | %RSD | Conc. of Peak area (ng/spot) | %RSD |
|--------------------------------|---------------|------|-----------------------------|------|
| 200                            | 536.31 ± 3.43 | 0.64 | 200                         | 534.69 ± 3.36 | 0.63 |
| 400                            | 693.68 ± 2.10 | 0.3  | 400                         | 695.23 ± 2.51 | 0.36 |
| 600                            | 872.83 ± 4.94 | 0.57 | 600                         | 873.18 ± 4.29 | 0.49 |
| Conc. of embelin (ng/spot)     |               |      | Conc. of Peak area (ng/spot) |      |
| 200                            | 6228.56 ± 13.47 | 0.22 | 200                         | 6232.71 ± 17.99 | 0.29 |
| 400                            | 9974.11 ± 25.02 | 0.25 | 400                         | 9983.26 ± 15.03 | 0.15 |
| 600                            | 13780.12 ± 18.92 | 0.14 | 600                         | 13755.90 ± 32.64 | 0.24 |
Under the stated experimental condition, minimum amount of analyte that could be detected is known as LOD which was found to be 75.24 ng/spot for gallic acid and 81.60 ng/spot for embelin. Minimum amount of analyte that could be quantified is known as LOQ which was found to be 227.99 ng/spot for gallic acid and 247.28 ng/spot for embelin. Result shows that the developed method is quite sensitivity.

The study of percent recovery reflects the accuracy of the method. The percent recovery study was performs by addition of known added amount of standard solution in the sample. The known amount of mixture of gallic acid and embelin were added in triplicates of three concentration levels viz. 50%, 100% & 150%. The percentage recovery was found to be 100.71%, 101.06% and 99.59% for gallic acid and 99.29%, 98.96% and 98.58% for embelin (Table 3).

| Formulation | Standard | Recovery level (%) | Amount in sample (ng) | Amount added (ng) | Recovered amount (ng) | % RSD | % Recovery | Average Recovery (%) |
|-------------|----------|--------------------|-----------------------|-------------------|----------------------|-------|------------|----------------------|
| MYPF        | Gallic Acid | 50                 | 200                   | 100               | 302.13               | 0.61  | 100.71     |                      |
|             |          | 100                | 200                   | 200               | 404.22               | 0.13  | 101.06     |                      |
|             |          | 150                | 200                   | 300               | 497.93               | 0.42  | 99.59      |                      |
| Embelin     |          | 50                 | 200                   | 100               | 297.87               | 0.65  | 99.29      |                      |
|             |          | 100                | 200                   | 200               | 395.85               | 0.36  | 98.96      |                      |
|             |          | 150                | 200                   | 300               | 492.89               | 0.71  | 98.58      |                      |

The %RSD of the peak areas were calculated for intentionally variation in chamber saturation time, variation in detecting wavelength and variation in mobile phase composition for 400 ng/spot for both marker compounds. The values of %RSD were less than 2 which indicated that there is no significant change in any parameters. Variations in the experimental parameters do not affect developed HPTLC method which proved that the developed method is robust (Table 4).

| Chamber saturation time | Variation | AUC ± SD | %RSD of AUC | R_f ± SD | AUC ± SD | %RSD of AUC | R_f ± SD |
|------------------------|-----------|----------|-------------|---------|----------|-------------|---------|
| 20 m                   | –10       | 682.69 ± 5.33 | 0.78 | 0.30 ± 0.01 | 9844.88 ± 17.71 | 0.18 | 0.66 ± 0.01 |
| 30 m                   | 0         | 695.34 ± 8.74 | 1.26 | 0.33 ± 0.01 | 9984.95 ± 13.56 | 0.14 | 0.67 ± 0.01 |
| 40 m                   | 10        | 707.67 ± 4.87 | 0.69 | 0.33 ± 0.01 | 10210.95 ± 15.09 | 0.15 | 0.67 ± 0.01 |
| Change in wavelength   | 244 nm    | –10       | 685.57 ± 4.44 | 0.65 | 0.29 ± 0.01 | 9924.67 ± 10.46 | 0.11 | 0.65 ± 0.01 |
|                        | 254 nm    | 0         | 695.34 ± 8.74 | 1.26 | 0.33 ± 0.01 | 9984.95 ± 13.56 | 0.14 | 0.67 ± 0.01 |
|                        | 264 nm    | 10        | 708.10 ± 6.50 | 0.92 | 0.33 ± 0.01 | 10099.38 ± 12.61 | 0.12 | 0.68 ± 0.01 |
| Change in mobile phase | Toluene: Ethyl | 4: 5: 0.5: 0.5 | 651.85 ± 6.55 | 0.96 | 0.36 ± 0.02 | 9807.05 ± 17.25 | 0.18 | 0.70 ± 0.03 |
|                        | Acetate: Methanol: | 5: 4: 0.5: 0.5 | 695.34 ± 8.74 | 1.26 | 0.33 ± 0.01 | 9984.95 ± 13.56 | 0.14 | 0.67 ± 0.01 |
|                        | Formic acid | 6: 3: 0.5: 0.5 | 708.56 ± 5.06 | 0.71 | 0.28 ± 0.01 | 10196.12 ± 16.43 | 0.16 | 0.65 ± 0.01 |

The HPTLC chromatogram shows that there is no interference with the peak of gallic acid and embelin. Therefore the method was specific. The presences of both marker compounds in sample track were confirmed by their R_f values with that of reference standard (Fig 3 and Fig 4). The chromatogram of in-house formulated MYPF and its market formulations (MYPF-M1, MYPF-M2 and MYPF-M3) showing the presence of gallic acid and embelin The peaks of individual standard in sample track were analyzed by comparing spectra. (Fig. 5, 6, 7 and 8). The overlay spectrum of standard gallic acid and embelin spots and gallic acid and embelin spots present in the samples were found to be overlap (Fig. 7). The spectra shows good resolution between gallic acid and embelin in the presence of other phytoconstituents confirms the specificity of the method.
ALAM, MISHRA., Orient. J. Chem., Vol. 36(1), 120-126 (2020) 125

Fig. 3. HPTLC chromatogram of standard gallic acid at 254nm

Fig. 4. HPTLC chromatogram of standard embelin at 254nm

Fig. 5. Densitogram of in-house formulated MYPF

Fig. 6. Densitogram of marketed MYPF-M1

Fig. 7. Densitogram of marketed MYPF-M2

Fig. 8. Densitogram of marketed MYPF-M3

Fig. 9. Overlay of standard gallic acid solution and standard embelin solution Quantification of Gallic acid and Embelin in in-house formulated MYPF and its marketed formulations

The amount of gallic acid and embelin in in-house formulated MYPF was found to be 46.57 ± 0.814 & 16.15 ± 0.180 μg/mg of MYPF with a %RSD of 1.75 and 1.11 respectively which showed highest among all tested marketed formulations (Table 5).

Table 5: Quantity of gallic acid and embelin in in-house formulated MYPF, MYPF-M1, MYPF-M2 and MYPF-M3 (mean ± SD, n=3)

| Formulation | Standard  | Quantity (μg/mg of formulation) | % RSD  |
|-------------|-----------|---------------------------------|--------|
| MYPF        | Gallic Acid | 46.57 ± 0.814                   | 1.75   |
|             | Embelin   | 16.15 ± 0.180                   | 1.11   |
| MYPF-M1     | Gallic Acid | 36.42 ± 0.551                   | 1.51   |
|             | Embelin   | 10.59 ± 0.158                   | 1.49   |
| MYPF-M2     | Gallic Acid | 26.14 ± 0.516                   | 1.97   |
|             | Embelin   | 8.48 ± 0.150                    | 1.77   |
| MYPF-M3     | Gallic Acid | 32.11 ± 0.426                   | 1.33   |
|             | Embelin   | 9.40 ± 0.098                    | 1.05   |

CONCLUSION

For biomarker (gallic acid and embelin) based characterization and standardization of MYPF, this invented HPTLC method was found to be most convenient, reliable and simple method. The developed HPTLC method is rapid, specific,
accurate, precise and robust. This is proved, efficient and reproducible method according to the
ICH guidelines for quantitative calculation of gallic acid and embelin in in-house formulated MYPF its
market formulations (MYPF-M1, MYPF-M2 and
MYPF-M3)^14-15. Reproducible, simple and efficient
HPTLC method for quantitative determination of
biomarkers present in MYPF has been developed.
This method showed good peak shape of gallic acid
and embelin. The present method of standardization
can be implemented in ayurvedic pharmaceutical
industries for regular quality control and quantitative
determination of gallic acid and embelin in MYPF.
This HPTLC provides a set quality standard for
identification, characterization and quantitative
estimation of biomarkers in Manibhadra Yoga
Polyherbal Formulation.

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Conflict of interest
No conflicts of interest.

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