INTRODUCTION

Modern pharmaceutical formulations are complex mixtures including, in addition to one or more medicinally active ingredients, a number of inert materials such as diluents, disintegrates, colors and flavours. To ensure quality and stability of the final product, the pharmaceutical analyst must be able to separate these mixtures into individual components prior to quantitative analysis. Moreover, comparison of the relative efficacy of different dosage forms of the same drug entity requires the analysis of the active ingredient in biological matrices such as blood, urine and tissue. Among the most powerful techniques available to the analyst for the resolution of these mixtures are a group of highly efficient methods collectively called chromatography (color-writing). Because this technique is involved so intimately in all aspects of pharmaceutical research and development, the pharmacist should possess a working knowledge of chromatographic principles and techniques.

Chromatographic separation relies on relative movement of two phases, similar to fractional distillation or counter current distribution. But in chromatography one phase is fixed (stationary phase) and other is mobile (mobile phase). The mobile phase passes over the stationary phase and transports components of the mixture at different speeds in the direction of the flow of mobile phase. The separation of components is a result of differential affinity of components for the mobile phase and a stationary phase.\(^1\)

**Definition:**

Tswett (1906) defined *Chromatography as the method in which the components of a mixture are separated on an adsorbent column in a flowing system*. Recently, the IUPAC has defined chromatography as:

“A method used primarily for the separation of the components of a sample, in which the components are distributed between two phases, one of which is stationary while other moves. The stationary phase may be a solid or a liquid supported on a solid or a gel, and may be packed in a column, spread as a layer or distributed as a film. The mobile phase may be gaseous or liquid”.

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Pharmacognostic, phytochemical and pharmacological investigation on *Pterospermum acerifolium* WILLD. (Sterculiaceae)
CLASSIFICATION OF CHROMATOGRAPHY

Chromatographic methods are generally classified according to the physical state of the solute carrier phase, that is the mobile phase. The classifications are represented in following charts a and b.

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b: Classification of chromatography according to mechanism of separation on stationary phase

The main divisions of chromatography, based on mobile phase, may also be subdivided according to the mechanism of solute interaction with the stationary phase. Two mechanisms, adsorption and partition, are the most commonly encountered for both, solution and gas mobile phase separations.

**Adsorption chromatography**, the stationary phase is a solid; the mobile phase containing the dissolved solutes passes over the surface of the stationary phase. Retention of the components and their consequent separation depends on the ability of the atoms on the surface to remove the solutes from the mobile phase and adsorb the temporarily by means of electrostatic forces. If the mobile is liquid, the process is called *liquid-solid chromatography (LSC)* but when the mobile is a gas, the method is called *gas-solid chromatography (GSC)*.

**Partition chromatography**, the stationary phase is liquid an inert solid material –such as silica gel, diatomaceous earth, or even the walls of the column itself-serves to support a thin layer liquid which is the effective stationary phase. As the mobile phase containing the solutes passes in close proximity to this liquid phase, retention and separation occur due to relative solubility of the analytes in the two fluids as

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determined by their partition coefficients. If the mobile phase is a liquid, this is called *
liquid-liquid chromatography (LLC)* and, if the mobile phase is a gas, the process is termed *
gas-liquid chromatography (GLC)*.

**Ion exchange chromatography**, the stationary phase consists of a polymeric matrix on to the surface of which ionic functional groups, such as carboxylic acid or quaternary amines, have been bonded chemically as the mobile phase passes over this surface, ionic solutes are retained by forming electrostatic chemical bonds with the functional groups. The mobile phases used in this type are always liquid.

**Size exclusion chromatography (Gel chromatography)**, the stationary phase is a polymeric substance containing numerous pores of molecular dimensions. Solutes whose molecular size is sufficiently small leave the mobile phase to diffuse into the pores. Larger molecules that will not be fit into the pores remains in the mobile phase and are not retained. This method is most suited to the separation of mixtures in which the solutes vary considerably in molecular size. The mobile phase in this type may be either liquid or gaseous.

**Affinity chromatography**, a specific ligand, such as an antibody, is bound to the inert stationary phase to achieve a highly selective separation. When a mixture of solutes containing a molecule that preferentially binds to the ligand, such as an antigen, is passes through the system, the antigen binds strongly to the ligand antibody and is retained, while the other solutes elute. The antigen then can be displaced and eluted in a purified state.

There has been continuous development in chromatography particularly in; techniques, materials, and requirement of instrumentation which has resulted in the efficient, reliable and sensitive chromatographic methods. The most conventional form of chromatographic technique is *Thin layer chromatography (TLC)* has provided a tool in separation of drugs from bulks, pharmaceutical formulations and biofluids.

**Thin layer chromatography (TLC)**

TLC is so widely used that it has become an essential technique for analyst and research workers. TLC, also known as *planar chromatography* or flat bed chromatography. Planar chromatography (PC) is a collective term including all analytical, micropreparative, and preparative separation methods where the mobile phase moves through the stationary phase (porous sorbents) in a planar arrangement.
It is equally applicable to drugs in their pure state, to those extracted from pharmaceutical formulations, to illicitly manufactured materials and to the biological samples. The most modern form of TLC is high performance thin layer chromatography.

**HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)**

HPTLC is a sophisticated form of thin layer chromatography. It also involves same theoretical principle of thin layer chromatography wherein substances are separated on the basis of their differential migration in a system of two phases on special type of plates. This technique is widely used in many fields both for qualitative and quantitative (identification and estimation) of constituents mixtures.

**Steps Involved In HPTLC**

The various steps are involved in TLC/HPTLC/planar chromatography. They are

1. Selection of TLC/HPTLC plates and sorbent
2. Activation of pre-coated plates
3. Sample preparation
4. Sample application
5. Pre-conditioning (Chamber Saturation)
6. Mobile phase
7. Chromatographic development
8. Detection of spot
9. Scanning and documentation
### 1. Selection of TLC/HPTLC plates and sorbent

Commonly available pre-coated plates with their applications

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Plate</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Silica gel 60F (unmodified)</td>
<td>More than 80% analysis are done on this layer.</td>
</tr>
<tr>
<td>2.</td>
<td>Aluminium oxide</td>
<td>Basic substances, alkaloids and steroids.</td>
</tr>
<tr>
<td>3.</td>
<td>High purity silica gel 60</td>
<td>Aflotoxins</td>
</tr>
<tr>
<td>4.</td>
<td>Cellulose (microcrystalline)</td>
<td>Amino acid, dipeptides, sugars, antibiotics and other labile compounds.</td>
</tr>
<tr>
<td>5.</td>
<td>PEI impregnated cellulose</td>
<td>Mono and oligonucleotides, co-enzymes, sugar phosphate.</td>
</tr>
<tr>
<td>7.</td>
<td>Silica gel chemically modified</td>
<td>Carboxylic acid, phenols, nucleotides, vitamins (B₈, B₆, B₁₂), uric acid, xanthine derivative.</td>
</tr>
<tr>
<td></td>
<td>(a) NH₂ (amino)</td>
<td>Pharmaceutical preservatives.</td>
</tr>
<tr>
<td></td>
<td>(b) CN (cyano)</td>
<td>Amino acids, dipeptides, lactones.</td>
</tr>
<tr>
<td></td>
<td>(c) CHIR</td>
<td>Hormones, steroids.</td>
</tr>
<tr>
<td></td>
<td>(d) DIOL</td>
<td>Plates impregnated with liquid paraffin, buffers, silver nitrate, ion exchange material, acid, bases, or detergents.</td>
</tr>
<tr>
<td></td>
<td>(e) Impregnated plates</td>
<td>Non-polar substances (lipids), fatty acids, carotenoids, steroids, cholesterol and its ester. Polar substances, basic and</td>
</tr>
<tr>
<td></td>
<td>(f) RP-2, RP-8 and RP-18</td>
<td></td>
</tr>
</tbody>
</table>
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Various forms of chromatographic development like ascending, descending, horizontal, continuous, gradient, multidimensional, can be tried. For HPTLC plates, migration distance of 5-6 mm is sufficient. After development, plates are removed from the chamber and dried to remove traces of mobile phase. Common problems encountered during chromatographic development are as follows,

(a) Tailing: This may occur due to the presence of traces of impurities or due to presence of more than one species of substances being chromatographed. This can be reduced by buffering the mobile phase system with acidic (1-2% acetic acid) or basic (ammonia) solution. It keeps material to be separated in non-ionic forms. Sometimes tailing may be due to overloading of sample plates.

(b) Diffusion: This is seen as zones on chromatographic plates. This may arise due to non-uniformity of mobile phase, longitudinal diffusion between mobile phase and stationary or due to no-equilibrium of stationary phase.

8. Detection of spot
Immediately after the development process is completed, the plates are removed from the chamber and dried to remove the traces of mobile phase. Generally detection can be done by iodine vapour in iodine chamber. Alternatively, detection can be done by visual inspection examination at 254 nm of ultra violet region in UV cabinet.

9. Scanning and documentation
The developed HPTLC plates are scanned at selected UV regions wavelength by the instrument and the detected spots are seen on computer in the form of peaks. The scanner converts band into peaks and peak height and area is related to the concentration of the substance on the spot. The peak height and area under the spot (curves) are measured by the instruments and are recorded as percent. Furthermore, the plates carry supplier’s name, batch number, chemical code etc. on the edge of the pre-coated plates. This helps in storing the data of individual plates for further use as well as for photo documentation and storage.

- Factors influencing the TLC/HPTLC separation and resolution of spots

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### Advantages of HPTLC

1. In HPTLC, simultaneous processing of sample and standard under similar conditions lead to better analytical accuracy and precision.
2. There is very less need of internal standard (I.S.)
3. Lower analysis time and less cost per analysis.
4. It is very simple to learn and the instrumentation is very easy to operate.
5. The sample preparation is very simple.
6. It involves very low maintenance cost.
7. Solvent used in HPTLC needs no prior treatment like filtration and degassing.
8. Solvents of analytical grade are suitable.
9. The mobile phase consumption for sample is extremely low.
10. It allows use of corrosive and UV absorbing mobile phase.
11. The system equilibrium time is small.
12. The stationary phase is usually not susceptible to sample poisoning.

Another most exploited chromatographic technique for analysis of drug sample from bulk, pharmaceutical formulations and biological fluid is high performance liquid chromatography.
VALIDATION OF ANALYTICAL METHOD

Validation of an analytical method is the process that establishes, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications.

When a method has been developed it is important to validate it to confirm that it is suitable for its intended purpose. The International Conference on Harmonisation (ICH) has provided definitions of validation issues included in “analytical procedures” for the fields of bioanalytical methodology, pharmaceutical and biotechnological procedures. Likewise the US pharmacopeia (USP) has published guidelines for method validation for analytical methods for pharmaceutical products. The most common validation parameters will be briefly described below.

1. ANALYTICAL PROCEDURE

The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to: the sample, the reference standard and the reagents preparations, use of the apparatus, generation of the calibration curve, use of the formulae for the calculation, etc.

2. SPECIFICITY

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

This definition has the following implications:

Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

Assay (content or potency): to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

3. ACCURACY
The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

4. PRECISION
The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

4.1. Repeatability
Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

4.2. Intermediate precision
Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

4.3. Reproducibility
Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

5. DETECTION LIMIT
The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

6. QUANTITATION LIMIT
The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.
7. LINEARITY
The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

8. RANGE
The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

9. ROBUSTNESS
The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

10. RUGGEDNESS
The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, analyst, instruments, lots of regents, elapsed assay times, assay temperature or days. Ruggedness is normally expressed as the lack of influence on the test results of operational and environmental variables of the analytical method. Ruggedness is a measure of reproducibility of test results under the variation in conditions normally expected from laboratory to laboratory and from analyst to analyst.

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EXPERIMENTAL

Instrumentation

HPTLC was performed with a Camag (Muttenz, Switzerland) Linomat V applicator, a Camag twin trough TLC chamber, a Camag TLC scanner 3, Camag Wincats software and a Hamilton (Reno, Nevada, USA) Syringe (25µL).

Chemicals

Rutin, gallic acid and quercetin purchased as a standard sample from Sigma Aldrich. Ethyl acetate, formic acid, glacial acetic acid were used as solvents to prepare the mobile phase. All the chemicals used were of Analytical Reagent Grade (S.D.Fine. Chem. Ltd., Mumbai). HPTLC analyses were performed on Merck 20 cm×10 cm silica gel 60 F254 HPTLC plates.

Quantitative Determination of Rutin in EAPAL, EAPAB and EAPAW

Plant Material

Leaves, bark and wood of *Pterospermum acerifolium* was collected from Nasik district of Maharashtra State (INDIA) and authenticated at Botanical Survey of India, Pune (voucher number- RASPTA1). The samples were powdered and passed through 40 mesh and stored at 25°C in air-tight containers till further use. Accurately weighed 1000g quantity each of powder of leaves, bark and wood of *Pterospermum acerifolium* were separately extracted with methanol by maceration which yields TMPAL, TMPAB and TMPAW. The methanolic extract was filtered through Whatmann Filter paper No.1. These extracts were fractionated with petroleum ether, chloroform and ethyl acetate. Ethyl acetate soluble fraction contains flavonoids. The pooled extracts were concentrated. EAPAL, EAPAB and EAPAW shows positive phytochemical test for flavonoids as describe by Evans, Brain and Turner.

Preparation of Standard Solutions

10 mg of standard rutin and quercetin and 1 mg of gallic acid was accurately weighed, quantitatively transferred into a 10 mL volumetric flask, dissolved in methanol and the volume was adjusted with the same solvent.

Sample preparation

100 mg/ml of TMPAL, TMPAB, TMPAW, EAPAL, EAPAB and EAPAW was placed in a stoppered volumetric flask. The sample was vortex mixed for 5 min and left to stand overnight at room temperature (28±2°C). The contents of the volumetric
flask were then filtered through a Whatman No.41 paper (E. Merck, Mumbai, India) and the clear supernatant solution was used for the assay.

**Preparation of Calibration Curve**

As recommended by the International Committee on Harmonization (ICH) guidelines [25-29], a calibration curve was established using six analytic concentrations 2–14 µg of rutin. Standard solutions were applied as bands by means of Linomat V, band length 8 mm, distance between two bands 11.3 mm, distance from the plate side edge 15 mm, and distance from the bottom of the plate 8 mm. Plates were developed to a distance of 8 cm beyond the origin with Solvent systems: Ethyl acetate: formic acid: glacial acetic acid: water (10:1:1:1:2.6) for rutin, cyclohexane: ethyl acetate: formic acid (4:6:1) for gallic acid and toluene : ethyl acetate : methanol (4.4 : 5 : 0.6) for quercetin in a vapour equilibrated Camag HPTLC twin trough chamber. The saturation time was 10 min for rutin and quercetin whereas 20 min for gallic acid. The plates of rutin, gallic acid and quercetin were quantified by linear scanning at 254 nm, 298 nm and 380 nm respectively. Lamp was D2. Calibration curve of was obtained by plotting peak areas verses concentration applied.

**Quantification of rutin in TMPAL, TMPAB, TMPAW, EAPAL, EAPAB and EAPAW**

6 µL each of sample solutions were applied in triplicate on a precoated silica gel 60 F\textsubscript{254} TLC plate (E. Merck) (0.25 mm thickness) with CAMAG Linomat V Automatic Sample Spotter. The plate was developed and scanned as mentioned above. The peak areas were recorded. The amount of rutin, gallic acid and quercetin in different samples was calculated using the calibration curve.

**Validation of the Proposed Method**

The proposed method was validated as per ICH guidelines.

**Linearity:**

A representative calibration curve of rutin, gallic acid and quercetin was obtained by plotting the peak area of rutin, gallic acid and quercetin against the concentration of rutin, gallic acid and quercetin respectively.

**Accuracy**

The accuracy was determined by standard addition method. To a fixed amount increasing amount of standard rutin, gallic acid and quercetin was added in the
calibration curve level. The Percentage recovery of rutin, gallic acid and quercetin was calculated at each level ($n = 3$).

**Inter-day and intra-day precision:**

Precision studies were performed by using standard solutions with the concentrations covering the entire calibration range. The precision of the method in terms of intra-day variation (%RSD) was determined by analyzing rutin, gallic acid and quercetin standard solutions in the calibration range three times on the same day. Inter-day precision (%RSD) was assessed by analyzing the standard rutin, gallic acid and quercetin solutions within the calibration range on three different days over a period of one week.

**Repeatability:**

Repeatability of measurement of peak area and peak height: Standard rutin, gallic acid and quercetin solution was spotted on a TLC plate, developed and dried. The separated spot was scanned for six times without changing plate position and RSD for measurement of peak area was computed.

**Sensitivity**

The sensitivity of measurement of rutin, gallic acid and quercetin using the proposed method was estimated in terms of the Limit of Quantitation (LOQ) and Limit of Detection (LOD). The LOQ and LOD were calculated by using the equations LOD = $3.3 \times N/B$ and LOQ = $10 \times N/B$, where $N$ is standard deviation of the peak areas of the drugs ($n = 3$), taken as a measure of noise, and $B$ is the slope of the corresponding calibration curve.

**Specificity:**

The overlay spectrum of standard rutin, gallic acid and quercetin spots with rutin, gallic acid and quercetin spots present in the samples were found to be similar or overlap. The peak purity of the rutin, gallic acid and quercetin was assessed by comparing the spectra at three different levels, viz. peak start, and peak apex and peak end positions of the spot.

**Robustness of the method**

By introducing small changes in the mobile phase composition, mobile phase volume and duration of mobile phase saturation, the effects on the results were examined. Robustness of the method was done in triplicate at a concentration level of $6 \mu g/spot$. 

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600ng/spot and 6μg/spot for rutin, gallic acid and quercetin respectively. The %R.S.D. was calculated.

**Ruggedness of the method**

It expresses the precision within laboratories variations like different days, different analyst, and different equipment. Ruggenedness of the method was assessed by spiking the standard 6 times with different analyst.

**Quantification of rutin, gallic acid and quercetin in TMPAL, TMPAB, TMPAW, EAPAL, EAPAB and EAPAW**

The contents of the rutin, gallic acid and quercetin was quantified using calibration curve of standard rutin, gallic acid and quercetin.
RESULTS AND DISCUSSION

Development of the optimum mobile phase

The TLC procedure was optimized with a view to quantify the herbal extract. Initially various mobile phases in varying ratios was tried. Finally, the different mobile phases consisting of Ethyl acetate: formic acid: glacial acetic acid: water (10:1.1:1.1:2.6) for rutin with $R_f = 0.28$, cyclohexane: ethyl acetate: formic acid (4:6:1) for gallic acid with $R_f = 0.31$ and toluene : ethyl acetate : methanol (4.4 : 5 : 0.6) for quercetin with $R_f = 0.52$ (Figure 5.2.1, Figure 5.2.2 and Figure 5.2.3). Well-defined spots were obtained when the chamber was saturated with mobile phase for 10 min at room temperature for rutin and quercetin whereas 20 min for gallic acid. The TLC plate was visualized under UV light at 254 nm, 298nm and 380nm respectively for rutin, gallic acid and quercetin without derivatization.

Method validation

The calibration plot for rutin, gallic acid and quercetin indicates the response is a linear in concentration range 2 -14 µg, 200-1400 ng and 2-14 µg respectively. The results were shown in Table 5.2.1. Results of recovery studies, listed in Table 5.2.2, were within acceptable limits (98.0 to 102.0%), indicating the accuracy of the method was good. The % RSD values for precision are depicted in Table 5.2.3. The measurement of the peak area at three different concentration levels showed % R.S.D less than 2 for inter- and intra-day variation, which suggested an excellent precision of the method, results were shown in Table 5.2.4. The low values of S.D. and % R.S.D obtained after introducing small deliberate changes in the developed HPTLC method indicated the robustness and ruggedness of the method and results were depicted in Table 5 and Table 6. The peak purity of rutin, gallic acid and quercetin was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot as shown in Figure 5.2.4, Figure 5.2.5 and Figure 5.2.6. The content of rutin, gallic acid and quercetin was quantified using calibration curve of rutin, gallic acid and quercetin. The Results obtained were shown in Table 5.2.7.

A rapid, simple, accurate and specific HPTLC method for quantitative estimation of rutin, gallic acid and quercetin present in the TMPAL, TMPAB, TMPAW, EAPAL, EAPAB and EAPAW has been developed and validated. The data could be used as a quality control standard. The method used in this work...
resulted in good peak shape and enabled good resolution of rutin, gallic acid and quercetin from other constituents of the plant material. Because recovery was in between 98% to 100%, there was no interference with the rutin, gallic acid and quercetin peak from other constituents present in the plant.
Table 5.2.1: Method validation parameters for the quantification of rutin, gallic acid and quercetin by HPTLC method

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Rutin</th>
<th>Gallic acid</th>
<th>Quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit of detection</td>
<td>0.2 (µg/spot)</td>
<td>200(ng/spot)</td>
<td>0.2 (µg/spot)</td>
</tr>
<tr>
<td>Limit of quantification</td>
<td>0.6 (µg/spot)</td>
<td>600(ng/spot)</td>
<td>0.6 (µg/spot)</td>
</tr>
<tr>
<td>Linearity range (µg/spot)</td>
<td>2-14 (µg/spot)</td>
<td>200-1400 (ng/spot)</td>
<td>2-14 (µg/spot)</td>
</tr>
<tr>
<td>correlation coefficient</td>
<td>0.9937</td>
<td>0.99705</td>
<td>0.9943</td>
</tr>
<tr>
<td>Slope</td>
<td>468.04</td>
<td>6.264</td>
<td>506.04</td>
</tr>
<tr>
<td>Intercept</td>
<td>812.23</td>
<td>258.4</td>
<td>455.57</td>
</tr>
</tbody>
</table>

Table 5.2.2: Results of Recovery Study

<table>
<thead>
<tr>
<th>Compound</th>
<th>Addition of standard (%)</th>
<th>Initial amount (ng)</th>
<th>Amount recovered ± S.D. [µg] n = 3</th>
<th>%Recovery</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>80</td>
<td>5000</td>
<td>3.99±0.027</td>
<td>99.87</td>
<td>0.697</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5000</td>
<td>4.98±0.008</td>
<td>99.75</td>
<td>0.178</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>5000</td>
<td>5.94±0.042</td>
<td>99.07</td>
<td>0.707</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>80</td>
<td>250</td>
<td>202.67±1.49</td>
<td>101.33</td>
<td>0.737</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>250</td>
<td>247.56±1.35</td>
<td>99.02</td>
<td>0.547</td>
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<tr>
<td></td>
<td>120</td>
<td>250</td>
<td>306.02±2.84</td>
<td>102.00</td>
<td>0.929</td>
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<tr>
<td>Quercetin</td>
<td>80</td>
<td>5000</td>
<td>4.03±0.011</td>
<td>100.8</td>
<td>0.274</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5000</td>
<td>4.96±0.028</td>
<td>99.14</td>
<td>0.584</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>5000</td>
<td>5.95±0.064</td>
<td>99.10</td>
<td>1.087</td>
</tr>
</tbody>
</table>

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### Table 5.2.3: Precision of rutin, gallic acid and quercetin

<table>
<thead>
<tr>
<th>compound</th>
<th>Concentration</th>
<th>Mean ±SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin (µg/spot)</td>
<td>7.95</td>
<td>7.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.09</td>
<td>8.02</td>
<td>0.597</td>
</tr>
<tr>
<td></td>
<td>8.02</td>
<td>8.06</td>
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<tr>
<td></td>
<td>8.01</td>
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</tr>
<tr>
<td>Gallic acid (ng/spot)</td>
<td>794.93</td>
<td>798.48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>798.65</td>
<td>803.93</td>
<td>0.474</td>
</tr>
<tr>
<td></td>
<td>804.58</td>
<td>802.8</td>
<td></td>
</tr>
<tr>
<td>Quercetin (µg/spot)</td>
<td>10.00</td>
<td>9.98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.97</td>
<td>10.07</td>
<td>0.869</td>
</tr>
<tr>
<td></td>
<td>10.18</td>
<td>10.14</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.2.4: Intra-day and Inter-day precision of rutin, gallic acid and quercetin

<table>
<thead>
<tr>
<th>Drug</th>
<th>Amount applied (µg/spot)</th>
<th>Amount found mean±S.D. (µg/spot)</th>
<th>%RSD n=3</th>
<th>Drug</th>
<th>Amount applied (µg/spot)</th>
<th>Amount found mean±S.D. (µg/spot)</th>
<th>%RSD n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>2</td>
<td>1.99±0.014</td>
<td>0.709</td>
<td>2</td>
<td>1.99±0.032</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8.05±0.035</td>
<td>0.44</td>
<td>8</td>
<td>8.09±0.012</td>
<td>0.151</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>14.02±0.076</td>
<td>0.546</td>
<td>14</td>
<td>14.06±0.034</td>
<td>0.244</td>
<td></td>
</tr>
<tr>
<td>Gallic acid</td>
<td>200</td>
<td>199.4±0.796</td>
<td>0.399</td>
<td>200</td>
<td>199.24±0.88</td>
<td>0.444</td>
<td></td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>801.01±1.18</td>
<td>0.14</td>
<td>800</td>
<td>801.11±1.153</td>
<td>0.191</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1200</td>
<td>1201.37±0.632</td>
<td>0.052</td>
<td>1200</td>
<td>1202.69±1.65</td>
<td>0.137</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>2</td>
<td>2.01±0.014</td>
<td>0.726</td>
<td>2</td>
<td>2.01±0.019</td>
<td>0.942</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6.03±0.009</td>
<td>0.163</td>
<td>6</td>
<td>6.02±0.01</td>
<td>0.182</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>12.06±0.081</td>
<td>0.676</td>
<td>12</td>
<td>12.06±0.097</td>
<td>0.811</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2.5: Results of ruggedness studies

<table>
<thead>
<tr>
<th>Ruggedness study</th>
<th>Rutin [%]</th>
<th>%RSD (n=5)</th>
<th>Gallic acid [%]</th>
<th>%RSD (n=5)</th>
<th>Quercetin [%]</th>
<th>%RSD (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyst I</td>
<td>99.69</td>
<td>1.06</td>
<td>100.16</td>
<td>0.202</td>
<td>101.32</td>
<td>0.800</td>
</tr>
<tr>
<td>Analyst II</td>
<td>99.32</td>
<td>0.577</td>
<td>99.99</td>
<td>0.225</td>
<td>100.58</td>
<td>0.226</td>
</tr>
</tbody>
</table>
Table 5.2.6: Results of robustness study

<table>
<thead>
<tr>
<th>Development distance [cm]</th>
<th>Rutin [%]</th>
<th>%RSD (n=5)</th>
<th>Gallic acid [%]</th>
<th>%RSD (n=5)</th>
<th>Quercetin [%]</th>
<th>%RSD (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>99.12</td>
<td>0.55</td>
<td>99.99</td>
<td>0.202</td>
<td>100.2</td>
<td>0.53</td>
</tr>
<tr>
<td>8</td>
<td>99</td>
<td>1.12</td>
<td>99.96</td>
<td>0.137</td>
<td>100.28</td>
<td>0.328</td>
</tr>
</tbody>
</table>

Table 5.2.7: Rutin, gallic acid and quercetin content in different extracts quantified by the TLC densitometric method described

<table>
<thead>
<tr>
<th>Samples</th>
<th>Content of Rutin (% w/w)</th>
<th>Content of Gallic acid (% w/w)</th>
<th>Content of Quercetin (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMPAL</td>
<td>0.053</td>
<td>0.1700</td>
<td>0.0098</td>
</tr>
<tr>
<td>EAPAL</td>
<td>2.87</td>
<td>1.185</td>
<td>1.55</td>
</tr>
<tr>
<td>TMPAB</td>
<td>0.077</td>
<td>0.206</td>
<td>0.176</td>
</tr>
<tr>
<td>EAPAB</td>
<td>6.7</td>
<td>1.407</td>
<td>2.72</td>
</tr>
<tr>
<td>TMPAW</td>
<td>0.028</td>
<td>0.218</td>
<td>0.013</td>
</tr>
<tr>
<td>EAPAW</td>
<td>7.99</td>
<td>1.211</td>
<td>1.75</td>
</tr>
</tbody>
</table>
Figure 5.2.1. HPTLC Chromatogram of Standard rutin Solution, measured at 254 nm, mobile phase Ethyl acetate: formic acid: glacial acetic acid: water (10:1.1:1.1:2.6)

Figure 5.2.2. HPTLC Chromatogram of Standard gallic acid Solution, measured at 298 nm and mobile phase cyclohexane: ethyl acetate: formic acid (4:6:1)
Figure 5.2.3. HPTLC Chromatogram of Standard Quercetin Solution, measured at 380 nm and mobile phase toluene : ethyl acetate : methanol (4.4 : 5 : 0.6)

Figure 5.2.4: Peak purity spectra of rutin in EAPAW, EAPAB, EAPAL scanned at 254 nm showing the peak-start, peak-apex and peak end positions.
Figure 5.2.5: Peak purity spectra of gallic acid in EAPAW, EAPAB, EAPAL scanned at 298 nm showing the peak-start, peak-apex and peak end positions.

Figure 5.2.6: Peak purity spectra of Quercetin in EAPAW, EAPAB, EAPAL scanned at 380 nm showing the peak-start, peak-apex and peak end positions.
Pharmacognostic, phytochemical and pharmacological investigation on *Pterospermum acerifolium* WILLD. (Sterculiaceae)
REFERENCES


Pharmacognostic, phytochemical and pharmacological investigation on _Pterospermum acerifolium_ WILLD. (Sterculiaceae)
22. Reviewer Guidance, Validation of Chromatographic Methods (CDER), Nov.1994

Pharmacognostic, phytochemical and pharmacological investigation on
Pterospermum acerifolium WILLD. (Sterculiaceae)


