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# Simultaneous Quantification Of Bioactive Compounds In Marketed Herbal Formulation By HPLC

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## ABSTRACT

Herbalism is one of finest practice used since ancient times to restore, cure and maintain human health. The practice has been commenced with the use of raw unprocessed form of herb and reached its pinnacle in the modern science with the use of pure isolated compound from plant material. The fundamental concern with such plant material and formulations associated with the plants are lack of standardization and safety evaluation. It is sole responsibility of manufacturer to substantiate the efficacy and safety of such product. Development of standardization parameter is tedious task due to presence multitudinous component and their variability in composition. Simultaneous quantification of multiple constituents in single formulation or extract is quite demanding task. The objective of the study was to develop and validate a HPLC method for Simultaneous determination of ten cardinal bioactive compounds i.e. Luteolin, Rutin, Hesperidin, Baicalin, Curcumin, Demethoxycurcumin, Bisdemethoxycurcumin, Piperine, Glycyrrhizin and Quercetin in marketed herbal suspension. The developed method was specific and precise with relative standard deviation $< 2.0\%$. Calibration curves showed excellent linear regressions ($R^2 > 0.99$). The recovery for components in herbal suspension was between 99.2 to 101%. The method is sensitive to quantify all components at minimum concentration viz. Baicalin- 7.3µg/mL,Rutin–15.12µg/mL,Hesperidin–11.38µg/mL, Luteolin-11.86µg/mL, Glycyrrhizin–5.0µg/mL, Quercetin – 29.92µg/mL, Piperine – 4.47µg/mL and Curcuminoids– 8.76 µg/mL. The developed method was accurate, precise, reproducible and appropriate for routine quantitative analysis of stated components.

**Keywords:** HPLC, Bioactive, Herbal, Method development, Validation

## INTRODUCTION

Nature has bestowed Plants with numerous beneficial constituents that could boon human health by enhancement the potential to fight against diseases and ailment. Polyherbal formulation comprises of multiple herbs associated with multifaceted chemical components in varying proportions Thus, quality control of such combinations in herbal formulations sets a great challenge[1]. Fingerprinting investigation has emerged as an important analytical method for quality control of herbal drugs. It offers essential characterization of a complex mixture with a quantitative extent of reliability and aims on the detection and stability evaluation of the components[2,3]. Modern analytical methods viz. High-performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC), and gas chromatography are exercised worldwide to obtain the fingerprint analysis of single as well as complex herbal drug products [4].This intrigued the development of a reliable protocol for quality assessment of the poly herbal formulation by using modern scientific analytical tools. Method development and optimization in liquid chromatography is an attractive field of research for academia and industry. Complex mixtures or samples required systematic method development involving accurate modelling of the retention behaviour of the analyte[5-8].Luteolin, 3′,4′,5,7-tetrahydroxyflavone, belongs to a group of naturally occurring compounds called flavonoids that are found widely in the plant kingdom. Flavonoids are polyphenols that play an important role in defending plant cells against microorganisms, insects, and UV irradiation[9]. Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside) is a flavonol, abundantly found in plants, such as passion flower, buckwheat, tea, and apple. It is a vital nutritional component of food stuff. Rutin, also called as rutoside, quercetin-3-rutinoside, and sophorin is a citrus flavonoid glycoside found in buckwheat[10,11]. Hesperidin (3,5,7-trihydroxyflavanone 7-rhamnoglucoside, hesperetin-7-*O*-rutino- side) belongs to flavanone compounds, one of the flavonoids subclasses. It has been recently extensively evaluated for its health-promoting and pharmacological effects and is used in a treatment of type 2 diabetes, cancer and cardiovascular diseases, neurological and psychiatric disorders, as well as a radio protector[12-16]. Baicalin is the main flavonoid glucoside of *Scutellariabaicalensis* Georgi, Lamiaceae, which has antiviral, bacteriostatic, antitumor, and anti-oxidative effects[17]. Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), also called diferuloylmethane, is the main natural polyphenol found in the rhizome of *Curcuma longa* (turmeric) and in others *Curcuma* spp.[18]. Curcuminoids, comprising curcumin, demethoxycurcumin (DMC), and bis demethoxy curcumin have been extensively reported to possess beneficial effects owing to their potent antioxidant and anti -

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inflammatory properties[19,20]. Piperine is a compound belonging to the alkaloids it is traditionally recommended for fevers and a variety of gastrointestinal conditions, as well as for neurological and broncho-pulmonary disorders i.e. asthma and chronic bronchitis [21]. Glycyrrhizin is a triterpenoid saponin found in *Glychyrrhiza glabra* (liquorice) Chemically, glycyrrhizin is a sulphated polysaccharide. Glycyrrhizin is having anti-atherogenic, anti-cancer, anti-diabetic, anti-microbial, antispasmodic, anti-inflammatory, and anti-asthmatic properties[22]. Quercetin is a natural flavonoid found abundantly in vegetables and fruits. There is growing evidence suggesting that quercetin has therapeutic potential for the prevention and treatment of different diseases, including cardiovascular disease, cancer, and neurodegenerative disease[23].Validation is an act of proving that any procedure, process, equipment, material, activity and system performs as expected under given set of conditions and also give the required accuracy, precision, sensitivity, ruggedness, etc. In recent years, the reporting of various active ingredients (i.e. marker profiling) have shown to be a useful method for standardization and quality control of herbal materials, especially when there is a lack of authentic standards for the identification of all active components present in these complex natural products[24]. The review of the literature portended the lack of appropriate method for simultaneous estimation of the selected bioactive in commercial formulations therefore development and validation of novel, precise, accurate, sensitive analytical techniques for the simultaneous estimation of these bioactive might be helpful in analysis of said compounds.

## MATERIALS AND METHODS:

### Chemical and Reagents

The analytical grades of organic solvents were procured from Merck specialities Pvt. Ltd. (Mumbai). All the analytes Luteolin, Rutin, Hesperidin, Baicalin, Curcumin, Demethoxycurcumin, Bisdemethoxycurcumin, Piperine, Glycyrrhizin and Quercetin has been procured from Panacea Phytoextract Pvt. Ltd. The marketed nutraceutical formulation (Vedicinals 9 Suspension) was purchased from the local market.

### Instrumentation and Chromatographic Conditions

Quantitative analysis was performed on a Waters HPLC system equipped with a HPLC quaternary pump, auto sampler, degasser, UV detector and column oven controlled by Empower 3 software, which was used for data analysis and processing. The HPLC conditions were optimized to achieve chromatograms with a good separation of adjacent peaks. Initially many mobile phase combinations were tried to resolve and standardize 10 compounds but

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due to their complex nature, satisfactory separation of individual compounds could not be achieved.MS compatible mobile phase at lower concentration and avoid using non-volatile ion pairing reagents & salts. Trifluoroacetic acid (boiling point – 72˚C and UV cutoff – 210nm) is used in mobile phase to make MS compatible mobile phase. Finally, gradient mobile phase afforded the best separation with achievement of distinct RT. Mobile Phase A: 0.5% Trifluoroacetic acid in Acetonitrile, Mobile Phase B: 0.5% Trifluoroacetic acid in Water and Diluent as Methanol used. Simple binary phase has been selecting with gradients and eliminated the filtration process of selection of mobile phase. In reverse phase chromatography the stationary phase is non-polar and the mobile phase is polar, causing polar peaks to generally elute earlier than non-polar peaks. Good resolution and peak shape observed on Poroshell EC-C18 with dimension 150*4.6mm, 2.7 µm column. Selection of an appropriate detection wavelength is necessary in order to detect all substances simultaneously and obtaining low LODs. All analytes of interest are having chromophore and their absorbance was measured over the wavelength of 200 to 800 nm using UV/PDA detector. Hesperidin, Rutin, Luteolin, Baicalin, Quercetin& Glycyrrhizin, have good absorbance at 260nm and Piperine, Curcumin- I, Curcumin -II, Curcumin- III have good absorbance at 370 nm. The spectra of compounds were obtained using a diode-array detector at a wavelength of 200 nm &800 nm with a run time of 40 min. The analytical work was performed at room temperature to prevent degradation of compounds.

**Table 1: Optimized Chromatographic Conditions**

| Column | Poroshell EC-C18 (150mm*4.6, 2.7µ) |
|---|---|
| Flow | 0.8 mL/min |
| Column temperature | 35˚C |
| Sampler temperature | 25˚C |
| Injection Volume | 10µL |
| Detector | 260nm & 370nm |

The gradient method used and the parameter given below.

**Table 2: Gradient Program**

| Time (in Minutes) | % Mobile Phase A | % Mobile Phase B |
|---|---|---|
| 0.01 | 15 | 85 |
| 5.00 | 27 | 75 |
| 6.01 | 30 | 70 |
| 6.50 | 30 | 70 |

| | | |
|---|---|---|
| 20.00 | 25 | 75 |
| 35.00 | 37 | 63 |
| 36.00 | 15 | 85 |
| 40.00 | 15 | 85 |

**Standard and Sample Preparation:**

The stock solutions of sample were prepared along with reference marker compounds, where Piperine - 0.0076 mcg/mL, Rutin - 0.375 mcg/mL, Quercetin- 0.55 mcg/mL, Luteolin - 0.105 mcg/mL, Baicalin – 0.105 mcg/mL, Curcumin – 0.525 mcg/mL, Glycyrrhizin – 0.265 mcg/mL, Hesperidin – 0.335 mcg/mL were prepared. In brief, the suspension sample and standard marker compound were weighed and transferred to suitable flask, then dissolved in methanol, after sonication the solutions were filtered through Nylon0.45 μ filter paper before injection.

**Method Development and Validation**

The novel high performance liquid chromatography method was developed to analyse the constituent present in formulation. Specificity, linearity, Limit of Detection, Limit of Quantitation, recovery & accuracy, precision, robustness, solution stability, filter interference study was taken in consideration as per guidelines of International Conference on Harmonization (ICH; Guideline, 2005). Analysis of reference compounds and analytes indicated that the method is specific. The five replicate standards for all 10 compounds were used to evaluate the % assay of sample. The developed method had good recovery with extraction accuracy of 98.1 to 102. It is found that the developed method is robust, that was calculated by introducing small but deliberate changes in certain chromatographic conditions (i.e. percentage,wavelength, flow rate and column temperature). The result of robustness study was ≤1.32%, indicated the developed method is well robust. The outcome of validation protocol prognosticated that method is specific, Linear, precise, accurate and sensitive adequate for the simultaneous quantification of 10 bioactive compounds in Herbal suspension. Figure 2 and 3 demonstrated the difficulty in concurrent separation. A comparative study has been to evaluate the separation efficacy of different columns *viz.* C8, C18, CN, and phenyl, among these, C18 column exhibited the highest level of separation efficiency. Experiments on the mobile phase demonstrated that the proportion of organic modifiers, such as acetonitrile or methanol, had a critical role in producing the best possible separation. The pH value had a crucial impact on the ionization of solutes, requiring the

employment of an acidic mobile phase to reduce ionization. Therefore, the most effective separation was accomplished by use

trifluoroacetic acid. The resolution and run duration of the instrument were improved by adjusting the gradient mode. The bioactive analyte peaks displayed clear resolution from neighbouring peaks, indicating exceptional peak symmetry and separation efficiency, as shown in Figures 2 and 3. The distinct chromophoric properties of these chemical groups allowed for straightforward identification by analysis of their UV diode-array absorption spectra. The method was critically evaluated of linearity, selectivity, accuracy, and precision, the outcome suggested that approach was propitious for analysing all 10 bioactive phytoconstituents. The analysis of the purity threshold and purity angle in the Empower software revealed that the procedure is highly specific for the bioactive molecule. The reported peaks are fully distinct from any other interfering substances.

A standard mixture of bioactive compounds was then analysed as a mixture and chromatographic conditions were optimized to maximize peak resolution. Elution of the bioactive compound from the column was in order of decreasing polarity of the mobile phase used: as the proportion of trifluoroacetic acid increased the retention times for the studied bioactive compound decreased and some of them eluted with solvent front. The greater the proportion of phosphate buffer, the better peak shapes and resolution of the studied compound. The separation pattern of the Rutin showed at $R_t$–7.026 min, Hesperidin showed at–8.941 min, Baicalin showed at–10.466 min, Luteolin showed at–13.687 min, Quercetin showed at–14.207 min, Glycyrrhizin at–28.80 min (Figure 1) and Piperine-31.398, Curcumin- I – 31.752, Curcumin –II – 32.021, Curcumin- III – 32.283 (Figure 2). The retention time of the bioactive compounds was reproducible, which makes the method most suitable for routine analysis. The peak asymmetry (T) and theoretical plate counts of all the four components were very close to the ideal value 1.2 and not less than 2000 respectively.

The correlation between the response of the detector and different concentrations of bioactive analytes was verified, as outlined in Tables 5-12. Table 4 displays the %RSD for intraday and inter-day studies. The results of intermediate precision evaluations, which involved different analysts, instruments, and days, indicated that these factors did not have a substantial impact on the differences in the results. After conducting these validation trials, it was confirmed that the approach has the ability to accurately quantify in our laboratory. The final phase of precision assessment involves shifting the emphasis towards scrutinizing result bias rather than just determining differences in precision, akin to inter-laboratory crossover studies, which will be our subsequent area of concentration. The table presents the accuracy, which

was evaluated by measuring the recovery of plant samples spiked with standards at two different concentration levels. Meticulous optimization of extraction conditions resulted in satisfactory recovery for each phytoconstituent. Therefore, this method, due to achieving favourable recovery and precision, can be recommended for the quantification of all bioactive constituents

**Selectivity**

The specificity pertains to the capacity of the method to accurately measure the response of the analyte in the presence of any interfering substances. As a result, the mixtures obtained from preparing the samples were analysed and the peaks of the substance being studied were evaluated for both their purity and distinction from the nearby peaks.

**Linearity**

In order to confirm the normal distribution of the data, a linearity assessment was performed by analysing the correlation between the concentrations of the analyte and the absorbance recorded by the UV-HPLC detector. The coefficient of determination ($r^2$) was computed using the method of least squares. Calibration curves were created using minimum five concentration of analyte. The objective of this technique was to determine the extent to which the linear regression model could account for the overall variability in the response.

**Precision**

The accuracy of each approach corresponds to the degree of variability within a series while analysing the same sample. A total of six authentic samples were examined on the same day (intra-day), while another six samples were examined on subsequent days (inter-day). Afterwards, the % RSD was computed by performing six injections of each sample into the HPLC.

**Recovery**

This parameter shows the proximity between the experimental values and the real ones. It ensures that no loss or uptake occurred during the process. The determination of this parameter was performed during the method by studying the recovery after a standard addition procedure, with two additional levels. Three replicate samples were prepared at different concentration by spiking the multi bioactive standard solution. In each additional level, three determinations were carried out and the recovery percentage was calculated in every case.

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## RESULT AND DISCUSSION

The development of quantitative analysis techniques is significantly hampered by the complexity of analysis procedures. The best approach is one that is simple enough for different operators in different labs to implement. But using a quantitative approach, attaining both accuracy and precision requires more complex procedures.

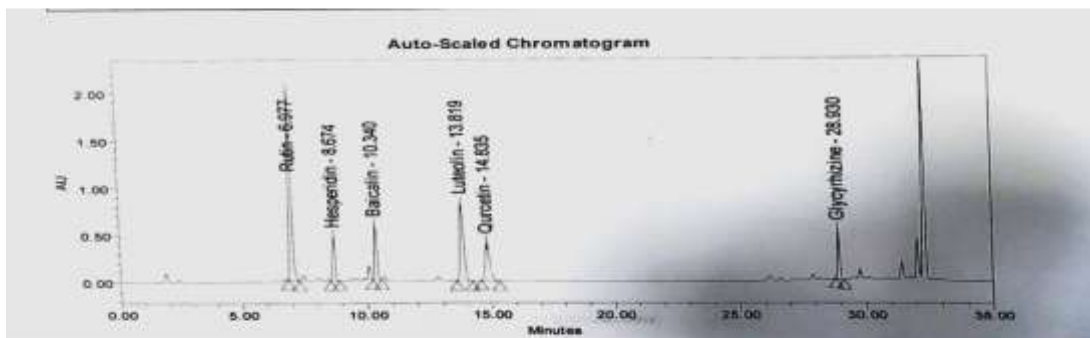


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**Figure 1:** Typical standard chromatogram extracted at 260 nm for Rutin, Hesperidin, Baicalin, Luteolin, Quercetin and Glycyrrhizin)

**Figure 2:** Typical standard chromatogram extracted at 370 nm for Piperine, Curcumin- I, Curcumin -II, Curcumin- III

### Specificity Parameters

The specificity of a chromatographic separation is described by the combined affinities that t he mobile phase and stationary phase exert on the sample components. A reliable chromatogr aphic assay also requires acceptable resolution between all the bioactive components, realistic retention times, theoretical plates and good peak symmetry. As the specificity results are described in table no 03 that the purity angle of each component is less than the purity threshold of corresponding peak, which shows the method is selective and specific.

**Table 3: Results of Specificity Parameters.**

| Peak Purity Parameters | Baicalin | Rutin | Hesperidin | Luteolin | Glycyrrhizin |
|---|---|---|---|---|---|
| Purity angle | 2.928 | 0.254 | 0.127 | 0.03 | 0.124 |
| Purity threshold | 3.601 | 2.224 | 2.913 | 3.920 | 8.620 |
| **Peak Purity Parameters** | **Quercetin** | **Pieprine** | **Curcumin - I** | **Curcumin - II** | **Curcumin - III** |
| Purity angle | 0.163 | 2.026 | 0.044 | 0.193 | 0.448 |
| Purity threshold | 7.538 | 2.958 | 0.228 | 0.257 | 3.863 |

**Precision Analysis**

Mix Standard solution of Luteolin, Rutin, Hesperidin, Baicalin, Curcumin, Demethoxycurcumin, Bisdemethoxycurcumin, Piperine, Glycyrrhizin and Quercetin was prepared and injected six replicates into HPLC system. The area response along with % relative standard deviation (%RSD) observed on replicate injection indicates the adequate reproducibility and thereby the precision of the system. Method precision was determined by measuring repeatability and ruggedness (intermediate precision – different day, by different analyst and on different make of instrument) for all the bioactive compounds at concentration mentioned above. The cumulative assay results were compared. The % RSD of assay results between two analyst and different days is found less than 2.0 of all the bioactive compounds, which shows that the developed method is precise.

**Table 4: Method Precision & Intermediate Precision**

| Sample | Baicalin | Rutin | Hesperidin | Luteolin | Glycyrrhizin | Quercetin | Piperine | Curcuminoids |
|---|---|---|---|---|---|---|---|---|
| 1 | 102.3 | 97.8 | 98.7 | 102.5 | 99.8 | 100.2 | 104.9 | 108.3 |
| 2 | 100.3 | 98.8 | 99.7 | 101.1 | 97.3 | 102.9 | 103.9 | 109.6 |
| 3 | 99.7 | 99.9 | 99.7 | 100.5 | 99.5 | 101.2 | 106.7 | 110.5 |
| 4 | 98.9 | 98.4 | 102.3 | 99 | 99.9 | 100.3 | 104.9 | 112.2 |
| 5 | 100.1 | 101.4 | 102.6 | 98.9 | 102.1 | 102.1 | 104.8 | 113.5 |
| 6 | 102.3 | 102.3 | 100.6 | 99.7 | 100.1 | 98.9 | 106.4 | 109.9 |
| 7 | 101.3 | 99.7 | 101.1 | 99.9 | 102.1 | 100.2 | 108.1 | 112.9 |
| 8 | 100.7 | 99.7 | 98.5 | 102.2 | 101.1 | 103.1 | 104.3 | 113.1 |
| 9 | 101.2 | 100.5 | 98.2 | 100.3 | 100.5 | 99.9 | 105.6 | 110.1 |
| 10 | 100.1 | 103.1 | 100.9 | 100.9 | 102.9 | 99.1 | 103.9 | 108.3 |
| 11 | 101.1 | 102.1 | 101.4 | 101.4 | 101.4 | 101.4 | 104.5 | 109.8 |
| 12 | 103.1 | 101.2 | 102.3 | 102.3 | 102.3 | 102.3 | 106.7 | 110.2 |
| **Average** | **100.93** | **100.41** | **100.50** | **100.73** | **100.75** | **100.97** | **105.39** | **110.70** |
| **Std. Dev.** | **1.13** | **1.43** | **1.43** | **1.10** | **1.53** | **1.40** | **1.31** | **1.63** |
| **%RSD** | **1.12** | **1.43** | **1.42** | **1.09** | **1.52** | **1.38** | **1.24** | **1.47** |

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**Results of Linearity Study**

Linearity of the method was obtained in the range shown in the table 5 to table 12. A series of seven solutions at low and high concentration levels (from 25% to 200% of the nominal concentration levels) were prepared, each solution was injected into HPLC system and the regression was calculated by least squares. Peak areas were calculated and the results interpolated on the calibration graph for each bioactive compound. The correlation coefficient for each bioactive compound is more than 0.99 and slope of the curve, were indicates the linearity of the test method.

**Table 5: Linearity of Baicalin**

| Linearity Level (%) | Concentration (mcg/mL) | Baicalin |
|---|---|---|
| 25 | 26.12 | 652605 |
| 50 | 52.24 | 1325211 |
| 75 | 78.32 | 1977816 |
| 100 | 105.23 | 2650421 |
| 125 | 131.26 | 3343126 |
| 150 | 157.85 | 3995632 |
| 200 | 211.23 | 5306842 |
| | **Slope** | **25194.13** |
| | **corr.** | **0.999** |
| | **r2** | **0.999** |

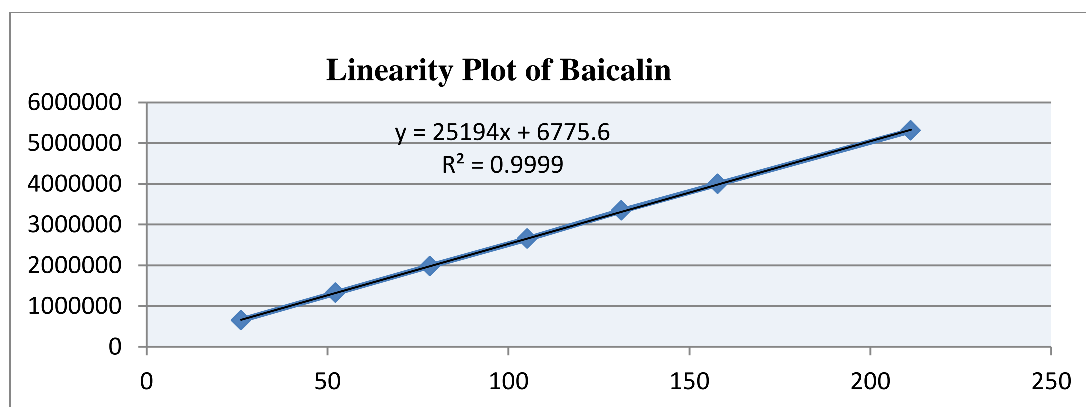


**Table 6: Linearity of Rutin**

| Linearity Level (%) | Conc. (mcg/mL) | Rutin |
|---|---|---|
| 25 | 93.67 | 1912034 |
| 50 | 187.34 | 3824069 |
| 75 | 281.34 | 5736103 |
| 100 | 375.50 | 7648137 |
| 125 | 471.20 | 9560171 |
| 150 | 560.30 | 11472206 |
| 200 | 750.81 | 15296274 |
| | **Slope** | **20384.55** |

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| | | |
|---|---|---|
| | **corr.** | **1.000** |
| | **r2** | **1.000** |

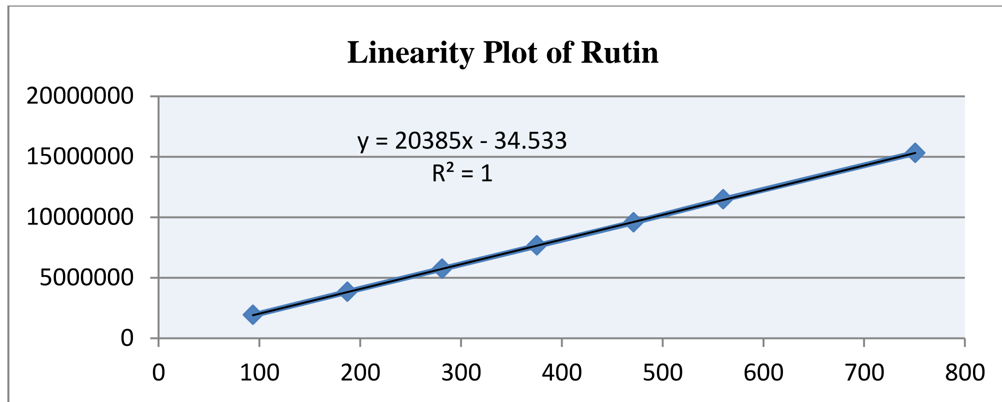


**Table 7: Linearity of Hesperidin**

| Linearity Level (%) | Conc. (mcg/mL) | Hesperidin |
|---|---|---|
| 25 | 83.73 | 436283 |
| 50 | 167.45 | 876565 |
| 75 | 252.10 | 1311848 |
| 100 | 335.46 | 1749130 |
| 125 | 420.50 | 2176413 |
| 150 | 503.19 | 2673695 |
| 200 | 672.34 | 3499260 |
| | **Slope** | **5231.24** |
| | **corr.** | **0.999** |
| | **r2** | **0.999** |

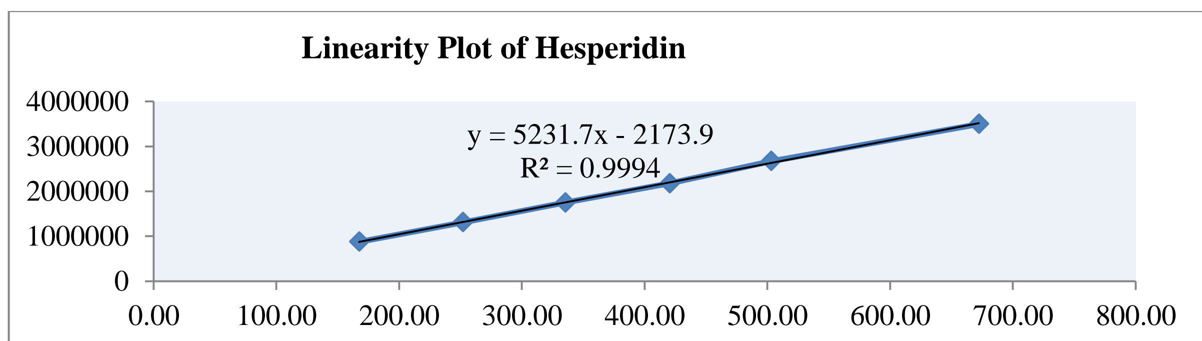


**Table 8: Linearity of Luteolin**

| Linearity Level (%) | Conc. (mcg/mL) | Luteoline |
|---|---|---|
| 25 | 28.83 | 1096860 |
| 50 | 57.65 | 2153721 |
| 75 | 86.35 | 3271581 |
| 100 | 115.60 | 4347441 |
| 125 | 143.20 | 5456301 |

| | | |
|---|---|---|
| 150 | 172.40 | 6545162 |
| 200 | 232.56 | 8701882 |
| | **Slope** | **37569.68** |
| | **corr.** | **0.999** |
| | **r2** | **0.999** |

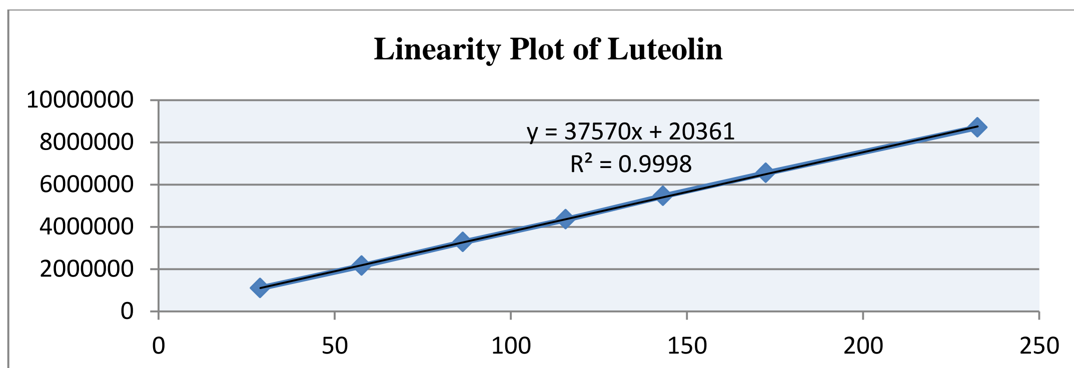


**Table 9: Linearity of Glycyrrhizin**

| Linearity Level (%) | Conc. (mcg/mL) | Glycyrrhizin |
|---|---|---|
| 25 | 66.63 | 410544 |
| 50 | 133.25 | 821088 |
| 75 | 199.45 | 1231632 |
| 100 | 264.50 | 1642176 |
| 125 | 331.20 | 2052720 |
| 150 | 397.80 | 2463264 |
| 200 | 530.66 | 3284352 |
| | **Slope** | **6198.06** |
| | **corr.** | **1.000** |
| | **r2** | **1.000** |

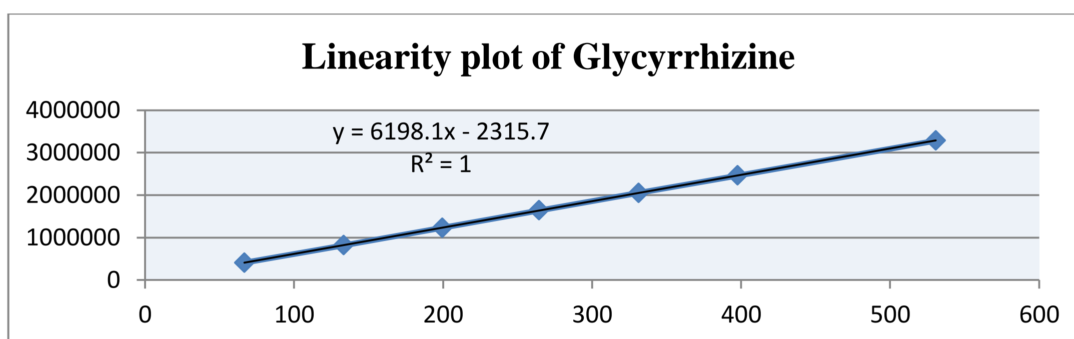


**Table 10: Linearity of Quercetin**

| Linearity Level (%) | Conc. (mcg/mL) | Quercetin |
|---|---|---|
| 25 | 138.27 | 577332 |
| 50 | 276.54 | 1168923 |
| 75 | 413.65 | 1752385 |
| 100 | 552.30 | 2349846 |
| 125 | 693.45 | 2987308 |

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| | | |
|---|---|---|
| 150 | 829.45 | 3624769 |
| 200 | 1106.80 | 4698792 |
| | **Slope** | **4302.22** |
| | **corr.** | **0.999** |
| | **r2** | **0.999** |

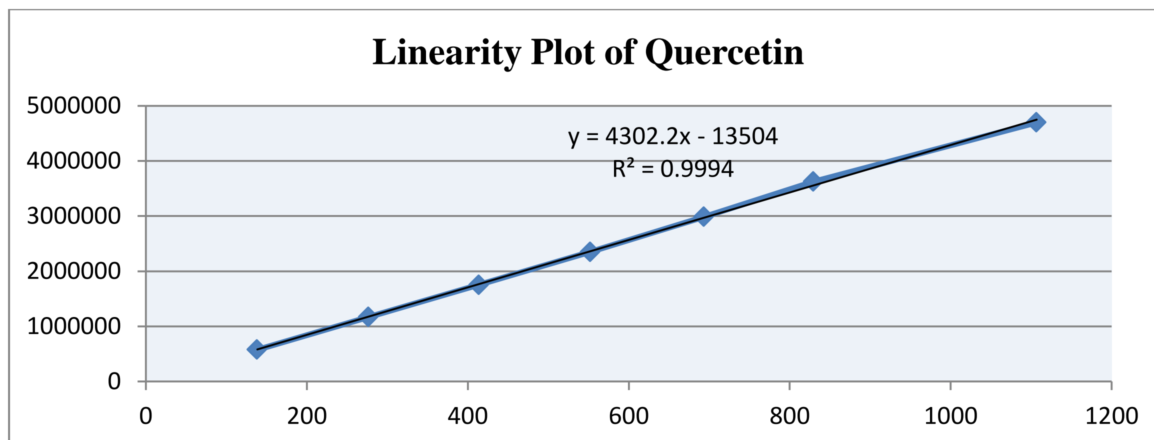


**Table 11: Linearity of Piperine.**

| Linearity Level (%) | Conc. (mcg/mL) | Piperine |
|---|---|---|
| 25 | 1.92 | 53357 |
| 50 | 3.83 | 107614 |
| 75 | 5.80 | 155470 |
| 100 | 7.66 | 209027 |
| 125 | 9.60 | 266584 |
| 150 | 11.60 | 345541 |
| 200 | 15.50 | 420054 |
| | **Slope** | **27881.12** |
| | **corr.** | **0.996** |
| | **r2** | **0.993** |

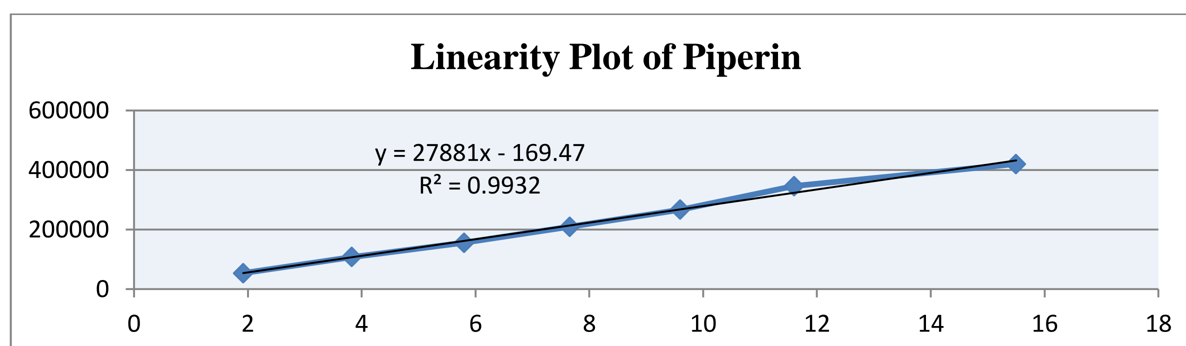

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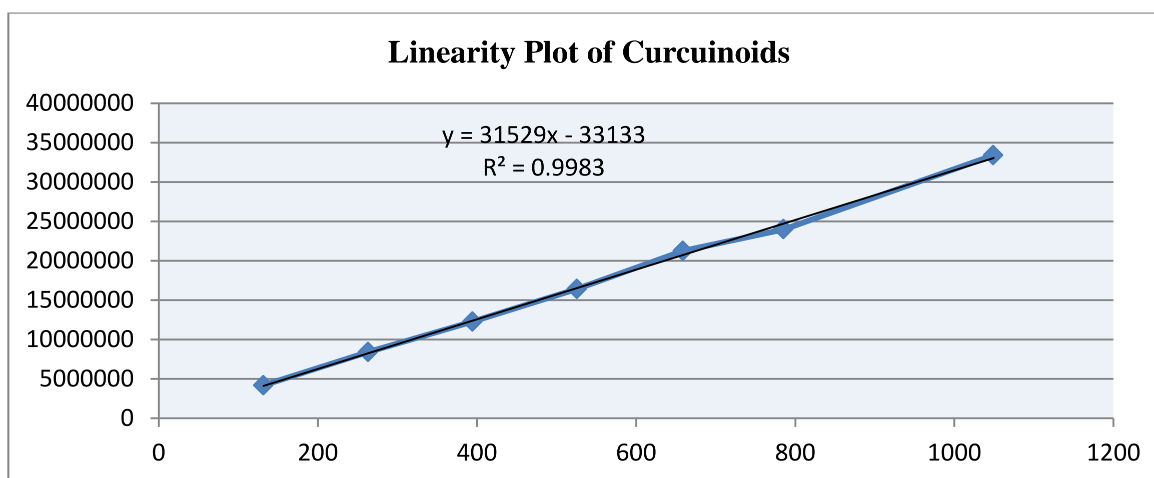
**Table 12: Linearity of Curcuminoids**

| Linearity Level (%) | Conc. (mcg/mL) | Curcuminoids |
|---|---|---|
| 25 | 131.60 | 4182351 |
| 50 | 263.20 | 8406703 |
| 75 | 394.50 | 12267054 |
| 100 | 525.66 | 16397405 |
| 125 | 658.90 | 21236756 |
| 150 | 785.60 | 23974508 |
| 200 | 1049.25 | 33386221 |
| | **Slope** | **31528.50** |
| | **corr.** | **0.9992** |
| | **r2** | **0.9983** |




**Accuracy/Recovery**

The accuracy of described method was evaluated by addition of known amounts of standard bioactive compounds from 50% level to 200% level. The recovery data was determined by comparing the areas obtained with that of standard solution analysed according to the method . All of these compounds were in good agreement with the added amount and recovered amount of the bioactive compounds. The results indicated the accuracy of the developed method.

**Table 13: Accuracy/Recovery**

| % Level | Baicalin | Rutin | Hesperidin | Luteolin | Glycyrrhizin | Quercetin | Piperine | Curcuminoids |
|---|---|---|---|---|---|---|---|---|
| 50 | 98.2 | 99.0 | 101.1 | 100.3 | 98.5 | 101.3 | 98.5 | 98.9 |
| 100 | 100.3 | 101.9 | 98.2 | 98.5 | 99.1 | 101.9 | 101.4 | 99.3 |
| 150 | 101.0 | 102.0 | 100.5 | 101.6 | 101.8 | 101.7 | 100.1 | 98.4 |
| 200 | 102.3 | 101.9 | 101.6 | 99.6 | 98.1 | 98.6 | 101.4 | 101.9 |
| **Average** | **100.5** | **101.2** | **100.4** | **100.0** | **99.4** | **100.9** | **100.4** | **99.6** |
| **SD** | **1.71** | **1.47** | **1.50** | **1.30** | **1.67** | **1.54** | **1.38** | **1.56** |
| **% RSD** | **1.71** | **1.45** | **1.50** | **1.30** | **1.68** | **1.52** | **1.37** | **1.57** |

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**Limit of Detection and Quantification**

Limit of detection and quantification were calculated from signal to noise ratio of all bioactive compound's chromatograms. From the results the detection limits (µg/mL), 1.50 to 8.99 found as mentioned in below table. The quantification limits (µg/mL), 4.74 and 29.97 found as mentioned in below table. The results indicated that the method is sensitive to detect and quantify the bioactive compounds.

**Table 14: Limit of Detection (LOD) & Limit of Quantitation (LOQ).**

| Parameters | Baicalin | Rutin | Hesperidin | Luteolin | Glycyrrhizin | Quercetin | Piperine | Curcumin |
|---|---|---|---|---|---|---|---|---|
| Correlation coefficient | 0.999 | 0.999 | 0.998 | 0.999 | 1.000 | 0.999 | 0.997 | 0.999 |
| Slope (m) | 25194.13 | 20384.55 | 5231.24 | 37569.10 | 6198.06 | 4302.22 | 27874.67 | 31528.5047 |
| Intercept (y) | 6775.52 | -34.63 | 7437.00 | 20457.29 | -2299.68 | -28909.08 | -164.98 | -22427.28 |
| STEYX | 18399.03 | 30827.78 | 5951.39 | 44545.05 | 3101.15 | 12892.66 | 13200.47 | 8837.19 |
| **LOD(µg/mL)** | **2.19** | **4.54** | **3.41** | **3.56** | **1.50** | **8.99** | **1.42** | **2.89** |
| **LOQ(µg/mL)** | **7.30** | **15.12** | **11.38** | **11.86** | **5.00** | **29.97** | **4.74** | **8.76** |

**Filter Interference Study:**

Filter interference study was performed, as validation of method, by using different make filters (0.45 µm Nylon filter and 0.45 µm PVDF filter) for standard and sample preparation, The results present in table no. 12 indicated that there is no interference from 0.45 µm Nylon filer and it is suitable for filter the sample and standard.

**Table 15: Results of Filter Interference Study**

| **Baicalin** | **% Difference** | **Glycyrrhizin** | **% Difference** |
|---|---|---|---|
| Centrifuged | NA | Centrifuged | NA |
| 0.45 µm Nylon filter | 0.40 | 0.45 µm Nylon filter | 0.20 |
| 0.45 µm PVDF filter | 0.87 | 0.45 µm PVDF filter | 1.19 |
| **Rutin** | **% Difference** | **Quercetin** | **% Difference** |
| Centrifuged | NA | Centrifuged | NA |
| 0.45 µm Nylon filter | 0.06 | 0.45 µm Nylon filter | 0.30 |
| 0.45 µm PVDF filter | -0.30 | 0.45 µm PVDF filter | 0.24 |
| **Hesperidin** | **% Difference** | **Piperine** | **% Difference** |
| Centrifuged | NA | Centrifuged | NA |
| 0.45 µm Nylon filter | 0.68 | 0.45 µm Nylon filter | 0.45 |
| 0.45 µm PVDF filter | 0.33 | 0.45 µm PVDF filter | -0.16 |
| **Luteolin** | **% Difference** | **Curcuminoids** | **% Difference** |
| Centrifuged | NA | Centrifuged | NA |
| 0.45 µm Nylon filter | 0.69 | 0.45 µm Nylon filter | 1.14 |
| 0.45 µm PVDF filter | 1.11 | 0.45 µm PVDF filter | 0.45 |

**Robustness of Method**

The robustness of the method was checked by deliberately varied chromatographic conditions, such as change in wavelength and flow rate, no significant change in assay was observed. With change in wavelength of study from260 nm± 2nm to 370 nm± 2 nm, change in response of peaks was observed but no impact on system suitability, assay results & elution order. Flow rate has been studied at 0.7 mL/min. and 0.9 mL/min.**,** No changes observed in elution order & change retention time of peaks was observed and resolution of peaks was decreased, so concluded that method is sensitive to flow.

## CONCLUSION:

A unique method for simultaneous quantification of bioactive compounds luteolin, rutin, hesperidin, baicalin, curcumin, demethoxycurcumin, bisdemethoxycurcumin, piperine, glycyrrhizin and quercetin in marketed herbal formulation has been developed and validated. Each step of method has been vigilantly controlled to reach out the best possible separation and quantification. The method is unique, appropriate, accurate, precise, selective and rugged for the routine analysis of selected phytoconstituents in any such marketed formulation containing one or all stated constituents. The method followed linearity, repeatability, intra and inter-day precision, accuracy and reliability. The devised method could be used for simultaneous quantitative determination of all mentioned bioactive compounds in single run and would be used to supersede conventional methods of analysis of such compounds. The developed method could be used for marker-based standardization of herbal formulations in pharmaceutical industries.

**Conflict of interest:** No conflict of interest

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