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# Journal of Pharmacy and Chemistry

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# Analytical Method Development and Validation for the Simultaneous Estimation of Olmesartan Medoxomil and Hydrochlorothiazide in Combined Tablet Dosage form by RP-HPLC

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## ABSTRACT

A simple, reproducible and efficient reverse phase high performance liquid chromatographic method was developed for simultaneous estimation of olmesartan medoxomil (OLM) and hydrochlorothiazide (HCTZ) in combined tablet dosage form. Formulation containing OLM with HCTZ are used as antihypertensive angiotensin II receptor blocker. Chromatography was performed on a 250 mm x 4.6 mm, 5- $\mu$ m particle size, C<sub>18</sub> Thermo Hypersil Keystone ODS column with a 40:60 (v/v) mixture of buffer and acetonitrile as a mobile phase and the pH was adjusted to 3.2 by adding dilute phosphoric acid. The detection of the combined dosage form was carried out at 265 nm and a flow rate employed was 1 ml min<sup>-1</sup>. The retention times were 4.99 & 3.02 min for olmesartan medoxomil and hydrochlorothiazide respectively. Linearity was obtained in the range of 40 to 200  $\mu$ g mL<sup>-1</sup> for olmesartan medoxomil and 12.5 to 250  $\mu$ g mL<sup>-1</sup> for hydrochlorothiazide, with a correlation coefficient of 0.9996 and 0.9998 respectively. The result of the analysis was validated statistically and recovery studies confirmed the accuracy and precision of the proposed method.

**Keywords:** Olmesartan Medoxomil, Hydrochlorothiazide, Reverse Phase – High Performance Liquid Chromatography, Simultaneous Estimation.

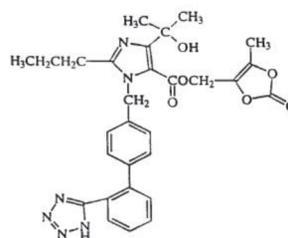
## Introduction

Olmesartan medoxomil belongs to angiotensin II receptor blocker effective in lowering blood pressure in hypertensive patients. Chemically it is known as 2,3-dihydroxy-2-butenyl 4-[1-hydroxyl-1-methylethyl]-2-propyl-1-[p(o-1 H-tetrazol-5 ylphenyl) benzyl] imidazole-5-carboxylate, cyclic 2,3-carbonate. Its molecular weight is 558.59 [1, 2, 10].

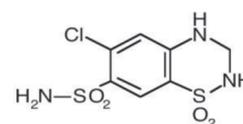
Hydrochlorothiazide is a diuretic of the class of benzothiadiazine widely used in antihypertensive pharmaceutical formulations, alone or combination with other drugs, which decreases active sodium reabsorption and reduced peripheral vascular resistance. It is chemically 6-chloro-3,4-dihydro-2H-1,2,4-benzothiazine-7-sulfonamide 1,1-dioxide and was successfully used as one content in association with other drugs in the treatment of hypertension. Its molecular weight is 297.7 [7, 10].

There are very few methods appearing in the literature for the simultaneous estimation of angiotensin II receptor antagonist and hydrochlorothiazide in

tablets [3,5,9]. Since these methods were based on HPLC, Capillary electrophoresis and UV derivative spectrophotometry [4,6,8], the procedure was inconvenient for determination and run time were rather long. Thus, an attempt was made to develop a simple, precise, accurate and economical RP-HPLC method for the simultaneous estimation of olmesartan medoxomil and hydrochlorothiazide in tablets. The chemical structures of olmesartan medoxomil and hydrochlorothiazide are shown in Fig. 1.



Olmesartan medoxomil



Hydrochlorothiazide

**Fig.1: The chemical structure of olmesartan medoxomil and hydrochlorothiazide.**

*\*Address for correspondence*

## Materials and Methods

### Chemicals and Reagents

HPLC- grade acetonitrile and potassium dihydrogen ortho phosphate analytical grade were procured from Rankem (Mumbai, India) and pure standards of olmesartan medoxomil (99.26%) and hydrochlorothiazide (99.89%) were obtained as gift samples from MICROLABS (Hosur, India). Hydrochloric acid was procured from E. Merck (Mumbai, India). HPLC grade water was procured from Ranbaxy (Mumbai, India).

### Instrumentation and Chromatographic Conditions

Chromatography was performed with a Shimadzu (Japan) LC-10ATVp isocratic pump, an UV-VIS detector (SPD-10AVa) and a rheodyne 9013 injector with 20- $\mu$ L loop, C<sub>18</sub> Thermo Hypersil ODS column (250 x 4.6mm, 5- $\mu$ m particles) was used for chromatographic separation under suitable condition. Detection was carried out at 265 nm and the software used was Shimadzu Class VP. The mobile phase was a 40:60 (v/v) mixture of freshly prepared buffer (potassium dihydrogen ortho phosphate and triethylamine) and acetonitrile. The mobile phase was filtered through 0.45  $\mu$  membrane filter and sonicated before use. The flow rate of mobile phase was maintained at 1 mL min<sup>-1</sup>. The column temperature was maintained at ambient temperature. The detection wavelength was 265 nm for both drugs. The injection volume was 20  $\mu$ L and total run time was 10 min. The peaks were identified by retention time; a typical chromatogram is shown in Fig. 2.

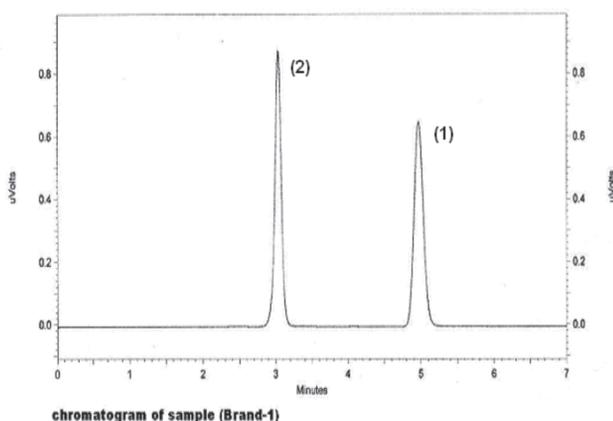


Fig.2: Typical chromatogram obtained from olmesartan medoxomil and hydrochlorothiazide.

### Preparation of standard solution for calibration Plots

Weigh accurately about 0.100g of olmesartan medoxomil working standard and 0.0625g of hydrochlorothiazide transferred to a 100 ml volumetric flask. Then, add 30 ml of 0.1N HCL, sonicated to dissolve the content and make up the volume with diluents (acetonitrile & water (80:20)). Filter through 0.45 $\mu$  membrane filter paper. Dilute 5ml of resulting solution to 50 ml with diluent to give a concentration

of 100  $\mu$ g mL<sup>-1</sup> of olmesartan medoxomil & 62.5  $\mu$ g mL<sup>-1</sup> of hydrochlorothiazide. Stock solution was diluted with mobile phase to give working standard solution containing 40 to 200  $\mu$ g mL<sup>-1</sup> of olmesartan medoxomil and 12.5 to 250  $\mu$ g mL<sup>-1</sup> hydrochlorothiazide. These standard solutions were injected for construction of calibration plots by plotting drug peak - area ratio (y) for each of the drug against concentration (x). Analysis was performed at ambient temperature. The retention times of olmesartan medoxomil and hydrochlorothiazide under these conditions were 4.99 and 3.02 min respectively.

### Assay procedure

Twenty tablets, each containing OLM (20 mg) and HCTZ (12.5 mg) were weighed, finely powdered, and an amount of powder sample equivalent to 100 mg of olmesartan medoxomil and 62.5mg hydrochlorothiazide was taken in 100 mL volumetric flask and dissolved in 30 ml of 0.1N HCL and extracted by sonication to ensure complete solubility of the drug. The excipients were separated by filtration. The mixture was then made up to 100 mL with diluents, thoroughly mixed and filtered through a 0.45 $\mu$  membrane filter. The filtrate concentration of olmesartan medoxomil was 100  $\mu$ g mL<sup>-1</sup> and for hydrochlorothiazide was 62.5  $\mu$ g mL<sup>-1</sup> from tablets. Then the solutions were injected five times into the column. Each injection was replicated five times. From the peak area, the drug content in the tablets was qualified using the regression equation obtained from the pure samples. A typical chromatogram obtained from a sample solution is shown in Fig. 2.

## Results and Discussion

### Method development

The objective of this study was to develop simultaneous estimation of two components under isocratic conditions. The mobile phase used was the mixture of acetonitrile with buffer in different ratios. Finally a mixture of acetonitrile- buffer (pH 3.2) in the ratio of 60:40 (v/v), proved to be effective mixture than the other mixture used for the separation. Then the flow rate tested includes 0.4, 0.8, 1.0, 1.2 and 1.5 ml. Among the flow rates 1.0 ml was selected for the assay because of better resolution of the peaks. The mentioned chromatographic conditions revealed to provide better resolution between olmesartan medoxomil and hydrochlorothiazide in a reasonable time of 4.99 and 3.02 min, respectively. The optimum wave length for detection was 265 nm, no indigenous interfering compounds eluted at the retention times of the drugs.

### Validation of the Method

The method was validated, in accordance with ICH guidelines, for linearity, accuracy, precision, specificity, sensitivity, ruggedness, and robustness.

## Linearity

Linearity was assessed with the aid of serially diluted calibration solutions as mentioned above. The standards were injected separately. Calibration graphs were plotted on the basis of triplicate analysis of each calibration solutions. Linear correlations were obtained over the range studied, with correlation coefficients  $r^2$  0.99 for the drugs. In case of tablets, the regression equation was  $y = 26035x$  ( $R^2=0.9996$ ) for olmesartan medoxomil and  $y = 22522x$  ( $R^2=0.9998$ ) for hydrochlorothiazide.

## Precision

The precision of the method was done by replicate ( $n=5$ ) analysis of tablet preparations. The precision was also studied in terms of intra-day changes in peak area of drug solution on the same day and on three different days over a period of one week. The intra-day and inter-day variation was calculated in terms of percentage relative standard deviation and the results are given in Table. 1.

## Accuracy

Accuracy was determined by the method of standard additions at three different levels, by multiple level recovery studies. Solution containing  $100 \mu\text{g mL}^{-1}$  OLM and  $62.5 \mu\text{g mL}^{-1}$  HCTZ for tablets was prepared from the stock solutions and was spiked with amounts of the standard drugs equivalent to 50, 100 and 150% of the amounts present in the original solution. The spiked concentration of 50, 100 and 150  $\mu\text{g mL}^{-1}$  OLM and 31.5, 62.5 and  $125 \mu\text{g mL}^{-1}$  HCTZ respectively. The percentage recovery was found to be 98% to 101% for OLM and 99% to 101% for HCTZ respectively. These solutions were then analyzed for recovery studies and consistent values by replicated injections cum analysis. Results for determination of precision and accuracy are presented in Table. 1.

TABLE-1

### Results from Validation and System suitability studies

| Method Characteristics               | Olmesartan medoxomil | Hydrochlorothiazide |
|--------------------------------------|----------------------|---------------------|
| Theoretical plates                   | 3969                 | 5476                |
| Resolution                           | 2.45                 | 0                   |
| Linearity range ( $\mu\text{g/ml}$ ) | 40-200               | 12.5-250            |
| Percentage recovery (%)              | 99.64                | 99.42               |
| Correlation coefficient ( $R^2$ )    | 0.9996               | 0.9998              |
| Accuracy (%RSD)                      | 1.0343               | 1.0599              |
| Intraday Precision (%RSD)            | 0.4099               | 0.3608              |
| Interday Precision (%RSD)            | 0.2779               | 0.9159              |
| LOD ( $\mu\text{g}$ )                | 0.255                | 0.851               |
| LOQ ( $\mu\text{g}$ )                | 0.297                | 0.99                |
| Standard Error                       | $\pm 0.1686$         | $\pm 0.0487$        |

## Specificity

Specificity was tested against standard compounds and against potential interferences in the presence of placebo. No interference was detected at the retention time of OLM and HCTZ in sample solution. Peak purity for OLM and HCTZ was tested by comparing spectra acquired at the start (S), apex (A), and end (E) of the peaks.

## Recovery

Recovery was determined by spiking the formulation with standards of each drug equivalent to 50, 100, and 150 % of the amount originally present. Average recoveries ranged from 98 to 100, as reported in Table. 1.

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

The LOD and LOQ for analyses were estimated by injecting a series of dilute solutions with known concentration. LOD and LOQ were calculated by using following equations.

$$\text{LOD} = 3.3 \text{XSyx/b}; \text{LOQ} = 10.0 \text{XSyx/b}$$

Where Syx is residual variance due to regression; b is slope.

LOD and LOQ for OLM were  $0.255 \mu\text{g mL}^{-1}$  and  $0.297 \mu\text{g mL}^{-1}$  respectively and for HCTZ were  $0.851$  and  $0.99 \mu\text{g mL}^{-1}$  respectively.

## Stability

To demonstrate the stability of both standard and sample solutions during analysis, both solutions were analyzed over a period of 24 h at room temperature. The results showed that for both solutions, the retention times and peak areas of OLM and HCTZ remained almost unchanged ( $\text{RSD} < 2.0\%$ ) indicating that no significant degradation occurred within this period, i.e. both solutions were stable for at least 24 h, which was sufficient to complete the whole analytical process. Sample solution were then stored at 4 and  $25^\circ \text{C}$  and checked after three days of storage. When results were compared with those from freshly prepared sample in each case no significant degradation occurred within the indicated period.

## Ruggedness and Robustness

The ruggedness of the method was determined by using different instrument (Waters 2489) and different column (Symmetry  $\text{C}_{18}$ ) of similar type. The robustness of the method was determined by making slight changes in the chromatographic conditions (buffer  $\text{pH} \pm 0.5$ , flow rate  $\pm 0.2$  min). Again there was no marked change in the chromatograms. These results indicated that the method was rugged and robust with regard

to these conditions. When mobile phase composition was changed by  $\pm 10\%$ , however, proper resolution could not be achieved; separation of the drugs was very sensitive to mobile phase ratio.

### System Suitability

System suitability tests are an integral part of chromatographic method. They were used to verify that the reproducibility of the chromatographic system is adequate for the analysis. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared standard stock solution of olmesartan medoxomil and hydrochlorothiazide. In addition, standard deviation of olmesartan medoxomil and hydrochlorothiazide standards were evaluated by injecting mixed standard of both olmesartan medoxomil and hydrochlorothiazide (100 and 62.5  $\mu\text{g/mL}$ ). All the parameters are shown in Table. 2

**TABLE-2**

### Results from validation and System suitability studies

| Formulation          | Labeled amount (mg per dose) | Amount found (mg per dose) | Percentage Recovery |
|----------------------|------------------------------|----------------------------|---------------------|
| Olmesartan medoxomil | 20                           | 19.96                      | 99.8                |
| Hydrochlorothiazide  | 12.5                         | 12.51                      | 100.08              |

### Conclusion

The proposed RP-HPLC method for simultaneous estimation of olmesartan medoxomil and hydrochlorothiazide in combined tablets dosage forms

is simple, precise, specific and highly accurate and less time consumption for analysis could be recorded. So, it can definitely be employed for the routine analysis. Hence this RP-HPLC method is suitable for quality control of raw materials and formulations, and also for dissolution studies. It can be used for bioequivalence studies in plasma.

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# Synthesis and antimicrobial activity of some pyrazoline derivatives

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## ABSTRACT

The objective of present study was to synthesize some new derivatives of pyrazoline and investigation of their antimicrobial property. 1-H-3-(4-bromo phenyl)-5- (substituted phenyl) pyrazoline was used as a precursor to synthesize some biologically active heterocyclic derivatives. Reaction of 1-(4-bromo phenyl) 3-(substituted phenyl) prop-2-en-1-one with hydrazine hydrate gave 1-H-3-(4-bromo phenyl)-5-(substituted phenyl)Pyrazoline, which on reaction with benzoyl chloride in pyridine has given 1-benzoyl-3-(4-bromophenyl) 5- (substituted phenyl) pyrazoline, while the former on reaction with acetic acid yielded 1-acetyl (4-bromo phenyl) 5-(substituted phenyl) pyrazoline derivatives. Several derivatives have been synthesized and were screened for their antimicrobial efficacy against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. Antifungal activity was performed against *Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporum* and *Candida albicans*. The present study revealed that most of the synthesized compounds have shown good to moderate antimicrobial activity.

**Key words:** Pyrazoline, antifungal activity, antibacterial activity.

## Introduction

Pyrazolines are well known and important nitrogen-containing five –membered heterocyclic compounds. Various methods have been used for their synthesis [1-4]. Various substituted pyrazolines and their derivatives are important biological agents and a significant amount of research work has been done on this class. In particular they are used as antitumor [5] antibacterial, antifungal, antiviral, antiparasitic, antitubercular and insecticidal agents [6-14]. Some of these compounds have also possesses anti-inflammatory, anti-diabetic, anaesthetic and analgesic properties [15-18]. Moreover, pyrazolines have played a crucial part in the development of theory in heterocyclic chemistry and also used extensively as synthons in organic synthesis [19-23].

## Materials and Methods

All the melting points have been determined in an open capillary and are uncorrected. The completion of reactions was monitored by TLC. The IR spectra have been recorded in KBr pellets on a Shimadzu 8201PC spectrophotometers ( $\nu_{\max}$  in  $\text{cm}^{-1}$ ),  $^1\text{H}$  NMR spectra on a AVANCE II 400 NMR Spectrophotometer (Chemical shift in  $\delta$  ppm), elemental analysis has been carried out on a Carlo Erba 1108 model analyzer.

### Synthesis of 1 (4-bromo phenyl)-3-substituted phenyl prop-2-en-1-one (I)

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*p*-Bromo acetophenone (0.01mol) was dissolved in ethanol. Aromatic substituted aldehyde (0.01mol) was added and the solution was heated to boiling.

To this hot solution 40% NaOH was added with constant stirring, A yellow orange coloured mass was obtained which was kept overnight and acidified by 10% HCl, washed with 10%  $\text{NaHCO}_3$  followed by water and recrystallized from ethanol I(a), m.p. 205°C, yield 69%. M.F.  $\text{C}_{15}\text{H}_{10}\text{O}_3\text{NBrCl}$  and M.W. 287. 687.

IR ( $\nu_{\max}$  in  $\text{cm}^{-1}$ ): 3050 (C-H stretching), 1548 (asymmetric stretching in -CO-CH=CH-), 1672 (C=O stretching), 1180 (C-N str.), 1600 (Ar- $\text{NO}_2$  syn. str.).

$^1\text{H}$  NMR  $\text{CDCl}_3$  in  $\delta$  ppm 6.42 (unsym, m, nitro sub. benzene ring), 6.02 (sym., m., chloro sub benzene ring, 2.56 (s, CH = CH).

### Synthesis of 1-H-3 (4-bromo phenyl)-5-(substituted phenyl) pyrazoline (II).

4-Bromo substituted chalcone (I) dissolved in ethanol (25ml) and hydrazine hydrate (0.02mol) was added to it. The reaction mixture was refluxed for 2 hrs, concentrated and allowed to stand overnight. The resulting solid which separated out was recrystallized from ethanol. m.p. 109°C, yield 72% M.F.,  $\text{C}_{15}\text{H}_{11}\text{N}_3\text{O}_2\text{BrCl}$ , M.W., 300.716. IR (nujol,  $\text{cm}^{-1}$ ): 315.29 (Ar-H stretching), 2905.69, (Ali,C-H stretching), 928.72 (N-O stretching), 835.16 (N-N stretching), 1675.19 (C=N stretching), 1320.16 (C-N stretching), 1170.65 (C-O stretching), 3401.65 (N-H stretching), 759.69 (Ar-Cl stretching).

6.03 (sym, m., chloro sub-benzene ring) 6.51 (unsym., m, bromo sub benzene ring) 7.39  $\delta$  (s, N-H), 2.54  $\delta$  (s, CH<sub>2</sub>), 2.41 $\delta$  (s, CH).

### Synthesis of 1-acetyl-3-(4-bromophenyl)-5-(substituted phenyl) pyrazoline (III)

Compound II and acetic acid (10 ml) were refluxed for 2 hrs. The reaction mixture was then concentrated, allowed to cool. The solid filtered, washed with water and recrystallized from ethanol to get III (a), (yield 75%, m.p.186).

IR (nujol, cm<sup>-1</sup>): 3049 (Ar-H stretching), 2915 (Alk C-H stretching), 1549 (C=N stretching), 1270 (C-N stretching), 1165 (C-O stretching), 921 (N-O stretching), 829 (N-N stretching), 401(C-H bending out of plane).

<sup>1</sup>HNMR (CDCl<sub>3</sub> in  $\delta$  ppm) 6.54 (unsym., m, bromo sub. benzene ring) 6.08 (sym., m, chloro sub.benzene ring) 2.48 ( $\delta$  CH<sub>2</sub>), 2.21 ( $\delta$  CH), 4.02 (s, COCH<sub>3</sub>).

### Synthesis of 1-benzoyl-3-(4-bromo phenyl)-5-(substituted phenyl) pyrazoline (IV)

1-H-3(4-bromo phenyl) 5-(4-chlorophenyl) pyrazoline II- was dissolved in pyridine and stirred at room temperature for 1hr, after which the reaction mixture was treated with cold dilute HCl. The resulting solid was filtered, washed successively with water, cold NaOH (2%) and water and finally recrystallized from glacial acetic acid to obtain IV (a), (yield 79% m.p. 185°C).

IR (nujol, cm<sup>-1</sup>): 3010 (Ar C-H stretching), 2845(Alk C-H stretching), 1672(C=N stretching), 1319 (C-N stretching), 1165(C-O stretching), 915 (N-O stretching), 830 (N-N stretching), 1319 (C=N stretching), 1165 (C-O stretching), 1402(C-H bending in plane.),659(C-H bending out of plane.).

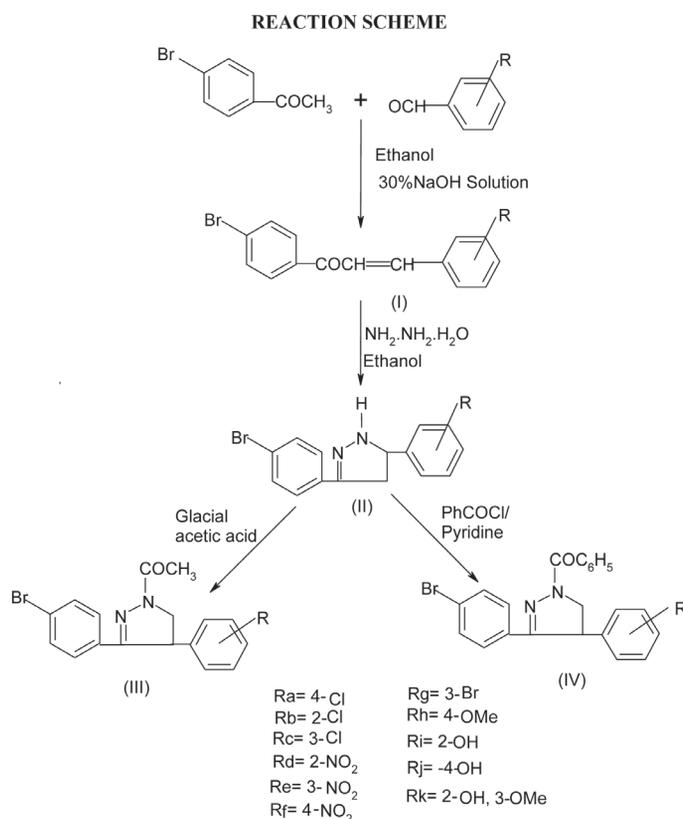
<sup>1</sup>HNMR (CDCl<sub>3</sub> in  $\delta$  ppm), 6.23 (unsym., m, bromo substituted benzene ring) 6.41 (sym., m, chloro sub.benzene ring), 3.02 (s, CH<sub>2</sub>) 2.46 (s, C-H), 7.38 (m, COC<sub>6</sub>H<sub>5</sub>).

### Antimicrobial Activity of synthesized pyrazoline derivatives:

In the present study the filter paper disc diffusion plate method <sup>24</sup> was employed to evaluate the antimicrobial activity, in vitro.

The medium was first inoculated with a loopful of broth culture of the organism and shaken for uniform distribution. It was poured into petridish. Discs soaked in test sample solution were placed over the seeded medium and pressed so that all parts of disc come in contact with the medium. The seeded plates were incubated at 28 °C for 32 hrs in case of bacteria and 37°C for 72 hrs in case of fungi. Same procedure was adopted for standard drugs. The activity was determined by using 50ppm and 100ppm solution of synthesized pyrazoline derivatives. 50ppm and 100ppm solutions of the standard drugs, Streptomycin (for bacteria) and Amphotericine-B (For fungi) were also prepared for comparison. Related data given in table 2 and 3.

## REACTION SCHEME



**Table-1**  
**Physical characterization data of synthesized pyrazoline derivatives(IIIa-IIIk)and( IVa-IVk)**

| Comp. | Molecular formula  | Mol. wt. | Yield % | M.P. | % of   |       |        |       |        |       |
|-------|--|----------|---------|------|--------|-------|--------|-------|--------|-------|
|       |  |          |         |      | C      |       | H      |       | N      |       |
|       |  |          |         |      | Calcu. | Found | Calcu. | Found | Calcu. | Found |
| III-a | C <sub>17</sub> H <sub>14</sub> N <sub>2</sub> OBrCl             | 377.65   | 75      | 186  | 54.01  | 53.97 | 3.70   | 3.67  | 7.41   | 7.38  |
| III-b | C <sub>17</sub> H <sub>14</sub> N <sub>2</sub> OBrCl             | 377.65   | 76      | 136  | 54.01  | 53.97 | 3.70   | 3.67  | 7.41   | 7.38  |
| III-c | C <sub>17</sub> H <sub>14</sub> N <sub>2</sub> OBrCl             | 377.65   | 71      | 145  | 54.01  | 53.97 | 3.70   | 3.67  | 7.41   | 7.38  |
| III-d | C <sub>17</sub> H <sub>14</sub> N <sub>3</sub> O <sub>3</sub> Br | 388.19   | 69      | 158  | 52.59  | 52.48 | 3.63   | 3.57  | 10.82  | 10.78 |
| III-e | C <sub>17</sub> H <sub>14</sub> N <sub>3</sub> O <sub>3</sub> Br | 377.65   | 74      | 160  | 52.59  | 52.48 | 3.63   | 3.57  | 10.82  | 10.78 |
| III-f | C <sub>17</sub> H <sub>14</sub> N <sub>3</sub> O <sub>3</sub> Br | 377.65   | 78      | 165  | 52.59  | 52.48 | 3.63   | 3.57  | 10.82  | 10.78 |
| III-g | C <sub>17</sub> H <sub>14</sub> N <sub>2</sub> OBr <sub>2</sub>  | 422.09   | 73      | 190  | 48.37  | 48.31 | 3.34   | 3.28  | 6.62   | 6.58  |
| III-h | C <sub>18</sub> H <sub>17</sub> N <sub>2</sub> O <sub>2</sub> Br | 373.22   | 72      | 180  | 57.92  | 57.86 | 4.59   | 4.51  | 7.50   | 7.47  |
| III-i | C <sub>17</sub> H <sub>15</sub> N <sub>2</sub> O <sub>2</sub> Br | 361.21   | 81      | 168  | 56.52  | 56.45 | 4.74   | 4.68  | 7.75   | 7.71  |
| III-j | C <sub>17</sub> H <sub>15</sub> N <sub>2</sub> O <sub>2</sub> Br | 361.21   | 75      | 170  | 56.52  | 56.45 | 4.74   | 4.68  | 7.75   | 7.71  |
| III-k | C <sub>18</sub> H <sub>18</sub> N <sub>2</sub> O <sub>3</sub> Br | 390.22   | 76      | 182  | 55.40  | 55.34 | 4.64   | 4.59  | 7.17   | 7.13  |
| IV-a  | C <sub>22</sub> H <sub>16</sub> N <sub>2</sub> OBrCl             | 439.72   | 80      | 184  | 60.09  | 59.97 | 3.66   | 3.61  | 6.37   | 6.33  |
| IV-b  | C <sub>22</sub> H <sub>16</sub> N <sub>2</sub> OBrCl             | 439.72   | 79      | 189  | 60.09  | 59.97 | 3.66   | 3.61  | 6.37   | 6.33  |
| IV-c  | C <sub>22</sub> H <sub>16</sub> N <sub>2</sub> OBrCl             | 439.72   | 81      | 182  | 60.09  | 59.97 | 3.66   | 3.61  | 6.37   | 6.33  |
| IV-d  | C <sub>22</sub> H <sub>16</sub> N <sub>3</sub> O <sub>3</sub> Br | 450.26   | 78      | 170  | 58.68  | 58.59 | 3.58   | 3.52  | 9.33   | 9.27  |
| IV-e  | C <sub>22</sub> H <sub>16</sub> N <sub>3</sub> O <sub>3</sub> Br | 450.26   | 76      | 167  | 58.68  | 58.59 | 3.58   | 3.52  | 9.33   | 9.27  |
| IV-f  | C <sub>22</sub> H <sub>16</sub> N <sub>3</sub> O <sub>3</sub> Br | 450.26   | 75      | 176  | 58.68  | 58.59 | 3.58   | 3.52  | 9.33   | 9.27  |
| IV-g  | C <sub>22</sub> H <sub>16</sub> N <sub>2</sub> OBr <sub>2</sub>  | 484.17   | 74      | 140  | 54.57  | 54.48 | 3.33   | 3.27  | 5.78   | 5.74  |
| IV-h  | C <sub>23</sub> H <sub>19</sub> N <sub>2</sub> O <sub>2</sub> Br | 435.29   | 69      | 190  | 63.46  | 63.39 | 4.39   | 4.34  | 6.43   | 6.39  |
| IV-i  | C <sub>22</sub> H <sub>17</sub> N <sub>2</sub> O <sub>2</sub> Br | 421.27   | 68      | 167  | 62.72  | 62.67 | 4.06   | 4.01  | 6.64   | 6.59  |
| IV-j  | C <sub>22</sub> H <sub>17</sub> N <sub>2</sub> O <sub>2</sub> Br | 421.27   | 70      | 168  | 62.72  | 62.67 | 4.06   | 4.01  | 6.64   | 6.59  |
| IV-k  | C <sub>23</sub> H <sub>20</sub> N <sub>2</sub> O <sub>3</sub> Br | 452.29   | 72      | 185  | 61.07  | 60.96 | 4.45   | 4.39  | 6.19   | 6.15  |

## Results and Discussion

The reaction of p-bromo acetophenone and aromatic substituted aldehyde in ethanol gave 1-(4-bromo phenol 3-substituted phenyl prop-2en-1 one I (a-k). In this step chalcones were formed. The compounds I (a-k) on treatment with hydrazine hydrate in ethanol gave 1-H-3-(4-bromo phenyl)-5-(4-chloro phenyl) pyrazoline II (a-k) which were refluxed with acetic acid have given 1-acetyl-3-(4-bromophenyl) 5-(substituted phenyl) pyrazoline III (a-k).

Compounds II (a-k) were stirred with benzoyl chloride in pyridine at room temperature and gave 1-benzoyl-3-(4-bromo phenyl)-5-(substituted phenyl) pyrazoline IV (a-k).

Both series of pyrazoline derivatives III(a-k) and IV(a-k) have been screened for their antibacterial activity against *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*

and *Staphylococcus aureus* at two different concentrations 50 ppm and 100 ppm respectively by filter paper disc plate method and antifungal activity against *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum* and *Candida albicans* by filter paper disc plate method at two different concentrations (50 ppm and 100 ppm). Standard antifungal drug Amphotericine-B and antibacterial drug Streptomycine have also been screened under the similar conditions for comparison.

On going through the result of antimicrobial activity of III (a-k) and IV (a-k) showed that IIIa (4-Cl), IIIb(2-Cl), IIIc (3-Cl), IIIId (2-NO<sub>2</sub>), IVa (4-Cl), IVi (2-OH), IVj (4-OH) & IVk (2-OH,3-CH<sub>3</sub>) are highly active against selected bacteria and fungi and rest of the pyrazoline derivatives have shown good to moderate activity.

Table-2

Antibacterial activity of the synthesized pyrazoline derivatives III(a-k) and IV (a-k) against various bacteria at two different concentration (in ppm)

| Comp. | <i>B. subtilis</i> |      | <i>E.coli</i> |      | <i>K.pneumoniae</i> |      | <i>S.aureus</i> |      |
|-------|--------------------|------|---------------|------|---------------------|------|-----------------|------|
|       | 50                 | 100  | 50            | 100  | 50                  | 100  | 50              | 100  |
| III-a | ++++               | ++++ | +++           | +++  | ++                  | ++   | ++              | ++++ |
| III-b | ++                 | +    | ++            | ++   | +                   | ++   | ++              | +++  |
| III-c | +++                | +++  | +             | ++   | +++                 | ++++ | ++              | +++  |
| III-d | +++                | ++++ | +             | ++   | ++                  | ++   | +               | ++   |
| III-e | ++                 | +    | +             | ++   | ++                  | +    | +               | ++   |
| III-f | +                  | ++   | -             | +    | +                   | ++   | ++              | +++  |
| III-g | -                  | ++   | -             | +    | -                   | +    | +               | ++   |
| III-h | ++                 | ++   | +             | ++   | +                   | ++   | ++              | +++  |
| III-i | -                  | +    | +             | ++   | ++                  | ++   | +               | ++   |
| III-j | +                  | +    | +             | ++   | ++                  | +++  | +               | ++   |
| III-k | ++                 | +    | +++           | +++  | ++                  | +++  | ++              | +++  |
| IV-a  | +++                | ++++ | +++           | +++  | ++++                | ++++ | ++              | +++  |
| IV-b  | ++                 | ++   | +             | ++   | ++                  | ++   | ++              | +++  |
| IV-c  | ++                 | ++   | +             | ++   | +                   | +    | -               | -    |
| IV-d  | +                  | -    | ++            | +++  | +                   | +    | ++              | ++   |
| IV-e  | -                  | -    | +             | ++   | +                   | +    | ++              | ++   |
| IV-f  | +                  | +    | +             | ++   | ++                  | ++   | ++              | ++   |
| IV-g  | ++                 | +    | +             | ++   | ++                  | ++   | +               | +    |
| IV-h  | ++                 | ++   |               | +    | +                   | ++   | ++              | ++   |
| IV-i  | ++++               | +    | +             | ++   | ++                  | +++  | +++             | ++++ |
| IV-j  | ++                 | ++   | +++           | +++  | +                   | +++  | ++              | +++  |
| IV-k  | ++                 | +    | +             | ++   | +                   | ++   | +               | +    |
| std.  | +++                | ++++ | +++           | ++++ | +++                 | ++++ | ++++            | ++++ |

#### Std:Streptomycin

++++ = strongly active range > 19; +++ = moderately active range < 12-18; ++ = weakly active range 8-12, +, - = Inactive range > 8

Thus we conclude that synthesized pyrazoline derivatives may act as good antimicrobial agents.

#### Acknowledgement

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Table-3

Antifungal activity of the synthesized pyrazoline derivatives III (a-k) and IV (a-k) against various fungi at two different concentrations (in ppm).

| Comp. | <i>A. flavus</i> |      | <i>A. niger</i> |      | <i>F. oxysporum</i> |      | <i>C.albicans</i> |      |
|-------|------------------|------|-----------------|------|---------------------|------|-------------------|------|
|       | 50               | 100  | 50              | 100  | 50                  | 100  | 50                | 100  |
| III-a | +++              | ++++ | +++             | ++++ | ++                  | +++  | ++                | +++  |
| III-b | +++              | ++++ | +               | ++   | ++                  | ++   | ++                | +++  |
| III-c | +++              | +++  | +++             | +++  | +++                 | +++  | ++                | +++  |
| III-d | ++               | +++  | ++              | ++   | ++                  | ++   | +                 | ++   |
| III-e | ++               | ++   | +               | ++   | ++                  | ++   | +                 | ++   |
| III-f | +                | +    | +               | +++  | +                   | ++   | +                 | ++   |
| III-g | +                | ++   | ++              | +++  | +                   | ++   | ++                | +++  |
| III-h | +                | +    | ++              | ++   | +                   | ++   | +                 | ++   |
| III-i | +                | +    | ++              | ++   | +                   | ++   | +                 | ++   |
| III-j | +                | ++   | +               | +    | +                   | ++   | +                 | ++   |
| III-k | +                | +    | +               | +    | +                   | +    | +                 | ++   |
| IV-a  | +++              | ++++ | ++              | +++  | +++                 | +++  | +                 | ++   |
| IV-b  | +                | ++   | ++              | +    | +                   | ++   | ++                | +++  |
| IV-c  | +                | ++   | +               | ++   | ++                  | ++   | +                 | +++  |
| IV-d  |                  | +    | +               | ++   | ++                  | ++   | +                 | ++   |
| IV-e  | +                | ++   | +               | ++   | +                   | ++   | ++                | +++  |
| IV-f  | +                | +    | +               | ++   | +                   | ++   | +                 | ++   |
| IV-g  | +                | ++   | +               | +    | +                   | +    | +                 | ++   |
| IV-h  | +                | ++   | +               | ++   | ++                  | ++   | +                 | +    |
| IV-i  | ++               | ++   | ++              | +++  | ++                  | +    | ++                | +++  |
| IV-j  | +++              | ++++ | +++             | +++  | +++                 | +++  | +                 | ++   |
| IV-k  | ++               | +++  | +               | ++   | ++                  | ++   | +++               | ++   |
| std.  | +++              | ++++ | +++             | ++++ | ++++                | ++++ | +++               | ++++ |

**Std :Amphotericine-B**

++++ = strongly active range > 19; +++ = moderately active range < 12-18; ++ = weakly active range 8-<12, +, - = Inactive range > 8.

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# Development and validation of bio-analytical method for quantification of pantoprazole in human plasma using LC-MS/MS

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## ABSTRACT

A simple, sensitive and selective method for the determination of Pantoprazole using rapid high - performance liquid chromatography / positive electrospray ionization - tandem mass spectroscopy. The method consists of extraction with Acetonitrile followed by the analysis of the sample by liquid chromatography-mass spectroscopy (LC-MS/MS) in selective reaction monitoring mode using electrospray ionization mode (ESI). Chromatography was performed on a C<sub>18</sub> reverse phase column, Methanol: Acetonitrile: 10mM ammonium acetate (45:45:10) as a mobile phase. The assay exhibited a linear dynamic range of 100 to 3050ng/ml for Pantoprazole in human plasma. Stability assessment was also included. A run time of 5.0 min for each sample made it possible to analyse healthy volunteers participating in pharmacokinetics drug-drug interaction studies.

**Key words:** LC-MS/MS, Human Plasma, Bioanalytical, Pantoprazole, Validation.

## Introduction

The proton-pump inhibitor pantoprazole sodium inhibit gastric acid by blocking the H<sup>+</sup> / K<sup>+</sup> Adenosine triphosphatase enzyme system (the proton pump) of the gastric parietal cell [1].

Pantoprazole sodium is chemically sodium 5-(difluoromethoxy)-2-[[[3, 4-dimethoxy-2-pyridinyl) methyl] sulfinyl]-1H-benzimidazole sesquihydrate. It is used for short-term treatment of erosion and ulceration of the esophagus [2]. The pantoprazole sodium oral dosage forms are supplied in enteric-coated tablets.

Different analytical methods are reported in the literature for the assay of pantoprazole sodium in dosage forms and in biological fluids including spectrophotometry [3-8], TLC [9], HPTLC [10-12].

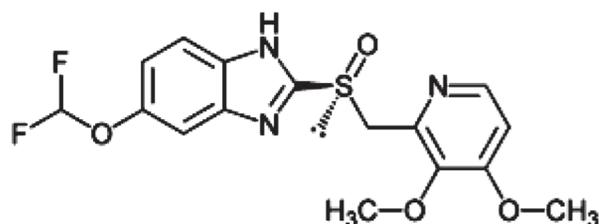
In this report, we describe a highly sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method developed and validated for the quantification of Pantoprazole in human plasma and utilizing a single-step extraction and a chromatographic separation. It is essential to establish an assay capable of quantifying Pantoprazole at lower concentrations. At the same time, it is expected

that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of Pantoprazole.

## Experimental

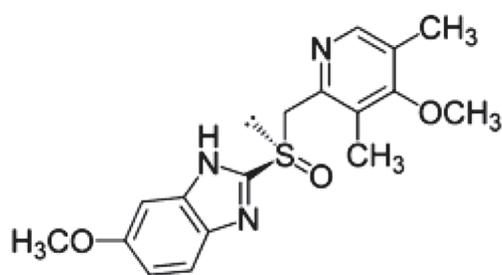
### Chemicals and Solvents

Pantoprazole drug substance and Omeprazole (Internal Standard) was obtained from Inventis Drug Delivery Systems (Hyderabad, India). The chemical structures are represented in Fig.1. HPLC-grade Methanol and Acetonitrile was purchased from JT Bakers. Ammonium Acetate purchased from Merck (Worli, Mumbai, India). HPLC-grade water from a Milli-Q water system Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.



(A) Pantoprazole

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(B) Omeprazole

Fig.1. Chemical Structures for Pantoprazole and the Omeprazole (IS)

### LC/MS/MS instrument and conditions

The high-performance liquid chromatography (HPLC) SILHTC system (Shimadzu Corporation, Kyoto, Japan) is equipped with LC-20 AD VP binary pump, a DGU20A3 Degasser, and a SIL-HTC auto sampler equipped with a CTO-10AS VP thermostat column. The chromatography was on Thermo BDS Hypersil C18, (5  $\mu$ m, 4.6 x 50mm) at a temperature of 30°C. The isocratic mobile phase composition was a mixture of 45:45:10 ACN: MeOH: 10 mM Ammonium acetate, which was pumped at a flow rate of 0.5 mL/min. Mass spectrometric detection was performed on a TSQ Quantum Discovery MAX triple quadrupole instrument (Thermo Finnigan, USA) using the Selective reaction monitoring (SRM) mode. A turbo electrospray ionization (ESI) interface in positive mode was used. The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed on LC Quan 2.5.6. Software package (Thermo).

Table-1

Main working parameters of the tandem mass spectrometer

| Parameters                           | Value                                |
|--------------------------------------|--------------------------------------|
| Spray voltage                        | 3500                                 |
| Sheath gas pressure                  | 35                                   |
| Auxiliary gas pressure               | 20                                   |
| Capillary temperature                | 300                                  |
| Turbulence offset                    | 101 & 108 (Analyte and IS)           |
| Skimmer offset                       | 0 (Analyte) and -10 (IS)             |
| Collision energy                     | 11 (Analyte) and 13 (IS)             |
| Polarity                             | Positive                             |
| Mode of analysis                     | SRM                                  |
| Ion transition for Pantoprazole, m/z | 433.160 $\pm$ 0.5/ 251.990 $\pm$ 0.5 |
| Ion transition for Omeprazole, m/z   | 346.037 $\pm$ 0.5/198.000 $\pm$ 0.5  |

### Sample preparation

Standard stock solutions of Pantoprazole (1 mg/mL) and the IS (1 mg/mL) were separately prepared in Methanol. Working solutions for calibration and controls were prepared by appropriate dilution in 90:10; Methanol: Water. The IS working solution (500 ng/mL) was prepared by diluting its stock solution with diluent (90:10) methanol: water. Working solutions (0.2 mL) were added to drug-free human plasma (9.8 mL) as a bulk, to obtain Pantoprazole concentration levels of 100.745, 201.490, 402.979, 805.959, 1465.380, 1953.839, 2442.299 and 3052.874 ng/mL, as a single batch at each concentration. Quality control (QC) samples were also prepared as a bulk on an independent weighing of standard drug, at concentrations of 121.460 (LLOQ), 253.042 (low), 1265.212 (medium) and 2108.686 ng/mL (high), as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in Ria Vials (Tarson, 5 mL) and stored in a freezer at below -80°C until analyses. A plasma sample (0.250 mL) was pipetted into a 2-mL Centrifuge tube, 50- $\mu$ L IS working solution (500 ng/mL) were added. After vortex mixing for 10 secs, a 1.0-mL aliquot of the extraction solvent, Acetonitrile, was added and the sample was vortex-mixed for 10 mins. Centrifuged the Centrifuge tubes at 14,000 rpm at 10°C for 10 min, transferred approximately 0.5 mL of supernatant liquid to pre-label HPLC vials and a 10- $\mu$ L aliquot was injected into the chromatographic system.

### Bioanalytical method validation

A calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma processed with the IS) and eight non-zero samples covering the total range 100–3050 ng/mL, including the lower limit of quantitation (LLOQ). The calibration curves were generated using the analyte to IS peak area ratios by weighted ( $1/x^2$ ) least-squares linear regression on consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient ( $r$ ) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the above criteria, including acceptable LLOQ and upper limit of quantification. The within-batch precision and accuracy were determined by analyzing six sets of QC samples in a batch. The between batch precision and accuracy were determined by analyzing six sets of QC samples on three different batches. The QC samples were randomized daily, processed and analyzed in a position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of the batch. The acceptance criteria for within- and between-batch precision were 20% or better for LLOQ and 15% or better for the other concentrations, and the accuracy was 100  $\pm$  20% or better for LLOQ and 100  $\pm$  15% or better for the other concentrations. Recovery of Pantoprazole from the

extraction procedure was determined by a comparison of the peak area of Pantoprazole in spiked plasma samples (six each of low, medium and high QCs) with the peak area of Pantoprazole in samples prepared by spiking extracted drug-free plasma samples with the same amounts of Pantoprazole at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted QC samples (n = 6) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography. The stability of the analyte and IS in human plasma under different temperature and timing conditions, as well as their stability in the stock solutions, was evaluated. QC samples were subjected to short-term room temperature conditions, to long-term storage conditions (-80°C), and to freeze/thaw stability studies. All the stability studies were conducted at two concentration levels (253.042 and 2108.686 ng/mL as low and high values) with five determinations for each.

## Results and discussion

### Mass Spectrometry

The analysis of Pantoprazole from human plasma is of major interest in pharmaceutical research. Pharmacokinetic applications require highly selective assays with high sample throughput capacity. Quantification of drugs in biological matrices by LC/MS/MS is becoming more common due to the improved sensitivity and selectivity of this technique. The Parent and product ion mass spectra's of Pantoprazole and the IS are shown in Fig. 2.  $[M+H]^+$  was the predominant ion in the Q1 spectrum and was used as the precursor (Parent) ion to obtain product ion spectra. The collisionally induced dissociation (CID) mass spectrum of Pantoprazole shows the formation of characteristic product ions at m/z 251.990. The CID mass spectrum of

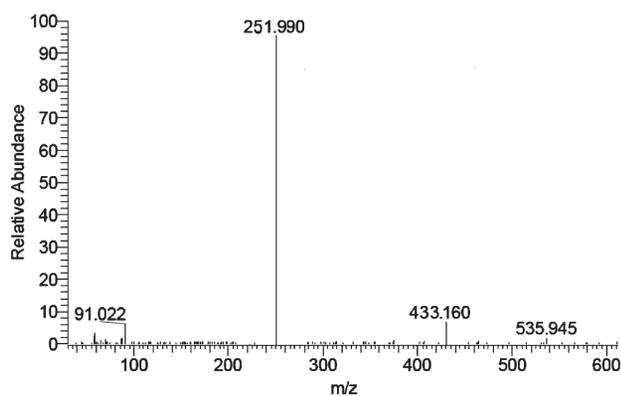
the IS shows the formation of characteristic product ions at m/z 198.000. The most sensitive mass transition was from m/z 433.160 to 251.990 for Pantoprazole and m/z 346.037 to 198.000 for the IS. LC/SRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus, the SRM technique was chosen for the assay development. The SRM state file parameters were optimized to maximize the response for the analyte. The parameters presented in Table 1 are the result of this optimization.

### Method development

The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analyte and IS, as well as a short run time. It was found that a mixture of ACN: MeOH: 10 mM Ammonium acetate (45:45:10) could achieve this purpose and was finally adopted as the mobile phase. The proportion of organic solvent eluted the analyte and the IS at retention times of 3.0 and 2.7 min, respectively. A flow rate of 0.5 mL/min produced good peak shapes and permitted a run time of 5.0 min. Precipitation (Extraction method) was used for the sample preparation in this work. Precipitation can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effect in LC/MS/MS analyses. Acetonitrile was found to be optimal, which can produce a clean chromatogram for a blank plasma sample. The average absolute recoveries of Pantoprazole from spiked plasma samples was 81.2 % and the recovery of the IS was 74.6% at the concentration used in the assay (500 ng/mL). Recoveries of the analytes and IS were good, and it was consistent, precise and reproducible. Therefore, the assay has proved to be robust in high-throughput bioanalysis.

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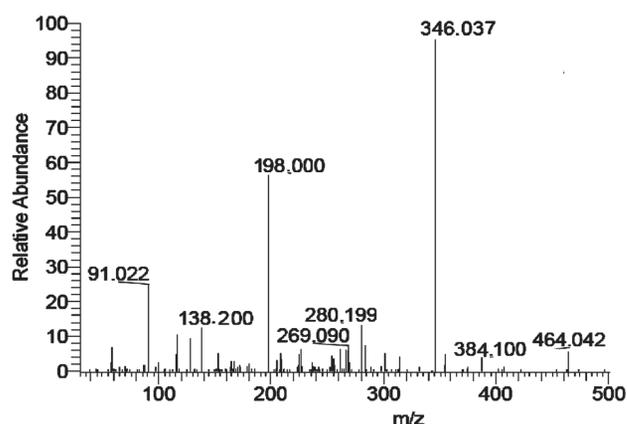
1.83e6



(a) Pantoprazole Full Scan mass spectra

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1.83e6



(b) Omeprazole Full scan mass spectra

Fig.2: Full-scan Positive ion turbo spray parent and product ion mass spectra's

Choosing the appropriate internal standard is an important aspect to achieve acceptable method performance, especially with LC/MS/MS, where matrix effects can lead to poor analytical results. Ideally, isotopically labeled internal standards for all analytes should be used, but these are not commercially available. Therefore, we opted for Omeprazole commercially available. In addition its retention behavior is similar to that of the target analyte. Clean chromatograms were obtained and no significant direct interferences in the SRM channels at the relevant retention times were observed. However, in ESI, signal suppression or enhancement may occur due to co-eluting endogenous components of the sample matrix. All validation experiments in this assay were performed with matrices obtained from different individuals. As all data fall within the guidelines, we conclude that the degree of matrix effect was sufficiently low to produce acceptable data, and the method can be considered as valid.

### Assay performance and validation

The eight-point calibration curve was linear over the concentration range 100–3050 ng/mL. The calibration model was selected based on the analysis of the data by linear regression with intercepts and weighting factors (1/x, 1/x<sup>2</sup> and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a 1/x<sup>2</sup> weighting factor. Linear regression equation for the calibration curve is  $y = mx + C$  here  $y$  is the peak area ratio of the analyte to the IS and  $x$  is the concentration of the analyte. Table 2 summarizes the calibration curve results and Fig.3 illustrates the eight-point calibration curve for the Pantoprazole. The selectivity of the method was examined by analyzing six blank human plasma extract, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte. Excellent sensitivity was observed for a 10- $\mu$ L injection volume. The SRM chromatograms obtained for an extracted plasma sample are depicted in Fig. 4.

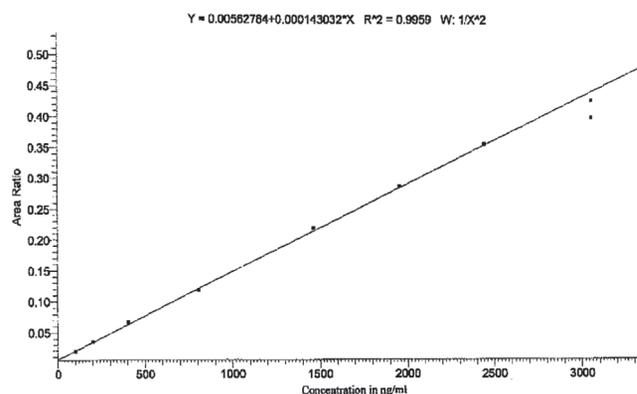


Fig.3. Calibration curve for the Pantoprazole

The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision, and was found to be 100 ng/mL in human plasma. The mean response for the analyte peak at the assay sensitivity limit was ten-fold greater than the Mean response for the peak in eight blank human plasma samples at the retention time of the analyte. The precision and accuracy for within-batch and between-batch experiments at the High, middle, low and LOQ QC quantification levels were summarized in Table 3. For the within and between batch experiments the precision and accuracy for the analyte met the acceptance criteria ( $d \leq 15\%$ ). These results suggest that samples with concentrations greater than the upper limit of the calibration curve can in this way be assayed to obtain acceptable data.

### Stability studies

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (around 7hrs). Samples were extracted and analyzed as described above, and the results are given in Table 4. These results indicate reliable stability behavior under the experimental conditions of the

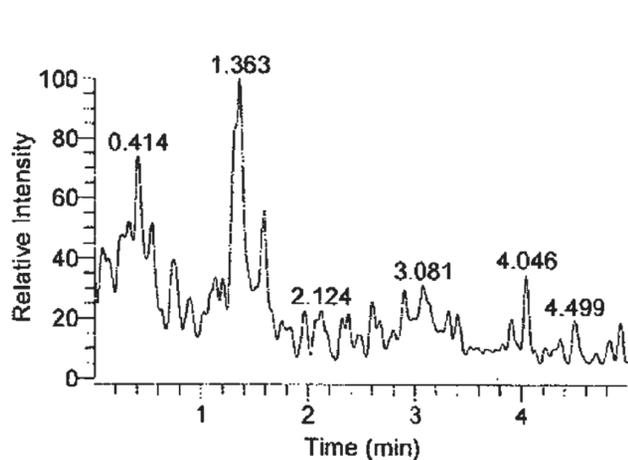
Table-2

Precision and accuracy data of back-calculated concentrations of calibration samples for Pantoprazole in human plasma (SD: standard deviation)

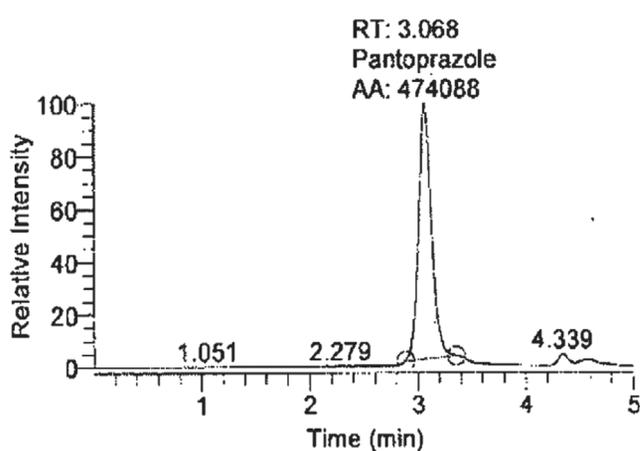
| STD ID | Nominal Conc. | Mean      | $\pm$ S.D. | Precision. | %Accuracy |
|--------|---------------|-----------|------------|------------|-----------|
| STD 1  | 100.745       | 99.7580   | 1.27951    | 1.3        | 99.0      |
| STD 2  | 201.490       | 203.0475  | 6.58682    | 3.2        | 100.8     |
| STD 3  | 402.979       | 418.3543  | 22.55363   | 5.4        | 103.8     |
| STD 4  | 805.959       | 784.7000  | 14.38963   | 1.8        | 97.4      |
| STD 5  | 1465.380      | 1462.6878 | 43.79327   | 3.0        | 99.8      |
| STD 6  | 1953.839      | 2005.9825 | 22.43186   | 1.1        | 102.7     |
| STD 7  | 2442.299      | 2318.9955 | 125.44499  | 5.4        | 95.0      |
| STD 8  | 3052.874      | 3101.5010 | 94.68520   | 3.1        | 101.6     |

**Table - 3**  
**Precision and accuracy of the method for determining Pantoprazole concentrations in plasma samples**  
**(SD: standard deviation)**

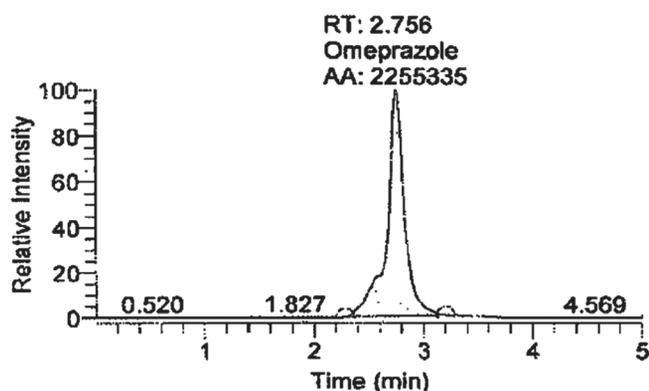
| Concentration | With-in batch precision (n=6) |                             |                                | Between-batch precision (n=6) |                             |                                |
|---------------|-------------------------------|-----------------------------|--------------------------------|-------------------------------|-----------------------------|--------------------------------|
|               | Concentration found           | Precision (%) added (ng/mL) | Accuracy (%) (mean±SD) (ng/mL) | Concentration found           | Precision (%) added (ng/mL) | Accuracy (%) (mean±SD) (ng/mL) |
| 2108.686      | 1998.5450± 57.14613           | 2.9                         | 94.8                           | 1979.3988±72.10275            | 3.6                         | 93.9                           |
| 1265.212      | 1315.5742± 73.27192           | 5.6                         | 104.0                          | 1284.4758± 67.27331           | 5.2                         | 101.5                          |
| 253.042       | 268.3358± 23.18471            | 8.6                         | 106.0                          | 257.2022± 17.98790            | 7.0                         | 101.6                          |
| 121.460       | 105.5967± 3.54046             | 3.4                         | 86.9                           | 114.9192± 6.61289             | 5.8                         | 94.6                           |



(A) Blank without drug and IS



(B) Pantoprazole



(C) Omeprazole

**Fig.4: SRM chromatograms for (A). Blank (with out Drug and IS), (B) Pantoprazole and the (C) IS resulting from analysis**

regular analytical procedure. The stability of QC samples kept in the auto sampler for 33 hrs was also assessed. The results indicate that solutions of Pantoprazole and the IS can remain in the auto sampler for at least 33 hrs without showing significant loss in the quantified values, indicating that samples should be processed within this period of time (Table 4). The data representing the stability of Pantoprazole in plasma at two QC levels over three freeze/thaw cycles are given in Table 4. These tests indicate that the analyte is stable in human plasma for three freeze/thaw cycles, when stored at below -80°C and thawed to room temperature. The stability study of Pantoprazole in human plasma showed reliable stability behavior, as the means of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicate that storage of Pantoprazole in plasma samples at below -80°C is adequate, and no stability-related problems would be expected during routine analyses for pharmacokinetic, bioavailability or bioequivalence studies.

**Table-4**  
**Stability of Pantoprazole in human plasma**

| Sample Concentration<br>(ng/mL) (n=6)                  | Concentration<br>found (ng/mL) | Precision<br>(%) | Stability<br>(%) |
|--|--------------------------------|------------------|------------------|
| Short –term (Bench top) stability for 07 hrs in plasma |                                |                  |                  |
| 2108.686   | 2031.3572                      | 1.7              | 108.5            |
| 253.042  | 261.5762                       | 3.4              | 113.0            |
| Freeze-thaw cycles                                     |                                |                  |                  |
| 2108.686   | 1918.849                       | 1.6              | 97.7             |
| 253.042  | 240.7655                       | 4.9              | 97.7             |
| Auto sampler stability for 33 hrs                      |                                |                  |                  |
| 2108.686   | 2048.8035                      | 2.0              | 101.2            |
| 253.042  | 260.4475                       | 6.8              | 96.1             |

The stability of the stock solutions was tested and established at room temperature for 7 hrs and for 3 days under refrigeration (-4°C). The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

### Conclusions

In summary, a method is described for the quantification of Pantoprazole in human plasma by LC/MS/MS in Positive ESI mode using SRM and fully validated according to commonly accepted criteria [13-14]. The current method has shown acceptable precision and adequate sensitivity for the quantification of Pantoprazole in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies. The desired sensitivity of Pantoprazole was achieved with an LLOQ of 100 ng/mL, which has within and between batch coefficients of variance (CVs), respectively. Many variables related to the electrospray reproducibility were optimized for both precision and sensitivity to obtain these results. The simplicity of the assay and use of rapid precipitation extraction and sample turnover rate of 5.0 min per sample make it an attractive procedure in high-throughput bioanalysis of Pantoprazole. The validated method allows quantification of Pantoprazole in the 100–3050 ng/mL range.

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# Anti-inflammatory Activity of Ripe Fruits of *Ficus Racemosa* Linn

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## ABSTRACT

Alcoholic extract of the ripe fruits of *Ficus racemosa* Linn. was screened for anti-inflammatory activity in carrageenan induced rat paw edema at the dose levels of 250 mg/kg and 500mg/kg respectively and compared with the standard drug Diclofenac sodium 10mg/kg. The result were found to be significant ( $p < 0.001$ ) when compared to control.

**Key words:** *Ficus racemosa*, Diclofenac sodium, Anti-inflammatory activity.

## Introduction

*Ficus racemosa* Linn. (Moraceae) syn. *Ficus glomerata* commonly known as 'Gular', 'Umar' or 'Jagya dumbar' [1]. It is a tree up to 18 mt. height and frequently met throughout India, but commonly found in south India from sea level to about 1800mt. in evergreen forest and near deciduous forest.

As a medicinal plant *Ficus racemosa* mentioned in the Charak Samhita and Susrata. Fruits are astringent, refrigerant, stomachic, vermifugal, antidiabetic and also use in diarrhea, dyspepsia, haemorrhages, glandular inflammation and carminative [2].

*Ficus racemosa* has been reported to possess antidiarrhoeal, antibacterial, antipyretic, hypoglycaemic and antifilarial activities. The bark and leaf extract of the plant has been also reported for the anti-inflammatory activity. Hence present study, evaluation of anti-inflammatory activity of ripe fruit extract of *Ficus racemosa* was performed.

## Materials and Methods

### Collection of plant materials

The ripe fruits of the *Ficus racemosa* was collected from the forest of Kaladungi, district Nainital, Uttarakhand and authenticated by Dr H.D. Pandey, Botanist of Jim Corbet Jaidi buti uddyan, Kaladungi and preserved in the herbarium of the department.

### Preparation of alcoholic extract

The ripe fruits were shade dried. After complete drying they were processed to a coarse powder and macerated with 95% alcohol for 3 to 4 days. The alcoholic

extract was collected and dried to obtain a brown sticky viscous mass and used for present investigation [3].

### Phytochemical screening

Preliminary phytochemical screening of ethanolic extract of ripe fruits of *Ficus racemosa* revealed the presence of alkaloids, phytosterols and sugars. These constituents were confirmed by a thin layer chromatography [4].

### Animal used

Healthy albino rats of wister strain of either sex weighing between 100-150gm were taken. They were housed in standard condition of temperature ( $25 \pm 2^\circ\text{C}$ ) in animal house college of pharmacy, IFTM Moradabad. They were fed with the standard pellet diet (Hindustan lever rat pellets) and water ad libitum. The animal experiment was approved by animal ethical institutional committee.

### Anti-inflammatory activity

The alcoholic extract was evaluated for the anti-inflammatory by carrageenan induced rat paw edema method. They were fasted overnight prior to the experiment. However they had free access to water before experiment. They were divided into four groups having six rats in each group. The first group was administered 1ml of vehicle orally and served as control. The second group was given standard drug Diclofenac sodium (10mg/kg) intraperitoneally. The third and fourth groups were orally administered with 250 and 500mg/kg alcoholic extract respectively. After 30min of dosing, all the rats were injected with 0.1ml 1% w/v solution of carrageenan in saline in the subplantar region of left hind paw. The right paw served as reference. The paw volume was measured at one hour interval till 3 hrs.

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The anti-inflammatory activity was expressed as % inhibition of edema =  $[1-V_t/V_c]*100$ , where  $V_t$  and  $V_c$  are the edema volume of carrageenan infected paw of the drug treated group and control group respectively [5].

### Statistical analysis

The result were analysed by using one way analysis of variance (ANOVA), Dunnett's test. P values less than 0.001 were considered as significant ( $p < 0.001$ ) [6].

### Discussion and Conclusion

The preliminary phytochemical study reveals that presence of tannins, alkaloids, flavanoids, triterpenoids and sterols [7].

The result of anti-inflammatory activity of ripe fruit extracts of *Ficus racemosa* against carrageenan induced

inflammation is shown in table-1 and figure-1. A significant decrease in the paw oedema was observed after 3 hrs. The % inhibition of oedema at the dose level of 500mg/kg fruit extract, 250mg/kg fruit extract and standard diclofenac sodium was 53.2%, 34% and 54.3% respectively.

Generally early phase of acute-inflammation is due to release of histamine and serotonin stored in the cells and late response is due to stimulatory effect on the synthesis of prostaglandins (PG). Alcoholic extracts of *Ficus racemosa* showed significant anti-inflammatory activity at 3 hrs against carrageenan infection showed the *Ficus racemosa* acted similar to the diclofenac sodium (standard drug) and it can be suggested that these results provides pharmacological evidence for its folklore claim as an anti-inflammatory agent.

TABLE-1

| Treatment group   | Dose Mg/kg | Increase in Paw volume in ml |              |                 |                 | % Inhibition in oedema |
|-------------------|------------|------------------------------|--------------|-----------------|-----------------|------------------------|
|                   |            | 30 min                       | 60min (1 hr) | 120 min (2 hrs) | 180 min (3 hrs) |                        |
| Control(vehicle)  | -          | 0.17±0.01                    | 0.32±0.01    | 0.40±0.012      | 0.47±0.02       | -                      |
| DiclofenacSodium  | 10 mg/kg   | 0.12±0.01**                  | 0.14±0.01*** | 0.19±0.012*     | 0.22±0.01*      | 54.3%                  |
| Alcoholic extract | 250 mg/kg  | 0.13±0.11***                 | 0.22±0.02    | 0.28±0.15*      | 0.32±0.01*      | 34%                    |
| Alcoholic extract | 500 mg/kg  | 0.11±0.01**                  | 0.10±0.02    | 0.19±0.008      | 0.21±0.001*     | 53.8%                  |

All values are given in mean ±SEM (n=6)

\* $p < 0.001$ , \*\* $p < 0.01$ , \*\*\* $p < 0.1$  as compared to control (ANOVA followed by Dunnett's test)

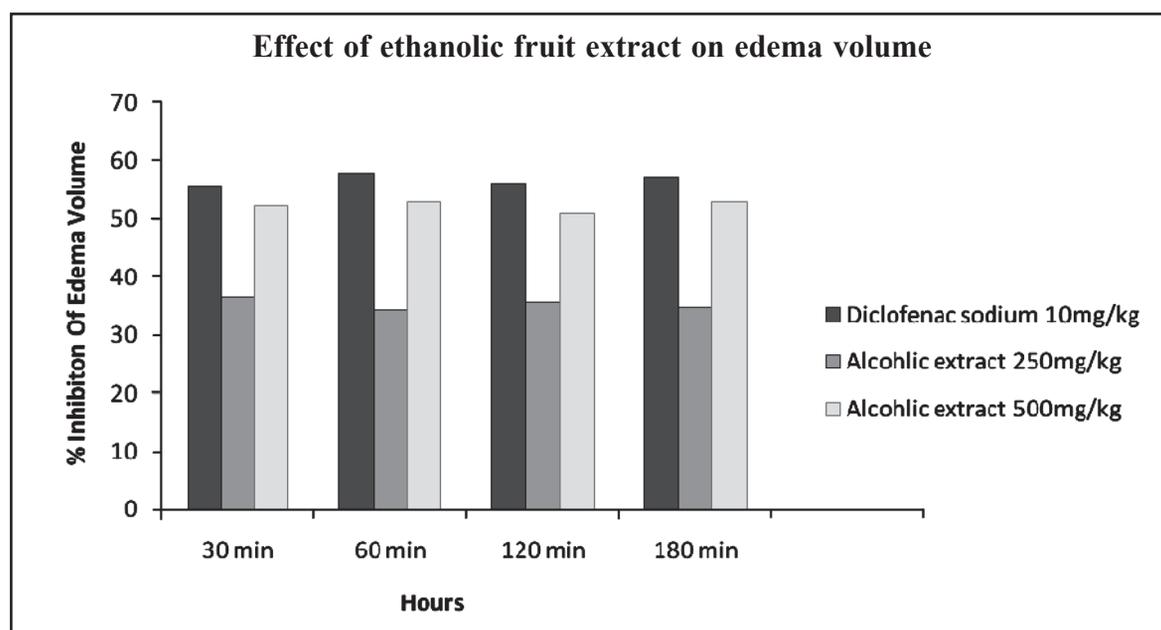


Figure-1

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# Development and Validation of High Performance Liquid Chromatography Tandem Mass Spectrometric Method for Quantification of Aceclofenac in Human Plasma

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## ABSTRACT

A fast, sensitive and specific LC-MS/MS method for the determination of Aceclofenac in human plasma has been developed and validated over the range of 0.106 µg/ml to 14.060 µg/ml ( $r^2 > 0.999$ ). Samples (200 µL) were buffered (pH 6.8), extracted using acetonitrile and 5 µL of sample extract was injected into the LC-MS/MS system. Analysis was performed using anal micro C18 (4.6 X 50 mm, 50µm, 60A<sup>o</sup>) column by gradient elution at a flow rate of 0.350 ml/min over a 2min's run-time. Retention times of Aceclofenac and Diclofenac (Internal Standard) were observed at 1.20 and 1.21 min's respectively. Detection was achieved by using Thermo, Triple Quadruple mass spectrometer, in positive electro spray ionization mode. Ion transitions were monitored using MRM (multiple reaction monitoring) for drug (m/z 354 → 250) and for IS (m/z 296.1 → 215). This validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic, bioavailability or bioequivalence studies.

**Key words:** LC-MS/MS, Human Plasma, Bioanalytical, Aceclofenac, Validation.

## Introduction

Aceclofenac [1] is an orally administered phenyl acetic acid derivative with effects on a variety of inflammatory mediators. Aceclofenac contains not less than 99.0% and not more than the equivalent of 101.0 percent of 2-[[2-[2-(2, 6-dichlorophenyl) amino] phenyl] acetyl] oxy] acetic acid. It is a white crystalline powder. It is an effective analgesic and anti-inflammatory agent. Aceclofenac is rapidly and completely absorbed after oral administration, peak plasma concentrations are reached 1 to 3 hours after an oral dose. Aceclofenac is metabolized to a major metabolite, 4'-hydroxyaceclofenac and to a number of other metabolites including 5-hydroxyaceclofenac, 4'-hydroxydiclofenac, diclofenac and 5-hydroxydiclofenac. Renal excretion is the main route of elimination of aceclofenac with 70 to 80% of an administered dose found in the urine, mainly as the glucuronides of aceclofenac and its metabolites of each dose of aceclofenac, 20% is excreted in the faeces. The plasma elimination half-life of the drug is approximately 4 hours.

Several chromatographic methods have been reported

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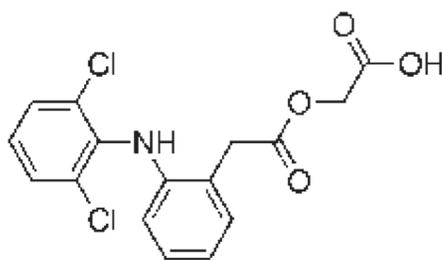
for the quantification of the Aceclofenac in human plasma [2-4]. Here we are reporting a highly sensitive Liquid chromatography/ Tandem Mass spectrometry (LC-MS/MS) method developed [5] and validated for the quantification of aceclofenac in human plasma [6-8].

## Materials and Methods

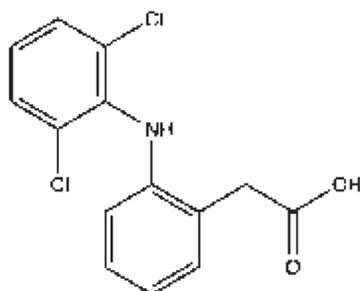
### Chemicals

Aceclofenac (99.98%) was supplied by Amoli organic pvt ltd, Gujarat. Diclofenac (99.00%) was supplied by Inventis drug delivery systems, Hyderabad. The chemical structures are represented in Fig.1. Methanol and acetonitrile (HPLC grade) was obtained from J. T. Bakers and ammonium acetate was from Merck. Purified water (HPLC grade) was prepared by using Sartorius water purification system and drug free control plasma was obtained from body care labs, Hyderabad. Analytical column anal micro C18 obtained from AC anal micro (I) pvt ltd.

LC-MS/MS instrument and conditions: The High performance liquid chromatography [9-10] (HPLC) system (Shimadzu Corporation, Kyoto, Japan) is equipped with LC- 20AD VP binary pump, a DGU 20 A3 degasser and a SIL – HTC auto sampler equipped with a CTO – 10 AS Vp thermo stated column. The chromatography was on anal



**Aceclofenac**



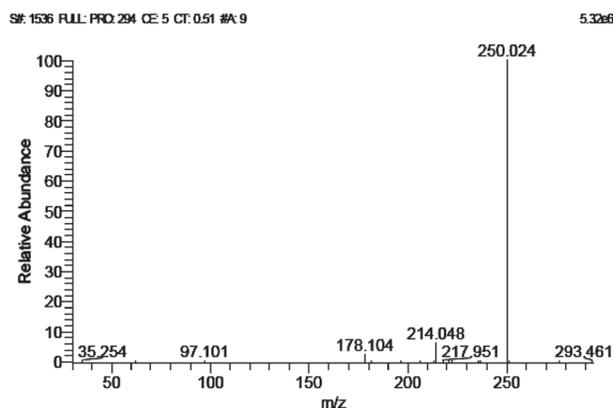
**Diclofenac**

micro C18 (4.6 X 50 mm, 50 $\mu$ m, 60A<sup>o</sup>) column at a temperature of 20<sup>o</sup> C. The gradient mobile phase was a mixture of methanol and 10mM ammonium acetate (80:20), which was pumped at a flow rate of 0.350ml/min.

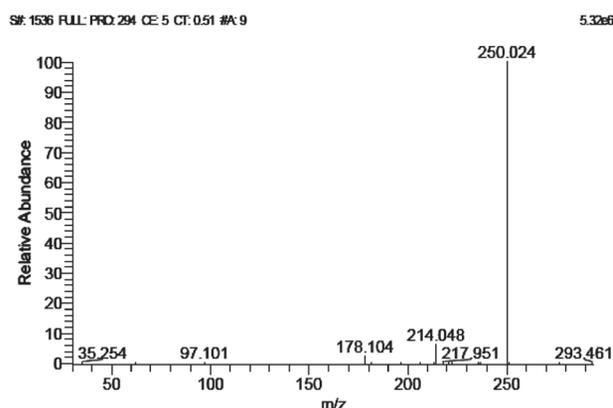
Mass spectroscopic [11] detection was performed on a Triple quadrupole instrument (Thermo, TSQ Quantum discovery max) using Multiple reaction monitoring (MRM) by Electro spray ionization in positive mode.

The analysis of aceclofenac from human plasma is of major interest in pharmaceutical research. Pharmacokinetic applications require highly selective assays with high sample throughput capacity. Quantification of drugs in biological matrices by LC-MS/MS is becoming more common due to the improved sensitivity and selectivity of this technique. The product ion mass spectra of aceclofenac and the diclofenac (IS) are shown in Fig. 2. [M-H]<sup>+</sup> was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The collisionally induced dissociation (CID) mass spectrum of Aceclofenac shows the formation of characteristic product ions at m/z 250.024. The CID mass spectrum of the diclofenac (IS) shows the formation of characteristic product ions at m/z 215.055. The most sensitive mass transition was from m/z 354.000 to 250.024 for aceclofenac and m/z 296.100 to 215.055 for the Diclofenac (IS). LC/MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM state file parameters were optimized to maximize the response for the analyte. The main working parameters of the mass spectrometer are

summarized in Table 1. Data processing was performed by LC Quan version 2.5.6 software package.



**(a) Aceclofenac product ion mass spectra**



**(b) Diclofenac (IS) product ion mass spectra**

**Fig.2: Full-scan product ion mass spectra's for analyte and IS**

**Table-1  
Main working parameters of the mass spectrometer**

| Parameters                          | Value                                |
|-------------------------------------|--------------------------------------|
| Spray voltage                       | 3500                                 |
| Sheath gas pressure                 | 15                                   |
| Auxiliary gas pressure              | 15                                   |
| Capillary temperature               | 270                                  |
| Tubulence offset                    | 100 (Analyte and IS)                 |
| Skimmer offset                      | 14 (Analyte) and 1 (IS)              |
| Collision energy                    | 06 (Analyte) and 21 (IS)             |
| Polarity                            | + Ve                                 |
| Mode of analysis                    | MRM                                  |
| Ion transition for Aceclofenac, m/z | 354.000 $\pm$ 0.5/ 250.024 $\pm$ 0.5 |
| Ion transition for Diclofenac, m/z  | 296.100 $\pm$ 0.5/215.055 $\pm$ 0.5  |

### Preparation of solutions

Aceclofenac stock solution (2mg/mL) was prepared by accurately weighing the aceclofenac and dissolution in the appropriate volume of methanol to give a final concentration of 2 mg/mL. Aceclofenac intermediate solution (2µg/mL) was prepared by diluting the Aceclofenac stock Solution with methanol. The aceclofenac intermediate solution was serially diluted as appropriate with 50:50 Methanol: Water for preparation of spiking solutions.

Diclofenac stock solution (0.5mg/mL) was prepared by accurately weighing the diclofenac and dissolution in the appropriate volume of methanol to give a final concentration of 0.5 mg/mL. A diclofenac dilution (50 µg/mL) was prepared by diluting the diclofenac stock Solution (0.5 mg/mL) with 50:50 Methanol: Water.

All solutions were stored in the refrigerator (2°C - 8°C) when not in use. Concentration of calibration and quality control bulk samples which were prepared as shown in table 2. The calibration control bulk samples were divided in to aliquots in ria vials (Tarson, 2mL) and stored in a freezer at below -70°C until analyses.

Plasma samples (0.2mL) were pipetted in to a 2 mL centrifuge tubes and then 50µL of IS working solution (50µg/mL) was added. After vortex mixing 600µL acetonitrile was added and vortexed. The samples were centrifuged at 14000 rpm at 10°C for 10 min. and 0.5 µL aliquot was injected in to the chromatographic system.

### Bioanalytical Method Validation

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose” (International Conference on Harmonization Guideline Q2A) [12]. “Method validation is the process of demonstrating that analytical procedures are suitable for their intended use” (US Food and Drug Administration Draft Guidance for Industry, 2000) [13].

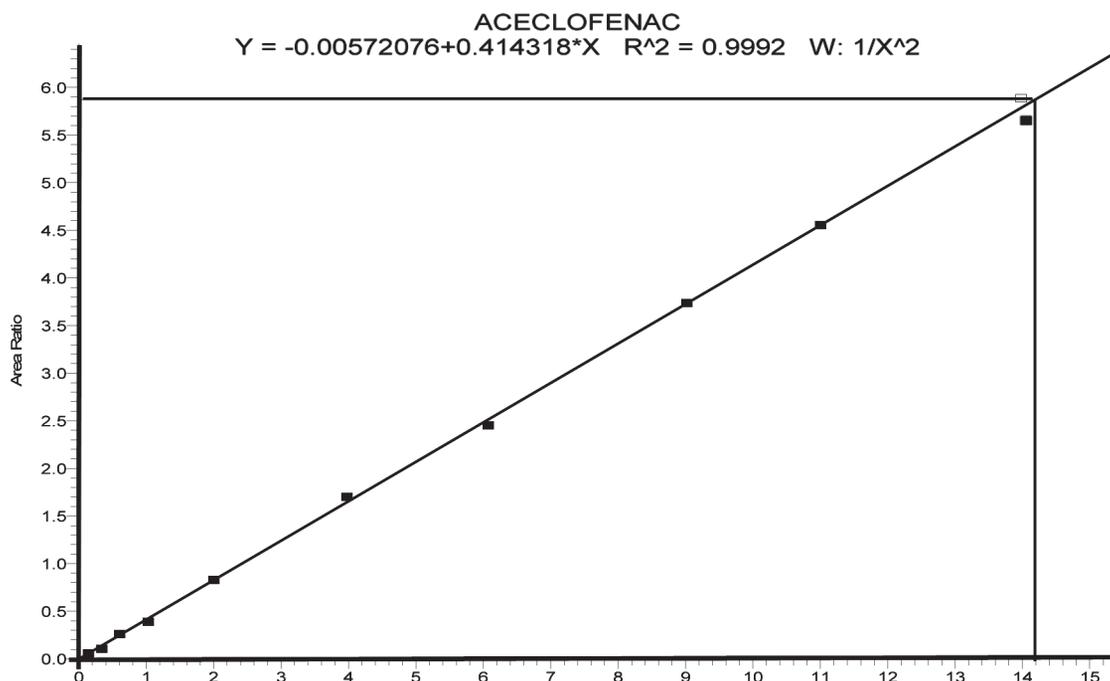
The ten-point calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma sample processed with the IS) and ten non-zero samples covering the total range 0.106 µg/ml to 14.060 µg/ml, including the lower limit of quantitation (LLOQ). The calibration curves were generated using the analyte to IS peak area ratios by weighted ( $1/x^2$ ) least squares linear regression [14] on consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient ( $r^2$ ) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the above criteria, including acceptable LLOQ and upper limit of quantification. The Calibration curve obtained for Aceclofenac depicted in Fig. 3.

The within-batch precision and accuracy were determined by analyzing six sets of QC samples in a batch. The between-batch precision and accuracy were determined

**Table-2**  
**Preparation of CC and QC samples**

| Concentration of Bulk Spiking solution (µg/ml) | Spiking Vol. of Bulk spiking solution (ml) | Total volume (ml) | CC Std values in plasma (µg/ml) | CC STDs ID |
|--|--|-------------------|---------------------------------|------------|
| 702.999  | 0.2  | 10                | 14.060                          | STD 10     |
| 551.854  | 0.1  | 5                 | 11.037                          | STD 9      |
| 452.520  | 0.1  | 5                 | 9.050                           | STD 8      |
| 303.189  | 0.1  | 5                 | 6.064                           | STD 7      |
| 201.620  | 0.1  | 5                 | 4.032                           | STD 6      |
| 100.810  | 0.1  | 5                 | 2.016                           | STD 5      |
| 50.405   | 0.1  | 5                 | 1.008                           | STD 4      |
| 30.243   | 0.1  | 5                 | 0.605                           | STD 3      |
| 15.122   | 0.1  | 5                 | 0.302                           | STD 2      |
| 5.293  | 0.2  | 10                | 0.106                           | STD 1      |
| 526.743  | 0.5  | 25                | 10.535                          | HQC        |
| 326.581  | 0.5  | 25                | 6.532                           | MQC1       |
| 146.961  | 0.5  | 25                | 2.939                           | MQC2       |
| 16.166   | 0.5  | 25                | 0.323                           | LQC        |
| 6.628  | 0.5  | 25                | 0.133                           | LOQ QC     |

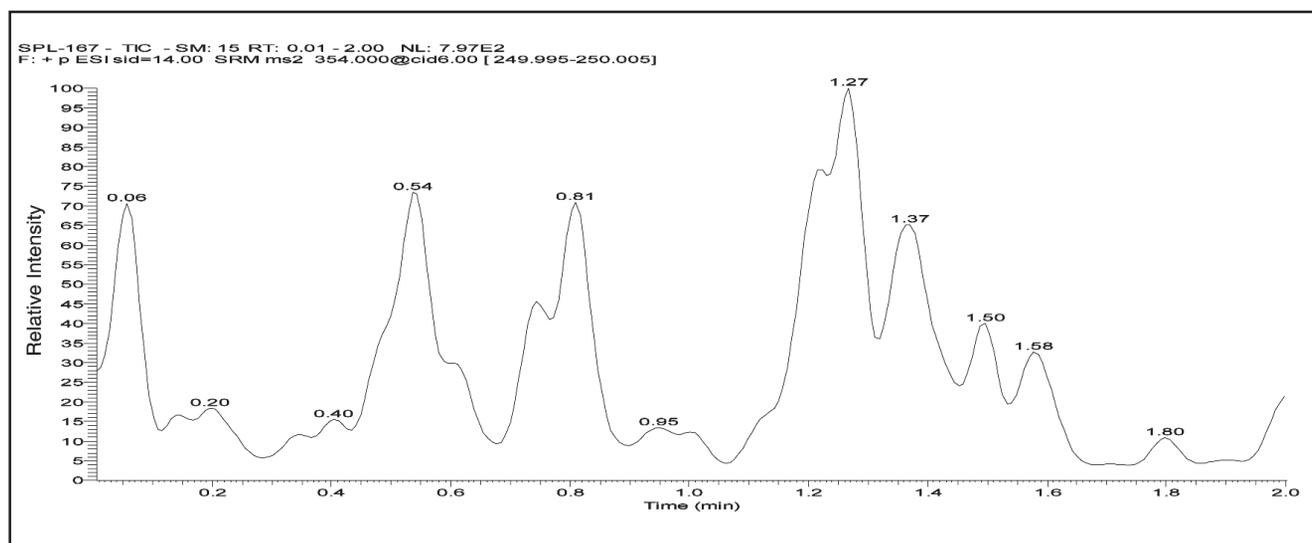
Figure: 3



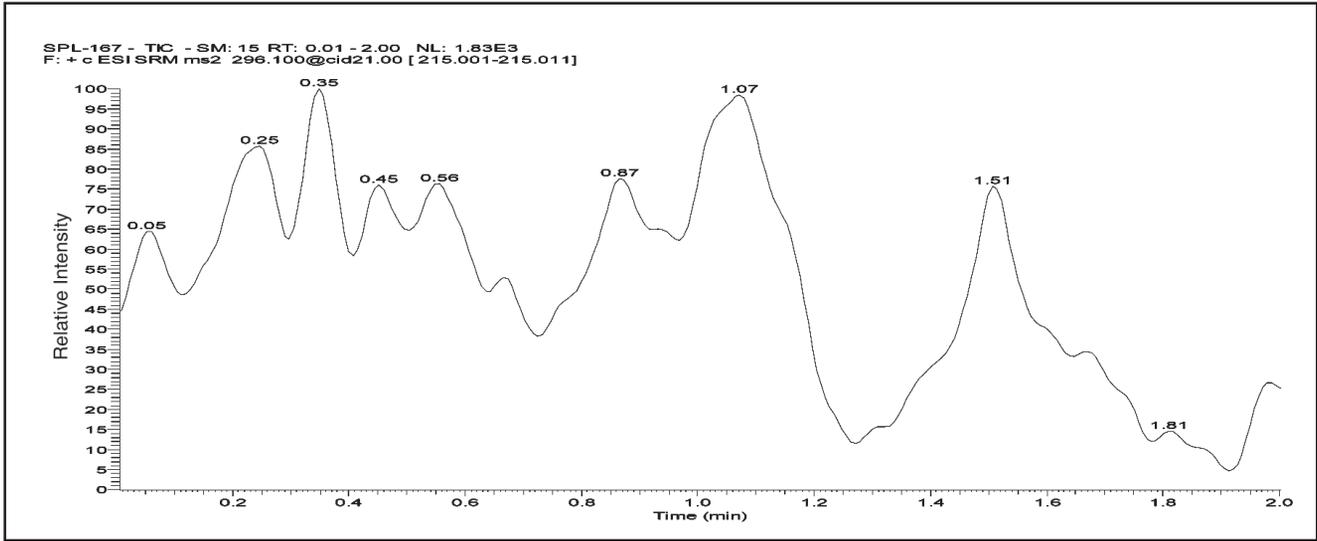
by analyzing six sets of QC samples on three different batches. The QC samples were randomized daily, processed and analyzed in a position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of the batch. The acceptance criteria for within- and between-batch precision were 20% or better for LLOQ and 15% or better for the other concentrations, and the accuracy was  $100 \pm 20\%$  or better for LLOQ and  $100 \pm 15\%$  or better for the other concentrations.

Recovery of aceclofenac from the extraction procedures was determined by a comparison of the peak area of

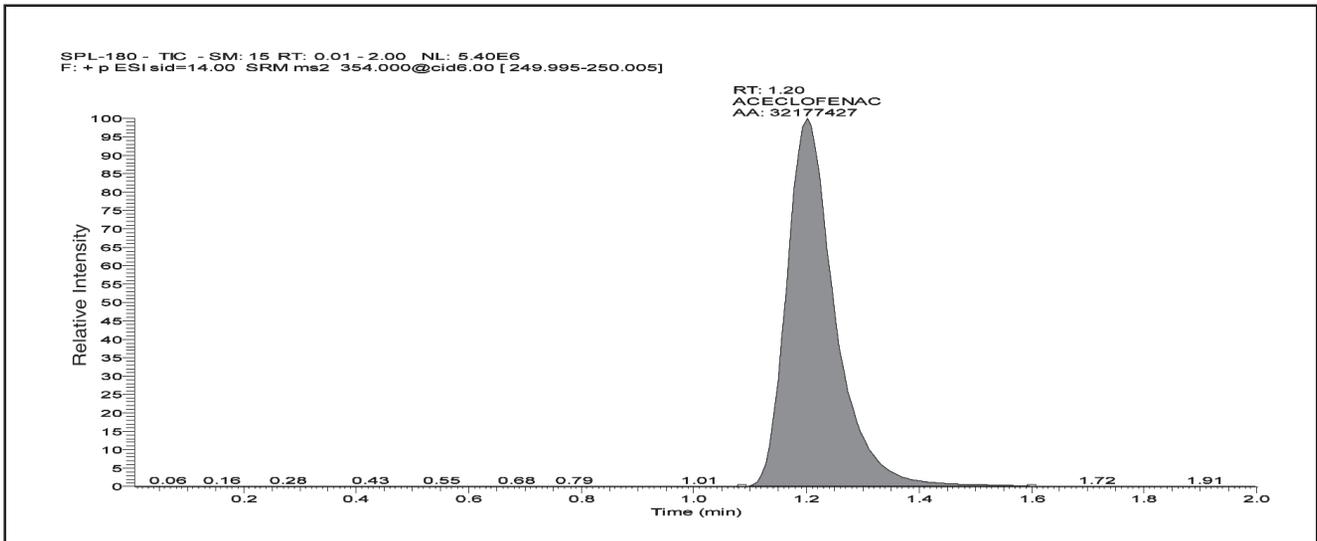
aceclofenac in spiked plasma samples (six of each low, medium and high QCs) with the area of aceclofenac in samples prepared by spiking extracted drug-free plasma samples with the same amounts of aceclofenac at the step immediately prior to chromatography. Similarly, recovery of diclofenac (IS) was determined by comparing the mean peak areas of extracted QC samples ( $n=6$ ) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma with the same amounts of diclofenac (IS) at the step immediately prior to chromatography. The SRM chromatograms obtained for extracted plasma with Analyte and IS, without Analyte, IS are depicted in Fig. 4.



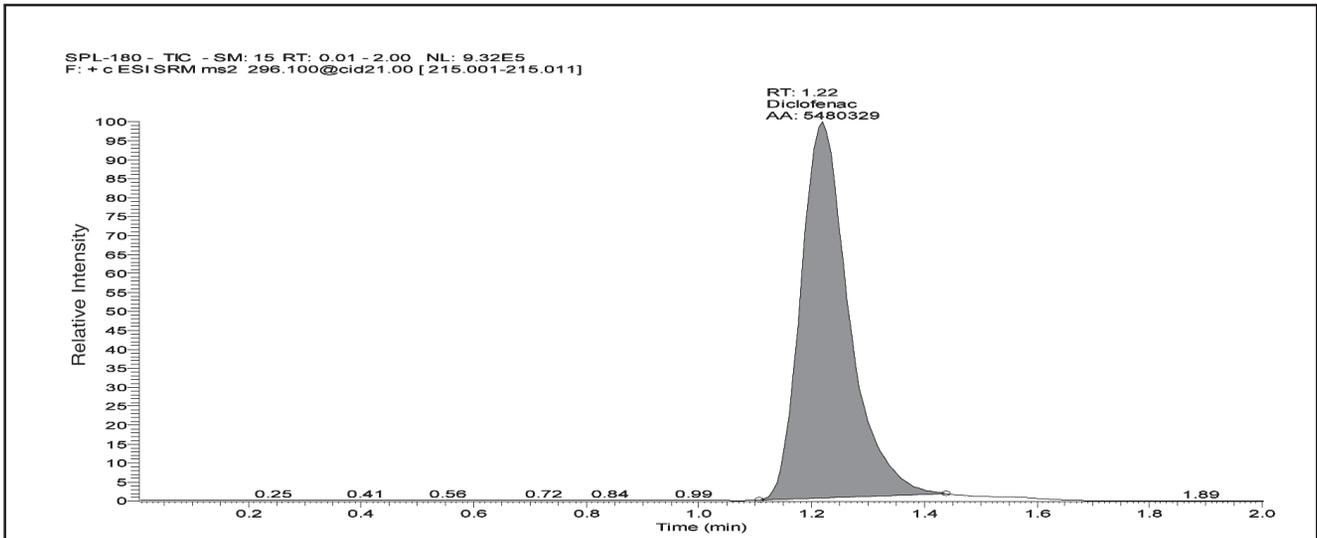
Extracted blank plasma with out Analyte



Extracted blank plasma with out IS



Extracted blank plasma with Aceclofenac



Extracted blank plasma with Diclofenac

The stability of the analyte and IS in human plasma under different temperature and timing conditions, as well as their stability in the stock solutions, was evaluated. QC samples were subjected to short-term room temperature conditions, to long-term storage conditions (-80°C), and to freeze-thaw stability studies. All the stability studies were conducted at two concentration level (10.535 µg/ml and 0.323 µg/ml as low and high values) with six determinations for each.

## Results and Discussion

All validation experiments in this assay were performed

with matrices obtained from different individuals. In addition, Validation. As all data fall within the guidelines, we conclude that the degree of matrix effect was sufficiently low to produce acceptable data, and the method can be considered as valid. The results are given as summary of validation in table 3. An LC-MS/MS method for the determination of unchanged Aceclofenac from human plasma has been successfully validated over of 0.106 µg/mL – 14.016 µg/mL using Diclofenac as internal standard.

The reproducibility, specificity and high sensitive of this method allows for the selective and reliable

**Table-3**  
**Validation Summary**

| Experiment   | Parameter  | Limits               | Results           |
|--|--|----------------------|-------------------|
| Linearity  | Range:- 0.106 µg/mL – 14.016µg/mL                    | $r^2 > 0.98$         | $r^2 > 0.99$      |
| Sensitivity  | Precision and Accuracy                               | $\leq 20$ & 80%–120% | 5.6 and 100.3%    |
| Precision  | Intrabatch (LQC, MQC, HQC)                           | $\leq 15$            | 1.5 – 6.5         |
|  | Intrabatch (LOQ QC)                                  | $\leq 20$            | 7.5               |
|  | Interbatch / Global (LQC, MQC, HQC)                  | $\leq 15$            | 2.1 – 7.3         |
|  | Interbatch / Global (LOQ QC)                         | $\leq 20$            | 6.8               |
| Accuracy   | Intrabatch (LQC, MQC, HQC)                           | 85%–115%             | 100.1% - 102.3%   |
|  | Intrabatch (LOQ QC)                                  | 80%-120%             | 98.3%             |
|  | Interbatch / Global (LQC, MQC, HQC)                  | 85%–115%             | 99.7% - 101.7%    |
|  | Interbatch / Global (LOQ QC)                         | 80%-120%             | 96.2%             |
| Ruggedness   | Linearity  | $r^2 > 0.98$         | $r^2 > 0.99$      |
|  | Precision (LQC, MQC, HQC)                            | $\leq 15$            | 2.1 – 6.8         |
|  | Accuracy (LQC, MQC, HQC)                             | 85%-115%             | 96.5%- 103.8%     |
|  | Precision (LOQ QC)                                   | $\leq 20$            | 4.8               |
|  | Accuracy (LOQ QC)                                    | 80%-120%             | 106.1%            |
| Recovery   | Drug and ISTD  | NA                   | 58.9% and 39.1%   |
| Dilution integrity:  | Accuracy (1/5 <sup>th</sup> and 1/10 <sup>th</sup> ) | 85%–115%             | 111.8% and 112.6% |
| Short Term Stock solution Stability of Drug and ISTD (6 hrs 15 minutes)            |  | 90%-110%             | 99.1% and 98.4%   |
| Long Term Stock solution Stability of Drug and ISTD (03 days 01 hrs and 45minutes) |  | 90%-110%             | 101.4% and 99.7%  |
| Freeze Thaw - Cycle III HQC and LQC  |  | 85%-115%             | 99.3% and 99.6%   |
| Bench Top - (06 hrs 15 minutes) HQC and LQC  |  | 85%-115%             | 97.1% and 103.8%  |
| Post Processing Stability – (27 hrs 13 minutes) HQC and LQC                        |  | 85%-115%             | 96.7% and 96.5%   |
| Interim Stability – (13 hrs 03 minutes) HQC and LQC                                |  | 85%-115%             | 102.3% and 99.5%  |
| Long term Stability of drug in Matrix (90 days 18 hrs 30 min) HQC and LQC          |  | 85%-115%             | 98.7% and 99.4%   |
| Anticoagulant Effect   | Precision (HQC and LQC)                              | $\leq 15$            | 3.2 and 2.5       |
|  | Accuracy (HQC and LQC)                               | 85%-115%             | 103.8% and 96.6%  |

determination of Aceclofenac in human plasma. The precision and accuracy data gives a result, which were within the acceptance limits. Consistent recoveries were observed for LQC, MQC1, MQC2 and HQC. The method is specific enough in the presence of different anticoagulants (CPDA and EDTA) and matrices collected from different sources. This method can be used for quantification of Aceclofenac in human plasma in routine and bioequivalence studies.

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# ***In-vitro* screening of antimicrobial activity of *Cyanotis fasciculata* Var. *Fasciculata***

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## **ABSTRACT**

Traditional uses of *Cyanotis fasciculata* var. *fasciculata* listed during literature survey, hinted about its possible antimicrobial property. To provide scientific basis for their traditional usage, cup-plate diffusion method was used to determine antifungal activity. Further, a pilot study was made by cup-plate diffusion method to assess antibacterial activity, being encouraged by the results, a macro broth dilution assay was done to determine MIC values of various extracts against some common hospital pathogens viz., *E.coli*, *S. aureus*, *E. faecalis*, *K. pneumoniae*. The chloroform extract of *C. fasciculata* appeared most promising among four extracts taken.

**Key words:** Antimicrobial activity, *Cyanotis fasciculata*, MIC values, DIZ.

## **Introduction**

Emergence of microbial resistance against potent antibiotics has posed a tough challenge to chemotherapy. So, worldwide invention for new antimicrobial agents is on progress with unprecedented vigour. Among the sources searched currently, medicinal plants have drawn priority. Because, plants produce huge variety of secondary metabolites to defy invasions by microbes & insects, of which many possess antimicrobial properties. Presently, we have selected plants of *Cyanotis fasciculata* (family: commelinaceae) to screen for antimicrobial properties.

Juice from succulent leaves of the plant is used by some tribes to treat superficial mycoses and mouth sores [1,2]. The hydroalcoholic extract of entire plant is reported to be useful in lymphatic leukemia and possess diuretic [3], antiviral activities [4].

## **Materials and Methods**

### **Plant material**

The plants of *Cyanotis fasciculata* var., *fasciculata* were collected from Fort-hill top of Bellary, Karnataka in the month of September and were authenticated by Dr.Kotresh, Department of Botony, Karnataka university, Dharwad, Karnataka.

The voucher specimens of these plants were preserved

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in the herbarium of the pharmacognosy department of this institution.

### **Preparation of extracts**

The plants were air-dried, as such in shade and were pulverized in a mechanical grinder to cottony lumps; it was extracted with petroleum ether, chloroform, hydroalcohol (70%), and methanol by soxhlation, exhaustively; extracts were dried in Rotary Vacuum Evaporator and stored in air tight containers at 4°C.

### **Preparation of test samples**

Formulation of petroleum ether and chloroform extracts were made by dissolving extracts in 5% dimethyl sulphoxide (DMSO). Whereas hydroalcoholic and methanolic extracts were added with distilled water, such that the final stock solutions were of 500 mg/ml concentration. Further, two fold serial dilutions of various extracts were reconstituted from them.

### **Determination of MIC and DIZ**

MIC values of each of four extracts mentioned above against selected bacterial strains were determined by macro broth dilution assay method [5]. Two fold serial dilution of the extracts of *C. fasciculata* (1–500mg /ml) were prepared in tubes with Mueller Hinton Broth as diluent. Duplicate tubes of each dilution were seeded with 0.1ml of the known stains of test organisms to the standard concentration ( $5 \times 10^5$  cfu/ml) procured from Department of microbiology, SDM-RCR, Dharwad, Karnataka. Ciprofloxacin (1mg/ml) was taken as experimental positive

control. The tubes were incubated at 37°C for 24 hours. The lowest concentration of the extract showing absence of growth and clarity was taken as the MIC.

Antifungal activity was determined by measuring Diameter of the Inhibition Zone(DIZ) in mm, using cup-plate method against *C.albicans* and *A.fumigatus*. The culture plates were incubated at 37°C for 72 hours, prior to calculation of the averages of triplicate readings [6].

## Results and Discussions

The results of elaborate macro tube dilution assay of four extracts are consolidated in Table 1. The assay revealed that the chloroform extract of *C. fasciculata* possess broad spectrum antimicrobial activity. Notably, it appeared highly effective on a notorious hospital pathogen; *S.aureus*. Whereas, organisms wise, *K. pneumoniae* remained highly sensitive to all extracts. Unexpectedly, petroleum ether extract of *C. fasciculata* showed good anti-microbial activity. Which may be attributed to the phytosterols present in the extract.

Although the juice of fresh leaves of *C. fasciculata* is reported to be used by tribes to treat skin fungal diseases, the DIZ values against *C. albicans* & *A. fumigatus* found in the present study were not very encouraging, because

extracts did not produce significant DIZs at concentrations less than 100 mg/ml. However, it can be concluded that the chloroform extract exerted more of antibacterial action than antifungal action, whereas petroleum ether extract appeared vice versa (Table 2). So, fractionation of chloroform and petroleum ether extract of *C. fasciculata* to ascertain antimicrobial-phytochemical is under progress.

## Acknowledgement

The authors wish to thank Chairman and Principal, T.V.M. College of Pharmacy, Gandhi Nagar, Bellary for the facilities provided for the study.

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**Table-1**  
**MIC Values of different extracts of *Cyanotis fasciculata* against test organisms. (mg /ml)**

| Extracts              | Test organisms    |                      |             |                          |
|-----------------------|-------------------|----------------------|-------------|--------------------------|
|                       | E.Coli ATCC 10536 | S. aureus ATCC 11632 | E. faecalis | K. pneumoniae ATCC 10031 |
| Pet. Ether            | 62.5              | 250                  | R           | 31.25                    |
| Chloroform            | 62.5              | 16                   | 31.25       | 4                        |
| Hydro alcoholic (70%) | 250               | 31.25                | 31.25       | 4                        |
| Methanolic            | 62.5              | 250                  | R           | 4                        |

R – Resistant.

**Table 2**  
**Antifungal activity of different extracts of *Cyanotis fasciculata* against test organisms. (DIZ in mm)**

| Extracts              | C.albicans ATCC 1013 |      |      | A.fumigatus |      |      |
|-----------------------|----------------------|------|------|-------------|------|------|
|                       | 500                  | 250  | 100  | 500         | 250  | 100  |
| Pet. Ether            | 25mm                 | 21mm | 16mm | 20mm        | 16mm | 13mm |
| Chloroform            | 24mm                 | 20mm | 16mm | 18mm        | 16mm | 12mm |
| Hydro alcoholic (70%) | R                    | R    | R    | R           | R    | R    |
| Methanolic            | 19mm                 | 17mm | 14mm | 14mm        | 12mm | R    |

R – Resistant.



# Antioxidant and Antiulcer Potential of *Mallotus philippensis* (Lam.) Mull-Arg leaf extract

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## ABSTRACT

The study was aimed at evaluating the antiulcer and *invitro* antioxidant activities of the different extracts of the leaves of *Mallotus philippensis* (Lam.) Mull-Arg (Family: Euphorbiaceae). The fruits and bark of the plant have been used medicinally to treat ulcers and tapeworms infestations. Also the seeds are used for wound healing. Antiulcerogenic activity of the methanolic extract of *Mallotus philippensis* (*mp*) (100 and 200 mg/kg *b.w.* orally) was evaluated using Aspirin plus Pylorus ligation induced acute gastric ulcer model. The antioxidant activity of the chloroform, ethyl acetate and methanolic extracts of *mp* has been assayed using *invitro* methods like 2,2-diphenyl-1-1-picrylhydrazylhydrate (DPPH) and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation (ABTS) assays. There was a significant dose dependent decrease in ulcerative lesion index produced in rats as compared to the standard drug Ranitidine (150mg/kg, *b.w.* orally). The reduction in gastric fluid volume, total acidity and an increase in pH of the gastric fluid in the *mp* treated group, in aspirin plus pyloric ligated rats proved the antisecretory activity of the extract. The extracts also exhibited significant free radical scavenging activity in the DPPH and ABTS assays. These results suggest that methanolic extract of the leaves of *mp* possesses potential antiulcer activity which may be attributed to its antisecretory and antioxidant mechanism of action.

**Keywords:** Antiulcer activity, Antioxidant, Aspirin plus Pyloric ligation, *Mallotus philippensis* leaves, 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical, 2,2-diphenyl-1-1-picryl-hydrazylhydrate (DPPH) radical.

## Introduction

Peptic ulcer is one of the most common gastrointestinal disorders, which causes a high rate of morbidity particularly for the population of non- industrialized countries [1]. It is one of the major gastro-intestinal disorders, which occur due to an imbalance between the offensive (gastric acid secretion) and defensive (gastric mucosal integrity) factors [2]. Thus, drug therapy of peptic ulcers has been commonly targeted at either counteracting the aggressive factors or stimulating defensive factors. The synthetic drugs presently used as antiulcer agents confers several side effects like impotence, gynaecomastia, hyperplasia and haemopoietic changes. As a result more herbal drugs are being explored for offering better treatment for peptic ulcers. There are evidences for participation of reactive oxygen species in the etiology and pathophysiology of human diseases such as neurodegenerative disorders, inflammation, viral infections, autoimmune gastro intestinal inflammations and

gastric ulcers [3]. It has been reported that many drugs possessing potent antioxidant activity are effective in healing experimentally induced gastric ulcers [4]. *Mallotus philippensis* (Lam.) Mull-Arg (kamala tree) is a small to medium-sized monoecious tree, up to 25m tall of the family Euphorbiaceae . Kamala tree is widespread, from the western Himalaya, through India, Sri Lanka, to southern China, Taiwan and the Ryukyu Islands, Burma (Myanmar), Thailand, and throughout Malaysia to Australia [5].

Kamala is used as anthelmintic and an extract of kamala in hexachlorethane may be useful in treating liver fluke in cattle.<sup>6</sup> Kamala is also known to affect the fertility of animal and man. All parts of the tree can be applied externally to treat parasitic infections of the skin. The leaves are bitter, cooling, stimulates appetite, causes flatulence and constipation [6]. The bark and fruits have been used traditionally to treat ulcers and tapeworms infestations. The pound leaves or seeds are used for fungal infections. Also the seeds are used for wound healing. Based on the traditional uses, the objective of the present study was to study the *invitro* antioxidant and potential

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antiulcer or ulcer protective efficacy of the methanolic extract of *Mallotus philippensis*.

## Materials and Methods

### Collection of Plant Material

The leaves of *Mallotus philippensis* were collected in the month of April / June from Nilgiris district, Tamil Nadu. The plant was identified, confirmed and authenticated by comparing with voucher specimen available at Survey of medicinal Plants & Collection Unit, Department of AYUSH, Ministry of Health and Family Welfare, Govt. of India, Emerald by Field Botanist, Dr. S. Rajan.

The leaves were cut into small pieces and dried in hot air oven maintained at a temp of 42-45° C. The dried material was then pulverized separately into coarse powder by a mechanical grinder. The resulting coarse powder was then used for extraction.

### Preparation of Extracts

The powdered leaves were subjected to successive hot extraction in soxhlet apparatus with various solvents in increasing order of polarity, such as Petroleum Ether, Chloroform, Ethyl Acetate, Methanol and Water. The extracts were filtered, and dried at 40°C under vacuum and the yields of Petroleum Ether, Chloroform, Ethyl Acetate, Methanol and Water extracts were 1.2%, 1.3%, 1.8%, 1.78% and 3.2% respectively [7].

### Phytochemical screening

Preliminary phytochemical screening of the powdered leaves extracts was performed to detect the presence of alkaloids, phenolics, flavonoids, saponins, glycosides and carbohydrates.

### Evaluation of Antiulcer Activity

#### Animals

Albino rats of Wistar strain of either sex weighing between 150-200g were used. They were housed in standard cages at room temp. (25±2°C) and provided with food and water ad libitum. The animals were deprived of food for 24 hrs before experimentation, but had free access to drinking water. The study was conducted after obtaining institutional ethical committee clearance bearing the number DSCP/PhD/PCol/IAEC/08/08.

#### Drugs and Chemicals

Chemicals of standard grade were purchased from S.D Fine Chemical Pvt. Lt. India, Merck Specialties Pvt. Lt. and Ranitidine, as a standard drug for these pharmacological studies was supplied by Dr. Reddy Laboratory, Hyderabad.

#### Acute Toxicity Studies

Six rats were fasted overnight and were administered a single oral dose (2000 mg/kg, b.w.) of *Mallotus*

*philippensis* leaf extract. After the administration of the extract, food was withheld for further 3–4 hrs. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 hrs and daily thereafter for a period of 14 days. Once daily, cage side observations including changes in skin and fur, eyes and mucous membrane (nasal) and respiratory rate, circulatory (heart rate and blood pressure), autonomic (salivation, lacrimation, perspiration, piloerection, urinary incontinence, and defecation) and central nervous system (ptosis, drowsiness, gait, tremors and convulsion) changes were observed. Mortality, if any, was determined over a period of 2 weeks [8].

### Selection of the Dose of the Extract

LD<sub>50</sub> was done as per OECD guidelines for fixing the dose for biological evaluation. The LD<sub>50</sub> of the extracts as per OCED guidelines falls under class four values with no signs of acute toxicity at 2000 mg/kg b.w.

The biological evaluation was carried out at doses of 100 and 200mg/kg b.w.

### Evaluation of Antiulcer Activity [9]

The antiulcer activity of the methanolic extract of leaves of *Mallotus philippensis* (MPME) was evaluated by Aspirin + Pylorus ligation induced Gastric ulcer model.

MPME and standard antiulcer drug, Ranitidine were prepared in 0.5% sodium carboxy methyl cellulose (CMC) suspension as vehicle and administered orally once daily at a volume of 10ml/kg b.w. The animals were divided into four groups, consisting of six each. Group I received Aspirin alone (200mg/kg, p.o.). Group II and III received MPME orally at the doses of 100 and 200 mg/kg b.w. respectively for 7 days. Group IV received Ranitidine orally at the dose of 150mg/kg b.w. for 7 days. From days 5 to 7, animals of all groups received orally an aqueous suspension of Aspirin at a dose of 200mg/kg, 2 hrs after the administration of the respective drug treatment. Animals in all the groups were fasted for 18 hrs after the respective assigned treatment and were anaesthetized with anaesthetic ether. The abdomen was opened by a small midline incision below the xiphoid process and pylorus portion of stomach was lifted and ligated (Shay). Precaution was taken to avoid traction to the blood supply. The stomach was sutured with interrupted sutures. 4 hrs after pylorus ligation, the rats were sacrificed and the stomach was removed. The gastric contents were collected, centrifuged and the volume of the supernatant was expressed as ml/100g b.w. Total acidity was determined by titrating with 0.1 N NaOH using Topffer's reagent using phenolphthalein as an indicator. The total acidity was expressed as μ equiv./100 g/4 hrs. The stomachs were then opened along the greater curvature and were washed slowly under running tap water and put on a glass slide and observed under 10 X magnification for ulcers. The ulcers were scored [10] as follows.

- 0 = Normal coloured stomach
- 0.5 = Red colouration
- 1 = Spot ulcer
- 1.5 = Haemorrhagic streaks
- 2.0 = ulcers  $\geq 3$  but  $\leq 5$
- 3.0 = ulcers  $\geq 5$

Mean ulcer score for each animal was expressed as ulcer index

% Ulcer protection was calculated according to the standard formula by

$$\% \text{ Ulcer protection} = \frac{(\text{Ulcer Index in Control} - \text{Ulcer index in Test})}{\text{Ulcer Index in Control}} \times 100$$

The gastric juice was collected and then centrifuged. The volume and pH was recorded. Free acidity and total acidity of the gastric juice were estimated.

### Statistical Analysis

Values were expressed as mean  $\pm$  SEM. The experimental mean values were compared statistically with that of vehicle control mean values using One Way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test. The analysis was carried out using Graph Pad Prism software V.4. p values less than 0.5 were considered to be statistically significant.

### DPPH assay [11]

The antioxidant activity of the extracts of the plant was assessed by the *invitro* method by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay.

To 200  $\mu$ l of DPPH solution, 10  $\mu$ l of each of the test sample or the standard solution was added separately in wells of the microtitre plate. The final concentration of the test and the standard solutions used were 1000, 500, 250, 125, 62.5, 31.25 and 15.625 and 7.8  $\mu$ g /ml. The plates were incubated at 37 °C for 30 mins and the absorbance of each solution was measured at 490 nm using Elisa reader against the corresponding test blanks. IC<sub>50</sub> values were calculated and compared with that of ascorbic acid and rutin which were used as standards.

### ABTS assay [12]

Free radical scavenging activity of ABTS [2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] radical cation was measured using the spectrophotometric method

ABTS (54.8mg) was dissolved in 50 ml of distilled water to 2 mM concentration and potassium persulphate (17mM, 0.3 ml ) was added. The reaction mixture was left to stand at room temperature overnight in dark before usage. To 0.2 ml of various concentrations of the extracts or standards was added, 1.0 ml of distilled DMSO and 0.16 ml of ABTS solution to make a final volume of 1.36 ml.

Absorbance was measured spectrophotometrically after 20 minutes at 734 nm. IC<sub>50</sub> values of the crude extracts was calculated and compared with that of ascorbic acid and rutin which were used as standards.

## Results

### Phytochemical Studies

In the qualitative phytochemical analysis carbohydrates, saponins, flavonoids and proteins were found to be present in the leaf extracts of the plant. Fixed oils and fats were present in all the extracts except aqueous extract. Only ethyl acetate extract showed the presence of alkaloids. Saponin glycosides were not present in any of the extracts. Flavones and flavonoids in varying proportions were present in all the extracts except pet.ether extract.

### Behavioral and Acute toxicity Studies

All the extracts of *mp* at 500, 1000 and 2000 mg/kg *b.w.* dose level did not show any significant behavioural alterations and toxicity in the initial hrs (1<sup>st</sup> four hrs). However, invariably in all the extracts slight irritability was noticed. Moreover extracts produced some dose dependent sedation in the initial hours, 72 hrs observations showed no mortality. Therefore it was concluded that for upto 2000mg/kg *b.w* there is no toxicity and hence for further pharmacological studies the biological evaluations were carried out at doses of 100 and 200 mg/kg *b.w*

### Aspirin + Pylorus ligation (SHAY) Rat Model

In the control group the ulcer parameters were evident and indicate that Aspirin + pylorus ligation rat model method was effective enough to produce gastric ulcers. Animals in the control group showed a significant (p < 0.01) increase in the ulcer index and acid secretory parameters like gastric volume, pH, free and total acidity when compared with those of vehicle treated group. In the rats of this group, a number of spot ulcers and haemorrhagic streaks were also observed. Administration of MPME produced significant (p < 0.01) decrease in ulcer index in a dose dependent manner. All the ulcers of scores 0.5, 1.5, 2.0 and  $\leq 3$  and  $\geq 5$  were observed. The extract also significantly reduced the gastric volume, total acidity and increase in pH of the gastric fluid, proving its antisecretory activity. MPME at a dose of 100 and 200 mg/kg *b.w.* showed protection index of 60.8 % and 67.17 % respectively, where as Ranitidine showed protection index of 85.13% at a dose of 150mg/kg *b.w.*

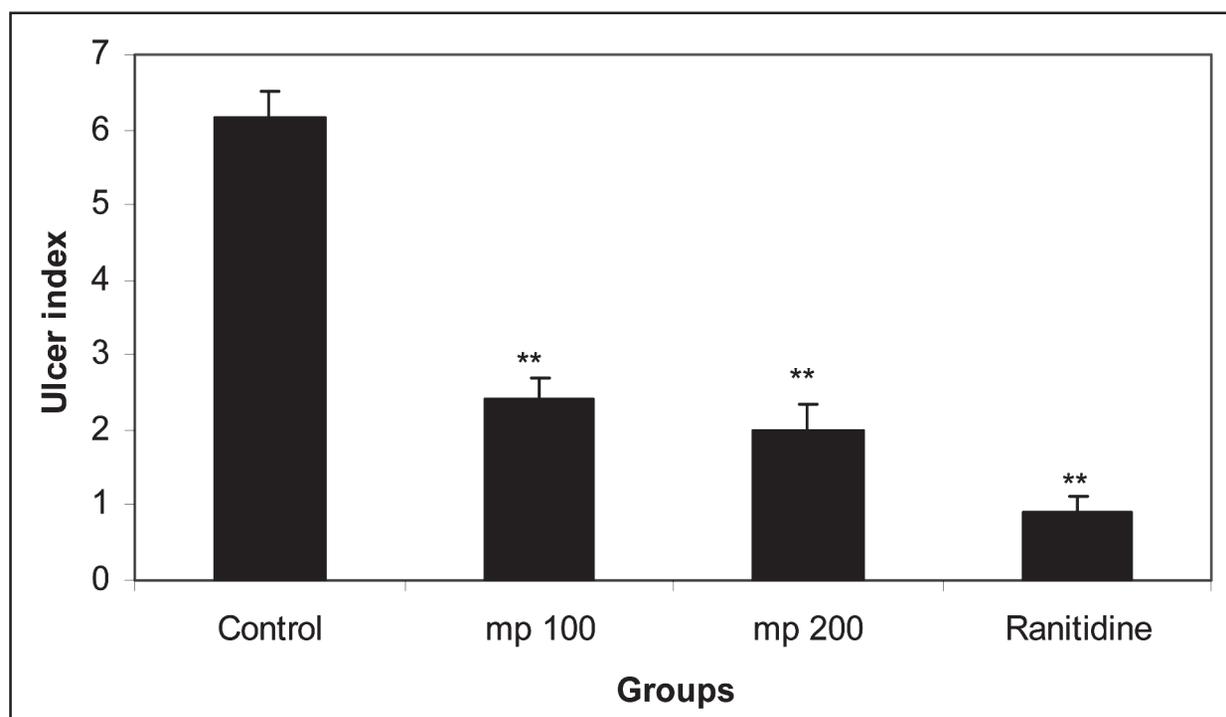
### In-vitro antioxidant study

Some of the extracts tested exhibited potent antioxidant activity with low IC<sub>50</sub> values in the scavenging of ABTS and DPPH radicals. The IC<sub>50</sub> values by the DPPH method were found to be 75 $\mu$ g/ml and 12.2  $\mu$ g/ml for the ethyl acetate and methanol extracts of *Mallotus philippensis*. Other extracts did not possess potent antioxidant activity by this method. The IC<sub>50</sub> values by the ABTS method were found to be 12 $\mu$ g/ml, 1 $\mu$ g/ml, and 0.42  $\mu$ g/ml for the

**Table-1**  
**Effect of methanolic extract of *Mallotus philippensis* leaves on Aspirin + pyloric ligation induced gastric ulceration in rats**

| Treatment  | Dose (mg/kg b.w) | Gastric volume (mg/100g)      | pH                 | Free acidity ( $\mu$ equiv/100g/4hrs) | Total acidity ( $\mu$ equiv/100g/4hrs) | Ulcer index       | % Inhibition |
|------------|------------------|-------------------------------|--------------------|---------------------------------------|--|-------------------|--------------|
| Control    | 200              | 2.65 $\pm$ 0.31               | 1.43 $\pm$ 0.12    | 61.01 $\pm$ 3.1                       | 80.84 $\pm$ 3.8                        | 6.17 $\pm$ 0.35   | -            |
| <i>Mp</i>  | 100              | 2.07 $\pm$ 0.23 <sup>NS</sup> | 2.88 $\pm$ 0.17**  | 33.98 $\pm$ 1.5**                     | 52.26 $\pm$ 3.2**                      | 2.42 $\pm$ 0.27** | 60.81        |
| <i>Mp</i>  | 200              | 1.720 $\pm$ 0.23*             | 4.017 $\pm$ 0.11** | 28.97 $\pm$ 1.0**                     | 44.09 $\pm$ 2.0**                      | 2.0 $\pm$ 0.34**  | 67.17        |
| Ranitidine | 150              | 1.52 $\pm$ 0.07**             | 4.44 $\pm$ 0.29**  | 21.13 $\pm$ 2.9**                     | 31.93 $\pm$ 3.5**                      | 0.917 $\pm$ 0.2** | 85.13        |

Values are mean  $\pm$ S.E.M, n=6, NS-not significant, \*p < 0.05 and \*\*p < 0.01 Vs control



**Fig.1: Comparison of Ulcer Index of various groups in Aspirin + Pylorus ligation Rat Model**

**Table-2**  
**DPPH and ABTS radical scavenging activity of the different extracts of *Mallotus philippensis***

| Sl. No. | Sample / Std          | IC <sub>50</sub> Values *( $\mu$ g/ml) |                     |
|---------|-----------------------|--|---------------------|
|         |                       | ABTS radical cation                    | DPPH radical cation |
| 1       | Chloroform Extract    | 12 $\pm$ 0.52                          | > 500               |
| 2       | Ethyl Acetate Extract | 1 $\pm$ 0.33                           | 75 $\pm$ 0.56       |
| 3       | Methanolic Extract    | 0.42 $\pm$ 0.035                       | 12.2 $\pm$ 0.68     |
| 4       | Ascorbic Acid         | 11.25 $\pm$ 0.49                       | 4.2 $\pm$ 0.44      |
| 5       | Rutin                 | 0.51 $\pm$ 0.048                       | 16.9 $\pm$ 0.66     |

\*All values are  $\pm$  SEM of three determinations.

chloroform, ethyl acetate and methanol extracts of *Mallotus philippensis* respectively. The IC<sub>50</sub> values of the methanolic extract were found to be comparable to the standards used indicating potent antioxidant activity.

## Discussion

The study revealed a significant antiulcer effect of a methanolic extract of *Mallotus philippensis* in experimental models of gastric ulceration induced by pyloric ligation combined with a non-steroidal anti-inflammatory drug aspirin.

Peptic ulcer and gastritis have multi-etiological factors. It is widely accepted that a major underlying factor of this disorder is the generation of free radicals. There is substantial evidence that oxygen derived free radicals play an important role in the pathogenesis of the injury of various tissues including the digestive system [13,14]. Also, involvement of oxygen derived free radicals such as superoxide anion, hydrogen peroxide and hydroxyl radical are well established in the pathogenesis of ischaemic injury of the gastrointestinal mucosa and in other models of mucosal damage induced by non-steroidal anti-inflammatory drugs [15]. Aspirin-induced ulcer is mediated through tissue damaging free radicals<sup>16</sup> which are produced from the conversion of hydroperoxyl to hydroxyl fatty acids, which leads to cell destruction. The hydroperoxyl fatty acids are generated from the degeneration of mast cells and generalized lipid peroxidation accompanying cell damage [17]. Many reports have demonstrated that most injury of gastric mucosa can be reduced by pre-treatment with scavengers of reactive oxygen species.<sup>18</sup> In folk medicine, *mp* leaves have been used for the treatment of ulcers, wounds, etc. The methanolic extract was found to have more potent antioxidant activity in the DPPH and the ABTS assay, when compared to the other extracts. Hence this extract was subjected to antiulcer studies.

The present study reveals that the methanolic extract treated groups showed a significant increase in gastric juice pH, reduced the gastric volume, free acidity and total acidity when compared to control in the aspirin plus pyloric ligation model. This effect was comparable to Ranitidine treated group. The extract decreased the ulcer index more effectively in a dose dependent manner. Hence an antisecretory mechanism may be involved.

The different mechanisms for antiulcer effects suggested are antisecretory activity on pepsin and acid mucosal protection by increased mucous synthesis, prostaglandin level, protective coating and radical scavenging. The methanolic crude extract showed radical scavenging activity which may be partly responsible for the antiulcer activity of the extract.

The results of our study prove that the crude methanolic extract of *mp* possess antiulcer activity against experimentally induced acute gastric ulcer models. Hence

it can be suggested that the antiulcer activity of the extract may be attributed to its antisecretory and antioxidant activities. Further *in vivo* antioxidant studies are in progress to study the exact mechanism of action.

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# Isolation and characterization of plumeiridine-5-o-glucoside, an antioxidant active principle from *Plumeria acutifolia* Poir

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## ABSTRACT

Plumeiridine-5-o-glucoside, an iridoid was isolated from the ethanolic extract of *Plumeria acutifolia* Poir. stem bark by bioassay-guided fractionation using silica column chromatography and HPLC. The protective *in vitro* antioxidant activity of plumeiridine-5-o-glucoside was investigated. The ethanolic extract (EE) and active fractions (FR5 and SF3) showed significant ( $P < 0.05$ ) and active compound (AC1) showed very significant ( $P < 0.01$ ) *in vitro* antioxidant activity in terms of DPPH free radical scavenging activity.

**Keywords:** *Plumeria acutifolia*, Plumeiridine-5-o-glucoside, Silica column chromatography, IR, LCMS and NMR.

## Introduction

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism. They are continuously produced by the body during respiration and some cell-mediated immune functions. These free radicals are also generated from environmental pollutants, cigarette smoke, automobile exhaust fumes, radiation and pesticides. Free radicals when accumulated in cells cause cumulative damage of proteins, lipids, DNA, carbohydrates and membranes, resulting in oxidative stress [1]. Oxidative stress causes food deterioration, aging and a wide range of human diseases including Alzheimer's disease [2], Parkinson's disease [3], diabetes [4], rheumatoid arthritis [5], cancers [6] and neurodegenerative [7] and cardiovascular [8] diseases. Recently, interest has considerably increased in identifying naturally occurring antioxidants to replace synthetic antioxidants as they cause toxic side effects such as cancers [9]. Many antioxidant compounds derived from plants have been identified as free radical or active oxygen scavengers [10]. Therefore, plant-derived antioxidants are now receiving a special attention [11].

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*Plumeria acutifolia* Poir. (Apocyanaceae), familiarly known as temple tree or tree of love, is extensively cultivated in India. The stem bark of this plant has been employed against abscess, gonorrhoea and fevers. The juice from the stem bark of this plant is rubifacient and antirheumatic. It has been applied to treat carious teeth, ulcers and wounds [12]. In this study, we isolated, characterized and elucidated the structure of an antioxidant active principle from the ethanolic extract of *Plumeria acutifolia* Poir. stem bark by bioassay-guided fractionation and investigated its antioxidant activity in terms of DPPH radical scavenging activity.

## Materials and Methods

### Preparation of plant material

*Plumeria acutifolia* Poir. was collected from Nallamalai forest, Nandyal, Andhra Pradesh, India. The plants were identified from the herbarium records of Department of Botany, S.K. University, Anantapur and voucher specimens were deposited. The stem bark of the plant was shade dried and ground into powder. About 1 kg of powdered material was Soxhlet extracted with n-hexane, ethyl acetate and ethanol successively. The ethanol extract was filtered using

Whatman filter paper (No.1) and then concentrated under vacuum at 40 °C using a Rotary Evaporator (Buchi, Germany). The residue obtained was stored at -20 °C prior to analysis.

### Bio-assay guided fractionation

A portion of crude ethanol extract (EE) which showed significant ( $P < 0.05$ ) *in vitro* antioxidant activity was subjected to fractionation by flash column chromatography using silica gel (230–400 mesh size) (Merck, Germany) (Tables 1, 2 and 3). The EE (63 g) was dissolved in a minimum amount of methanol and adsorbed on silica (180 g). Column was prepared using 600 g of silica gel (230–400 mesh size) and eluted with the mixture of chloroform–methanol (2000 ml) in gradient manner to give twelve fractions of 500 ml each. Fraction 5 (FR 5) (1.5 g) which showed significant ( $P < 0.05$ ) *in vitro* antioxidant activity was then adsorbed onto 3.5 g silica and placed on 25 g of silica gel (230–400 mesh size) in a column. Gradient elution was effected with chloroform–methanol mixture (150 ml) and a total six fractions of 50 ml each were collected. Fraction 3 (SF1) (0.4 g) which showed significant ( $P < 0.05$ ) *in vitro* antioxidant activity was again adsorbed on to silica (2.0 g) and chromatographed over 20 g of silica gel (230–400 mesh size). The gradient elution was effected with ethyl acetate–methanol mixture (100 ml) and each time 30 ml of fractions were collected. The fraction three (AC1) showed very significant ( $P < 0.01$ ) *in vitro* antioxidant activity. The pure compound (AC1) was obtained by direct crystallization with methanol. Purity of the fractions was analyzed by TLC and HPLC.

### Characterization and Structural Determination of AC1

Characterization and structural determination of AC1 was established on the basis of spectral studies. Infra red spectra were recorded on Perkin–Elmer model 283 B and 297 double beam spectrophotometers by the KBr pellet method and  $\nu$  values were given in  $\text{cm}^{-1}$ . High Performance Liquid Chromatography (HPLC) coupled with Mass Spectrometry (MS) and DAD–UV detection was performed on LC/MSD (Agilent 1100 series) equipped with an ESI and a quadrupole ion trap. Mobile phase was 50% acetonitrile in water. The ionization conditions adopted for analyses were;

ESI: Source voltage 3–6 kV, nebuliser 20–40 psi, dry gas 12 L/min, dry temperature 300 °C, capillary voltage 10–30 V and heated capillary temperature 200 °C. Nuclear Magnetic Resonance (NMR) spectra were used to elucidate the structure of compounds and also for routine analyses of the fractions.  $^1\text{H}$  and  $^{13}\text{C}$  NMR, COSY (COrrelation SpectroscopY), HSQC (Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) spectra were recorded on a Bruker-AMX 400 instrument at 400 ( $^1\text{H}$ ) and 100 ( $^{13}\text{C}$ ) MHz. The shifts are

indicated in ppm, with tetramethyl silane (TMS) used as an external standard.

### *In Vitro* antioxidant activity: DPPH assay

Radical scavenging activity was determined by a spectrophotometric method based on reduction of a methanol solution of 1, 1–diphenyl–2–picryl hydrazyl (DPPH) [13, 14]. EE, FR5, SF1 and AC1 (400  $\mu\text{g}$ ) were mixed with 0.4 ml of 0.5 mM DPPH in ethanol, and final volume was adjusted upto 2.0 ml with ethanol. Mixtures were vigorously shaken and left in dark for 30 min. Absorbance was measured at 517 nm using ethanol as a blank. One ml of 0.5 mM DPPH diluted in 4 ml of ethanol was used as control. Inhibition of DPPH radical in percent (I %) was calculated using the following equation.

$$I \% = (A_{517}^{\text{control}} - A_{517}^{\text{Sample}} / A_{517}^{\text{control}}) \times 100$$

Concentration of extracts/fractions/active compound providing 50% inhibition ( $\text{IC}_{50}$ ) was calculated from the graph plotted inhibition percentage against oil concentration. Synthetic antioxidant butylated hydroxytoluene (BHT) was used as a positive control and tests were carried out in triplicate.

### Statistical analysis

Results were expressed as mean  $\pm$  standard deviations (S.D). Differences were accepted as statistically significant when  $P < 0.05$ . Statistical analysis was performed by analysis of variance (ANOVA). SPSS package was used for statistical analysis.

## Results and Discussion

### Bio-assay guided fractionation

The EE was obtained as a brown viscous residue (18% w/w). When this residue was subjected to column chromatography over silica gel and eluted with the mixture of chloroform–methanol in gradient manner, FR 5 resulted from the eluant of chloroform–methanol mixture (7:3) showed significant ( $P < 0.05$ ) *in vitro* antioxidant activity. FR 5 was noticed to possess a mixture of compounds on TLC plates and from HPLC analysis. Separation of FR 5 was carried out by chromatography over silica gel column using chloroform–methanol mixture in increasing polarity, as eluant. The fraction SF1 which was eluted with chloroform–methanol mixture (2:3) showed significant ( $P < 0.05$ ) *in vitro* antioxidant activity. SF1 (0.45 g) was obtained as a dark brown gummy residue and showed 3 distinct spots ( $R_F = 0.86, 0.73$  and  $0.14$ ) on TLC plates. Separation of SF1 was again carried out by column chromatography over silica gel and the active fraction AC1 was obtained from the eluant of ethyl acetate–methanol mixture (95: 5). AC1 showed very significant ( $P < 0.01$ ) *in vitro* antioxidant activity. The results of chromatography are shown in tables 1, 2 and 3.

**Table-1**  
**Silica gel column chromatography of EE**

| Eluant (Chloroform:methanol) | Fractions | Active fractions | Yield (g) |
|------------------------------|-----------|------------------|-----------|
| 100 : 0                      | 01        | -                | 0.25      |
| 95 : 5                       | 02        | -                | 1.30      |
| 90 : 10                      | 03        | -                | 0.88      |
| 80 : 20                      | 04        | -                | 13.0      |
| 70 : 30                      | 05        | FR 5             | 14.0      |
| 60 : 40                      | 06        | -                | 5.4       |
| 50 : 50                      | 07        | -                | 0.3       |
| 40 : 60                      | 08        | -                | 0.15      |
| 30 : 70                      | 09        | -                | 1.5       |
| 20 : 80                      | 10        | -                | 2.1       |
| 10 : 90                      | 11        | -                | 0.1       |
| 0 : 100                      | 12        | -                | 0.5       |

**Table-2**  
**Silica gel column chromatography of FR 5**

| Eluant (Chloroform: Methanol) | Fractions | Active fractions | Yield (g) | Rf*             |
|-------------------------------|-----------|------------------|-----------|-----------------|
| 100 : 0                       | 01        | -                | 0.01      | 0.86, 0.73&0.14 |
| 85 : 15                       | 02        | -                | 1.0       |                 |
| 70 : 30                       | 03        | SF1              | 0.45      |                 |
| 60 : 40                       | 04        | -                | 0.02      |                 |
| 50 : 50                       | 05        | -                | 0.04      |                 |
| 0 : 100                       | 06        | -                | 0.05      |                 |

\* Solvent system used for TLC = Chloroform: Methanol (2: 3)

**Table-3**  
**Silica gel column chromatography of SF1**

| Eluant (Ethyl acetate:methanol) | Fractions | Active fractions | Yield (g) | Rf*  |
|---------------------------------|-----------|------------------|-----------|------|
| 100 : 0                         | 01        | -                | 0.01      | 0.86 |
| 97 : 3                          | 02        | -                | 0.03      |      |
| 94 : 6                          | 03        | AC1              | 0.15      |      |
| 90 : 10                         | 04        | -                | 0.04      |      |
| 85 : 15                         | 05        | -                | 0.08      |      |
| 75 : 25                         | 06        | -                | 0.04      |      |
| 50 : 50                         | 07        | -                | 0.05      |      |

\* Solvent system used for TLC = Ethyl acetate: Methanol (1: 4) & (4:1)

**Characterization of AC1:**

Compound AC1 : Plumieridin – 5 – O – glucoside  
 Physical shape : Colourless amorphous powder  
 Amount isolated : 125 mg  
 Melting point : 185 – 190 °C  
 Molecular formula : C<sub>21</sub>H<sub>26</sub>O<sub>12</sub>

IR (KBr)  $\nu$  max : 3376 (OH), 1757 (Lactone), 1695, 1597 (Pyran), 1436, 1353, 1289, 1226, 1181, 1106, 1076, 1037, 1003, 865, 750, 638 cm<sup>-1</sup>.

<sup>1</sup>H NMR (400 MHz, DMSO) (Table 4):  $\delta$  1.27 (3H, d, *J*=6.6 Hz, CH<sub>3</sub>-1), 6.70 (1H, d, *J* = 1.9 Hz, H-4), 2.8 (1H, *J* = 9.3, 1.5 Hz, H-5), 2.40 – 2.42 (1H, m, H-6), 2.98-3.03 (1H, m, H-7), 6.26 (1H, d, *J* = 6.0 Hz, H-8), 5.53 (1H, d, *J* = 6.0 Hz, H-9), 7.27 (1H, d, *J* = 1.5 Hz, H-11), 4.39 (1H, dq, *J* = 6.6, 1.5 Hz, H-14), 1.27 (3H, d, *J* = 6.6 Hz, H-15), 5.1 (1H, d, *J* = 7.3 Hz, H-16), 3.73-3.16 (5H, m, H-17, 18, 19, 20).

<sup>13</sup>C NMR (100 MHz, DMSO) (Table 4) :  $\delta$  52.0 (COOCH<sub>3</sub>), 163.0 (C-3), 129.1 (C-4), 48.0 (C-5), 44.1 (C-6), 40.7 (C-7), 142.7 (C-8), 129.5 (C-9), 97.8 (C-10), 148.6 (C-11), 138.1 (C-12), 171.0 (C-13), 61.5 (C-14), 22.6 (C-15), 99.6 (C-16), 76.3 (C-17), 72.8 (C-18), 69.4 (C-19), 78.0 (C-20), 61.0 (-O-).

LC-MS [M+H] <sup>+</sup>: 470  
 [M+Na] <sup>+</sup>: 493  
 [M+K] <sup>+</sup>: 509.09

**Structural elucidation Of AC1 (Plumieridin-5-O- glucoside)**

The active compound from fraction AC1 was obtained as a colourless amorphous powder (125 mg) from ethyl acetate, m.p. 185-190°C and assigned a molecular formula of C<sub>21</sub>H<sub>26</sub>O<sub>12</sub>, which showed an [M+H] <sup>+</sup> ion peak at *m/z* 470 in LC-MS spectrum. In <sup>13</sup>C NMR spectrum, the carbons that resonated at  $\delta$  148.6 (C-11), 138.1 (C-12) and 171.0

**Table-4**

**<sup>1</sup>H and <sup>13</sup>C NMR spectral data (ppm, DMSO-d<sub>6</sub>) and HMBC correlations for active Compound AC1 (Plumieridin-5-O-glucoside)**

| Position | <sup>1</sup> H NMR <sup>a</sup> (Mult: <i>J</i> (Hz)) | <sup>13</sup> C NMR <sup>b</sup> | HMBC             |
|----------|---|----------------------------------|------------------|
| 1        | 1.27 (d, 6.6)   | 52.0 (COOCH <sub>3</sub> )       |                  |
| 2        | –   |                                  |                  |
| 3        | –   | 163 (C)                          | C-4,6,7          |
| 4        | 6.70 (d, 1.9)   | 129.1 (CH)                       | C-3              |
| 5        | 2.8 (dd, 9.3, 1.5)                                    | 48.0 (CH)                        | C-4,6,7,10       |
| 6        | 2.4-2.42 (m)  | 44.1 (CH)                        | C-10,11          |
| 7        | 2.98-3.03 (m)   | 40.7 (CH)                        | C-3,8,9,10       |
| 8        | 6.26 (d, 6.0)   | 142.7 (CH)                       | C-7,9,10         |
| 9        | 5.53 (d, 6.0)   | 129.5 (CH)                       | C-6,7,8,10       |
| 10       | –   | 97.8 (C)                         |                  |
| 11       | 7.27 (d, 1.5)   | 148.6 (CH)                       | C-10,12,13,14,15 |
| 12       | –   | 138.1 (C)                        |                  |
| 13       | –   | 171 (C)                          |                  |
| 14       | 4.39 (dq, 6.6, 1.5)                                   | 61.5 (CH)                        | C-11,12,15       |
| 15       | 1.27 (d, 6.6)   | 22.6 (CH <sub>3</sub> )          | C-12,14          |
| 16       | 5.1 (d, 7.3)  | 99.6 (CH)                        |                  |
| 17       |   | 76.3 (CH)                        |                  |
| 18       |   | 72.8 (CH)                        |                  |
| 19       | 3.73-3.16 (m)   | 69.4 (CH)                        |                  |
| 20       |   | 78.0 (CH)                        |                  |
| 21       |   | 78.9 (CH <sub>2</sub> )          |                  |
| 22       |   | 61.0 (-O-)                       |                  |

<sup>a</sup> <sup>1</sup>H directly linked to <sup>13</sup>C determined from HSQC

<sup>b</sup> Multiplicities were obtained from DEPT experiments

(C-13) ppm could be ascribed to an  $\alpha$ ,  $\beta$ -unsaturated carbonyl structure, as HMBC interaction between H-11 and C-13, in addition to the strong absorption band at 1757  $\text{cm}^{-1}$  in IR spectrum, was also supportive of the presence of the lactone in active compound AC1 (Table 4).

In the  $^1\text{H}$  NMR spectrum, the methine proton bearing a hydroxyl group at  $\delta$  4.39 (dq,  $J = 6.6, 1.5$  Hz, H-14) ppm was coupled with a proton at  $\delta$  7.27 (d,  $J = 1.5$  Hz, H-11) ppm and with a methyl group at  $\delta$  1.27 (d,  $J = 6.6$  Hz, H-15) ppm. Further, HMBC correlations between H-15 and C-12, H-14 and C-11 and H-11 and C-14 indicated that the 1-hydroxyethyl fragment was the  $\alpha$ -side chain of the 5-membered  $\alpha$ ,  $\beta$ H-unsaturated lactone (Table 4). Two groups of proton signals with  $\delta$  6.26 (d,  $J = 6.0$  Hz, H-8) and 5.53 (d,  $J = 6.0$  Hz, H-9) ppm, were assigned to the double bond of the cyclopentene with the help of the HMBC experiment. HMBC correlations observed between H-7 and C-9 and C-10, between H-8 and C-10 and between H-9 and C-6 and C-7 established the presence of the cyclopentene moiety in active compound.

Besides the five degrees of unsaturation of the revealed structural fragment of AC1, the remaining segment of AC1 should be a tetrahydropyran sharing the two  $\text{sp}^3$  carbons of the cyclopentene as in plumieride, as indicated by the NMR spectra. Thorough illustration of the proposed hydrated pyran ring was accomplished mainly by the HMBC signals. In HMBC spectrum, H-4 interacted with C-5. All the above HMBC information proved that the tetrahydro pyran ring of AC1 had the same connectivities as those of plumieride. The chemical shift of 52.0 ppm was ascribed to C-3, which bears a  $\text{COOCH}_3$  substituent. The HMBC correlations are listed in Table 4.

In addition, the  $^1\text{H}$  NMR spectrum showed a series of signals between  $\delta$  3.73 – 3.15 which can be attributed to a sugar moiety. The coupling constant ( $J = 7.3$  Hz) of the anomeric proton located at  $\delta$  5.09 and  $^{13}\text{C}$  NMR chemical shifts of the sugar carbons [15] ( $\delta$  99.6, 76.3, 72.8, 69.4 and 78.0) revealed the presence of  $\beta$ -O-glucoside unit in AC1 (Table 4). The structure of AC1 (Fig. 1) was in good agreement with that of plumieride, the iridoid glycoside isolated from *P. rubra* heartwood [16].

### In Vitro antioxidant activity

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [17]. The reduction capability of the DPPH radical is determined by the decrease in its absorbance at 570 nm induced by antioxidants. It is due to the reaction between antioxidant molecules and radicals which results in the scavenging of the radical by hydrogen donation and is visualized as a discoloration from purple to yellow [18, 19].

In this study, EE, FR5, SF1 and AC1 strongly reduced the stable DPPH radical with the  $\text{IC}_{50}$  values of 60, 43, 31 and 10.5  $\mu\text{g}/\text{ml}$ , respectively compared to synthetic antioxidant BHT (19  $\mu\text{g}/\text{ml}$ ).

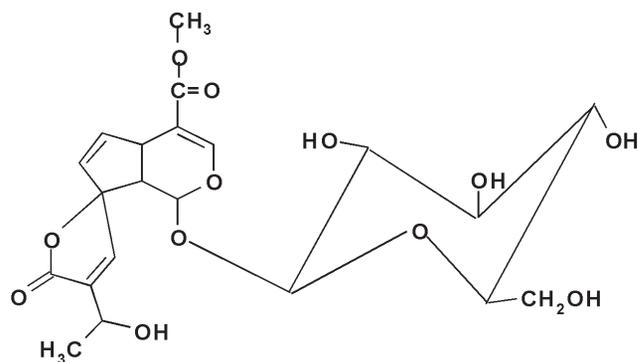


Fig. 1. Chemical structure of AC1 (Plumeiridine-5-o-glucoside)

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