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Drug Monitoring and Toxicology

Nootropic Activity of Morus Alba

Highlights

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Discovering Thoughts, Inventing Future

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PHARMA, DRUG DISCOVERY, TOXICOLOGY & MEDICINE



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Drug Monitoring and Toxicology: Quantification of Antifungal Drug Voriconazole in Human Plasma and Serum by High-Performance Liquid Chromatography with Fluorescence Detection

By Dr. Peter H. Tang

University of Cincinnati College of Medicine

Abstract- This paper describes a simple and rapid high-performance liquid chromatographic (HPLC) method with fluorescence detection (FL) for the determination of voriconazole concentration in human plasma and serum. Ketoconazole is selected as the internal standard. Acetonitrile alone is used to precipitate protein and extract voriconazole and ketoconazole in human plasma and serum using a single dilution step procedure. Following protein precipitation and extraction, voriconazole and ketoconazole in the extract are quantitated by injecting directly onto the HPLC system. Limit of quantitation and linearity (0.1-10 µg/mL) of the method adequately cover the therapeutic range for appropriate drug monitoring. This method has shown some essential improvements such as allowing a small portion of the extract to be analyzed (10 µL) and completing an isocratic chromatography in <7 min per injection when compared to most published HPLC/FL and HPLC/UV methods. This method would be of interest to analytical and clinical laboratories equipped with the HPLC/FL systems because it employs simple, rapid, and cost-effective procedures without time-consuming solvent evaporation and residual reconstitution.

Keywords: *antifungal drug, voriconazole, ketoconazole, HPLC, fluorescence.*

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Drug Monitoring and Toxicology: Quantification of Antifungal Drug Voriconazole in Human Plasma and Serum by High-Performance Liquid Chromatography with Fluorescence Detection

Dr. Peter H. Tang

Abstract- This paper describes a simple and rapid high-performance liquid chromatographic (HPLC) method with fluorescence detection (FL) for the determination of voriconazole concentration in human plasma and serum. Ketoconazole is selected as the internal standard. Acetonitrile alone is used to precipitate protein and extract voriconazole and ketoconazole in human plasma and serum using a single dilution step procedure. Following protein precipitation and extraction, voriconazole and ketoconazole in the extract are quantitated by injecting directly onto the HPLC system. Limit of quantitation and linearity (0.1-10 $\mu\text{g/mL}$) of the method adequately cover the therapeutic range for appropriate drug monitoring. This method has shown some essential improvements such as allowing a small portion of the extract to be analyzed (10 μL) and completing an isocratic chromatography in <7 min per injection when compared to most published HPLC/FL and HPLC/UV methods. This method would be of interest to analytical and clinical laboratories equipped with the HPLC/FL systems because it employs simple, rapid, and cost-effective procedures without time-consuming solvent evaporation and residual reconstitution.

Keywords: antifungal drug, voriconazole, ketoconazole, HPLC, fluorescence.

I. INTRODUCTION

Voriconazole (2R,3S-2-(2,4-difluorophenyl)-3-(5-fluoropyrimidin-4-yl)-1-(1H-1,2,4-triazol-1-yl)butan-2-ol) (Figure 1) is a triazole antifungal drug that is generally used to treat invasive fungal infections including invasive aspergillosis, invasive candidiasis, fusariosis, scedosporiosis, and severe fungal corneal infections. Voriconazole exhibits its antifungal activity by inhibiting fungal cytochrome P450-dependent 14α -sterol demethylase (1), an enzyme responsible for ergosterol biosynthesis, which leads to disruptions of the structure and function of the fungal cell membrane.

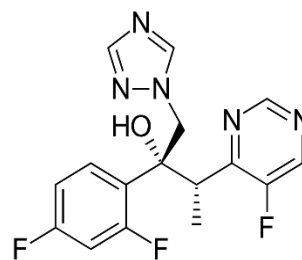


Figure 1: Chemical structure of voriconazole

Voriconazole is metabolized primarily by hepatic cytochrome P450 isoenzyme 2C19 (2), where CYP2C19 contributes largely to pharmacokinetic variability. This drug appears to display non-linear pharmacokinetics, most likely due to saturation of metabolism (3). Many patient factors such as body weight, age, sex, food, drug interactions, and hepatic disease state affect voriconazole plasma or serum concentrations, which leads to significant variability (4-11). In virtue of wide variability of voriconazole plasma or serum concentrations within and between patients, the use of therapeutic drug monitoring is strongly recommended. Determination of voriconazole concentration in plasma or serum is a most useful way for adjusting antifungal drug dosage, individualizing and improving the treatment regimen, and resulting in better efficacy and minimal side effects.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been the most powerful technique used for the determination of voriconazole concentration. LC-MS/MS or LC-MS methods require small sample sizes (12-17) and are superior in sensitivity and specificity. However, the purchase, maintenance, and running costs of LC-MS/MS and LC-MS are high. In contrast, high-performance liquid chromatography (HPLC) methods with fluorescence (FL) or ultraviolet (UV) detection (18-26) allow cost-effective operations and appropriate sensitivities for clinically relevant drug concentrations (1-5.5 $\mu\text{g/mL}$). A validated HPLC/UV method (26) has been previously developed at the Cincinnati Children's Hospital Medical Center (Cincinnati, OH) and used routinely for measuring voriconazole concentration in human plasma and

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serum. The validated HPLC/UV method has provided satisfactory service for therapeutic drug monitoring of voriconazole in patients. To meet increasing demands of drug monitoring and challenges from combination of antifungal therapy, an accurate and sensitive fluorescence detection for the determination of voriconazole concentration in human plasma or serum has come to the forefront. The development of an alternative assay is particularly critical to therapeutic drug monitoring of voriconazole, which accurately monitors plasma or serum drug concentrations in real time and allows more flexibility in managing test schedules and better turnaround time. In this paper, a new HPLC/FL method for the determination of voriconazole concentration in human plasma and serum is described.

II. EXPERIMENTAL

Chemicals and reagents: Voriconazole (analytical standard grade), ketoconazole (pharmaceutical secondary standard grade), trichloroacetic acid (TCA), trifluoroacetic acid (TFA), zinc sulfate heptahydrate and ammonium acetate were obtained from Sigma (St. Louis, MO). HPLC-grade acetone, acetonitrile, ethanol, methanol and n-propanol were purchased from Fisher Scientific (Hampton, NH). Lyphochek® Therapeutic

Drug Monitoring Control was from Bio-Rad (Hercules, CA).

Patient plasma or serum samples used for this study were de-identified and approved by the IRB. Normal pooled human plasma or serum (analyte-free) were used as blank. Ammonium acetate solution (0.1 M) was prepared by dissolving 7.7 g ammonium acetate in a 1000 mL deionized water. The mobile phase consisted of 0.1 M ammonium acetate solution, acetonitrile and TFA (409:590:1, v/v/v).

Instrumentation: Automated Hitachi Chromaster™ system (Tarrytown, NY) was equipped with Model 5110 quaternary pump, Model 5210 autosampler, Model 5310 column oven and Model 5440 FL detector. The EZChrom Elite® software was used for monitoring output signal and processing result. The analytical column was a 250-mm x 4.6-mm ODS HYPERSIL column (Thermo Scientific, Sunnyvale, CA) with 5-μm spherical particles connected to a Security Guard (Phenomenex, Torrance, CA) equipped with C18 cartridge (4-mm x 3-mm). MAGNA nylon filter (0.2-μm, 47-mm diameter) was from GE Water & Process Technologies (Minnetonka, MN) for the filtration of mobile phase. Details of HPLC settings are described in Table 1.

Table 1: HPLC Settings

Parameters	Conditions
Analytical column	ODS HYPERSIL, 5-μm, 250 x 4.6 mm
Mobile phase	Ammonium acetate buffer:acetonitrile:TFA (409/590/1, v/v/v)
Mode	Isocratic elution
Column oven	45° C
Autosampler	5° C
Flow rate	1.1 mL/min
Injection volume	10 μL
Detector settings	254 nm (excitation) and 372 nm (emission)
Chromatographic time	<7 min

Preparation of calibrator and quality control samples in plasma: Stock solutions of voriconazole and ketoconazole were prepared separately by dissolving pure chemical in acetonitrile to have a concentration of 100 μg/mL. Two series of calibrators for voriconazole in the range of 0.1–10 μg/mL were prepared separately by diluting the stock solution of voriconazole with normal pooled human plasma and serum, respectively. Quality controls (QCs) in normal pooled human plasma or serum were similarly prepared containing 0.3, 1, 4, and 8 μg/mL. All solutions were stored at refrigerator for 24 hrs, an aliquot of 100 μL calibrator or QC sample was then dispensed in a 1.5-mL polypropylene screw-top tube. Internal standard ketoconazole solution (1 μg/mL) was prepared in acetonitrile from the ketoconazole stock

solution. All solutions and samples were stored at –20 °C until required.

External standard calibration for voriconazole and ketoconazole: In these experiments, two series of calibrators for both voriconazole and ketoconazole were prepared separately by diluting their stock solutions with acetonitrile to produce final concentrations in the range of 0.1-10 μg/mL. External standard calibration curves for voriconazole and ketoconazole were individually generated by HPLC analysis of their fluorescent responses, respectively. Experiments were performed in triplicate. Linearity was assessed by a least squares linear regression of the analyte peak height versus the analyte concentration.

Protein precipitation and extraction procedures: To evaluate the protein precipitation and extraction efficiency of selected reagents, normal pooled human plasma or serum fortified with voriconazole (0.3, 1, 4, and 8 $\mu\text{g/mL}$) or ketoconazole (1 $\mu\text{g/mL}$) were used. Ketoconazole solution was prepared by diluting its stock solution (100 $\mu\text{g/mL}$) with normal pooled human plasma to give a final concentration of 1 $\mu\text{g/mL}$ and refrigerated for 24 hrs prior to analysis. The following reagents were selected and prepared as protein precipitants: TCA (10%, w/v), zinc sulfate heptahydrate (10%, w/v), acetone, acetonitrile, ethanol, methanol and n-propanol. Each precipitant was added to the fortified plasma or serum containing either voriconazole or ketoconazole in volume ratio of 3:1 in triplicate. Mixtures were vortexed for 1 min and centrifuged for 10 min at 10,000 rpm. The supernatant was transferred to an autosampler vial, capped, and 10 μL was injected directly onto the HPLC system. Concentrations of voriconazole and ketoconazole in supernatant were determined by using the external standard calibration curves for both analytes and compared to that of unextracted analytes where extraction efficiency = $\frac{[\text{extracted analyte}]}{[\text{unextracted analyte}]} \times 100$.

Internal standard calibration for voriconazole: Frozen calibrators of voriconazole (0.1–10 $\mu\text{g/mL}$) prepared in human plasma or serum in 1.5-mL polypropylene screw-top tubes were thawed at room temperature. The internal standard ketoconazole in acetonitrile, 100 μL of 1 $\mu\text{g/mL}$, was then added to each tube followed by 200 μL of acetonitrile. Mixtures were processed in the same manner as the fortified plasma and serum described in the aforementioned section for extraction and protein precipitation. Internal standard calibration curve for voriconazole was generated by a least squares linear regression of the voriconazole-internal standard peak height ratio versus the voriconazole concentration.

Sample preparation: The internal standard ketoconazole in acetonitrile, 100 μL of 1 $\mu\text{g/mL}$, was added to 100 μL each of patient plasma or serum, normal pooled human plasma or serum, or QC, and followed by adding 200 μL of acetonitrile. Mixtures were processed in the same manner as the fortified plasma described in the aforementioned section for extraction and protein precipitation procedures. The isolated supernatant (10 μL) was injected directly onto the HPLC system for measuring voriconazole and ketoconazole. After analyzing the voriconazole-ketoconazole peak height ratio, voriconazole concentration was determined from the internal standard calibration curve for voriconazole.

Selectivity: To explore possible interference, ten serum samples from patients treated with other drugs and the Lyphochek® Therapeutic Drug Monitoring Control were examined. Human blood and lyophilized products contains endogenous components, metabolites, decomposition products, concomitant medication or

exogenous xenobiotics. These subjects contained multiple substances such as acetaminophen, amikacin, amiodarone, amitriptyline, caffeine, carbamazepine, chloramphenicol, cortisol, cyclosporine, desipramine, digoxin, disopyramide, estriol, ethosuximide, felbamate, flecainide, gabapentin, gentamicin, haloperidol, imipramine, lacosamide, lamotrigine, levetiracetam, lidocaine, lithium, methotrexate, methsuximide, micafungin, milrinone, mycophenolic acid, mycophenolic acid glucuronide, N-acetylprocainamide, netilmicin, nortriptyline, oxcarbazepine, phenobarbital, phenytoin, posaconazole, primidone, procainamide, propranolol, quinidine, rufinamide, salicylate, T3, T4, theophylline, tobramycin, tricyclic antidepressants, thyroid-stimulating hormone, valproic acid, vancomycin and zonisamide. These subjects were treated as patient samples and processed in the same manner as described in the sample preparation section.

Accuracy and precision: Accuracy and precision of within-run or between-run were evaluated by six times processing and analyses of the LLOQ and four QCs (0.3, 1, 4 and 8 $\mu\text{g/mL}$). Accuracy was expressed as percentage of the deviation of mean from the true value, determined with the formula $\frac{[\text{mean measured concentration} - \text{true concentration}]}{\text{true concentration}} \times 100$, whereas precision was expressed as coefficient of variation (CV) calculated as follows: $[\text{CV}\% = (\text{standard deviation}/\text{mean of measured values}) \times 100]$. Criteria for accuracy is within $\pm 15\%$ deviation from the actual value except at the lower limit of quantitation (LLOQ), where it should not deviate by more than 20% (27). Criteria for precision is within 15% of the CV% except for the LLOQ, where it should not exceed 20% of the CV% (27).

Reproducibility and stability: To evaluate between-run reproducibility, the LLOQ and four QCs were analyzed on different days. Stabilities of voriconazole and ketoconazole in QCs were evaluated under a variety of storage and handling conditions: freeze-thaw cycles at room temperature; bench-top stability experiments were conducted at room temperature under normal laboratory light for up to 24 hrs; long-term stability (stored samples at $-20\text{ }^\circ\text{C}$ for 5 days and then thawed for 30 min at room temperature); the stability of stock solutions of voriconazole and ketoconazole were examined; and finally the stability of processed samples sitting on the bench-top for up to 24 hrs were examined.

Cross-validation: Cross-validation was performed on 30 patient samples which were separated into paired sets and stored at $-20\text{ }^\circ\text{C}$ until required. One set of samples was submitted to the HPLC/UV analysis. The second set of patient samples were analyzed using the current method. To confirm the reliability of this method, paired results from the two different methods were compared.

III. RESULTS AND DISCUSSION

Chromatography: Typical chromatograms obtained from a normal pooled human plasma, the 1.25 µg/mL calibrator, and a representative plasma of patient treated with voriconazole are shown in Figure 2. Peaks of voriconazole and internal standard ketoconazole were

eluted at ~4.0 and ~5.7 min, respectively. A chromatography was completed in <7 min. The current method allows good resolutions of voriconazole and ketoconazole without ambiguity in identification in the chromatogram.

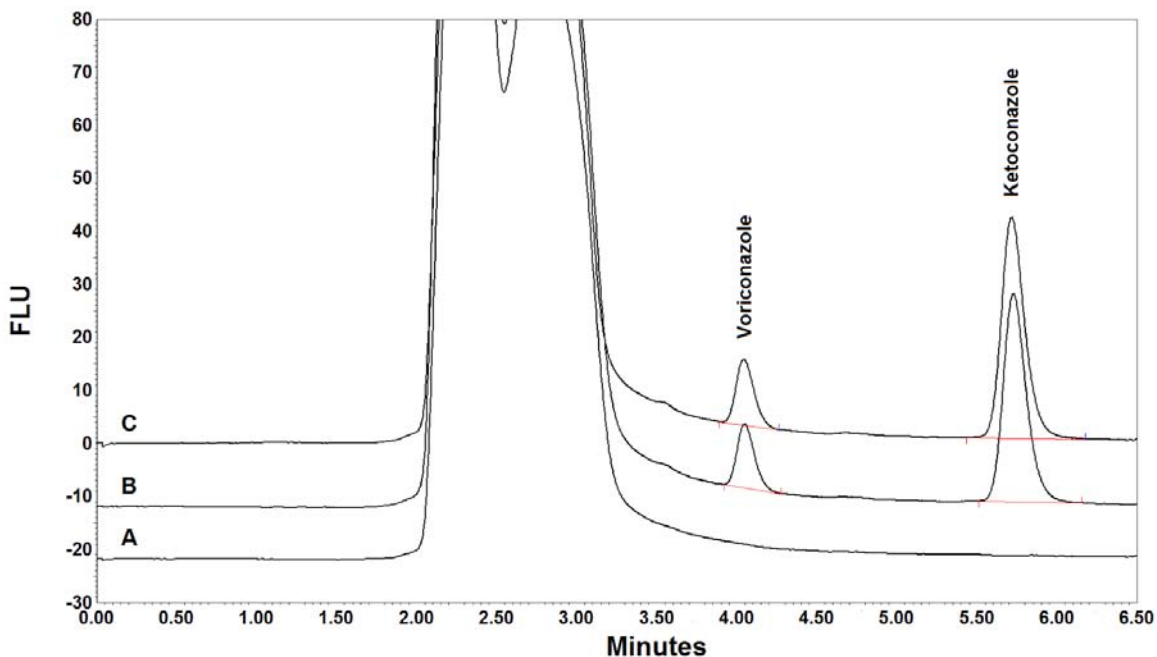


Figure 2: Chromatograms obtained from a normal pooled human plasma (A), the 1.25 µg/mL calibrator (B), and a representative plasma of patient treated with voriconazole (C) are shown. The retention times of voriconazole and ketoconazole were ~4.0 and ~5.7 min, respectively

Analytical method validation: Method validation was carried out according to the FDA Guidance for Industry Bioanalytical Method Validation (27). The fundamental parameters for the current bioanalytical method validation included selectivity, accuracy, precision, sensitivity, reproducibility, and stability. Measurements for voriconazole and ketoconazole in plasma and serum were validated. In addition, the stability of voriconazole in fortified samples was determined.

For selectivity, human plasma and serum obtained from ten patients were evaluated. In addition, lyophilized products such as the Lyphochek® Therapeutic Drug Monitoring Controls were also examined. Samples free of voriconazole and ketoconazole did not show any interference with the voriconazole and ketoconazole signals. None of other drugs tested showed chromatographic interference with voriconazole or ketoconazole. Selectivity was also ensured at the LLOQ. The ability of the current method to differentiate and quantify both voriconazole and ketoconazole in the presence of other components in human plasma and serum was proven.

Quantitative recovery of voriconazole in human plasma and serum has recently been achieved by using

methanol as an extracting solvent in a single dilution step (26). Most recently, a single dilution step procedure for simultaneous protein precipitation and analytes extraction has been proved as an excellent sample preparation for acquiring fast sample clean-up and disruption of protein-drug binding (28). It is evident that significant loss of analyte due to adsorption at the precipitate exists during protein precipitation. Therefore, addition of internal standard during sample preparation was necessitated to compensate the loss of analyte. After investigating several compounds, ketoconazole was found to best fit as an internal standard in this study. According to Polson *et al.* (29), the most efficient protein precipitants for protein removal were zinc sulfate, acetonitrile and TCA. These three precipitants were found to remove plasma protein effectively at 2:1 and greater volumes of precipitant to plasma. Therefore, the precipitant to plasma volume ratio of 3:1 was chosen in the current study. Seven protein precipitants were evaluated for their extraction efficiencies of voriconazole (4 µg/mL) and ketoconazole (1 µg/mL) in human plasma. The concentrations of extracted voriconazole and ketoconazole were determined by using the external standard calibration curves. The extraction efficiency

results for these protein precipitants are shown in Table 2. Each extraction efficiency is an average of three replicates.

Table 2: Comparison of extraction efficiency for various protein precipitants

Precipitants*	% Analyte extraction efficiency** (% RSD)		
	Voriconazole	Ketoconazole	Average
Acetonitrile	96.2 (1.5)	93.7 (0.8)	95.0
Methanol	95.6 (1.1)	90.5 (0.7)	93.1
Ethanol	93.9 (0.8)	90.9 (0.7)	92.4
Acetone	89.1 (1.9)	90.3 (1.1)	89.7
n-Propanol	89.6 (1.2)	87.7 (1.5)	88.6
Zinc sulfate (10%, w/v)	22.5 (2.8)	26.4 (2.0)	24.5
TCA (10%, w/v)	43.7 (2.0)	3.0 (1.5)	23.3
*Each precipitant was carried out three times (n = 3).			
**% Analyte extraction efficiency = ([extracted analyte]/[unextracted analyte]) x 100.			

With the exception of zinc sulfate and TCA, all protein precipitants were on average at least 88% effective in extraction. Both zinc sulfate and TCA had decreased concentrations of voriconazole and ketoconazole in the supernatant due to the loss of analytes by adsorption at the precipitate. Because voriconazole and ketoconazole are more soluble in organic solvents, both analytes were effectively extracted by the organic solvents. Acetonitrile, aside from being excellent in precipitating protein, was chosen based on its optimal extraction efficiency toward voriconazole and ketoconazole. It is believed that recovery pertains to the extraction efficiency of an analytical method. Recovery of voriconazole from human plasma and serum were carried out in triplicate by comparing the analytical results for extracted drug at four concentrations (0.3, 1, 4 and 8 µg/mL) with unextracted drug. Recoveries of voriconazole from plasma and serum were similar at four concentrations, and the mean recovery of voriconazole was in the range 94.7-98.1%.

The internal standard calibration curve of voriconazole prepared in plasma was linear ($r^2 > 0.99$) over the concentration range of 0.1 to 10 µg/mL. The slopes of internal standard calibration curves in the six different preparations for voriconazole were practically the same. The mean linear regression equation of internal standard calibration curve was $y = 8.615x - 0.0302$, where y represents the concentration of voriconazole and x represents the ratio of voriconazole peak height to that of the internal standard. The LLOQ was 0.1 µg/mL, whereas the limit of detection (LOD) was 0.04 µg/mL. The internal standard calibration curve prepared in serum was $y = 8.628x - 0.0413$, while the internal standard calibration curve prepared in acetonitrile was $y = 8.128x - 0.013$. All calibration curves were linear ($r^2 > 0.99$). However, matrix effects

were detected when compared the slopes of calibration curves prepared in plasma or serum with that prepared in acetonitrile ($p < 0.05$). Since the plasma and serum calibration curves were parallel, no matrix effects was detected between plasma and serum. As such, the linear regression of the plasma curve was used to derive the voriconazole concentrations in human plasma and serum.



Table 3: Within-run and between-run precision and accuracy

Sample	Nominal ($\mu\text{g/mL}$)	Found ($\mu\text{g/mL}$)	Accuracy (% Bias)	Precision (% CV)
Within-run variability (n = 6 at each concentration)				
LLOQ	0.10	0.098 \pm 0.008	-2.5	8.2
QC1	0.30	0.295 \pm 0.022	-1.7	7.5
QC2	1.00	1.01 \pm 0.035	1.4	3.5
QC3	4.00	4.08 \pm 0.053	2.1	1.3
QC4	8.00	8.01 \pm 0.196	0.2	2.4
Between-run variability (n = 6 at each concentration)				
LLOQ	0.10	0.097 \pm 0.014	-3.1	14.4
QC1	0.30	0.305 \pm 0.028	1.7	9.2
QC2	1.00	0.961 \pm 0.043	-3.9	4.5
QC3	4.00	4.03 \pm 0.207	0.85	5.1
QC4	8.00	7.89 \pm 0.461	-1.4	5.8

The accuracy and imprecision were evaluated at the LLOQ and four QC concentrations (Table 3). Bias was calculated as the found minus the nominal concentration, expressed as a percentage of the nominal concentration. Imprecision was calculated as within- and between- runs coefficient of variation (CV). The bias for LLOQ and QCs were <4%. Imprecision was <15% at the LLOQ and QC concentrations, as indicated

by both within- and between- runs. For QCs, within-run precision was between 1.3% and 7.5% and between-run precision was between 4.5 and 9.2%. Overall accuracy was between 96.1% and 102.1%. The minimal deviation of the mean from the true value indicates the excellent accuracy of the method. Table 4 provides the method reproducibility performed by five technicians for patient samples. Overall CVs were less than 9%.

Table 4: Method reproducibility performed by five technicians

Sample	Replicate	Found ($\mu\text{g/mL}$)	Precision (% CV)
Patient #1	5	1.23 \pm 0.101	8.2
Patient #2	5	6.86 \pm 0.349	5.1
Patient #3	5	3.05 \pm 0.146	4.8
Patient #4	5	0.994 \pm 0.085	8.6
Patient #5	5	4.41 \pm 0.192	4.4
Patient #6	5	2.58 \pm 0.154	6

The stability of QC samples at -20°C was evaluated at 2-week intervals for 3 months. The stability of stock standard solutions at -20°C for 6 months was evaluated. The effects of freezing and thawing on voriconazole were studied using QC samples, which were subjected three free-thaw cycles before analysis (freeze samples at -20°C and then thaw for 30 min at room temperature). The stability of the processed samples sitting at 5°C (the temperature of the autosampler) for 24 hrs were evaluated. Voriconazole and internal standard ketoconazole were stable in all storage and handling conditions.

voriconazole concentrations ranging from 0.1 to 7.2 $\mu\text{g/mL}$. Results from the current method were compared with data generated using a validated HPLC-UV method (26) served as the reference. These data are shown in Figure 3. The least squares linear regression equation for correlation where y is the current method and x is the reference method described here was $y = 1.024x - 0.018$ where $r^2 = 0.981$ with a standard error value of 0.027.

Cross-validation: A cross-validation was also carried out, using a total of 30 de-identified patient samples with

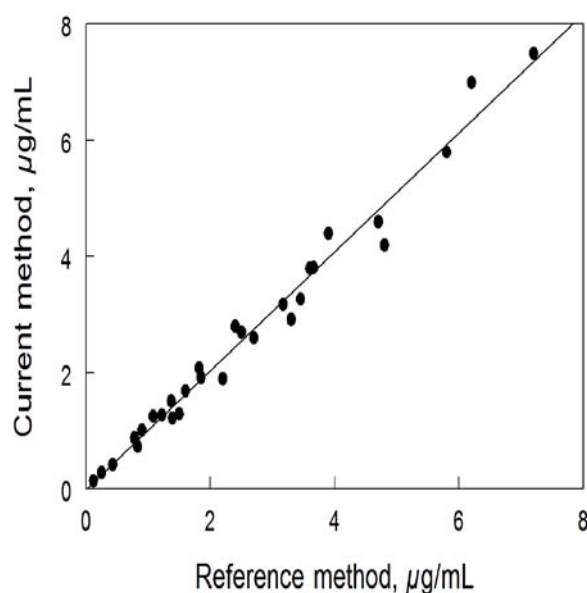


Figure 3: Method comparison of results obtained with 30 de-identified patient samples are plotted with regression statistics. The voriconazole plasma concentrations ($\mu\text{g/mL}$) are shown for each sample analyzed in both methods

Analytical method comparison: Up to now, three HPLC/FL methods (18-20) for measuring voriconazole in biological fluids have been previously described. Michael *et al.* (18) reported the first HPLC-FL method for the determination of voriconazole in 0.3 mL of human plasma or saliva by using an internal standard UK-115 794 which was not readily available. Human sample was extracted twice with *n*-hexane-ethyl acetate, two extracts were then combined and evaporated to dryness. The residue was reconstituted and 20 μL of reconstituent was injected onto the HPLC system with a run time of 12 min. Heng *et al.* (19) described a procedure to manipulate 4 mL of human bronchoalveolar lavage fluid by using a freeze dryer. After 48 hrs of drying, the lyophilized powder was reconstituted with water and extracted with acetonitrile. Following centrifugation, an aliquot (70 μL) of the extract was injected onto the HPLC system. Ogata *et al.* (20) used ethyl acetate to extract voriconazole and naproxen (as an internal standard) from 0.2 mL of human plasma. After protein precipitation, tedious procedures such as evaporation of ethyl acetate supernatant to dryness and reconstitution of residue with mobile phase were used for sample preparation prior to HPLC analysis. Finally, a lengthy chromatography of longer than 22 min per injection was required for the analysis. In comparison with the reported HPLC/FL methods, the current method employs a single dilution step with acetonitrile for protein precipitation and extraction. Once again, a single dilution step procedure proved to be the most rapid and simplest procedure for sample preparation. The current method only needed a small injection volume (10 μL) and small sample volume (0.1 mL) to achieve the LLOQ of 0.1 $\mu\text{g/mL}$ (1 ng on column). Injection of a small portion of extract (10 μL) has allowed the HPLC system

to load ~1500 injections without replacing pre-filter columns. It is cost-effective and time-saver, because the replacements of pre-filter columns are frequently required after loading up to ~700 injections with large portions of extract (20 μL or greater) per injection. Finally, the current method completes a chromatographic run in <7 min per injection with the optimized conditions.

IV. CONCLUSION

A validated HPLC/FL method for the determination of voriconazole concentration in human plasma or serum has been described. Ketoconazole is readily available and has been successively used as an internal standard. The current method uses acetonitrile as protein precipitant and extraction solvent in a single dilution step procedure which provides rapid sample clean-up and excellent extraction efficiency. By avoiding complex liquid-liquid extraction, tedious solid-phase extraction, evaporation of extract or supernatant, and residual reconstitution procedures, the current method substantially decreases set-up time. This method is simple, rapid, sensitive, accurate and practical for use in the analytical and clinical laboratories for therapeutic drug monitoring of voriconazole in human plasma and serum.

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Role of Heme-Oxygenase-1 in Attenuated Cardioprotective Effect of Ischemic Preconditioning in Hyperlipidemic Myocardium

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Abstract- Nitric Oxide (NO) is responsible for cardioprotective effect of ischemic preconditioning (IPC). Heme Oxygenase-1 (HO-1) facilitates release of NO by disrupting caveolin-eNOS complex. Both, the expression/activity of HO-1 and the IPC mediated cardioprotection are decreased significantly in hyperlipidemia. In this study the role of HO-1 in attenuation of IPC- induced cardioprotective effect in hyperlipidemic rat was investigated. Hyperlipidemia was induced by feeding high fat diet to Wistar rats. Isolated Langendorff heart preparation model was used. Cardioprotective effect was assessed by myocardial infarct size measurement and release of Lactate Dehydrogenase (LDH), Creatine Kinase (CK-MB) in coronary effluent. Nitrite estimation was done to indirectly infer the level of cardiac NO production. In hyperlipidemic rat, IPC-induced cardioprotection and release of NO were significantly decreased. Perfusion with sodium nitrite (NO precursor) and pre-treatment with daidzein (DDZ) (caveolin inhibitor) and hemin (HO-1 inducer), alone or in combination significantly restored the attenuated cardioprotective effect of IPC in hyperlipidemic rats. Administration of zinc protoporphyrin (ZnPP), HO-1 inhibitor, significantly abolished the observed cardioprotection in hemin pre-treated hyperlipidemic rat. The significant restoration of the attenuated cardioprotective effect of IPC following induction of HO-1 by hemin in hyperlipidemia was observed. The results indicated that attenuation of IPC-induced cardioprotective effect may be due to the decrease in HO-1 induced NO release in hyperlipidemic rat heart.

Keywords: heme oxygenase-1, ischemic preconditioning, hyperlipidemic rat heart, hemin, daidzein.

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Role of Heme-Oxygenase-1 in Attenuated Cardioprotective Effect of Ischemic Preconditioning in Hyperlipidemic Myocardium

Atinderpal Kaur ^α, Tapan Behl ^σ, H. N. Yadav ^ρ & PL Sharma ^ω

Abstract- Nitric Oxide (NO) is responsible for cardioprotective effect of ischemic preconditioning (IPC). Heme Oxygenase-1 (HO-1) facilitates release of NO by disrupting caveolin-eNOS complex. Both, the expression/activity of HO-1 and the IPC mediated cardioprotection are decreased significantly in hyperlipidemia. In this study the role of HO-1 in attenuation of IPC-induced cardioprotective effect in hyperlipidemic rat was investigated. Hyperlipidemia was induced by feeding high fat diet to Wistar rats. Isolated Langendorff heart preparation model was used. Cardioprotective effect was assessed by myocardial infarct size measurement and release of Lactate Dehydrogenase (LDH), Creatine Kinase (CK-MB) in coronary effluent. Nitrite estimation was done to indirectly infer the level of cardiac NO production. In hyperlipidemic rat, IPC-induced cardioprotection and release of NO were significantly decreased. Perfusion with sodium nitrite (NO precursor) and pre-treatment with daidzein (DDZ) (caveolin inhibitor) and hemin (HO-1 inducer), alone or in combination significantly restored the attenuated cardioprotective effect of IPC in hyperlipidemic rats. Administration of zinc protoporphyrin (ZnPP), HO-1 inhibitor, significantly abolished the observed cardioprotection in hemin pre-treated hyperlipidemic rat. The significant restoration of the attenuated cardioprotective effect of IPC following induction of HO-1 by hemin in hyperlipidemia was observed. The results indicated that attenuation of IPC-induced cardioprotective effect may be due to the decrease in HO-1 induced NO release in hyperlipidemic rat heart.

Keywords: heme oxygenase-1, ischemic preconditioning, hyperlipidemic rat heart, hemin, daidzein.

1. INTRODUCTION

Coronary artery disease is a leading cause of morbidity and mortality worldwide [1, 2]. Inadequate blood flow to the myocardium leads to ischemia and, early reperfusion is necessary for the viability of myocardium [3]. Reperfusion after a prolonged period of ischemia is not without risk, it damages the myocardium, which is known as ischemia-reperfusion injury [4, 5]. Ischemic preconditioning (IPC), is a powerful endogenous cardioprotective phenomenon in which short intermittent cycles of sublethal ischemia, followed by reperfusion before the

subsequent prolonged ischemic insult, improves the tolerance against ischemia-reperfusion-induced injury [6, 7]. IPC mediated cardioprotection has been documented in various species including human beings [7, 8].

IPC produces cardioprotection by stimulating the generation of various endogenous ligands which bind to their respective G-protein coupled receptors [9, 10] and initiate a signalling cascade i.e., activation of PI-3K/Akt [11], phosphorylation of eNOS, generation of NO and by opening of mito K_{ATP} channel [12, 13]. The cardioprotective effect of IPC is attenuated in hyperlipidemic myocardium and it may be due to decreased HO-1 [14, 15], impairment of K_{ATP} channel [16] impairment of PI-3K/AKT pathway [17, 18] and altered activation of JAK/STAT and MAPK, GSK-3 β [19, 20]. Hence, the mechanism involved in attenuation of cardioprotective effect of IPC in hyperlipidemic myocardium, remain to be elucidated.

Caveolae are the specialised membrane domains which serve as organizing centres for cellular signal transduction [21]. Various signalling molecules like src-like kinases, tyrosine kinase, members of Ras-MAPK cascade and eNOS [22] are localized within caveolae. Caveolin is also a well known negative regulator of eNOS and these results in decreased availability of NO [23, 24] which is responsible for cardioprotective effect of IPC [13]. It has been reported that expression of caveolin is upregulated in hyperlipidemic myocardium [25].

Heme-oxygenase is the rate-limiting enzyme in the biochemical pathway responsible for catabolism of heme into ferrous (Fe^{++}) ion, carbon monoxide, and biliverdin, the latter being subsequently converted into bilirubin by biliverdin reductase [26]. HO-1 is localized in the membrane caveolae and the inner leaflet of the plasma membrane where it is interacts with caveolin [27]. In transgenic mice, the overexpression of Heme-oxygenase-1, conversely regulates the expression of caveolin [25]. Moreover, HO-1 facilitates release of NO by disrupting association of caveolin with eNOS [25]. It has been reported that a decrease in the cardiospecific expression of HO-1 exacerbates the ischemia reperfusion-induced injury [26], while upregulation of HO-1 produces cardioprotection against ischemia-reperfusion induced injury [27]. Transgenic mice

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expressing cardiac-specific HO-1 are resistant, while the heart of HO-1 knock-out mice is more susceptible to ischemia-reperfusion-induced injury [28]. In hyperlipidemia, the expression and activity of HO-1 is reduced [29] whereas the increase in HO-1 in hyperlipidemic rats is associated with activated eNOS [30]. Therefore, the present study was designed to investigate the role of Heme- Oxygenase-1 in attenuated cardioprotective effect of IPC in hyperlipidemic rat hearts.

II. MATERIALS AND METHODS

Daidzein (0.2mg/Kg/s.c) (Enzo Life Sciences International, Inc., USA) was dissolved in 10% Dimethyl Sulphoxide (DMSO) and then injected to the animals for 7 days after 8 weeks of high fat diet administration. Hemin (4mg/kg/i.p.) (Himedia Laboratories Pvt. Ltd., Mumbai) was dissolved in 0.2M NaOH and was injected 18 h before isolation of heart. Zinc Protoporphyrin (50µg/kg/i.p.) (Enzo Life Sciences International, Inc., USA) was dissolved in DMSO and injected 6 hr before hemin treatment [31]. TTC Stain, Tris-chloride buffer, sulphanilamide, phosphoric acid and sodium nitrite was purchased from CDH Pvt. Ltd., New Delhi. N-(1-Naphthyl) ethylenediamine dihydrochloride was purchased from Himedia Laboratories Pvt. Ltd., Mumbai. The LDH enzymatic estimation kit and CK-MB enzymatic estimation kit was purchased from Coral Clinical Systems, Goa, India. All other reagents used in this study were of analytical grade and always freshly prepared before use.

a) Animals

Age matched young male Wistar rats, weighing 180-250 g housed in animal house and provided 12 h light and 12 h dark cycle were used. They were fed on standard chow diet (Ashirwad Industries Ltd., Ropar, India) and provided water ad libitum. The experimental protocol was approved by the Institutional Animal Ethics Committee in accordance with the National (CPCSEA) Guidelines on the Use of Laboratory animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

b) Induction of experimental hyperlipidaemia

Male Wistar rats (180-250) were employed in the present study. Experimental hyperlipidemia was induced by high fat diet (corn starch 44.74 g, casein 14 g, sucrose 10 g, butter 20 g, fibre 5 g, mineral mix 3.5 g, vitamin mix 1 g, choline 0.25 g, terbutylhydroquinone 0.0008 g, cholesterol 1 g, cholic acid 0.5 g) for 8 weeks. Serum cholesterol and triglyceride level was estimated spectrophotometrically at 505 nm by PEG and GPO / PAP method (Trinder, 1969; Bucolo, 1973, Fossati, 1982) using enzymatic kits (Coral Clinical Systems, Goa, India). Serum cholesterol level 800- 1000 mg/dl and

serum triglyceride level 200- 300 mg/dl were considered to be hyperlipidemic.

c) Isolated rat heart preparation

Rats were administered heparin (500 IU/L, i.p) 20 min. prior to sacrificing the animal by cervical dislocation. Heart was rapidly excised and immediately mounted on Langendorff's apparatus [30]. Isolated heart was retrogradely perfused at constant pressure of 80 mmHg with Krebs-Henseleit buffer (NaCl 118 mM; KCl 4.7 mM; CaCl₂ 2.5 mM; MgSO₄.7H₂O 1.2 mM; KH₂PO₄ 1.2 mM; C₆H₁₂O₆ 11 mM), pH 7.4, maintained at 37°C bubbled with 95% O₂ and 5% CO₂. Flow rate was maintained at 7-9 ml/min. using Hoffman's screw. The heart was enclosed in double wall jacket, the temperature of which was maintained by circulating water heated at 37°C. Ischemic preconditioning was produced by closing the inflow of K-H solution for 5 min followed by 5 min of reperfusion. Four such episodes were employed. Global ischemia was produced for 30 min. followed by 120 min. of reperfusion. Coronary effluent was collected before ischemia, immediately, 5 min. and 30 min. after reperfusion for estimation of LDH, CK-MB and nitrite release [32].

d) Assessment of myocardial injury

The assessment of myocardial infarct size was done by using triphenyltetrazolium chloride (TTC) staining method. The heart was removed from the Langendorff's apparatus. Both the atria and root of aorta were excised and ventricles were kept overnight at -4°C temperature. Frozen ventricles were sliced into uniform sections of about 1-2 mm thickness. The slices were incubated in 1% w/v triphenyltetrazolium chloride stain (TTC stain) at 37°C in 0.2M Tris-chloride buffer for 30 min. The normal myocardium was stained brick red while the infarcted portion remained unstained. Infarct size was measured by the volume method [33]. LDH and CK-MB were estimated by using commercially available kits. Values of LDH and CK-MB were expressed in international units per litre (IU/L).

e) Nitrite estimation

Nitrite is stable nitrogen intermediate formed from the spontaneous degradation of NO. Unlike NO, nitrite can be measured easily and nitrite concentrations can be used to infer levels of NO production. Nitrite release in coronary effluent was measured. Greiss reagent 0.5 ml (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride in water) was added to 0.5 ml of coronary effluent. The optical density at 550 nm was measured using spectrophotometer (UV-1700 Spectrophotometer, Shimadzu, Japan). Nitrite concentration was calculated by comparison with spectrophotometer reading of standard solution of sodium nitrite prepared in K-H buffer [32].

f) *Experimental design (n= 72; each group contained 6 rats)*

Group 1 Sham control.



Group 2 Diadzein per se



Group 3 Hemin per se.



Group 4 Ischemia reperfusion control.



Group 5 Ischemic preconditioning control.



Group 6 IPC in sodium nitrite perfused normal rat hearts.



Group 7 Ischemic preconditioning in hyperlipidemic rat heart.



Group 8 IPC in sodium nitrite perfused hyperlipidemic rat hearts.



Group 9 IPC in diadzein pretreated hyperlipidemic rat heart.



Group 10 IPC in Hemin (4 mg/kg i.p) pretreated Hyperlipidemic rat heart.



Group 11 IPC in Diadzein and Hemin pretreated Hyperlipidemic rat heart.



Group 12 IPC in Zn protoporphyrin (50µg /kg i.p) and hemin (4mg/kg i.p) pretreated hyperlipidemic rat heart.



Diagrammatic representation of experimental protocol is shown. In all groups, isolated rat heart was perfused with K-H (Krebs-Hensleit) solution and allowed for 10 min of stabilization. Isolated rat heart preparation was stabilized for 10 min and then perfused continuously with K-H solution for 190 min.

g) *Data analysis and statistical procedures*

All values were expressed as mean ± standard deviation (S.D). Statistical analysis was performed using Graphpad Prism Software (5.0). The data obtained from the various groups were statistically analysed using student t-test, one-way analysis of variance (ANOVA), two way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. P < 0.05 was considered to be statistically significant.



III. RESULTS

a) *Effect of high fat diet on body weight*

The high fat diet was fed for 8 weeks to the rats and a significant increase in body weight was observed as compared to basal value (Fig. 1).

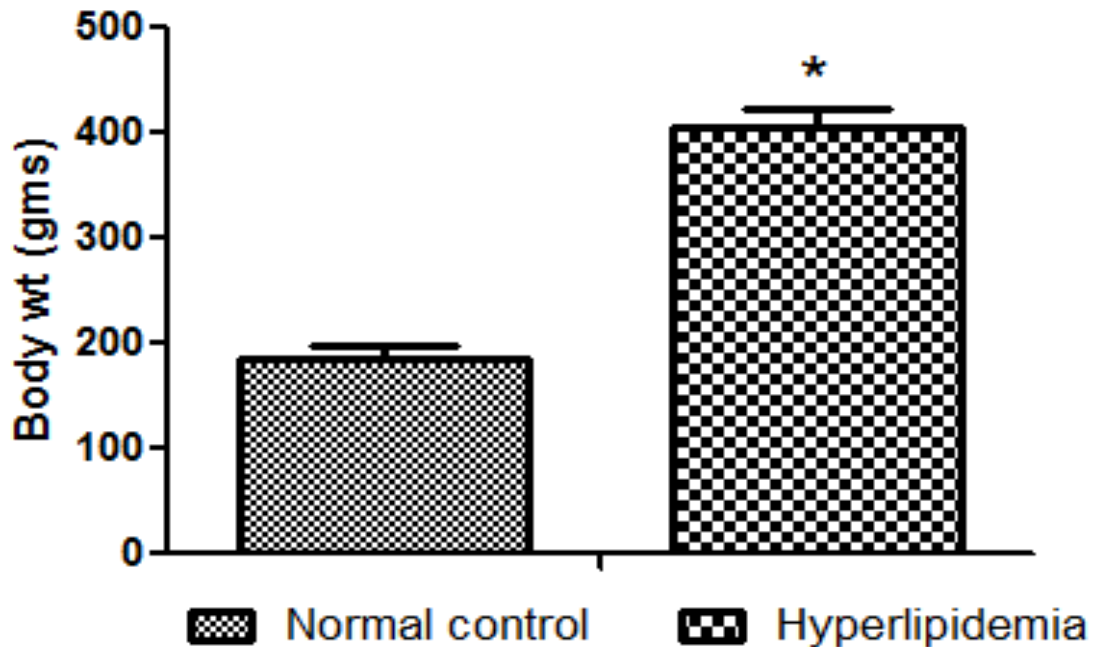


Fig. 1: Effect of high fat diet administration on serum body weights of rats.

Values were expressed as mean \pm S.D.

b) *Effect of high fat diet on serum cholesterol and triglyceride level*

After feeding high fat diet for 8 weeks to the rats, serum cholesterol and serum triglycerides level was estimated and a significant increase in serum cholesterol and serum triglyceride level was observed as compared to basal value. (Fig. 2)

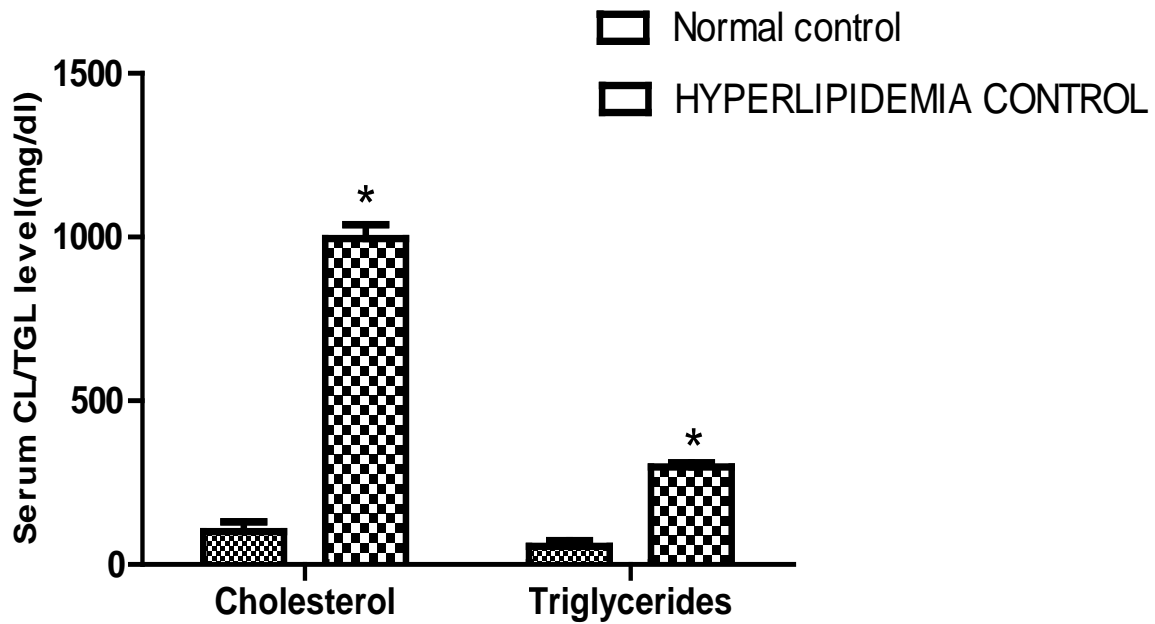


Fig. 2: Comparison of serum cholesterol and triglyceride level in normal and hyperlipidemic rat hearts.

Values are expressed as mean ± S.D.

c) Effect of ischemic preconditioning and pharmacological interventions on myocardial injury (Infarct size, LDH and CK-MB)

Global ischemia for 30 min followed by 120 min of reperfusion significantly increased the myocardial injury as compared to sham control. Four episodes of IPC significantly decreased I/R-induced increase in myocardial injury in normal rat heart. However, ischemic preconditioning failed to decrease the myocardial injury

in hyperlipidemic rat heart. Moreover, IPC induced decrease of myocardial injury was significantly restored in sodium nitrite perfused hyperlipidemic rat heart. Similar results were obtained in daidzein and hemin, administered alone or in combination. Administration of ZnPP significantly blocked the observed cardioprotective effect in hemin in hyperlipidemic rat (Fig. 3, 4, 5).

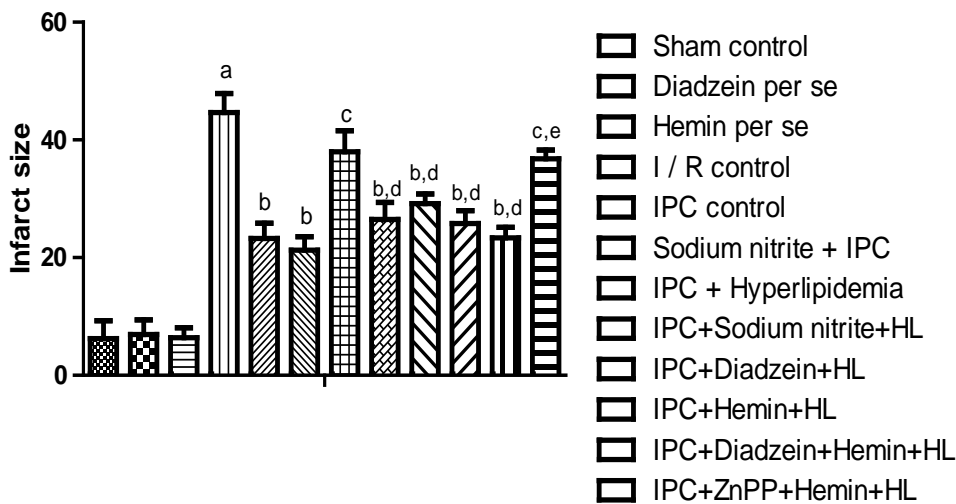


Fig. 3: Effect of pharmacological interventions on myocardial infarct size.

Values are expressed as mean ± S.D. a = p < 0.05 vs. Sham Control and basal value; b = p < 0.05 vs. I/R Control; c = p < 0.05 vs. IPC control; d = p < 0.05 vs. IPC in hyperlipidemic rat heart; e = p < 0.05 vs. IPC in hemin treated hyperlipidemic rat heart.

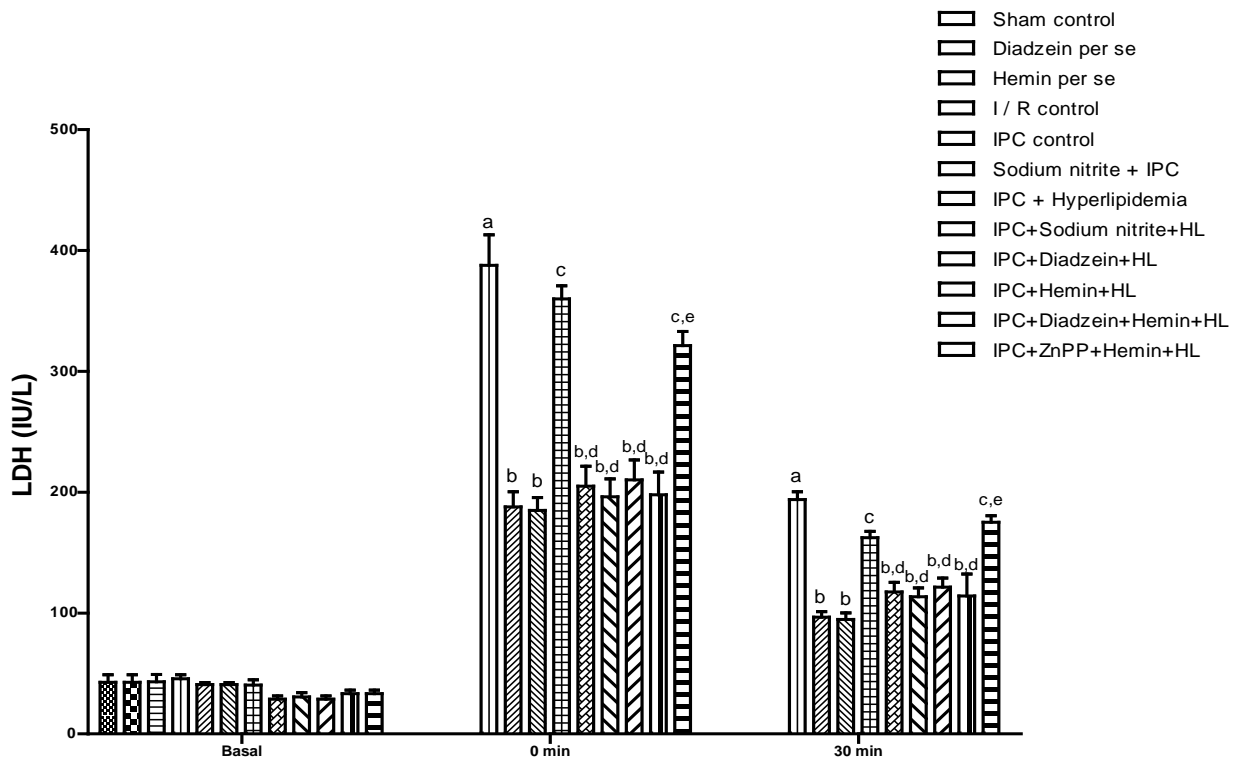


Fig. 4: Effect of pharmacological interventions on myocardial release of lactate dehydrogenase (LDH).

Values are expressed as mean \pm S.D. a = $p < 0.05$ vs. Sham Control and basal value; b = $p < 0.05$ vs. I/R Control; c = $p < 0.05$ vs. IPC control; d = $p < 0.05$ vs. IPC in hyperlipidemic rat heart; e = $p < 0.05$ vs. IPC in hemin treated hyperlipidemic rat heart.

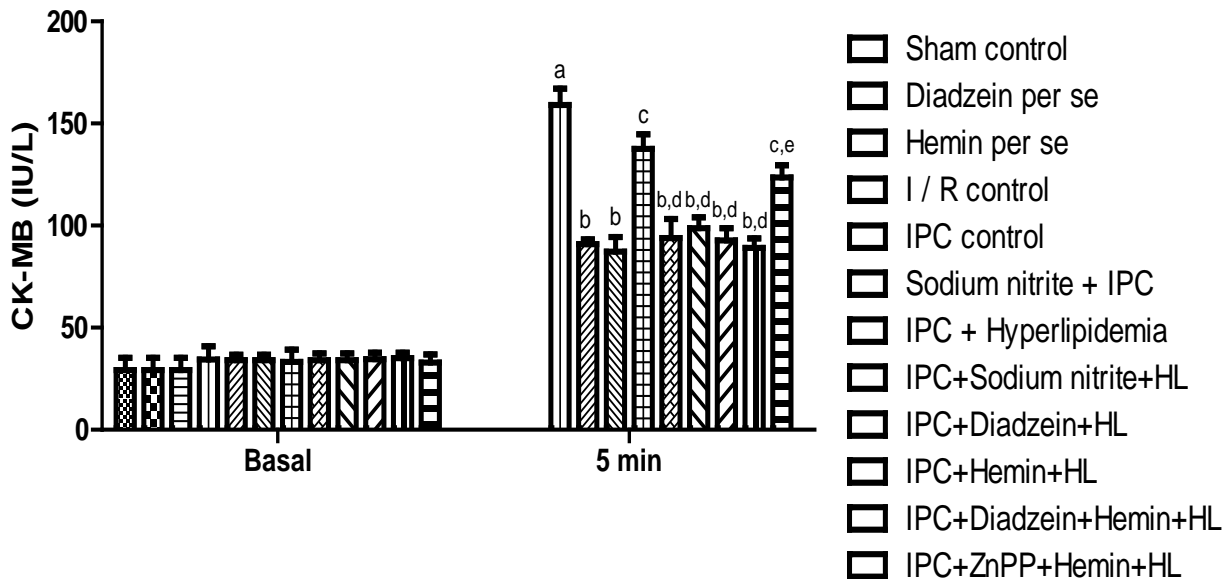


Fig. 5: Effect of pharmacological interventions on myocardial release of Creatine Kinase (CK-MB).

Values are expressed as mean \pm S.D. a = $p < 0.05$ vs. Sham Control and basal value; b = $p < 0.05$ vs. I/R Control; c = $p < 0.05$ vs. IPC control; d = $p < 0.05$ vs. IPC in hyperlipidemic rat heart; e = $p < 0.05$ vs. IPC in hemin treated hyperlipidemic rat heart.

d) Effect of ischemic preconditioning and pharmacological interventions on the nitrite release in coronary effluent

Global ischemia for 30 min followed by 120 min of reperfusion significantly decreased the nitrite release. Four episodes of IPC significantly restored the I/R induced decrease in nitrite release in normal rat heart. However, ischemic preconditioning failed to increase the

nitrite release in hyperlipidemic rat heart. Moreover, IPC induced increase of nitrite release was significantly restored in sodium nitrite perfused hyperlipidemic rat heart. Pre-treatment with daidzein and hemin, alone or in combination also restored it. Furthermore, administration of ZnPP significantly abolished the restored cardioprotective effect of hemin in hyperlipidemic rat (Fig.6).

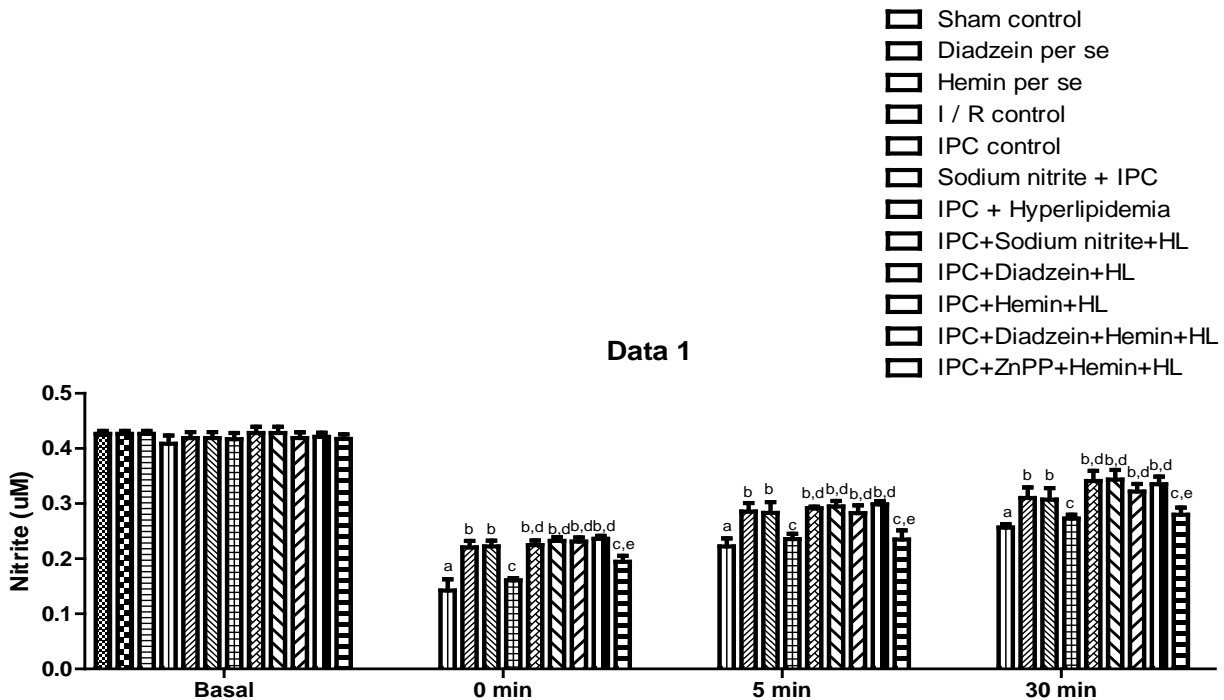


Fig. 6: Effect of pharmacological interventions on myocardial release of nitrite.

Values are expressed as mean ± S.D. a = p < 0.05 vs. Sham Control and basal value; b = p < 0.05 vs. I/R Control; c = p < 0.05 vs. IPC control; d = p < 0.05 vs. IPC in hyperlipidemic rat heart; e = p < 0.05 vs. IPC in hemin treated hyperlipidemic rat heart.

IV. DISCUSSION

This study was designed to investigate the role of HO-1 in attenuated cardioprotective effect of IPC in hyperlipidemic rat hearts. After a prolonged period of ischemia, reperfusion produces further damage to myocardium which is known as ischemia reperfusion injury. The ischemic preconditioning induced by four episodes of 5 min global ischemia and 5 min reperfusion was reported to produce cardioprotective effect in isolated rat heart preparation [34]. Our findings were in agreement with these phenomenon's. The cardioprotective effect of IPC had been reported to be significantly attenuated in hyperlipidemia. Our results were in accordance with these published studies [35].

Perfusion of sodium nitrite (NO donor) produces cardioprotection in isolated heart from normal rat, subjected to global ischemia [36]. In our study, perfusion of sodium nitrite in isolated hyperlipidemic rat heart followed by IPC, significantly restored the attenuated effect of IPC in diabetic myocardium.

Release of nitric oxide during the ischemic preconditioning was reported to produce cardioprotection against ischemia-reperfusion induced injury [12]. In our study, IPC significantly increased the release of NO (measured in coronary effluent), as compared to ischemia reperfusion control group. However, this IPC mediated increase in release of nitric oxide was significantly decreased in hyperlipidemic rat heart. Sodium nitrite perfusion in hyperlipidemic rat heart significantly restored the attenuated cardioprotective effect of ischemic preconditioning. Thus, the reduced release of NO in hyperlipidemic rat heart may be responsible for attenuation of cardioprotection mediated by IPC in hyperlipidemic rat. It was interesting to note that treatment with sodium nitrite did not enhance the cardioprotective effect of IPC in normal rat heart. This indicated that once IPC mediated increased generation of NO achieved the threshold for cardioprotection; addition of sodium nitrite was unable to further increase the myocardial protection by IPC.

Caveolae are 50-100 nm invaginated plasma membrane domains which serve as organizing centers of signal transduction [37]. Caveolins are proteins that form the structure of caveolar membrane, act as signalosomes for GPCR and other molecules such as NOS and Src-like kinases [38]. Increased expression of caveolin, leads to the, decreased phosphorylation of endothelial nitric oxide synthase and consequent decreased generation of nitric oxide. Further, it has been reported that expression of caveolin is upregulated in hyperlipidemic myocardium [39]. Thus, it may results in increased formation of Caveolin-eNOS complex, which decreases the availability of nitric oxide. It has been reported that NO is responsible for cardioprotective effect of ischemic preconditioning [40].

Upregulation of caveolin in diabetic rat heart may inhibit the activity of eNOS by making its complex which leads to a decrease in the release of NO [41]. Administration of daidzein increases the generation of nitric oxide by inhibiting the caveolin-eNOS complex and subsequent activation of the eNOS [42]. In our study, one week of pretreatment of hyperlipidemic rat with daidzein, a caveolin inhibitor [42], significantly restored the cardioprotective effect of ischemic preconditioning in hyperlipidemic rat heart, noted in terms of decrease in infarct size and release of LDH, CKMB, and also increase in the release of NO. Our findings were in agreement with reports from other laboratories [20].

Heme-Oxygenase-1 is localized in the membrane caveolae of the plasma membrane where it is interacts with caveolin [27]. It has been reported that a decrease in the cardiospecific expression of HO-1 exacerbates while an upregulation of HO-1 produces cardioprotection against ischemia-reperfusion injury [43]. HO-1 facilitates release of NO by disrupting complex of caveolin and eNOS [43]. The expression of HO-1 is diminished into hyperlipidemic myocardium. In our study, pretreatment with hemin, a heme-oxygenase-1 inducer, restored the decrease in release of nitric oxide and significantly restore the attenuated cardioprotective effect of ischemic preconditioning in hyperlipidemic rat heart.

Thus it was speculated that the attenuated cardioprotective effect of IPC in hyperlipidemic rat heart may be due to inhibition of eNOS by enhancing the binding of eNOS with caveolin, which leads to decrease in the release of nitric oxide. Also, administration of ZnPP, an inhibitor of HO-1, significantly blocked the observed cardioprotection and increase in release of NO in hearts of hemin pretreated hyperlipidemic rats. Furthermore, the restoration of the attenuated cardioprotective effect of IPC in hyperlipidemic rat heart by combination of daidzein and hemin was not greater than that observed when the drugs were administered alone. This suggested that these two drugs may be acting via the same mechanism i.e., NO pathway.

On the basis of above discussion it was clear that activation of heme-oxygenase-1 enzyme, by a specific inducer i.e. hemin, restored the cardioprotective effect of ischemic preconditioning in hyperlipidemic rat heart, by disrupting the caveolin-eNOS complex and there by enhancing the release of NO. Further, pretreatment with ZnPP, a specific heme-oxygenase-1 inhibitor, significantly blocked the restoration of cardioprotective effect of ischemic preconditioning in hemin pretreated hyperlipidemic rat heart. Therefore, it was concluded that attenuation of cardioprotective effect of ischemic preconditioning in hyperlipidemic rat heart, was due to impairment of HO-1 induced release of nitric oxide.

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Conflict of Interest: The authors declared no conflict of interest.

Abbreviations:

NO, Nitric Oxide;
IPC, ischemic preconditioning;
eNOS, endothelial nitric oxide synthase;
HO-1, Heme Oxygenase-1;
LDH, Lactate Dehydrogenase;
CK-MB, Creatine Kinase;
DDZ, Daidzein;
ZnPP, Zinc protoporphyrin;
K-H, Krebs-Henseleit

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Adaptogenic Activity of *Morus Alba* Extracts in Wistar Rats

By Dr. Somayeh Afsah Vakili & Syed Fayazuddin

Abstract- *Morus alba* was broadly used with a long history of traditional medicinal remedy for the regimen of various illnesses. The current investigation was designed to evaluate anti-stress activity of aqueous and ethanol extracts of *Morus alba*. Anti-stress of aqueous and ethanol extracts of *Morus alba* was estimated by inducing stress in rats through the forceful swimming. Homovanillic acid (HVA), urinary vanillylmandelic acid (VMA), 6- β -OH-cortisol, 5-hydroxyindoleacetic acid (5HIAA) and ascorbic acid were reckoned as non-invasive biomarkers to assess the adaptogenic activity. Daily oral administration of aqueous and ethanol extracts of *Morus alba* at dose of 200 and 400 mg/kg body weight one hour before the induction of stress to retard stress-induced urinary biochemical changes in a dose dependent manner. However, non-significant changes in the urinary excretion of Homovanillic acid (HVA), urinary vanillylmandelic acid (VMA), 6- β -OH-cortisol, 5-hydroxyindoleacetic acid (5HIAA) and ascorbic acid was perceived when compared to basal levels in normal animals.

Keywords: *morus alba*, stress, homovanillic acid, urinary vanillylmandelic acid, 6- β -OH-cortisol, 5-hydroxyindoleacetic acid, ascorbic acids.

GJMR-B Classification: NLMC Code: QV 4



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Adaptogenic Activity of *Morus Alba* Extracts in Wistar Rats

Dr. Somayeh Afsah Vakili ^α & Syed Fayazuddin ^ο

Abstract- *Morus alba* was broadly used with a long history of traditional medicinal remedy for the regimen of various illnesses. The current investigation was designed to evaluate anti-stress activity of aqueous and ethanol extracts of *Morus alba*. Anti-stress of aqueous and ethanol extracts of *Morus alba* was estimated by inducing stress in rats through the forceful swimming. Homovanillic acid (HVA), urinary vanillylmandelic acid (VMA), 6-β-OH-cortisol, 5-hydroxyindoleacetic acid (5HIAA) and ascorbic acid were reckoned as non-invasive biomarkers to assess the adaptogenic activity. Daily oral administration of aqueous and ethanol extracts of *Morus alba* at dose of 200 and 400 mg/kg body weight one hour before the induction of stress to retard stress-induced urinary biochemical changes in a dose dependent manner. However, non-significant changes in the urinary excretion of Homovanillic acid (HVA), urinary vanillylmandelic acid (VMA), 6-β-OH-cortisol, 5-hydroxyindoleacetic acid (5HIAA) and ascorbic acid was perceived when compared to basal levels in normal animals.

Keywords: *morus alba*, stress, homovanillic acid, urinary vanillylmandelic acid, 6-β-OH-cortisol, 5-hydroxyindoleacetic acid, ascorbic acids.

I. INTRODUCTON

Stress can be defined as the sum total of all the reaction of the body, which disorganise the normal physiological condition and result in a state of threatened homeostasis. Stress is an internationally conceded phenomenon fortified by advancement of industrialization in a demanding civilization. Thus every individual is likely to face stressful situation in day to day life (Selye, 1998). Stress is a stimulus that activates the hypothalamic pituitary adrenal (HPA) axis and Sympathetic Nervous System (SNS) and begets a physiological change. Physiological responses to stressful stimuli, including the increases in blood pressure, heart rate, body temperature and plasma concentration of adreno-corticotrophic hormone (ACTH), can be related to the stress induced activation of the SNS. Stress prompts synthesis and release glucocorticoids (corticosterone and cortisol) and monoamines such as epinephrine, dopamine, norepinephrine and serotonin which are characteristic

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stress hormones (Carrasco et al., 2003). Adaptogens are the substances that help organisms to adapt to unfavourable stressful conditions, which could be physical, chemical, biological or mental conditions (Rege et al., 1999). The prevalent objective of adaptogenic therapy is due to diminish stress reactions during the alarm phase of the stress response, inhibit or retard the state of exhaustion and consequently issue a certain level of protection against long-term stress (Wagner et al., 1994). *Morus alba* belongs to family *Moraceae* commonly called as white mulberry. This plant has been used traditionally as anti-asthma, antidiabetic (Singab et al., 2005), hypotensive (Fukai et al., 1985) and neuroprotective (Kang et al., 2006). The current investigation was carried out to assess the anti-stress activity of aqueous and ethanol extracts of *Morus alba*.

II. MATERIALS AND METHODS

a) Plant material and Preparation of extracts

The fruits of *Morus alba* were collected from Chennai, Tamil Nadu, India and authenticated by Green Chem, Bangalore, Karnataka, India, a voucher specimen (MAT-SIP-501) were preserved for future references. The fruits materials (1kg) was dried, powdered and extracted with water and ethanol (60-80°C) using soxhlet methods. The filtrate was evaporated at 70 °C in a vaccum dryer to give final yield 40.5g.

b) Chemicals

Homovanillic acid (CAS 306-08-1), urinary vanillylmandelic acid (VMA), 6-β-OH-cortisol, 5-hydroxyindoleacetic acid (5HIAA) and ascorbic acid was purchased from Sigma, ST Louis, MO, USA. Acetonitrile and methanol HPLC grade were supplied from Qualigens, Fischer Scientific, Mumbai. All other chemicals were analytical grade and obtained from local store of Visveswarapura Institute of Pharmaceutical Sciences.

c) Animals

Albino Wistar rats (150-200gm) of either sex obtained from the NIMHANS animal house, Bengaluru and were housed at room temperature in a well-ventilated animal house under 12 hrs light / dark cycle in polypropylene cages (29"x22"x14") with stainless steel grill top, bedded with paddy husk. The animals were maintained under standard conditions in an animal

house as per the guidelines of "Committee for the Purpose of Control and Supervision on Experiments on Animals" (CPCSEA) for at least one week prior to use. The rats had free access to standard rat chow and water *ad libitum*. The study protocol was approved by Institutional Animal Ethics Committee (IAEC), Visveswarapura Institute of Pharmaceutical Sciences, Bangalore. (Registration No: 152/1999, renewed in 2012).

d) *Calibration curves of HVA, VMA, 6-β-OH cortisol and 5-HIAA in urine by simultaneous HPLC determination*

Simultaneous HPLC determination of HVA, VMA, 6-β-OH cortisol and 5-HIAA in urine were determined to assess their standard value (Sreemantula et al., 2004). Different dilutions 25 ng/ml, 50 ng/ml, 100 ng/ml, 250 ng/ml, 500 ng/ml, 1000 ng/ml, 2500 ng/ml, 5000 ng/ml of the HVA, VMA, 6-β-OH cortisol and 5-HIAA were made with mobile phase from the working standards (1mg/ml) which consist of 250 ng/ml internal standard (Ethyl-3-hydroxy-4-methoxy-mandelate). These dilutions were spiked into the HPLC and calibration curves were plotted as peak area ratio vs. concentration. The peak area ratios of HVA, VMA, 6-β-OH cortisol and 5-HIAA to that of internal standard were calculated and substituted in the respective regression equations to estimate the amount of the metabolite present in the sample.

e) *Calibration curve for ascorbic acid*

Calibration curve for ascorbic acid was done according method of (Roe and Kuether, 1943). Standard solution of ascorbic acid (1mg/ml) was prepared by dissolving 10 mg of ascorbic acid in 10 ml of distilled water. By using this different concentration of ascorbic acid 5, 10, 20, 30, 40 μg/ml were prepared with 4% Trichloro Acetic acid (TCA). 10 ml of each of these were taken and mixed well with 0.375 gm of activated charcoal and filtered. 4% TCA was used for the blank. From the filtrate 1 ml was taken in to the test tube and a drop of thiourea was added to that. Then to those test tubes 1 ml of 2,4-Dinitro Phenyl Hydrazine (2,4-DNPH) was added and kept in incubator for about 3 hrs, maintained at 37°C. The tubes were then placed in the beaker containing ice and 5 ml of 95% sulphuric acid was added drop by drop within 1 min interval with intermittent mixing. Finally they were shaken and kept aside for 30 min. Then optical density was measured at 550 nm using spectrophotometer [Shimadzu (UV-1601)]. Standard curve was plotted by taking concentration of ascorbic acid on X-axis and optical density on Y-axis.

f) *Evaluation of anti-stress activity*

Rats of either sex weighing between 180–220 gm were divided into five groups (I, II, III, IV & V) each containing six animals. The 24 hr urine sample from each group were collected into two different beakers,

one containing 5 ml of 10% oxalic acid for the spectrophotometric determination of ascorbic acid at 550 nm and the other containing 0.5 ml of 6N hydrochloric acid for the determination of stress metabolites. The experimental protocol was divided into four phases: In the first phase of the experiment, 24 hr urine samples were collected in all the groups and subjected to analysis for HVA, VMA, 6-β-OH cortisol, 5-HIAA and ascorbic acid and the normal values were recorded for four consecutive days. In the second phase, after a recovery period of one week, the animals in each group were subjected to fresh water swimming stress individually. In this method, rats are forced to swim until exhausted in a cylindrical vessel of 60 cm height and 45 cm diameter containing water at room temperature (28°C). Water depth was always maintained at 40 cm. The 24 hr urine samples were collected in all the groups and subjected to analysis for HVA, VMA, 6-β-OH cortisol, 5-HIAA and ascorbic acid and the values were recorded for four consecutive days. In the third phase of the experiment, after a recovery period of one week, the experimental animals were administered as follows for four consecutive days. Group 1 rats served as normal control and received 2ml/kg distilled water, group 2,3 rats were administered orally with aqueous extracts of *Morus alba* at dose of 200 mg/kg and 400 mg/kg respectively. Group 4,5 rats received ethanol extracts *Morus alba* orally at dose of 200 mg/kg and 400 mg/kg respectively. In the final phase of the experiment, after a recovery period of one week, the administration of *Morus alba* extract were done as mentioned in the third phase, one hour prior to the daily induction of stress for four consecutive days while group I serving as control. The 24 hr urine samples were collected in all the groups and subjected to analysis for HVA, VMA, 6-β-OH cortisol, 5-HIAA and ascorbic acid and the values were recorded for four consecutive days to study the influence of the aqueous and ethanol extracts of *Morus alba* on the stress induced biochemical changes (Sreemantula et al., 2004).

g) *Statistical analysis*

The data were expressed as mean ± S.E.M. Statistical analysis was performed by using student's paired t-test, where the difference was considered significant if $p < 0.05$.

III. RESULTS

a) *Calibration curves of biomarkers for determination of standard value*

A typical chromatogram was manifested in Figure 1. The retention times of VMA, IS (Ethyl-3-hydroxy-4-methoxy-mandelate), 5-HIAA, 6-β-OH cortisol and HVA were found to be 4.46, 5.210, 9.31, 10.84 and 12.15 respectively. The standard graphs of HVA, VMA, 6-β-OH cortisol and 5-HIAA were displayed in Figure

2,3,4 and 5. Calibration curve for ascorbic acid was exhibited to estimate standard value in Figure 6.

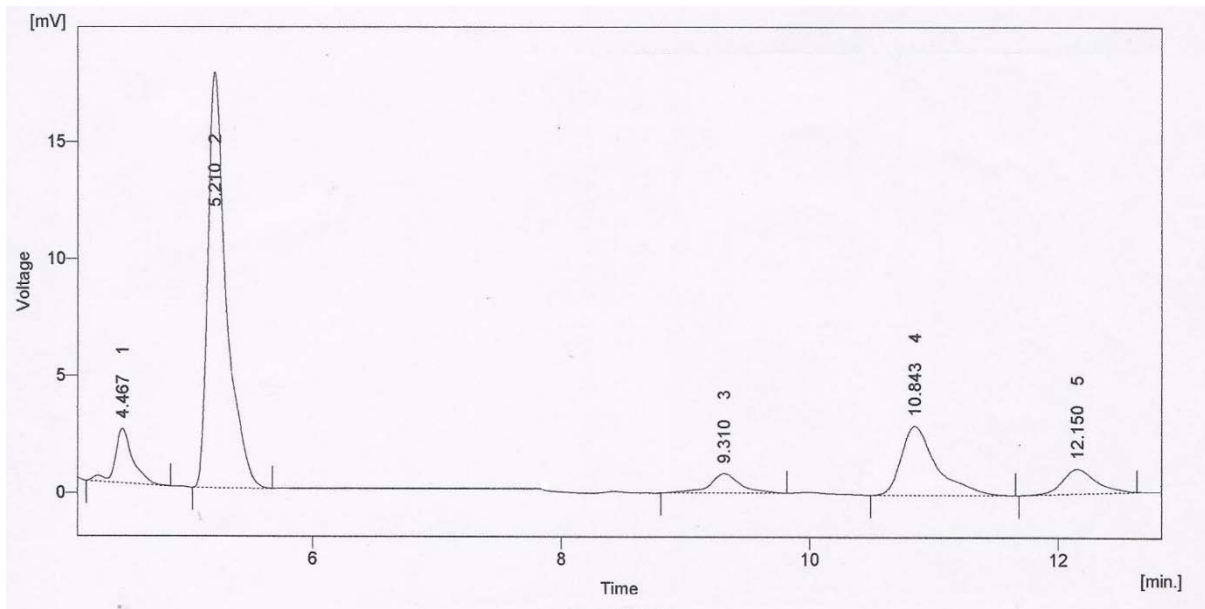


Figure 1: Chromatogram representing simultaneous method for the determination of standard HVA, VMA, 6- β -OH cortisol and 5-HIAA

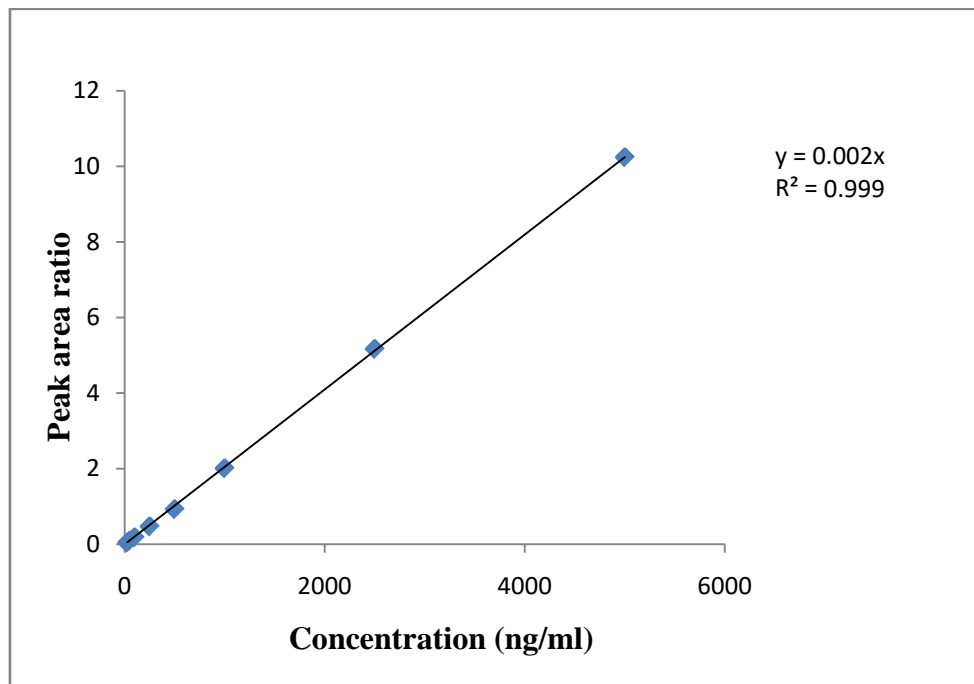


Figure 2: Standard graph of Homovanillic acid (HVA)

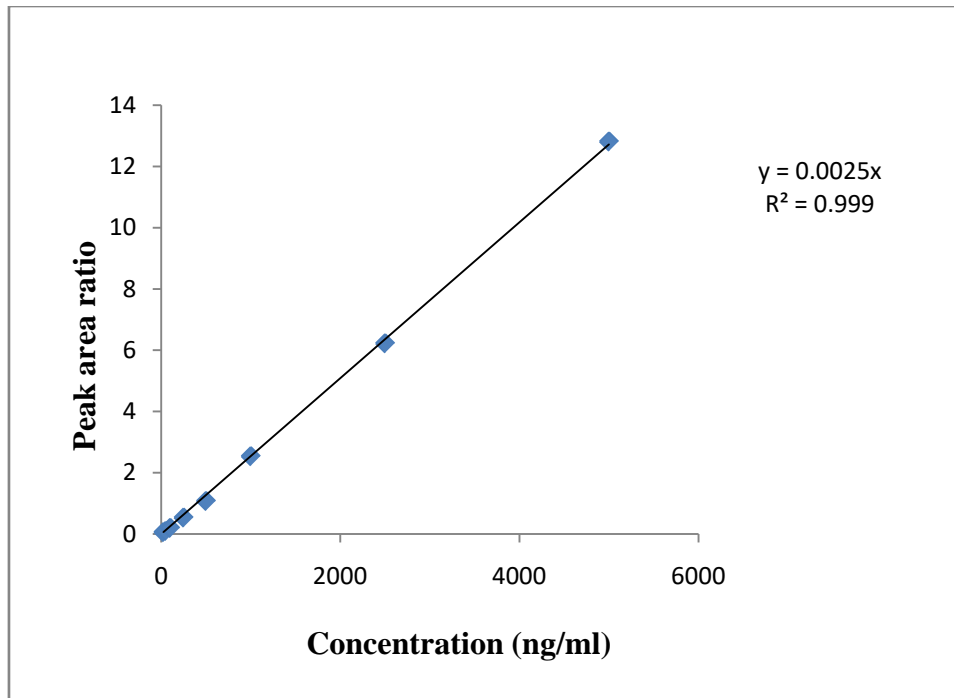


Figure 3: Standard graph of Vanillylmandelic Acid (VMA)

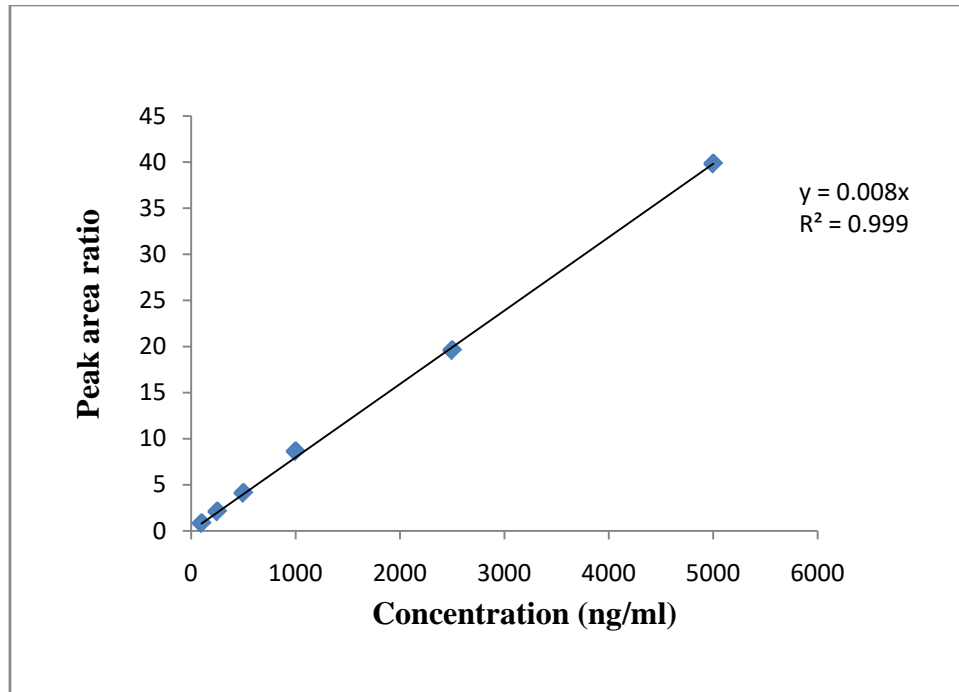


Figure 4: Standard graph of 6-β-Hydroxy Cortisol (6-β-OH cortisol)

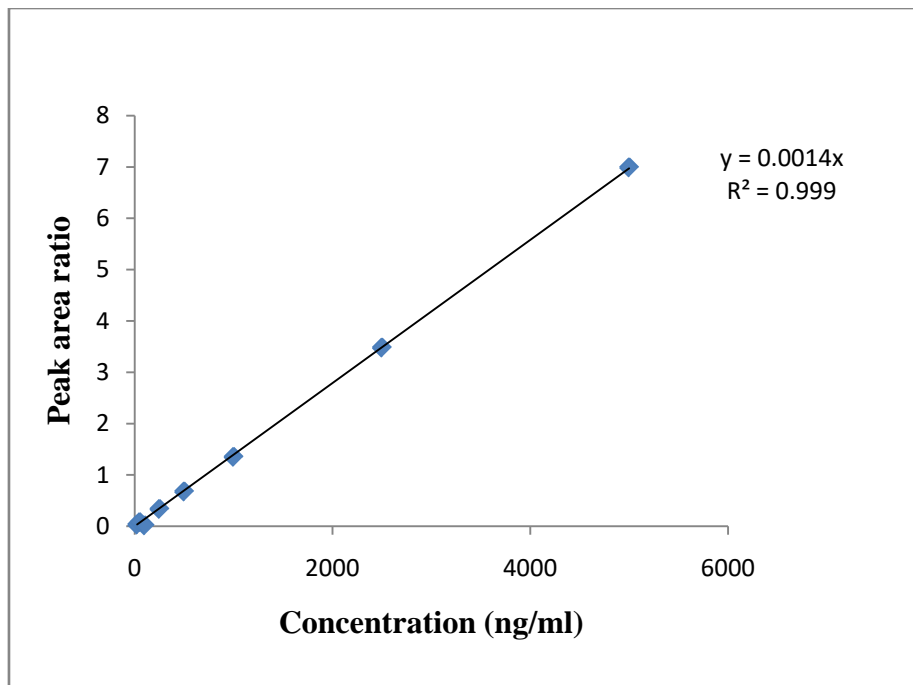


Figure 5: Standard graph of 5-Hydroxy Indole Acetic Acid (5-HIAA)

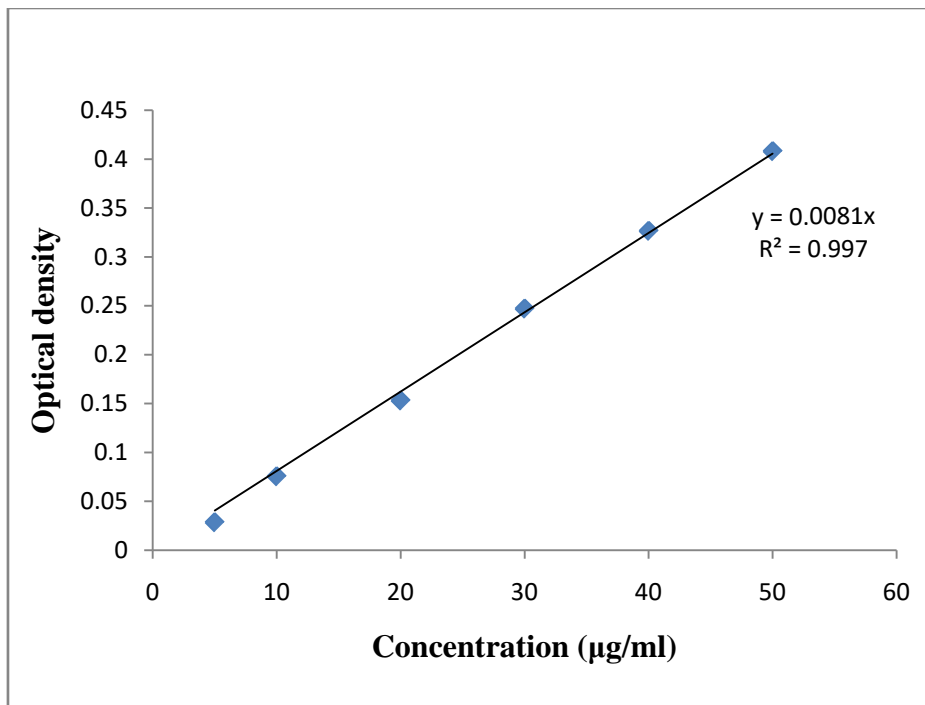


Figure 6: Standard graph of Ascorbic acid

b) Evaluation of anti-stress activity

Figures 7, 8, 9, 10 and 11 revealed the effect of aqueous and ethanol extract of *Morus alba* on urinary levels of biomarkers in normal and stress condition. There was variation in each biomarkers from group to group in normal state. The amount of VMA ($0.343 \mu\text{g} \pm 0.019$), 5-HIAA ($0.348 \mu\text{g} \pm 0.013$) and HVA ($0.113 \mu\text{g} \pm 0.009$) were low in group I in normal state and high amount of VMA ($0.431 \mu\text{g} \pm 0.017$), 5-HIAA ($0.463 \mu\text{g} \pm$

0.022) and HVA ($0.176 \mu\text{g} \pm 0.006$) were found in group IV in normal state. The level of 6- β -OH cortisol was low in group V ($0.424 \mu\text{g} \pm 0.032$) and high in group I ($0.557 \mu\text{g} \pm 0.010$), also the amount of ascorbic acid was low in group IV ($43.92 \mu\text{g} \pm 2.33$) and high in group I ($52.64 \mu\text{g} \pm 5.64$) in normal condition. Significant increase ($P < 0.05$) in urinary levels of VMA, 5-HIAA, 6- β -OH cortisol and HVA was noted in group I to V. Significant decrease ($P < 0.05$) in urinary levels of

ascorbic acid was observed in group I to V in stress condition. There were slight changes in VMA, 5-HIAA, 6- β -OH cortisol, HVA and ascorbic acid levels in urine of animals treated with aqueous and ethanol extracts of *Morus alba* in the normal state. There was variation from day to day and the variation is different from group to group. However observed the changes in the levels of the urinary metabolites when compared to normal basal

levels were found to be non-significant. Aqueous and ethanol extracts of *Morus alba* significantly ($P < 0.05$) diminished urinary levels of VMA, 5-HIAA, 6- β -OH cortisol and HVA in group II, III, IV and V, also significant increased ($P < 0.05$) in urinary ascorbic acid levels was perceived in group II, III, IV and V compared to their respective stress condition.

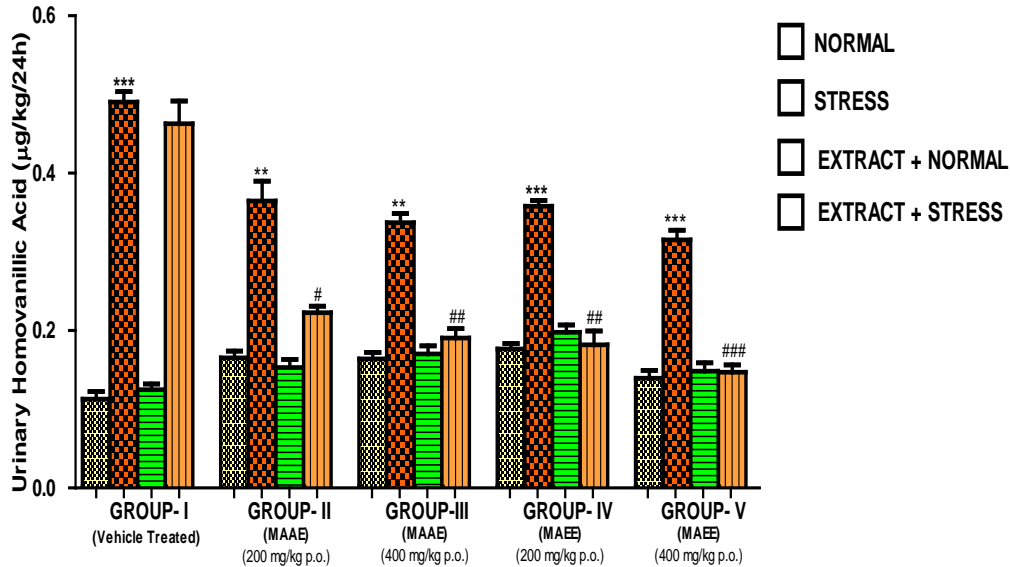


Figure 7: Influence of aqueous and ethanol extracts of *Morus alba* on 24h urinary levels of HVA in normal and stress induced rats. Each bar indicates the mean excretion of six animals. *** $P < 0.0001$, ** $P < 0.001$ significant difference from normal condition of the corresponding groups; ### $P < 0.0001$, ## $P < 0.001$, # $P < 0.01$ significant difference from stress condition of the corresponding groups.

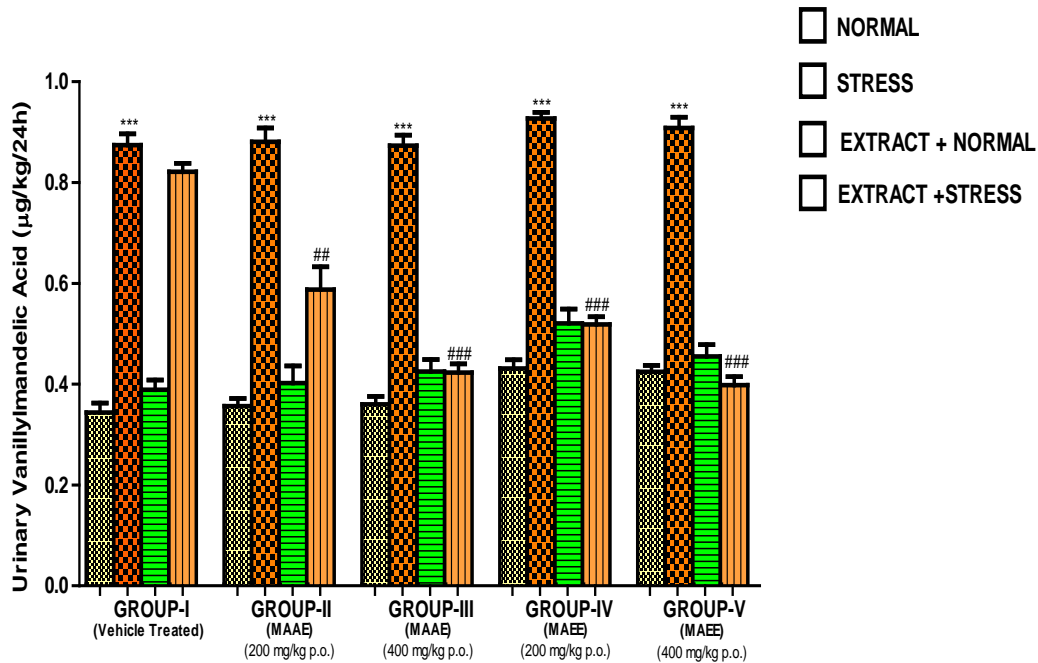


Figure 8: Influence of aqueous and ethanol extracts of *Morus alba* on 24h urinary levels of VMA in normal and stress induced rats. Each bar indicates the mean excretion of six animals. *** $P < 0.0001$ significant difference from normal condition of the corresponding groups; ### $P < 0.0001$, ## $P < 0.001$ significant difference from stress condition of the corresponding groups.

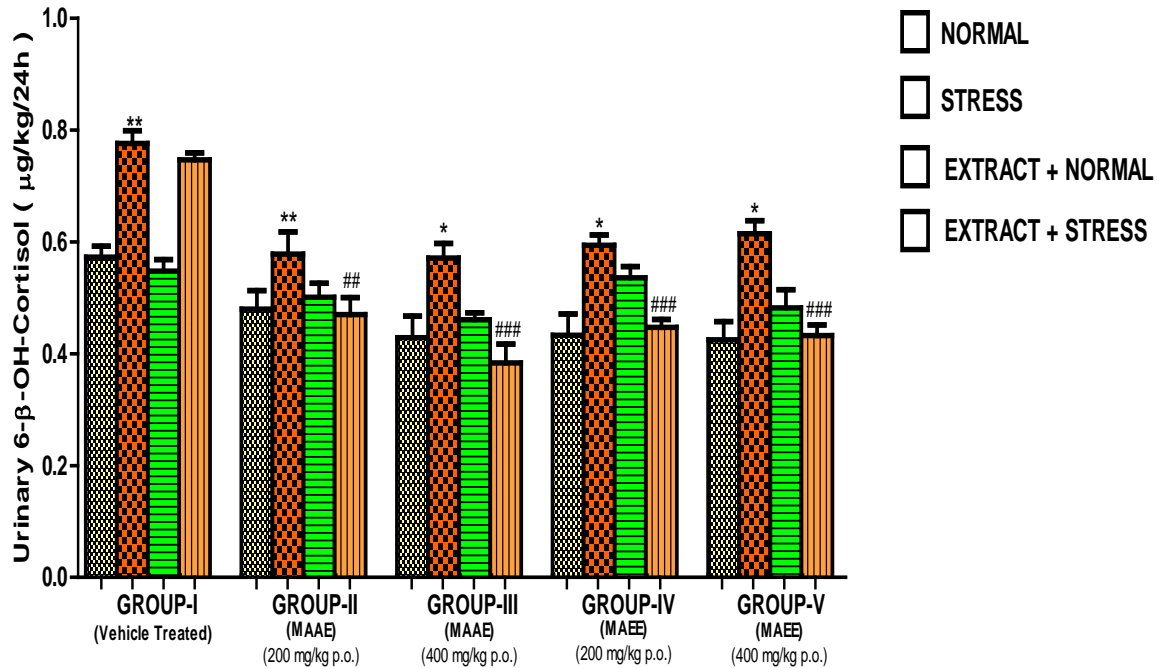


Figure 9: Influence of aqueous and ethanol extract of Morusalba on 24h urinary levels of 6-β-OH-Cortisol in normal and stress induced rats. Each bar indicates the mean excretion of six animals. ** $P < 0.001$, * $P < 0.01$ significant difference from normal condition of the corresponding groups; ### $P < 0.0001$, ## $P < 0.001$ significant difference from stress condition of the corresponding groups.

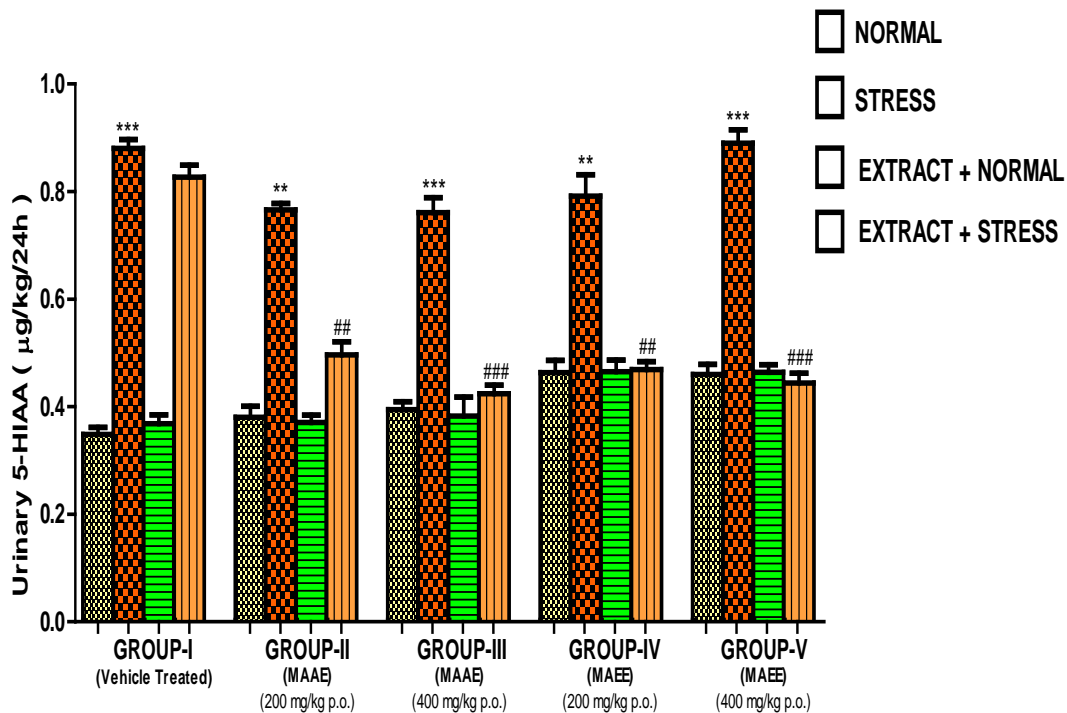


Figure 10: Influence of aqueous and ethanol extracts of Morusalba on 24h urinary levels of 5-HIAA in normal and stress induced rats. Each bar indicates the mean excretion of six animals. *** $P < 0.0001$, ** $P < 0.001$ significant difference from normal condition of the corresponding groups; ### $P < 0.0001$, ## $P < 0.001$ significant difference from stress condition of the corresponding groups.

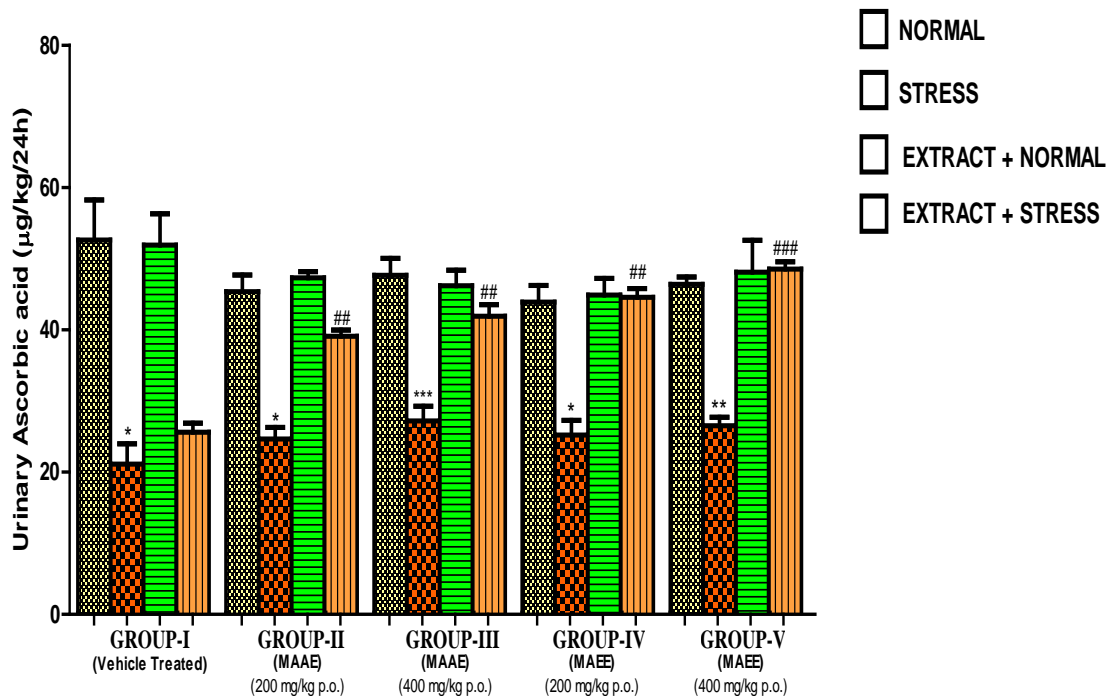


Figure 11: Influence of aqueous and ethanol extracts of *Morus alba* on 24h urinary levels of Ascorbic acid in normal and stress induced rats. Each bar indicates the mean excretion of six animals. *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.01$ significant difference from normal condition of the corresponding groups; ### $P < 0.0001$, ## $P < 0.001$ significant difference from stress condition of the corresponding groups.

IV. DISCUSSION

Stress represents reactions of the body to a stimulus that tends to modify homeostasis (Selye, 1998). Stress hormones are synthesised during stress condition for example the catecholamines (epinephrine and norepinephrine) produced by the SNS, and corticosteroids, produced by the ACTH stimulated adrenal cortex and glucocorticoid stimulated increase in serotonin are the major stress hormones (Uresin et al., 2004). In the current investigation, VMA as the peripheral metabolite of NA, 5-HIAA as the main metabolite of serotonin, 6- β -OH cortisol as metabolite of cortisol, HVA as the predominant metabolite of dopamine and ascorbic acid as a metabolite of glucose (in rats) were taken as an non-invasive biomarkers to display the increase in peripheral sympathetic activity during stress to assess the anti-stress activity of aqueous and ethanol extracts of *Morus alba*. The data indicated that VMA, 5-HIAA, 6- β -OH cortisol, HVA and ascorbic acid were excreted in urine daily at certain levels (basal values) as metabolites of NA, 5-HT, cortisol, DA and glucose respectively. The stress affected on the neurotransmitter levels and increased VMA, 5-HIAA, 6- β -OH cortisol, HVA and diminished ascorbic acid excretion. When aqueous and ethanol extracts of *Morus alba* administered to normal animals did not change VMA, 5-HIAA, 6- β -OH cortisol, HVA and ascorbic acid in comparison with basal values but prior administration of aqueous and ethanol extracts of *Morus alba* to stress induced rats

exhibited the reduction in urinary VMA, 5-HIAA, 6- β -OH cortisol, HVA and increased the ascorbic acid levels in dose dependent manner. The previous phytochemical evaluation of *Morus alba* divulged the presence of phenolic compounds such as flavonoids (Quercetin, rutin), tannin which could be expected to be responsible for anti-stress activity (Ayoola et al., 2011). As VMA is a metabolite of norepinephrine (NE) and NE is synthesized by dopamine. Previous reports exhibited that these phytochemicals can bind to the GABA_A-BZDS complex, consequently, enhance GABA level and decline dopamine and decrease plasma corticosterone level that lead to reduce level of VMA and 6- β -OH cortisol respectively (Patil et al., 2006). Phenolic compounds such as flavonoids showed the affinity towards D₂ receptor; hence they can block the dopamine receptor and decrease the serotonin which causes to decrease level of HVA (Samson et al., 2006). These active compounds can prevent activity of tryptophan hydroxylase enzyme which is involved in the biosynthesis of 5-HT, thus, they can reduce level of 5-HIAA ultimately (Singh et al., 1990). Ascorbic acid is also utilized as a co-factor in the biosynthesis of NE from DA may also attribute to low concentration of ascorbic acid in urine. Hence, effect of these bioactive compounds on reduction of dopamine synthesis that can influence on the increase of urinary level of ascorbic acid.

V. CONCLUSION

The present investigation manifest that aqueous and ethanol extract of *Morus alba* have anti-stress activity due to normalization of monoamines and glucocorticoids homeostasis by acting on HPA axis and SNS.

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Metformin: A Unique Herbal Origin Medication

By Nadin Yousef, Farah Yousef, Oussama Mansour & Jehad Herbali
Damascus University

Abstract- Many oral anti-hyperglycemic agents have been used for treatment of Type II diabetes (TY2D), yet Metformin, the only compound remained of biguanide derivates, continued to be used in this treatment. Its mechanism of action and pharmacokinetics are unique and significant. It has low side effects and no adverse incidences with healthy heart and kidney T2D, compared with its family group members. The previous properties made metformin tops the oral anti hyperglycemic agents list recommended from FDA in TY2D therapy. In this review paper, we aimed to point at bi-guanide development through time as glucose lowering agents, and show the magnificent characteristics of dimethylbiguanide; metformin.

GJMR-B Classification: NLMC Code: QV 767



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Metformin: A Unique Herbal Origin Medication

Nadin Yousef ^α, Farah Yousef ^σ, Oussama Mansour ^ρ & Jehad Herbali ^ω

Abstract- Many oral anti-hyperglycemic agents have been used for treatment of Type II diabetes (TY2D), yet Metformin, the only compound remained of biguanide derivatives, continued to be used in this treatment. Its mechanism of action and pharmacokinetics are unique and significant. It has low side effects and no adverse incidences with healthy heart and kidney T2D, compared with its family group members. The previous properties made metformin tops the oral anti hyperglycemic agents list recommended from FDA in TY2D therapy. In this review paper, we aimed to point at bi-guanide development through time as glucose lowering agents, and show the magnificent characteristics of dimethylbiguanide; metformin.

I. INTRODUCTION

a) Origin Background

In 1920s, guanidine based compounds were firstly known with their efficacy as anti-hyperglycemic agents ^[1], yet their origins go back to 1600s, when a plant; Galega officinalis, was commonly used in European Folklore to decrease the symptoms of what it's known today as type II diabetes (TY2D). ^{[2] [3]}

Although this plant, also known as Goat's Rue or French Lilac, was found, in late 1800s, to be full of guanidine and galagine; isoamylene guanidine, ^{[4] [5] [6] [7]} however the efficacy of guanidine to lower blood glucose levels was not proven in animals till 1918.

This invention struggled some challenges as the toxicity of guanidine decreased its chance as a potential hypoglycemic drug, and the discovery of safer guanidine based structure compounds was somehow not succeeded by scientists, as well. Moreover, that the era of insulin discovery and availability by that time downplayed guanidine effectiveness role in diabetes treatment. ^[1]

Through what the world had witnessed of crises in 1900s, Researches did not stop investigating G.

officinalis; there were still eyes on galagine as safer anti hyperglycemic agent than guanidine.

However, that was not for too long with the presence of diguanide compounds; decamethylene diguanide (Synthalin A[®]) and dodecamethylene diguanide (Synthalin B[®]) in the clinical use by the same period of time.

These diguanide compounds were more tolerated than guanidine compounds, but their hepatic toxicity reports in 1930s were the cause of not prescribing them any longer by medical professionals. ^[8]

Although many guanidine compounds were synthesized in 1929, but biguanide derivatives did not see the light till the later of 1950s. Phenofornin was discovered by Urgan and Shapiro and it was introduced to the medical usage in 1958 with DPI[®]. ^[9] While Buformin was discovered by Mehnert. ^[10]

In spite of these two proved greater efficacy than our unique compound, the lactic acidosis incidences that follow the use of them resulted in withdrawing them from the market in 1970s. ^{[11] [12] [13]}

Metformin; Dimethyl-biguanide, the only bi-guanide remained in TY2D remedy, was developed by Jean Sterne in France. His results were published in 1957 and he was the one who named metformin as "Glucophage"; glucose eater. ^{[14] [15]} Yet, American Food and Drugs Administration did not approve on metformin usage in the U.S market till 1995. ^[16]

b) Bi-guanide chemical development

Guanidine and galagine structures are shown in fig 1. Di-guanide compounds are presented in fig 2, and Biguanide compounds have the general structure shown in fig 3. These compounds' structures are shown in fig 4. ^[17] Their chemical and physical properties are shown in Table 1.

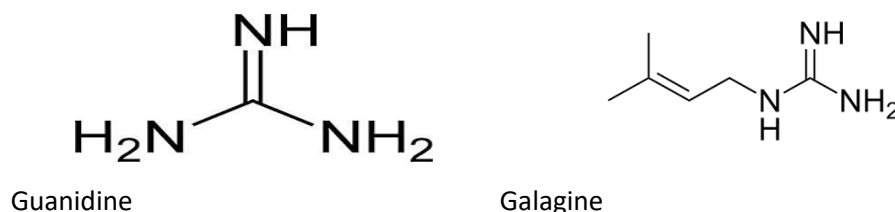


Figure 1: Guanidine and Galagine structures

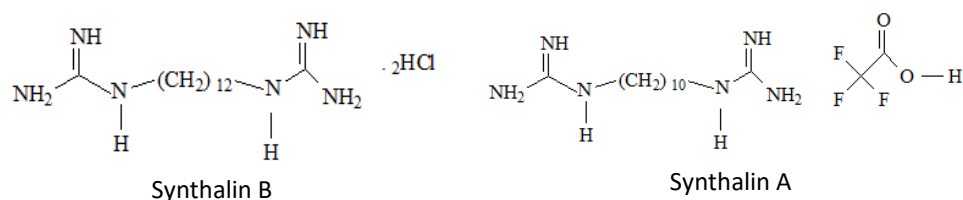


Figure 2: Di-guanide derivatives' structures

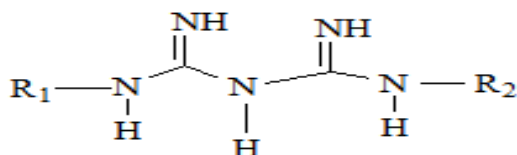


Figure 3: Biguanide derivatives' general structure

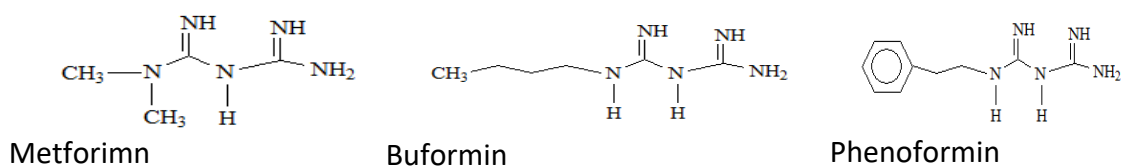


Figure 4: Biguanides' compounds

Table 1: Chemical properties of biguanide derivatives ^[18]

Compound Name	M. Weight	Log P	pK _a	Hydrogen bond Donner count	Hydrogen bond acceptor count	Dosage mg/day	Rel. potency
Synthalin A	370.23	N/A	N/A	5	7	N/A	N/A
Synthalin B	357.368	N/A	N/A	4	4	N/A	N/A
Buformin	157.221	-1.2	N/A	3	1	N/A	N/A
Phenoformin	241.723	-0.83	N/A	4	1	N/A	N/A
Metformin	129.167	-0.5	12.4	3	1	500/3	N/A

Metformin is the safest biguanide derivatives in use by TY2D patients.^[19] As can be inferred from metformin structure, it is a bi-guanide derivative, no (CH₂)_n chain separates between the two guanidine groups. R₁, R₂ are small alkyl groups (-CH₃).

On the other hand, di-guanide derivatives have been withdrawn from the market due to their adverse to toxic effects; Synthalins for their hepato-toxicity, while phenoformin and buformin; bi-guanide derivatives, for their lactic acidosis incidences.^[16]

As can be seen from their structures, having (-CH₂)₁₀ chain, (-CH₂)₁₂ chain separating between two guanidine groups as there are respectively in Synthalin A and Synthalin B, or (Ar-CH₂-CH₂-) moiety as it is in

phenoformin, might be responsible for the undesirable effects of these compounds. However, their anti-hyperglycemic efficacy were stronger than metformin.

This can indicate an inquiry that does shortening (CH₂)_n; n < 10, or having other small or cyclic moieties on the both terminal NH₂ can take a part in these compound efficacy or decrease the adverse effects biguanide compounds struggled with?! This hypothesis is still in question.

Recent research has proven that a number of other biguanide hydrochloride derivatives also have anti diabetic activity. One of them, actually has a di-oxyethyl benzene moiety in its chemical Skelton. Structures of these compounds are shown in fig (5).^[20]

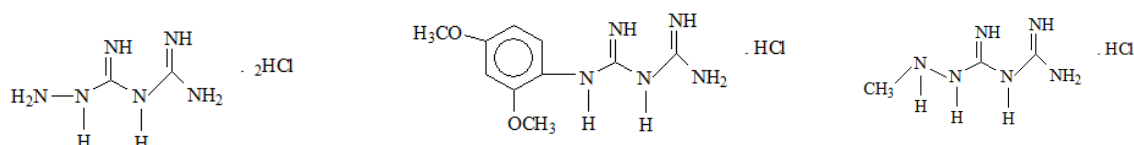


Figure 5: Biguanide derivatives work as anti diabetic agents

c) Metformin Synthesis

Shapiro had prepared metformin from the reaction between dimethylamine hydrochloride and dicyano diamide at 120-140 °C in 4 hrs time with 69% yield.^[21]

New route was used in this synthesis. This ecologically safe protocol using microwaves; 540W,

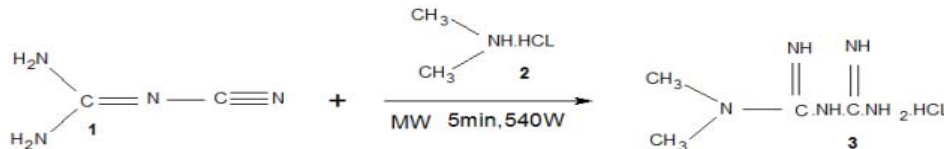


Figure 6: New synthesis route for metformin

d) Metformin unique Characteristics

i. Pharmacodynamics

According to American Diabetes Association, metformin is the first-line drug for treatment of T2D patients in the world. Metformin can be considered Sugar Regulator rather than anti-hyperglycemic agent.^{[23] [24]}

Its mechanism of action does not differ from phenofornin's. They both increase AMPK activation in hepatic cells.^{[5] [25] [26]} Recent research demonstrates that their pharmacodynamics play deeper role interacting with cellular bioactivity. they interact with Hepatic Mitochondrial Respiratory Chain Complex 1(H⁺ giant pump).^{[27] [28] [29]}

This results in decreasing the cellular production of ATP.^[30] In both ways, Energy sources in hepatic cells are reduced resulting in enhancing insulin receptor sensitivity toward insulin.^[31]

That makes the main peripheral targeted cells; skeletal muscle, and hepatic cells, stop glycogenesis operation and produce more insulin receptors and Glucose Transporter 4 (GLUT-4) to augment glucose inwardly transportation.^{[32] [33] [34] [35]}

ii. Pharmacokinetics

No information was provided about biguanide derivatives' pharmacokinetics but for Metformin. It is 50-60 % absorbed after an oral single dosage of (500, 850, 1000) mg. Its protein binding percentage is negligible. It is eliminated unchanged in the urine.^{[36] [37]} Its half-life is 6.2 hours with duration of action 8-12 hours.

iii. Superiority

Hundreds of efficacy studies verified metformin superiority to the rest of his family members, and other chemical groups, used in Type II diabetes therapy.^{[38] [39]} Likewise, toxicology experiments affirmed its narrow toxicity margin. Metformin advantage\ risk scale brought it to be the first drug in the guideline of initial remedy of Type II diabetes.^{[40] [41]}

As glucose blood levels become uncontrollable by time due to disease progression in TY2D and the life styles in many communities facilitates in this falling

shortens the reaction time; 5mins, reduce the amount used of solvents, to obtain a clean product with yield of 92% by optimum use of energy and tiny amounts of reacted materials. Fig (6)^[22]

back, many oral anti hyperglycemic agent combinations with metformin presented in the drug market.^{[42] [43] [44]}

Metformin combination with Sulfonyl-urea is very common. In fact, this double combination has proven more efficacy than if each drug was solely taken. Glucovans ® is the trade name of metformin and glyburide combination.^{[45] [46]}

Above all that, this ancient-modern small molecule can be used in adolescents and childhood, as TY2D since 1990 started to be caused not only in adults but also in children.^{[47] [48]}

e) Side Effects and Other benefits

Metformin has no significant side effects with its labeled dosage.^[49] The most frequently reported adverse events were digestive disturbance such as stomach ache, diarrhea, and vomiting. It also may cause headaches.

However, these undesired effects can be reduced when metformin is taken during meals, or as extended release pharmaceutical forms; metformin XR.^{[50] [51]} Furthermore, metformin reduces mortality compared to other anti-diabetic agents.^[52]

This magical bi-guanide compound distinguished itself from other oral anti hyperglycemic agents side effects. Its usage does not lead to clinical hypoglycemia, lactic acidosis, or changes in physical examinations. Yet, rare lactic acidosis reported in some cases where over dosage was taken.^{[53] [54]}

In other words, Metformin magnificent side effect in its modest losing weight assistant made it likable to be prescribed by medical professions for obese TY2D patients.^{[55] [56] [57]}

This significant usage was especially observed in obese children with or without Type II diabetes (TY2D) compared to diet. Proposed mechanism of this effect is by reducing glucose absorption levels.^{[58] [59] [60] [61] [62]}

This herbal origin drug is also used in poly cystic ovary.^{[63] [64]} Latest researches deepens its effectiveness in Cancer treatment, as well.^{[65] [66] [67]} Besides to that, metformin has proven anti stress efficacy that led it to be used in inflammatory cases, and anti-aging factor, too.^[68]

Furthermore, clinical studies verified its role in decreasing cardiovascular mortality by decreasing cholesterol blood levels and macro vascular cells' formation.^{[20] [69] [70]}

Metformin proved its positive role in metabolic syndrome patients, as well as it enhances neuron system health.^{[71] [72] [73]}

i. Contraindications

Heart failure is considered a potential contraindication for metformin usage in T2D treatment. Though lactic acidosis incidences are rare with metformin, yet this metabolic disturbance might be fatal in heart failure patients as it leads to hypo-perfusion.^{[74] [75]} Despite this potential risk, yet FDA did not announce heart failure as contraindication for metformin.^[76]

Because metformin is eliminated unchanged in the urine, renal failure is another metformin contraindication. However, the level of renal dysfunction was not determined in which to be considered risky for metformin usage or not to avoid lactic acidosis incidences.^{[77] [78] [79]}

ii. Drug Interactions

On the long term of taking, Metformin decreases the absorption of vitamin B₁₂.^{[80] [81]} Renal eliminated drugs such as digoxin, ranitidine, trimethoprim and many others also has interaction with metformin way of elimination.

iii. Prescriptions in the Market

Though metformin was shadowed from the time it was synthesized; 1920s, passing by its development in 1950s, till it was approved by FDA in 1995; nowadays, Metformin is categorized as essential drug in the World Health Organization's (WHO) lists for TY2D treatment.^[82]

Bristol-Myers Squibb Company ; an American pharmaceutical global company, got the protection patent of marketing metformin under the trade name Glucophage till 2002.^[83] Its prescriptions extended 50 million filled in the US alone, and Bristol-Myers Squibb earnings of this investment reached peak sales of US\$2.7 billion in 2001.^[84]

By the time the protection patent expired, many companies such as Lubrizol, Zydus Pharmaceuticals and others compete for marketing approved generics such as Glucophage, Glucophage XR, Riomet, Fortamet, Glumetza, Jentaducto, JANUMET® XR, Synjardy®, INVOKAMET™.

Due to this strong market completion, It is expected that metformin market value increase 2.1% in 2021 compared with its market value in 2014. It is estimated that metformin market value in 2014 was 660 million USD. As global statics indicate, this might reach 720 million USD in 2021.

II. CONCLUSION

We tried in this review to shed light on biguanide discovery history and the chemical

differences among this group compounds that are in a way or another responsible of their characteristics.

However, We look forward for more researches to be done in this field in aim to have as modified structures as it can be done to have better anti-hyperglycemic agents with less side effects and proven efficacy.

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Appraisal of Nootropic Activity of *Morus Alba* Extracts

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Abstract- Mulberry (*Morus alba* L) species is native to northern China and is widely cultivated various Asian countries such as Japan, Iran, etc. *Morus alba* was traditionally used in Chinese medicinal remedy for ailments of bronchitis, insomnia, constipation and inflammatory. The research was planned to appraisal nootropic activity of aqueous and ethanol extracts of *Morus alba* using conditioned avoidance response in rat and estimation of acetyl cholinesterase activity by Ellman's method in rats. Conditioned avoidance response was evaluated by using the Perspex chamber apparatus. Animals were treated with scopolamine butyl bromide (1mg/kg bw, i.p) thirty minutes before foot shock to produce amnesia. Animals were trained to jump on the pole to avoid shock with receiving daily oral dose of aqueous and ethanol extracts of *Morus alba* at dose of 200 and 400 mg/kg body weight one hour before the induction of foot shock. The esterase activity was measured by providing an acetyl thiocholine which cause to release thiocholine as result of cleaving by AChE. Thiocholine reduced 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to thionitrobenzoic acid which absorbed light at 412 nm. The groups treated with aqueous and ethanolic extracts of *Morus alba* were found to nootropic activity by reversing the scopolamine induced amnesia. Acetyl cholinesterase inhibitory activity of extracts of *Morus alba* were performed the supportive nootropic activity by enhancing the cognitive function.

Keywords: *morus alba*, nootropic activity, acetyl cholinesterase.

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Abstract- Mulberry (*Morus alba* L) species is native to northern China and is widely cultivated various Asian countries such as Japan, Iran, etc. *Morus alba* was traditionally used in Chinese medicinal remedy for ailments of bronchitis, insomnia, constipation and inflammatory. The research was planned to appraisal nootropic activity of aqueous and ethanol extracts of *Morus alba* using conditioned avoidance response in rat and estimation of acetyl cholinesterase activity by Ellman's method in rats. Conditioned avoidance response was evaluated by using the Perspex chamber apparatus. Animals were treated with scopolamine butyl bromide (1mg/kg bw, i.p) thirty minutes before foot shock to produce amnesia. Animals were trained to jump on the pole to avoid shock with receiving daily oral dose of aqueous and ethanol extracts of *Morus alba* at dose of 200 and 400 mg/kg body weight one hour before the induction of foot shock. The esterase activity was measured by providing an acetyl thiocholine which cause to release thiocholine as result of cleaving by AChE. Thiocholine reduced 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to thionitrobenzoic acid which absorbed light at 412 nm. The groups treated with aqueous and ethanolic extracts of *Morus alba* were found to nootropic activity by reversing the scopolamine induced amnesia. Acetyl cholinesterase inhibitory activity of extracts of *Morus alba* were performed the supportive nootropic activity by enhancing the cognitive function.

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I. INTRODUCTION

Nootropic compounds exhibit a novel class of psychotropic agents with selective facilitating neural activity on integrative function on the central nervous system, especially on intellectual performance, memory and learning capacity (Giurgea, 1973). Indian herbal remedies have been used in the treatment of epilepsy, cognitive dysfunction and insomnia (Bhanumathy et al., 2010) such as *Baccopa monniera* and *Centella asiatica* (Mohan et al., 2005). There is crucial evidence that stress can modify cognitive functions which can lead to various neurodegenerative disorders such as Parkinson's disease or Alzheimer's disease (Koppula and Choi, 2011).

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The central cholinergic system is surveyed to be main neurotransmitter involved in the modulation of cognitive functions. Acetyl cholinesterase has closed interrelation activity with cholinergic function and cognition. Consequently assessment of AChE activity can supply a vital correlation of cognitive function and cholinergic activity (Srikumar et al., 2004). The Indian system of medicine emphasizes use of herbs for neurodegenerative disorders (Jakka AI, 2016). Accordingly the current research was investigated for nootropic activity of aqueous and ethanol extracts of *Morus alba*.

II. MATERIALS AND METHODS

a) Plant material and Preparation of extracts

The fruits of *Morus alba* were collected from Chennai, Tamil Nadu, India and authenticated by Green Chem, Bangalore, Karnataka, India, a voucher specimen (MAT-SIP-501) were preserved for future references. The fruits materials (1kg) were dried, powdered and extracted with water and ethanol (60-80°C) using soxhlet methods. The filtrate was evaporated at 70°C in a vacuum dryer to give final yield 40.5g.

b) Chemicals

Scopolamine butyl bromide and piracetam procured from Stride acrolabs Ltd, Bangalore, India. Other chemicals were analytical up grade and acquired from local store of Visveswarapura Institute of Pharmaceutical Sciences.

c) Animals

Male albino wistar rats (180-200gm) acquired from the NIMHANS animal house, Bangalore. The animals were kept under standard conditions in an animal house as per the guidelines of "Committee for the Purpose of Control and Supervision on Experiments on Animals" (CPCSEA) for at least one week prior to use. The rats had free access to standard rat chow and water *ad libitum*. The study protocol was approved by Institutional Animal Ethics Committee (IAEC), Visveswarapura Institute of Pharmaceutical Sciences, Bangalore. (Registration No: 152/1999, renewed in 2012).

d) Conditioned avoidance response (Perspex chamber apparatus)

Animals were subjected to a training schedule individually by placing inside the perspex chamber of

the apparatus 60 minutes after oral administration. Buzzer was given followed by a shock through the grid floor. The rat had to jump on the pole to avoid foot shock. Jumping prior to the onset of the shock was considered as avoidance. The session was terminated after completion of 30 trials with an interval of 20–30 seconds given for each trial. This procedure was repeated at 24 h intervals until all groups reach 95 to 99% avoidance. After attaining complete training of a particular group, the animals were treated with a single dose of scopolamine butyl bromide (1 mg/kg body weight, i.p.), thirty minutes before the next day dosing. The training schedule was continued further with the daily doses of the aqueous and ethanol extracts of *Morus alba* until they returned to normal level from scopolamine induced amnesia (Cook and Weidley, 1957).

e) *Estimation of acetyl cholinesterase activity by Ellman's method*

Rats were decapitated; brains were removed rapidly and kept in ice-cold saline. Frontal cortex, hippocampus and septum were quickly dissected out on a petri dish chilled on crushed ice. The tissues were homogenized in 0.1m Phosphate buffer. Added 0.4 ml of the homogenates to 2.6 ml phosphate buffer and 100 μ l of DTNB. Absorbance was measured at 412 nm in a UV spectrophotometer. When absorbance reaches stable value, it was recorded as the basal reading. Added 20 μ l of acetyl thiocholine iodide and recorded the change in the absorbance for a period of 10 minutes. Change in the absorbance per minute was determined. The enzyme activity is calculated using the following formula (Srikumar et al., 2004):

$$R = 5.74 \times 10^{-4} \times A/CO$$

R= Rate in mole of substrate hydrolysed/minute/gm tissue

A= Change in absorbance/ minute

CO= Original concentration of the tissue (mg/ml)

f) *Treatment schedule*

For conditioned avoidance response in rat, wistar albino rats were divided into 5 groups consisting of 6 animals in each group. Group 1 rats served as normal control and received 2ml/100g bw distilled water, group 2,3 rats received aqueous extracts *Morus alba* orally at dose of 200 mg/kg and 400 mg/kg respectively. Group 4,5 rats were administrated orally with ethanol extracts of *Morus alba* at dose of 200 mg/kg and 400 mg/kg respectively and for estimation of acetyl cholinesterase activity by Ellman's method in rats, wistar albino rats were divided into 6 groups consisting of 6 animals in each group. Group 1 was vehicle group (Distilled water 2ml/100g bw). Group 2 received standard drug piracetam 200mg/kg i.p. Group 3,4 rats were administrated orally with aqueous extracts of *Morus alba* at dose of 200 mg/kg and 400 mg/kg respectively. Group 5,6 rats received ethanol extracts of

Morus alba orally at dose of 200 mg/kg and 400 mg/kg respectively.

g) *Statistical analysis*

The data were expressed as mean \pm S.E.M. Results were statistically analysed by using one way ANOVA followed by Dunnett's test and $p < 0.05$ was considered as statistically significant.

III. RESULTS

a) *Evaluation of the nootropic activity of aqueous and ethanol extracts of Morus alba using conditioned avoidance response (CAR) in rats*

Figure 1 exhibits the effects of aqueous and ethanol extracts of *Morus alba* on mean percentage of conditioned avoidance response after oral administration in rats. The CAR of rats treated with the aqueous and ethanol extract of *Morus alba* and vehicle increased gradually to 95% over eight to ten days. The percentage avoidance was higher in the groups administered with aqueous and ethanol extract of *Morus alba* compared to vehicle treated control group. The acquisition (time to achieve 95% CAR) for the groups treated with aqueous and ethanol extracts of *Morus alba* was quicker and found to be dose dependent. Animal in group II and III administered with aqueous extract of *Morus alba* at a dose of 200 mg/kg p.o and 400 mg/kg p.o have taken ten days and nine days respectively to reach the point of acquisition. Whereas animals in group IV and V administered with ethanol extract of *Morus alba* at a dose of 200 mg/kg p.o and 400 mg/kg p.o have taken nine days and eight days respectively to reach the point of acquisition. Administration of scopolamine produced amnesia as seen from reduction in the observed CAR. The amnesia was found to be greater in control group compared with the groups treated with aqueous and ethanol extract of *Morus alba* and was also found to be dose dependent. Animals treated with aqueous extract of *Morus alba* at a dose of 200 mg/kg and 400 mg/kg had taken five and four days whereas, group treated with ethanol extract of *Morus alba* at a dose of 200 mg/kg and 400 mg/kg had taken three days each to reach the point of acquisition after administration of scopolamine butylbromide. The control group had taken eleven days for retention and recovery from scopolamine induced amnesia.

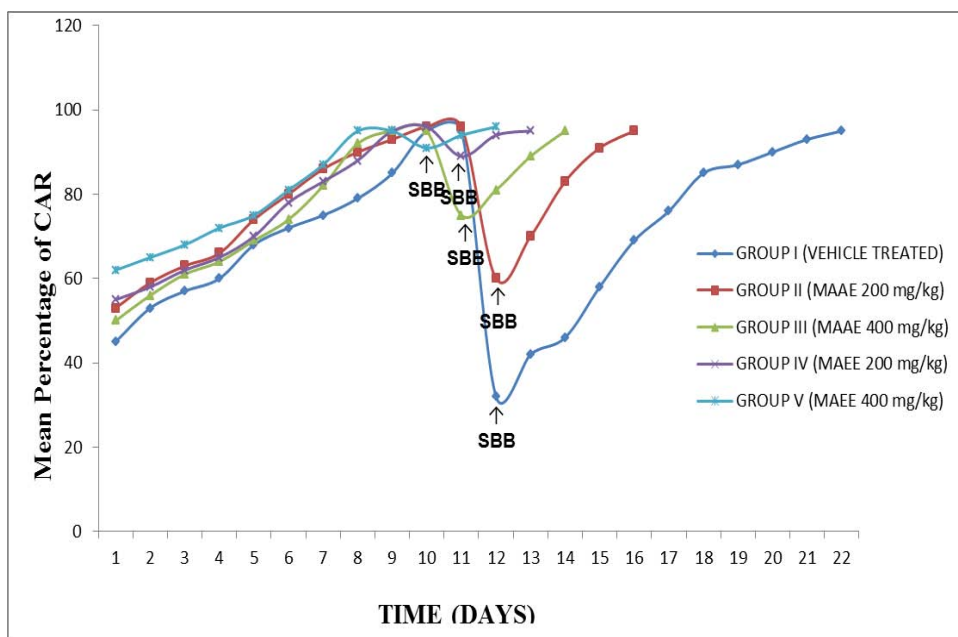


Figure 1: Effect of aqueous and ethanol extracts of *Morus alba* on mean percentage of conditioned avoidance response after oral administration in rats. Scopolamine butylbromide (SBB) was administered 30 minutes before next day dosing with the extracts after attaining complete acquisition. MAAE: aqueous extract of *Morus alba*, MAEE: ethanol extract of *Morus alba*.

b) Estimation of acetyl cholinesterase activity by Ellman's method

Figure 2 manifests the effects of aqueous and ethanol extracts of *Morus alba* on acetylcholinesterase (AChE) activity in rats' brain. The groups treated with aqueous and ethanol extract of *Morus alba* had indicated decrease in AChE activity as compared to control group. Control group had showed 7.460×10^{-7} $\mu\text{mol}/\text{min}/\text{g}$ tissue of acetylcholinesterase activity in rat brain. Prior administration with Piracetam (standard) had showed a significant reduction in acetylcholinesterase

activity 4.010×10^{-7} $\mu\text{mol}/\text{min}/\text{g}$. Prior administration of aqueous extract of *Morus alba* at dose of 200 mg/kg p.o and 400 mg/kg p.o have showed non-significant decrease in acetylcholinesterase activity 6.820×10^{-7} and 6.320×10^{-7} $\mu\text{mol}/\text{min}/\text{g}$ respectively as compared to control group. However, significant decline was observed in groups treated with ethanol extract of *Morus alba* at dose of 200 mg/kg p.o and 400 mg/kg p.o with acetylcholinesterase activity 4.940×10^{-7} $\mu\text{mol}/\text{min}/\text{g}$ ($P < 0.01$) and 4.540×10^{-7} $\mu\text{mol}/\text{min}/\text{g}$ ($P < 0.001$) respectively as compared to control group.

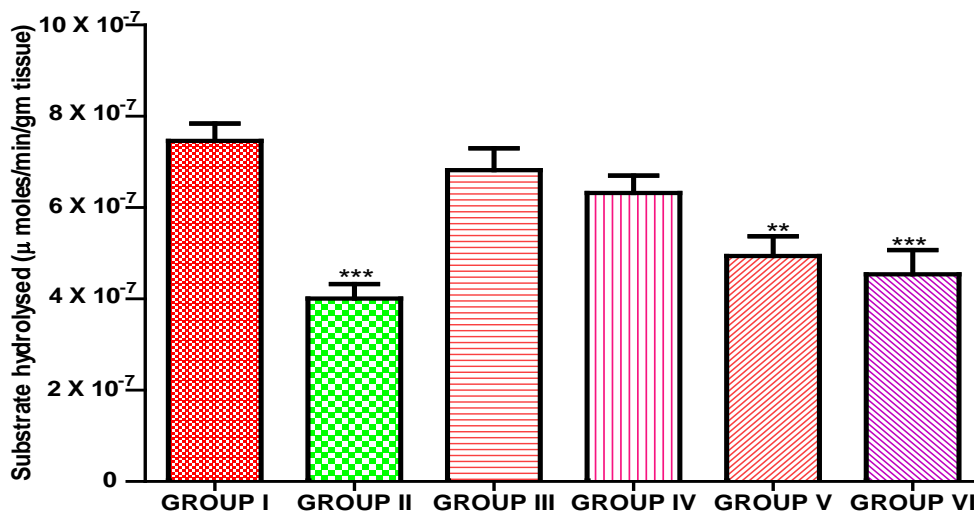


Figure 2: Effect of aqueous and ethanol extracts of *Morus alba* on acetylcholinesterase (AChE) activity in rat's brain. Data is expressed as mean \pm SEM. Statistical analysis was done by one-way ANOVA followed by Dunnett's test. ** $P < 0.01$ and *** $P < 0.001$ were considered statistically significant.

IV. DISCUSSION

The original definition for nootropics was laid out by Dr. Corneliu E. Giurgea in 1972 who also is the inventor behind Piracetam. The word itself is taken from the Greek language and is a combination of two words: "noos" (mind) and "tropein" (turning). It is literally translated as "towards the mind" or "affecting the mind", it means enhancement of learning and memory (Shivkumar et al., 2011). A nootropic drug is distinguished by activating of the higher integrative brain mechanisms directly which lead to enhance cortical vigilance, a telencephalic functional selectivity, and a particular efficiency in restoring deficient higher nervous activity (Giurgea, 1973). These drugs have particularly the intellectual performance, learning capacity and memory. Nootropics drug can work out by mechanism of action of increasing the brain supply of neurochemicals, improving brain oxygen supply or by stimulating nerve growth. The learning and memory is closely allied with functional status of central cholinergic system (Shivkumar et al., 2011). In the current investigation, administration of aqueous and ethanol extracts of *Morus alba* in *wistar* rats exhibited significant improvement in memory functions by reversing the scopolamine butyl bromide induced amnesia in learning and memory task performed on perspex chamber of the apparatus. The scopolamine model recommends that the cognitive deficits that can be observed after scopolamine treatment are directly associated to a decline in central cholinergic functions (Ellis and Nathan, 2001). The memory and learning is tightly related to the functional status of the central cholinergic system, the basal forebrain provides the major source of cholinergic input to the neocortex and hippocampus (Shivkumar et al., 2011). Previous literatures have shown that scopolamine impairs retrieval memory in rats and such amnesia is associated with elevated MDA and reduced GSH levels (Koppula and Choi, 2011). Since oxidative stress has been implicated in the pathophysiology of dementia, and also scopolamine has been reported to elevate rat brain oxidative stress, scopolamine-induced amnesia in rats could be used as a valid model to screen drugs with potential therapeutic benefit in dementia (Shivkumar et al., 2011). Nalini et al, correlated the improvement in learning and memory to the reduction in the levels of NE, DA and 5-HT (Nalini et al., 1995). The previous phytochemical investigation of *Morus alba* manifested the presence of phenolic compounds such as flavonoids (Quercetin, rutin), tannin which could be responsible for nootropic activity (Srikumar et al., 2004). NE is synthesized by dopamine. Previous reports displayed that these phytochemicals can diminish dopamine level and also these bioactive compounds can prevent activity of tryptophan hydroxylase enzyme which is involved in the biosynthesis of 5-HT (Bharani et al., 2010). ACh has a

crucial role in the enhancement of sensory perceptions and in sustaining attention. Damage to the cholinergic system has been exhibited to be possibly related to the memory deficits associated with alzheimer's disease. Inhibition of ACh hydrolysis may be achieved through the use of AChE inhibitors (Jagetia et al., 2004). Aqueous and ethanol extracts of *Morus alba* showed significant decrease in AChE activity in a dose dependent manner, hence maintaining the acetylcholine level which is responsible for memory. Flavonoids may mimic the actions of estrogens in the brain (Jager and Saaby, 2011) or may influence the synthesis of acetylcholine and neurotropic factors such as BDNF and nerve growth factor in hippocampus and frontal cortex. *Morus alba* contains flavonoids (Ayoola et al., 2011) as one of its active constituent which expected to be responsible for acetylcholine synthesis and improvement of memory.

V. CONCLUSION

The current research evinces that aqueous and ethanol extracts of *Morus alba* have nootropic activity so it can be appraised worthwhile for supportive therapy in memory deficits associated with alzheimer's disease or amnesia.

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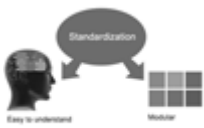
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