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RESEARCH ARTICLE

Optimization of Cardioprotective Potential of Various Concentrations of Medicinal Plants by Using Response Surface Methodology

Nadia Afsheen[1], Khalil-ur-Rehman[1], Nazish Jahan[2]*, Khalid Mahmood Khan[1] and Muhammad Anjum Zia[1]

[1]Department of Biochemistry; [2]Department of Chemistry, University of Agriculture, Faisalabad, Pakistan
*Corresponding author: nazishjahanuaf@yahoo.com

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ABSTRACT

This study was aimed to optimize the various concentrations of selected medicinal plants through Response Surface Methodology (RSM) in conjunction with Central Composite Design (CCD) to assess their therapeutic doses for cardioprotection. Dose response relation is an important tool to study pharmacological efficacy and therapeutic index of herbal medicines. In this study the toxicological assay of various concentrations of *Rauvolfia serpentina, Eletaria cardamom, Coriandrum sativum, Piper nigrum, Allium sativum, Crataegus oxyacantha* and *Terminalia arjuna* was performed prior to *in vivo* evaluation. The toxicological findings depicted that none of the selected medicinal plant showed any toxicity, therefore is declared to be safe for various cardiovascular disease. Instead of *in vivo* trial of hundreds of the possible doses, RSM suggested only five doses (80, 110, 140, 170 and 200 mg/kg b.wt) to explore cardioprotective potential of selected medicinal plants in rats. Blood samples were taken at different time intervals to analyze the cardiac markers (CK-MB, LDH and SGOT). These cardiac markers were statistically analyzed by "RSM" to get the optimal therapeutic dose of each selected medicinal plants. The results revealed that the *R. serpentina, C. oxyacantha, T. arjuna, E. cardamom, C. sativum, P. nigrum* and *A. sativum* showed maximum cardioprotection at corresponding concentration of 164, 172, 165, 190, 183, 186 and 170 mg/kg b.wt.

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INTRODUCTION

Cardiovascular disease (CVD) is a known ubiquitous cause of morbidity and has become a leading contributor of mortality in almost all countries. Not to speak of underdeveloped or developed countries, the developed nations have also not been able to control and provide the successful solution for the management and treatment of cardiovascular disorders (Aslam *et al*., 2015). Cardiovascular diseases cause 17.1 million fatalities each year and it will reach upto 20 million in 2020 (Zafar *et al.*, 2015). Among CVD, the Myocardial Infarction (MI) is the most dreaded menace. MI is prolonged ischemia with necrosis of myocytes due to interruption of blood supply to an area of heart (Kumar and Gurusamy, 2014; Tselios *et al*., 2016) which results in multiple biochemical alterations (Alamgeer *et al.*, 2015).

A number of herbs have been reported for their cardioprotective effect, which are safe and inexpensive (Beaulah *et al.*, 2014; John, 2014). Therefore, the abundantly available natural cardioprotective herbs have diverted the attention of entire world population towards the green source. These medicinal plants possess primary and secondary metabolites which are responsible for prevention and management of CVD (Beaulah *et al.*, 2014). However, dose response relation is not known for such cardioprotective herbs. Dose-response experiments based upon RSM should be routinely conducted in preclinical and clinical trials to find out the effective concentration of medicinal plants and the probability of a response (Yankov, 2010).

Preclinical and clinical trials need a number of experiments, of *in vitro* and *in vivo* nature for confirmation of any pharmaceutical product. This may involve a large number of experimental animals to be sacrificed, more cost of laboratory and animal trials and above all more times for series of experiments. Better results may be obtained through relatively small number

of laboratory trials, if these are statistically conjuncted with statistical tool RSM. Therefore, main advantage of RSM is to reduce the number of experiments necessary to conduct the research. Thus, it results in decrease in the consumption of materials and provide most appropriate dose of selected herb (Jing *et al.*, 2015). Keeping in view the above facts, in this study the dose response relation of seven selected medicinal plants was evaluated for their cardioprotective potential against salbutamol induced CVD in rats by using RSM.

MATERIALS AND METHODS

Collection of medicinal plants: Different parts of medicinal plants including roots of *Rauvolfia serpentina*, seeds of *Eletaria cardamom*, *Coriandrum sativum*, leaves of *Piper nigrum*, fruit of *Allium sativum*, *Crataegus oxyacantha* and bark of *Terminalia arjuna* were collected from University of Agriculture, Faisalabad. These parts of the plants were washed, dried, pulverized and sieved to get fine powder.

Preparation of herbal extract: The powdered plants were macerated in methanol and kept in orbital shaker for four days and the macerates were filtered. The filtrate was concentrated by rotary evaporator and dried by using lyophilizer (Jahan *et al.*, 2012).

***In vitro* assay:**

Toxicity assay: Prior to dose response evaluation of selected medicinal plants, the toxicological assay of selected medicinal plants was performed through "Hemolytic activity" and "Mutagenicity assay".

Hemolytic activity

Preparation of erythrocytes suspension: Blood sample was withdrawn from individual and centrifuged for three minutes at 1500 rpm. The supernatant was discarded and the pellet was rinsed by phosphate buffer of pH 7.2±0.2. The cells were resuspended in normal saline (Kumar *et al*., 2011).

Hemolytic activity: An equal volume of cell suspension and various concentrations of plants extracts (100, 500 and 1000 µg/mL) were mixed in PBS. The reaction mixtures were incubated for 30 min at 37°C in an incubator (MIR-254, Sanyo, Japan) and centrifuged for 10 min at 1500 rpm. The free hemoglobin in the supernatant was measured in UV-Vis spectrophotometer (Dynamica, Halo BD-20, Australia) at 540 nm. The percentage hemolysis was calculated by given formula:

$$\text{Hemolysis (\%)} = A_t - A_n / A_c$$

"A_t" is the absorbance of plants extract, "A_n" showed the absorbance of saline control and "A_c" is the absorbance of water control (Riaz *et al*., 2012).

Mutagenicity assay

Bacterial strain: The mutant strain *S. typhimurium* TA98 was maintained on nutrient agar and incubated at 37˚C for 18-24 hr before test.

Reagent mixture: Reagent mixture was prepared by mixing Devis Mingoili salt (21.62 mL), D-Glucose (4.75 mL), D-Biotin (1.19 mL), Bromocresol purple (2.38 mL) and L-Histidine (0.06 mL) aseptically in falcon tube.

Procedure: Herbal extracts, distilled water, reagent mixture and standard mutagen were mixed with the quantity presented in Table 1 and inoculated with homogenous culture broth of *S. typhimurium*. The contents of each tube were allotted into each well of micro titration plate and plates were incubated for four days at 37°C.

Interpretation of result: The turbid or yellow wells were marked as positive while purple wells as negative. Herbal extract would be considered to be mutagenic, if the numbers of positive well were considerably higher than background plate (Razak *et al*., 2007).

Dose response experiment

Selection of animals: The rats were acclimatized for one week under laboratory conditions. The husk in the cages was renewed thrice a week. All the animals were kept in Animal House, Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad.

Protocol: The rats were randomly divided into following groups. The rats in control group were fed with normal diet throughout the experimental period of 23 days. In positive control group the rats were treated with normal diet for 21 days and after that 80 mg/kg b.wt. of salbutamol was given orally twice at an interval of 24 hr. The preventive group included seven treatment groups; each treatment group was pretreated with different concentrations of its respective plant for three weeks. The concentrations of all treatment groups were suggested by "Central Composite Design" of Response Surface Methodology as given in Table 2. After three weeks the salbutamol (80 mg/kg b.wt.) were administered to all treatment groups for two consecutive days.

Biochemical analysis: The blood sampling was performed after 24 hr of administration of salbutamol. The cardiac biomarkers including Creatine Kinase-MB (CK-MB), Serum Glutamic-Oxaloacetic Transaminase (SGOT) and Lactate Dehydrogenase (LDH) were analyzed by using kit method through chemistry analyzer.

Statistical analysis: All the data was statistically analyzed by "Central Composite Design" of "Response surface methodology" Design-expert 7.0 (Raymond *et al*., 2016).

Table 1: Set up of the mutagenicity assay

Treatment	Volume (mL)				
	Mutagen standard	Plant extract	Reagent mixture	Distilled water	S. typhimurium
Blank	-	-	2.5	17.5	-
Background	-	-	2.5	17.5	0.005
Standard mutagen	0.1	-	2.5	17.4	0.005
Test samples	-	0.005	2.5	17.5	0.005

Table 2: The CCD for dose response experiment of selected medicinal plants against MI

Grps.	Plants	Conc. (mg/kg)	Grps.	Plants	Conc. (mg/kg)	Grps.	Plants	Conc. (mg/kg)
G1	*T. arjuna*	80		*P. nigrum*	110		*A. sativum*	200
	T. arjuna	80		*P. nigrum*	170		*A. sativum*	200
	T. arjuna	110		*P. nigrum*	200		*A. sativum*	140
	T. arjuna	170		*P. nigrum*	200	G6	*R. serpentine*	80
	T. arjuna	200		*P. nigrum*	140		*R. serpentine*	80
	T. arjuna	200	G4	*C. sativum*	80		*R. serpentine*	110
	T. arjuna	140		*C. sativum*	80		*R. serpentine*	170
G2	*C. oxyacantha*	80						
				C. sativum	110		*R. serpentine*	200
	C. oxyacantha	80		*C. sativum*	170		*R. serpentine*	200
	C. oxyacantha	110		*C. sativum*	200		*R. serpentine*	140
	C. oxyacantha	170						
				C. sativum	200	G7	*E. cardamom*	80
	C. oxyacantha	200		*C. sativum*	140			
							E. cardamom	80
	C. oxyacantha	200	G5	*A. sativum*	80		*E. cardamom*	110
	C. oxyacantha	140		*A. sativum*	80		*E. cardamom*	170
G3	*P. nigrum*	80		*A. sativum*	110		*E. cardamom*	200
	P. nigrum	80		*A. sativum*	170		*E. cardamom*	200
							E. cardamom	140

RESULTS

Toxicity assay

Hemolytic assay: Hemolytic assay of *R. serpentina*, *E. cardamom*, *C. sativum*, *P. nigrum*, *A. sativum*, *C. oxyacantha* and *T. arjuna* was performed against human erythrocytes using triton X-100 as positive control. The hemolytic activity of extracts of various plants at varying concentrations (100, 500 and 1000 µg/mL) is expressed as percentage hemolysis in Fig. 1. None of the plant extract showed any hemolytic effect against erythrocytes, so pharmacologically these plants are safe to use for human beings as a source of therapeutic drug. The *T. arjuna* and *C. oxyacantha* showed very low % age hemolysis as compared to other selected medicinal plants.

Mutagenicity assay (Ames test): The mutagenic probability of these selected medicinal plants was also evaluated by using test strain *S. typhimurium* TA 98. The standard *S. typhimurium* TA 98 showed considerable mutagenicity with high number (94/96) of positive wells (Table 3). The mutagenicity of all the medicinal plants was counted and compared with background plate (11/96 positive wells). The medicinal plants are assumed to be mutagenic if the number of positive wells is two folds higher as compared to the background plate. The *C. sativum*, *T. arjuna* and *R. serpentina* showed 8/96 while the *E. cardamom*, *A. sativum* and *P. nigrum* presented 16/96 yellow wells in microplate (Table 3). Among these the *C. oxyacantha* showed good results mean absolutely safe with no positive well in microplate.

Dose response experiment

Cardiac markers: The effect of different concentrations of medicinal plants against MI was evaluated by estimation of cardiac markers including CK-MB, SGOT and LDH (Fig. 2).

CK-MB: The positive control group, to which only salbutamol (80 mg/kg) was given, showed considerable elevation (296 IU/L) in the CK-MB level as compared to normal control group. The rats in different treatment groups were treated with various concentrations (80, 110, 140, 170 and 200 mg/kg) of medicinal plants. The effect of these medicinal plants on the level of CK-MB has been given graphically in Fig. 2(a). The increase in concentration from 80 to 170 mg/kg b.wt. of *T. arjuna*, *C. oxyacantha* and *R. serpentina* maintained the level of CK-MB gradually. The dose of 80 mg/kg b.wt. of *P. nigrum* could not maintain the level of CK-MB within limits in contrast to the same dose of other selected medicinal plants. However, the rats treated with *P. nigrum* at concentrations of 170 and 200 mg/kg depicted almost good impact in order to maintain the level of CK-MB. *A. sativum* and *E. cardamom* depicted the maximum preventive potential at the concentration of 200 mg/kg b.wt.

The experimental data was statistically analyzed by using RSM and the ANOVA (Table 4) explained that the quadratic model is significant (<0.05) for such an experiment. The F-value indicated the dose response interaction strength of CK-MB. The value of Predicted *R*-Squared was close to the Adjusted R-Squared value as expected. This may indicate a small block effect or a possible problem (Table 4).

SGOT: The normal control group illustrated 36 IU/L of SGOT while the positive control group, merely treated with salbutamol, showed 96 IU/L level of SGOT. The graphical presentation in Fig. 2(b) showed the response of different treatments on the level of SGOT against salbutamol induced MI. The *T. arjuna, C. oxyacantha* and *R. serpentina* depicted maximum protective potential at the concentration of 140 and 170 mg/kg. None of the concentrations of *P. nigrum* from 80 to 140 mg/kg b.wt. could maintain the level of SGOT as compared to normal control group. However, 170 mg/kg of *P. nigrum* might be considered as the effective concentration that may cope with complications related to MI.

The F-value in ANOVA indicated the significance (<0.05) of quadratic model for optimization of effective cardioprotective dose (Table 4). Moreover, the accuracy and reliability of the experiment was also confirmed by the coefficient of variation.

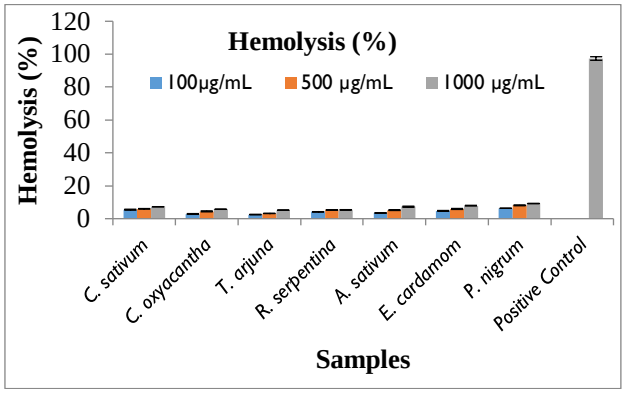

Fig. 1: % Hemolysis of extracts of selected medicinal plants at different concentrations.

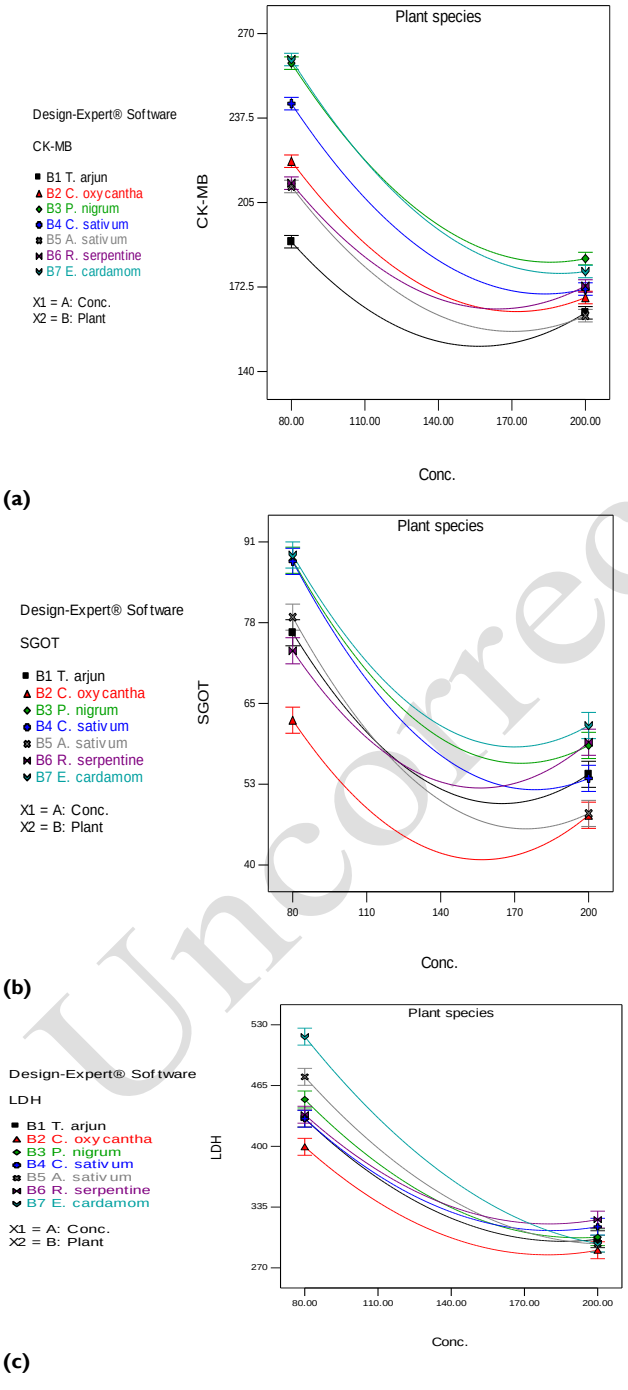

Fig. 2: Optimization of medicinal plants for CK-MB, SGOT and LDH against salbutamol induced MI.

Table 3: The mutagenicity of standard, Background and extracts of selected medicinal plants

Sr. #	Plant extracts	+ve/total	Results	Interpretation
1	Standards	94/96	+	Mutagenic
2	Background	11/96		
3	*C. sativum*	08/96	-	Non mutagenic
4	*C. oxyacantha*	01/96	-	Non mutagenic
5	*E. cardamom*	16/96	-	Non mutagenic
6	*P. nigrum*	16/96	-	Non mutagenic
7	*A. sativum*	16/96	-	Non mutagenic
8	*T. arjuna*	8/96	-	Non mutagenic
9	*R. serpentine*	8/96	-	Non mutagenic

LDH: The normal and positive control groups presented 250 and 519 IU/L level of LDH respectively. Different treatment groups were administered with varying concentrations of selected medicinal plants and their effects on the level of LDH have been presented in Fig. 2(c). The graphical presentation showed that the *T. arjuna* and *C. oxyacantha* presented the effective response to maintain the level of LDH at the concentration of 140 mg/kg during myocardial infarction. While *C. sativum*, *E. cardamom*, *A. sativum* and *R. serpentina* showed good results at the concentration of 200 mg/kg b.wt.

The ANOVA for quadratic model was presented in Table 4. The value of determination coefficient for LDH was 0.9832 which means that the calculated model was able to explain 98.32% of the results. Meanwhile, a relatively lower value of coefficient of variation showed a better precision and reliability of the experiment.

The RSM analyzed the experimental data and suggested the optimum concentration, 165, 157 and 164 mg/kg b. wt. for *T. arjuna*, *C. oxyacantha* and *R. serpentina* respectively that may sustain the level of cardiac markers near to normal. All the suggested doses by RSM were found very close to the experimentally proved findings (140-170 mg/kg b.wt). According to experimental approach the concentration of 170 mg/kg b.wt. of *P. nigrum* maintained the enzyme level against salbutamol induced MI while the response surface methodology suggested the concentration of 186 mg/kg that may able to maintain the enzymatic level near to normal. The RSM predicted the optimum concentration of 170 mg/kg for *A. sativum* while the experimental value depicted the concentration of 200 mg/kg to keep the level of cardiac markers within range. The RSM endorsed the concentration of 183 and 190 mg/kg for *C. sativum* and *E. cardamom* that was near to our experimental approach (200 mg/kg). The RSM gave the appropriate doses of selected medicinal plants, which would reduce the cost of expensive analysis methods and their associated numerical noise.

DISCUSSION

Preclinical dose response experiments are used to determine an effective and safe dose for use in humans and ultimately to achieve successful review of registration to support marketing approval (Robinson *et al.*, 2009).

In this study the dose response evaluation of selected medicinal plants including *R. serpentina*, *T. arjuna*, *C. sativum* and *E. cardamom*, *P. nigrum*, *A. sativum* and *C. oxyacantha* was carried out followed by *in vitro* characterization. *In vitro* toxicological evaluation of the selected medicinal plants was performed to confirm whether

Table 4: ANOVA for response surface methodology of CK-MB, SGOT and LDH as a function of independent variables

	Source	SS	Df	MS	F Value	Prob>F	
CK-MB	Model	45186.46	14	3227.60	437.43	<0.0001	Significant
	A-Conc.	25348.74	1	25348.74	3435.46	<0.0001	.
	B-Plant	11898.25	6	1983.04	268.76	<0.0001	
	AB	2697.00	6	449.50	60.92		
	A^2	5242.48	1	5242.48	710.50		
	Residual	250.87	34	7.38			
	Lack of Fit	250.37	20	12.52	350.52	<0.0691	Non Significant
	Pure Error	0.50	14	0.036			
	Cor Total	45437.33	48				
	R-Squared	0.9945	Standard Deviation				2.72
	Adj R-Squared	0.9922	Mean				190.84
	Pred R-Squared	0.9898	Coefficient of Variation (CV) %				1.42
	Adeq Precision	72.594	Prediction Error Sum of Squares (PRESS)				463.20
SGOT	Model	8591.59	14	613.69	114.98	<0.0001	Significant
	A-Conc.	4741.56	1	4741.56	888.41	<0.0001	.
	B-Plant	1909.99	6	318.33	59.64	<0.0001	
	AB	399.52	6	66.59	12.48		
	A^2	1540.52	1	1540.52	288.64		
	Residual	181.46	34	5.34			
	Lack of Fit	174.95	20	8.75	18.80	< 0.0864	Non Significant
	Pure Error	6.52	14	0.47			
	Cor Total	8773.05	48				
	R-Squared	0.9793	Standard Deviation				2.31
	Adj R-Squared	0.9708	Mean				62.34
	Pred R-Squared	0.9633	Coefficient of Variation (CV) %				3.71
	Adeq Precision	36.813	Prediction Error Sum of Squares (PRESS)				321.95
LDH	Model	2.076E+005	14	14828.25	142.17	<0.0001	Significant
	A-Conc.	1.655E+005	1	1.655E+005	1587.14	<0.0001	.
	B-Plant	15205.97	6	2534.33	24.30	<0.0001	
	AB	11629.36	6	1938.23	18.58		
	A^2	15226.65	1	15226.65	145.99		
	Residual	3546.09	34	104.30			
	Lack of Fit	3545.59	20	177.28	4963.82	<0.0781	Non significant
	Pure Error	0.50	14	0.036			
	Cor Total	2.111E+005	48				
	R-Squared	0.9832	Standard Deviation				10.21
	Adj R-Squared	0.9763	Mean				360.26
	Pred R-Squared	41.125	Coefficient of Variation (CV) %				2.83
	Adeq Precision	10.823	Prediction Error Sum of Squares (PRESS)				6086.98

the selected medicinal plants were toxic or not (Abudayyak *et al*., 2015), so that these may be used safely for therapeutic purpose of various CVD. The toxicological evaluation represented that none of the selected medicinal plants showed any toxicity. The hemolytic percentage of extracts was found to be increased with increase in concentration of extracts of plants. The phytochemicals constituents present in extracts of these medicinal plants might be responsible for their antihemolytic and non mutagenic property (Lakshmi *et al.*, 2014). The aqueous extracts of different *Acacia* species have also been screened against normal human erythrocytes which exhibited low to mild hemolytic effect (Sulaiman and Gopalakrishnan, 2013). Thus, the toxicological evaluation of medicinal plants is related to chemical composition and concentration of each plant extract (Zohra and Fawzia, 2014).

In dose response *in vivo* experiment the increase in cardiac markers in positive control group might be due to the reason that salbutamol causes leakage of cardiospecific enzymes from cardiomyocytes into the blood stream. It occurs as a result of collapse of cellular and subcellular compartments that reflect pathological alterations in myocardium (Khan *et al*., 2014; Aslam *et al*., 2015). Cardiospecific enzyme are existed in myocardium and released into the blood stream following myocytes injury and disintegration of the subcellular and cellular compartments (Mnafgui *et al*., 2015) hence considered as standard for diagnosis of MI (Jagannadha *et al*., 2010).

The RSM optimized the effective response of *T. arjuna*, *C. oxyacantha* and *R. serpentina* at the concentration of 165, 157 and 164 mg/kg b.wt to maintain the level of cardiac enzymes during CVD. RSM is an effective modeling tool to optimize the appropriate therapeutic various concentrations of medicinal plants (Zhao *et al*., 2012). The RSM technique can optimize complex processes because it allows more efficient interpretation of experiments as compared to other traditional method. The cardioprotective potential of *T. arjuna* is attributed to the presence of potent antioxidant compounds (Aslam *et al*., 2015). The *C. oxyacantha* is deemed as best known cardiotonic hence it helps to improve the blood supply to heart by dilating blood vessels and attenuating symptoms of heart failure (Zafar *et al*., 2015). Serpentine present in *R. serpentina* is useful to cure hypertension and CVD. *R. serpentina* is a hopeful herbal option due to the existence of considerable bioactive compounds in roots (Gawade *et al*., 2012). Plants containing flavanoids have been reported to possess strong antioxidant properties which is responsible for its cardio-protective potential (Abirami and Kanagavalli, 2013).

Conclusions: In dose response experiment, the said medicinal plants including *R. serpentina, C. oxyacantha, T. arjuna, E. cardamom, C. sativum, P. nigrum* and *A. sativum* showed maximum cardioprotection at the corresponding concentration of 164, 172, 165, 190, 183, 186 and 170 mg/kg. These doses could considerably minimize the elevated enzymatic levels as compared to other selected doses of plants.

Authors contribution: KR, NJ and KMK conceived the idea and designed the project. NA performed research work, wrote the protocol and draft of the manuscript. KR and AZ provided guidance related to clinical analysis. All authors read and approved the final manuscript.

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