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ORIGINAL ARTICLE

Evaluation of Cardioprotective Potential of Isolated Swerchirin against the Isoproterenol-Induced Cardiotoxicity in Wistar albino rats

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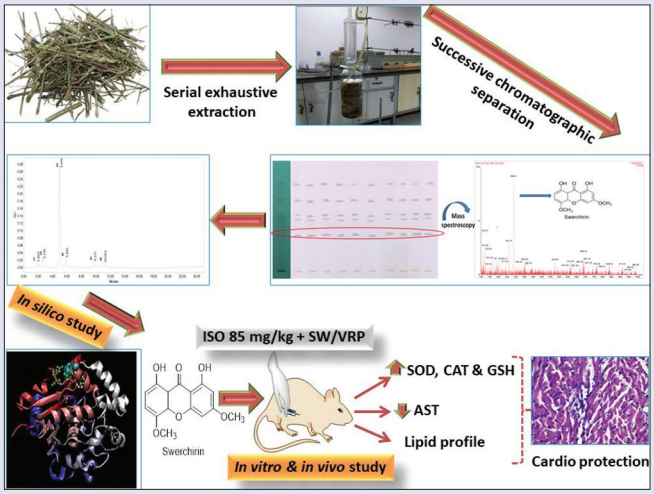
ABSTRACT

Background: Cardiotoxicity is one of the emerging health-care issues worldwide and mortality due to this is increasing exponentially. Oxidative stress, myocardial inflammation, and damage to the cardiac membrane are major causative attributes for this toxicity. Isoproterenol (ISO)-induced cardiotoxicity is a well-established and accepted model to estimate innovative cardioprotective agents, as it exhibits several morphological and biochemical aberrations in the myocardium of rat that is comparable with those detected in clinical practice. **Objectives:** In the present study, we explored the cardioprotective effect of swerchirin (SW) extracted from *Swertia chirayita* in the ISO model. **Materials and Methods:** SW was isolated from *S. chirayita*, characterized by high-performance thin-layer chromatography-mass spectrometry, ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR, and high-performance liquid chromatography techniques. The *in-vitro* study was performed for 2,2-Diphenyl-1-picrylhydrazyl and nitric oxide scavenging activity to explore the antioxidant capacity. *In silico* study was performed to ascertain the binding affinity of SW with antioxidant enzymes. The *in-vivo* study was performed to explore the cardioprotective activity in the ISO-induced cardiotoxic model. **Results:** The *in vitro* studies showed the significant antioxidant potential of SW whereas the *in silico* study revealed its effective binding into the catalytic pocket domain of superoxide dismutase and catalase. The *in vivo* study showed a significant improvement in antioxidant enzymes and a reduction in serum glutamic-oxaloacetate transaminase and lipid profile. Further, SW also significantly reversed the histopathological aberrations in a dose-dependent manner. **Conclusion:** Findings of the study showed a significant cardioprotective effect of SW against ISO-induced cardiac damage.

Key words: High-performance liquid chromatography, high-performance thin-layer chromatography-mass spectrometry, myocardial infarction, oxidative stress, swerchirin, *Swertia chirayita*

SUMMARY

- In the current study, we have explored the cardioprotective potency of swerchirin (SW) against Isoproterenol (ISO)-induced cardiotoxic manifestations in Wistar albino rats. SW was first extracted from *Swertia chirayita* and characterized by high-performance thin-layer chromatography-mass spectrometry, ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR and high-performance liquid chromatography techniques. Isolated SW was tested for antioxidant activity using *in-vitro* methods (2,2-Diphenyl-1-picrylhydrazyl and nitric oxide scavenging activity) and also explored for binding affinity against antioxidants enzymes, superoxide dismutase (SOD), and catalase (CAT), using *in silico* approach. Further to ascertain the cardioprotective potency of isolated SW in the *in vivo* study, three different doses of SW (6, 12, and 24 mg/kg) were used and compared with the standard drug verapamil (20 mg/kg). The outcome of the *in-vitro* study showed significant antioxidant activity of SW. *In silico* study showed effective binding affinity of SW into the catalytic pocket domain of SOD and CAT. *In-vivo* study showed reversal in the level of SOD, CAT, aspartate transaminase, TBARS, and histological aberrations toward the normal which otherwise got disturbed due to ISO toxicity. SW at the dose of 12 and 24 mg/kg showed dose-dependent cardioprotection, however, SW at the dose of 6 mg/kg failed to exhibit cardioprotection against ISO-induced cardiac damage. Nonetheless, based on the current findings, we propose to investigate a detailed mechanism of cardioprotection targetting various signaling pathways to establish the novelty of this drug.



Abbreviations used: CVDs: Cardiovascular diseases; ISO: Isoproterenol; SW: Swerchirin; HPTLC: High-performance thin-layer chromatography; MS: Mass spectrometry; NMR: Nuclear magnetic resonance; HPLC: High-performance liquid chromatography; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; NO: Nitric oxide; SOD: Superoxide dismutase; CAT: Catalase; ROS: Reactive oxygen species; BNPL: Bioactive Natural Product Laboratory; VRP: Verapamil; TLC: Thin-layer chromatography; ESI-MS: Electrospray ionisation mass spectrometry; DMSO: Dimethyl sulfoxide; PTFE: Polytetrafluoroethylene; ELISA: Enzyme-linked immunosorbent assay; PBS: Phosphate-buffered saline; PDB: Protein data bank; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals; SGOT: Serum glutamic-oxaloacetate transaminase; AST: Aspartate transaminase; TGs:

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Triglycerides; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; VLDL: Very-low-density lipoprotein; TC: Total cholesterol; H and E: Hematoxylin and eosin; MDA: Malondialdehyde; ANOVA: Analysis of variance; AMIT: Amitrole; SA: Salicylic acid; LCS: 1,4,5-dichloro-2-m-tolylpyridazin-3 (2H)-one; DDC: diethyldithiocarbamic acid.

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INTRODUCTION

Globally, cardiovascular diseases (CVDs) are one of the leading causes of death. The World Health Organization reported 17.8 million deaths due to CVDs during 2017 and it is expected to reach about 22.2 million by 2030.[1-3] The huge increase in the prevalence rate of CVDs necessitates deeper understanding and investigations of the problem to overcome the staggering health and economic burdens of CVDs.[4] Health-care providers and researchers have been exploring novel strategies to solve the problem for a long time but still, the success achieved is far from optimum.[5] For evaluation of the cardioprotective potential of drug candidates, the use of the animal model is more prevalent among various available strategies. In such models, cardiotoxicity is first induced then the cardioprotective potential of molecules is evaluated.[6] The ISO-induced model of CVD serves as a well-recognized, acceptable cardiotoxic model to assess the various novel cardioprotective agents as it exhibits similarity with those detected in human CVD.[7-9]

ISO causes myocardial stress due to the development of infarct-like necrosis and oxidative stress in the myocardium which leads to the production of reactive oxygen species (ROS), lipid peroxidation, and irreversible membrane injury as well as structural and biochemical anomalies.[10] Various herbs and their phytoconstituents are being investigated for their cardioprotective activities due to safety, efficacy, and cost-effectiveness.[11]

Xanthones, the polyphenolic compounds which commonly occur in plants belonging to the genus *Swertia*, family: Gentianaceae are rich sources of xanthonoids having extensive pharmacological actions.[12] Xanthones are potent antioxidant phytonutrients that promote a healthy cardiovascular system by neutralizing free radicals.[13] Swerchirin (SW) (1,8-dihydroxy-3,5-dimethoxy xanthone) is an important tetraoxygenated naturally occurring xanthone obtained from the plants of *Swertia chirayita* (Roxb. ex. Flem).[14] SW has been reported to possess hypoglycemic, hepatoprotective, chemo-preventive, hemopoietic, and anti-cancer.[15-20]

According to our literature survey, we found that no one has explored the cardioprotective effect of this noteworthy polyphenolic constituent. Therefore, we planned to isolate SW from *S. chirayita* and explore the scientific basis of its use for cardioprotective potential as a novel drug candidate.

MATERIALS AND METHODS

Collection and authentication of plant material

Dried crude whole plant material of the *S. chirayita* was procured from Universal Biotech 1313-Farash Khana, Delhi (India) and was authenticated through the standard procedure of Unani Pharmacopoeia of India.[21] The voucher specimen was deposited to Bioactive Natural Product Laboratory (BNPL), School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India (Specimen No.: BNPL/JH/PhD/09/2015/11).

Drugs and reagents

ISO hydrochloride (purity >99%) was acquired from Tokyo Chemical Industry Co., Ltd., Japan; verapamil (VRP) was acquired from Sun Pharmaceutical Industries Limited, Gurugram, Haryana, India. The other experimental solvents used were of analytical and laboratory grade.

Extraction and fractionation method

One kilogram of dried crude whole plant material of *S. chirayita* was pulverized to obtain a coarse powder that was used for extraction by the soxhlet apparatus. Initially, the plant material was defatted using 6 L of hexane for 8 h, the obtained marc was further extracted with 8 L ethanol for 15 h by soxhlet at 60°C. The mixture was filtered using Whatman's filter paper (0.45 µm) to remove the unwanted part. The filtrate was concentrated under vacuum pressure. The obtained mass was suspended in the optimum amount of distilled water. The resulting mixture was then fractionated with chloroform (4 × 300 ml) which was concentrated to half of its volume and fractionated with an aqueous solution of 5% sodium hydroxide. Thereafter, the aqueous layer was acidified with 10% hydrochloric acid to get free xanthone moiety. Further acidic mixture was extracted with chloroform (4 × 300 ml). With the help of vacuum pressure, the resulting chloroform fractions were pooled and concentrated. The chloroform residue was used for high-performance thin-layer chromatography (HPTLC) profiling to confirm the number of compounds present in fractionated mass.[22]

Isolation and identification of swerchirin using high-performance thin-layer chromatography-mass spectrometry technique

A stock solution (30 mg/ml) rich fraction was prepared in high-performance liquid chromatography (HPLC) grade methanol and followed by vortex and centrifuged at 1200 rpm for 10 min at 25°C ± 2°C. The resulting solution was filtered in another tube using a polytetrafluoroethylene (PTFE) membrane filter of 0.22 µm. Thereafter, with the help of the Camag Linomat-V (CAMAG, Switzerland) HPTLC system, 8 µL of the sample was applied individually on silica gel 60 F_{254} HPTLC plates (20 cm × 10 cm; Merck, Germany). The thin-layer chromatography (TLC) plate was developed up to 80 mm at 25°C ± 2°C in a presaturated TLC development chamber containing glacial acetic acid: ethyl acetate: toluene (1:3:6, v/v/v) as a mobile phase. The developed plate was dried and envisaged at 254 nm. As per the visibility of spots, five major spots were scratched out and dissolved into methanol to free the loaded compound with silica gel individually. The resulting solutions then proceeded for mass spectroscopy (MS).[23]

Characterization of swerchirin using mass spectrometry

In MS, the isolated samples were analyzed in the positive ion mode for the presence of SW by the electrospray ionization mass spectrometry (ESI-MS) electrospray ionization system (Waters Zspray™ Xevo TQD). The flow rate and column temperature were set at 0.4 ml/min and 40°C, respectively. The instrument was operated as per the guidelines of the proprietary manufacturer's software. The ion source was operated at an optimized temperature of 140°C, whereas desolvation gas flow and cone gas flow were adjusted at 750 L/h and 50 L/h, respectively.[24]

Characterization of swerchirin using nuclear magnetic resonance spectroscopy

The nuclear magnetic resonance (NMR) spectroscopical analysis of isolated compounds was performed as per the described method using Bruker Avance 400 and 100 MHz NMR spectrometer, respectively, with d_6-dimethylsulphoxide as solvent and tetramethylsilane as an internal standard. In brief, the above-isolated sample was dried under high vacuum pressure to remove traces of solvent. The resulting residue was dissolved in dimethyl sulfoxide (DMSO) as a standard solvent then the sample proceeded for spectroscopic analysis by NMR technique.[25,26]

Comparative high-performance thin-layer chromatography profiling

Further, comparative HPTLC profiling was done for SW. Simultaneous application of chloroform extract and the referenced compound on the HPTLC plate (10 × 10). The plate was developed in glacial acetic acid: ethyl acetate: and toluene (1:3:6, v/v/v) as a mobile phase. The developed plate was kept undisturbed, air-dried, visualized under 254 nm of UV light and, scanned for the analysis. The percentage purity of SW in chloroform extract was determined based on the peak area and peak percentage.[27]

Column chromatography for bulk isolation of swerchirin

The purification of SW from chloroform extracted mass was conducted using column chromatography. A total of 2 g chloroform extract was loaded on silica gel laid over the silica bed in the column. 230–400 mesh size silica gel was used for the packing of the column. The sample was then eluted with toluene and toluene with ethyl-acetate gradient, respectively. The elution rate was set at 3 ml/min. All the eluted fractions were collected in the test tubes (50 ml) until its 40 ml amount. The collected fractions were identified through TLC to target SW. The obtained residue of SW was further subjected to HPLC for its percentage purity.[17]

High-performance liquid chromatography profiling of swerchirin

One mg of SW was completely dissolved in HPLC grade methanol and then filtered into an HPLC vial using a PTFE membrane filter of 0.22 µm. The resulting solution then proceeded for present purity confirmation through the analytical HPLC profiling using the HPLC system (LC-2010 CHT series chromatographic system, Shimadzu, Japan) which consisted of a Model LC-10 ATVP binary pump and equipped with SPD-10 AVP UV–Visible spectrophotometric detection system. Class-VP 5.032 software was stated for routine drug analysis. All the chromatographic conditions were given as per the referenced protocol.[28]

2,2-Diphenyl-1-picrylhydrazyl antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) antioxidant potential of SW was determined as per the referenced protocol with some modifications.[29] In brief, 20 µL of SW solution of each concentration (10–500 µg/ml) and 180 µL of DPPH (0.01 mM) were mixed in each defined wells of the enzyme-linked immunosorbent assay (ELISA) plate. The obtained mixtures were kept undisturbed for 30 min in a dark place at room temperature and then absorbance was determined using an ELISA spectrophotometer at 517 nm. The measurements were taken in triplicate. The positive control was used as ascorbic acid and considered the efficacy of SW in the proportion of ascorbic acid. A calibration curve was plotted between concentrations and percentage scavenging activity.

Nitric oxide scavenging activity

Nitric oxide (NO) scavenging activity of SW was determined by using the referenced protocol with some modifications.[30] In brief, 0.5 ml of SW solution of each concentration (10–500 µg/ml), 2 ml of sodium nitroprusside (10 mM), and 0.5 ml of phosphate-buffered saline was mixed in testing tubes. The obtained mixtures were incubated at room temperature for 30 min in a dark place. Thereafter, 0.5 ml of the reaction mixture and, 1 ml sulphanilic acid reagent was mixed followed by the addition of 1 ml, 0.1% naphthyl ethylenediamine dihydrochloride. The resulting solution was remained undisturbed for 30 min and the measurement was taken at 540 nm in triplicate. The positive control was used as ascorbic acid. A calibration curve was plotted between percentage scavenging activity and the concentration of the SW.

In silico study

Receptor and ligand preparation

In silico docking studies were carried out by downloading the three-dimensional crystal structures of target protein catalase (CAT) (PDB ID 1QQW) and superoxide dismutase (SOD) (PDB ID 5YTO) from the protein data bank (PDB) (https://www.rcsb.org) for the antioxidant activity.[31,32] The recovered protein from the PDB was processed using discovery studio, heat atoms and water molecules were removed, processed proteins were saved in.pdb format. We performed molecular docking using the AutoDock Vina 4.2 tool.[33] We maximized the grid box size along the axis X = 62.383596, Y = 79.605938, Z = −27.086856 for 1QQW (site1) protein, X = 61.38, Y = 63.61, Z = −11.09 for 1QQW (site 2) protein, X = 67.38, Y = 59.61, Z = −36.09 for 1QQW (site 3) protein, X = 70.38, Y = 31.61, Z = −12.09 for 1QQW (site-small) protein X = 70.38, Y = 31.61, Z = −12.09 for 5YTO protein respectively, for SOD protein dimer grid box were created at centroid of Trp32B and Trp32H, X = 74.34050899999998, Y = 77.12746602083335, Z = −14.763381083333336. The grid box dimension was maintained at X = 30, Y = 30 and Z = 30. For this purpose, each ligand was prepared and optimized using the Pubchem database (https://www.ncbi.nlm.nih.gov/), then converted to the PDB file format. The validation of docking and the binding interaction between ligand and receptor were visualized by the Pymol tool.[34]

Validation of docking

The docking procedure was validated using this method; co-crystallized ligand (946) from protein (1QQW) was removed and re-docked into the active site using the AutoDock tool. It was done to ensure the ligand-binding exactly to the active site pocket to show less deviation compared to the co-crystallized complex. The re-docked complex was then superimposed onto the reference co-crystallized complex using the Pymol tool.

Experimental animals

Protocol project number 1217 was approved for the use of animals by the Jamia Hamdard Institutional Animal Ethics Committee, New Delhi, India. The experiment was executed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. In this study, male Wistar albino rats (200–250 g) were obtained from the Central Animal House Facility, Jamia Hamdard, New Delhi, India. The polypropylene cages were used to keep them and acclimatize with 12 h light and dark cycles at temperature 23°C ± 2°C with relative humidity (60% ± 5%) for a week. The standard commercial pellet diet was fed to all the groups of animals with water *ad libitum*.

Experimental design

After completion of 1 week of acclimatization, rats were divided into eight groups, each group having five rats ($n = 5$) and treatment was given as follow:

Group I (Vehicle control)

Administered vehicle (0.5 ml normal saline/day) per oral for 7 days and 0.5 ml normal saline/kg, s.c. on the 6th and 7th day of the treatment.

Group II (Isoproterenol treated)

Administered vehicle (0.5 ml normal saline) per oral for 7 days and 85 mg ISO/kg, subcutaneous on the 6th and 7th days of the treatment.

Group III (Swerchirin$_6$ + isoproterenol)

Administered SW (6 mg/kg/day) per oral for 7 days and 85 mg ISO/kg, subcutaneous on 6th and 7th days of the treatment.

Group IV (Swerchirin$_{12}$ + isoproterenol)

Administered SW (12 mg/kg/day) per oral for 7 days and 85 mg ISO/kg, subcutaneous on the 6th and 7th days of the treatment.

Group V (Swerchirin$_{24}$ + isoproterenol)

Administered SW (24 mg/kg/day) per oral for 7 days and 85 mg ISO/kg, subcutaneous on the 6th and 7th days of the treatment.

Group VI (Swerchirin$_{24}$ per se)

Administered SW (24 mg/kg/day) per oral alone for 7 days.

Group VII (Verapamil$_{20}$ + isoproterenol)

Administered VRP (20 mg/kg/day) per oral for 7 days and 85 mg ISO/kg, subcutaneous on the 6th and 7th days of the treatment.

Group VIII (Verapamil$_{20}$ per se)

Administered VRP (20 mg/kg/day) per oral alone for 7 days.

Biochemical estimations

The estimation of SOD and CAT was performed in myocardial tissue using the method of Marklund and Marklund,[35] and Claiborn,[36] respectively. Aspartate transaminase (AST)[37] and lipid profile (triglycerides [TGs]; high-density lipoprotein [HDL]; and total cholesterol [TC])[38] was estimated by kits which were commercially available (Span Diagnostics Limited, Surat, India) by following the instructions of the manufacturer. The amount of low-density lipoprotein (LDL) and very-LDL (VLDL) were evaluated by the formula (Friedewald's equation: VLDL = TGs/5 and LDL = TC - HDL – VLDL).[39]

Histopathology

Myocardial tissues of rats were fixed in 10% formalin, processed and impregnated with paraffin immediately after the sacrifice. The sections of myocardial tissue were then stained with hematoxylin and eosin (H and E) dye. The Meiji fluorescent microscope supported with a Lumenera camera was used for the capturing of photomicrographs and with the help of Infinity Analyze 3 Software (Lumenera Corp., Canada) the images were analyzed.[40]

Statistical analysis

For the statistical analysis of data, the Prism software package version-7 (GraphPad Software Inc., California, USA) was used. The data were expressed as the mean ± standard error of the mean. One-way analysis of variance (ANOVA) was carried out and the statistical comparisons among the groups were accomplished with Tukey's test. The statistically significant value was considered using the value of $P < 0.05$.

RESULTS

Extraction and fractionation

The dried plant material was defatted using hexane which gave highly viscous mass (82 g) and in ethanolic extraction, it further gave dark green viscous mass (143 g). The chloroform fractionated residue from the ethanolic extract was found to be 2.3 g with a slightly viscous nature which was used for the isolation and characterization of SW.

Isolation and characterization of swerchirin hyphenated high-performance thin-layer chromatography-mass spectrometry technique

In the HPTLC-MS technique for the estimation of SW, a presample applied HPTLC plate was developed and the scratched major spots were analyzed through MS and the spectral data revealed that the first spot laid at the R_f of 0.367 was identified as SW having m/z^{+1} 289.41. Furthermore, the sample was re-authenticated by ^{1}H-NMR and ^{13}C-NMR spectroscopy. The isolation and identification of SW through the hyphenated HPTLC-MS technique are shown in Figure 1.

Characterization of swerchirin using nuclear magnetic resonance spectroscopy

^{1}H-NMR and ^{13}C-NMR analysis was done successfully. The aromatic substitution pattern of the compound was determined by the presence of two meta-coupled doublets showing a *J* value of 2.4 Hz and two AB-type doublets exhibiting a *J* value of 8.4 Hz. The two doublets at δ 6.29 and 6.48 were consigned for H-2 and H-4, respectively, while two AB-type doublets at δ 7.17 and 6.62 were due to H-6 and H-7. Two singlets each of three protons that appeared at δ 3.82 and 3.87 indicated the presence of two methoxy groups in the molecules. Proton NMR showed two broad singlets also each at δ 11.91 and δ 11.32 that were assigned to two phenolic groups present in the molecule. The two methoxy groups present in the compound were further confirmed by ^{13}C-NMR chemical shift values that appeared at δ 55.32 and δ 58.02. ^{13}C-NMR also showed values that satisfied the characterization of 1,3,5,8-tetrasubstituted xanthone. The highly down fielded peak that appeared at δ 183.89 was assigned to the carbonyl carbon of the compound. The NMR spectroscopical data of SW is [Figure 2] shown in Table 1.

Comparative high-performance thin-layer chromatography profiling

Further, the comparative HPTLC profiling of chloroform extract was conducted successfully and the results revealed the percentage purity of SW 53% in the chloroform extract which was determined on the basis of peak area and peak percentage. In the comparative HPTLC profiling, the densitometry scanning was performed at 254 nm as shown in Figure 3.

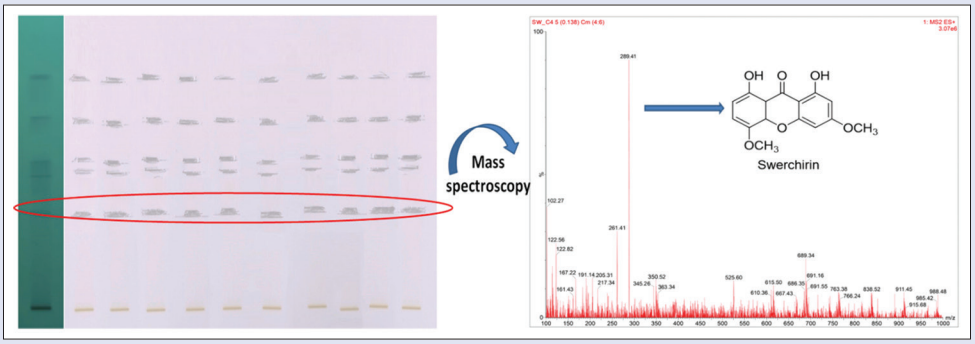

Figure 1: Isolation and identification of SW through hyphenated HPTLC-MS technique. HPTLC: High-performance thin-layer chromatography; MS: Mass spectrometry; SW: Swerchirin

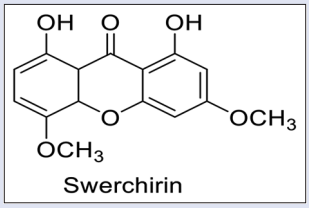

Figure 2: The structure of SW. SW: Swerchirin

Colum chromatography for bulk isolation of Swerchirin

For the isolation of SW from chloroform fractionated mass, column chromatography was performed. The fractions rich in SW from F52-68 were collected separately at 75% toluene gradient with ethyl acetate and dried under reduced pressure. The resulting residue of SW was found to be 83 mg and then subjected to HPLC for its percentage purity. The schematic representation of column chromatography is represented in Figure 4.

High-performance thin-layer chromatography profiling of swerchirin

In the analytical profiling of SW by HPLC, the resulting data from HPLC profiling reveals the elution or retention time (R_t) at 5.019 min with a percentage purity of 91% as shown in Figure 5.

2,2-Diphenyl-1-picrylhydrazyl antioxidant activity

DPPH antioxidant activity of SW was determined successively. The results have shown the significant and dose-dependent inhibitory potential of SW with a maximum inhibition of 87.25% ± 2.60% at 500 µg/ml and the IC_{50} value was found to be 109.1 ± 0.852 µg/ml. Whereas, ascorbic acid showed 91.35% ± 1.97% inhibitions at 500 µg/ml with IC_{50} value 77.75 ± 2.15 µg/ml. SW was found to possess statistically significant antioxidant properties with *P* value summary** and *P* value (one-tailed) 0.0041 with respect to ascorbic acid.

Nitric oxide scavenging activity

The scavenging potential of SW was then determined. The results revealed the significant and dose-dependent inhibitory potential of SW with a maximum inhibition of 72.25 ± 1.05% at 500 µg/ml with IC_{50} value 278.134 ± 0.852 µg/ml. Whereas, ascorbic acid showed 87.95 ± 1.01% inhibitions at 500 µg/ml with IC_{50} value 83.15 ± 0.92 µg/ml. SW was found to possess statistically significant antioxidant properties with *P* value summary** and *P* value (one-tailed) < 0.002 with respect to ascorbic acid.

Table 1: The findings from nuclear magnetic resonance spectroscopy

SW (1,8-dihydroxy-3,5-dimethoxy xanthone)		
C/H	δC	δH (J in Hz)
1	163.14	-
2	98.63	6.29, 1H, d (2.4)
3	168.51	-
4	92.97	6.48, 1H, d (2.4)
4a	158.72	-
4b	153.85	-
5	141.13	-
6	121.76	7.17, 1H, d (8.4)
7	110.24	6.62, 1H, d (8.4)
8	147.18	-
8a	107.26	-
9	183.89	-
9a	103.12	-
3-OMe	55.32	3.82, 3H, s
5-OMe	58.02	3.87, 3H, s
1-OH	-	11.91, 1H, s
2-OH	-	11.32, 1H, s

SW: Swerchirin

In silico study

Molecular docking studies were carried out on the different active sites of target proteins, CAT (PDB ID 1QQW) and SOD (PDB ID 5YTO) with SW ligand using the AutoDock software.

ProBis tool[41,42] showed that CAT enzyme contains eleven active binding sites (http://probis.cmm.ki.si/browse.php?job_id = 01072103958488) in the 1QQW receptor. In our in silico study, we used five top active sites of CAT. The docking results showed that out of five active binding sites only three sites (site-3, site-2, and site-small) were found to have significant interactions with SW ligand. *In silico* molecular docking on site-3 revealed that SW has binding affinity ΔG = −4.883 Kcal/mol, it was found to be a favorable binding site for SW as compared to the other ligands on the same docking site as well as other docking sites for the SW. The binding energy of reference inhibitors amitrole (AMIT) and salicylic acid (SA)[43] showed that the binding energy of reference inhibitor SA (ΔG = −2.062 Kcal/mol) is just half the binding energy of SW, whereas it is comparable to the binding energy of reference inhibitor AMIT (ΔG = −4.889), the chemical structure of AMIT have two protonated nitrogen's which could be contributing to form the salt bridge and additional week bonds with the acid-rich amino acid present in the receptor. The binding site-small has maximum binding energy for SW (ΔG= −7.84 Kcal/mol) but the size of the binding pocket is very small because of that the binding between the ligand and protein might be for a short period. The binding site-2 have comparable binding energy for the reference inhibitors salicylic

acid (ΔG = −4.192 Kcal/mol), amitrol (ΔG = −4.598 Kcal/mol), reference standard VRP (ΔG = −4.553 Kcal/mol), and SW (ΔG = −4.008 Kcal/mol) [Figure 6].

The *in silico* docking of SOD protein 5YTO dimer grid box was created at the centroid of Trp32B and Trp32H study revealed that the SW has a good docking score −4.602 Kcal/mol, which is comparable to the reference standard VRP −4.674 Kcal/mol, 1,4,5-dichloro-2-m-tolylpyridazin-3 (2H)-one (LCS) −4.462 and diethyldithiocarbamic acid (DDC) −3.468. The binding energy of the SW was ΔG = −7.025 Kcal/mol which is slightly less as compared to the LCS it could be because of the small size of LCS, but its docking score was less as compared to SW, whereas binding energy, as well as the docking score of SW, is better as compared to the reference inhibitors molecule.[44]

The docking studies revealed that ligand strongly binds with both CAT and dismutase enzymes which were responsible for the catalysis of oxidative reactions in the human body. Therefore, binding of the ligand could lead to the activation of an enzyme to carry out redox reactions [Figure 6].[31, 34,45]

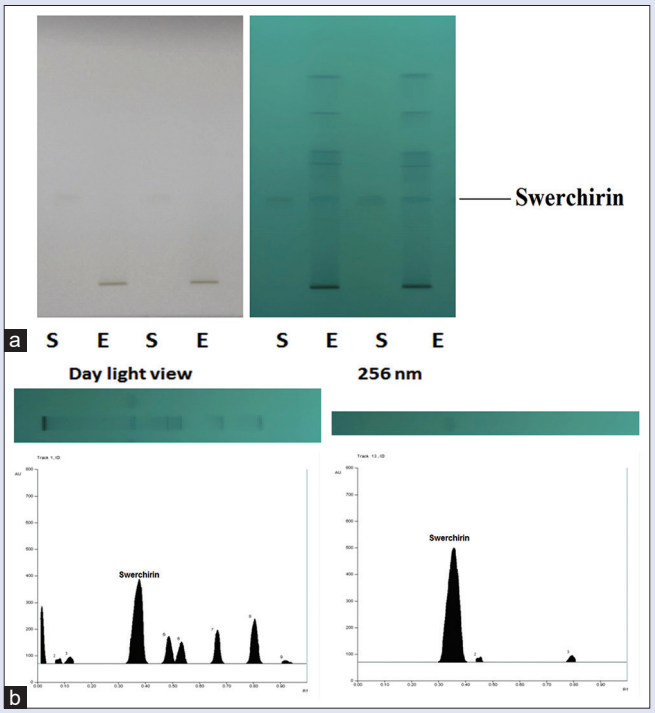

Figure 3: (a) Representing comparative HPTLC profiling view of chloroform fractionated extract and SW. (b) Representing a comparative HPTLC profiling chromatogram of chloroform fractionated extract and SW. HPTLC: High-performance thin-layer chromatography; SW: Swerchirin

Docking validation

The re-docking was done to examine the docking procedure and efficiencies. The protein (PDB ID 5YTO) co-crystallized ligand (946) was bound exactly to the active site with a good binding score of −4.887. Pro55, Glu127, Val 59, Lys57, Trp59, Val59, Glu48, Gln49, Lys50, Glu48, and Trp59 are the interacting amino acids in the active site. We performed the redocking of hem ligand against PDB (1QQW) using AutoDock 4.2. The active site of the protein was found to contain Phe64, Val74, Val73, His75, Met61, Arg72, Thr361, Tyr358, Ala357, Met350, Phe161, Pro158, Arg354, Phe153, His218, Leu299, Asn148, Gly131, Val146, Gly216, Ser217, Gly147, Ala133, Phe334, Ser114, Phe132, Ala333, Arg112, Ile332 interacting amino acids in the active site Pocket. The redocked complex was then superimposed onto the native co-crystallized ligand from PDB using Pymol.

Effect of swerchirin on isoproterenol-induced oxidative stress

Administration of ISO induced significant oxidative stress in the myocardial tissue by reducing the activity of SOD and CAT ($P < 0.001$). When we treated the rats with SW_6, no significant antioxidant effect was observed ($P > 0.05$ for SOD and CAT) whereas treatment with SW_{12}, SW_{24}, and VRP_{20} significantly reversed the activity of SOD ($P < 0.01$ for SW_{12} and $P < 0.001$ for SW_{24} and VRP_{20}, respectively) and CAT ($P < 0.01$ for SW_{12} and $P < 0.001$ for SW_{24} and VRP_{20} respectively) toward normal as shown in Figure 7.

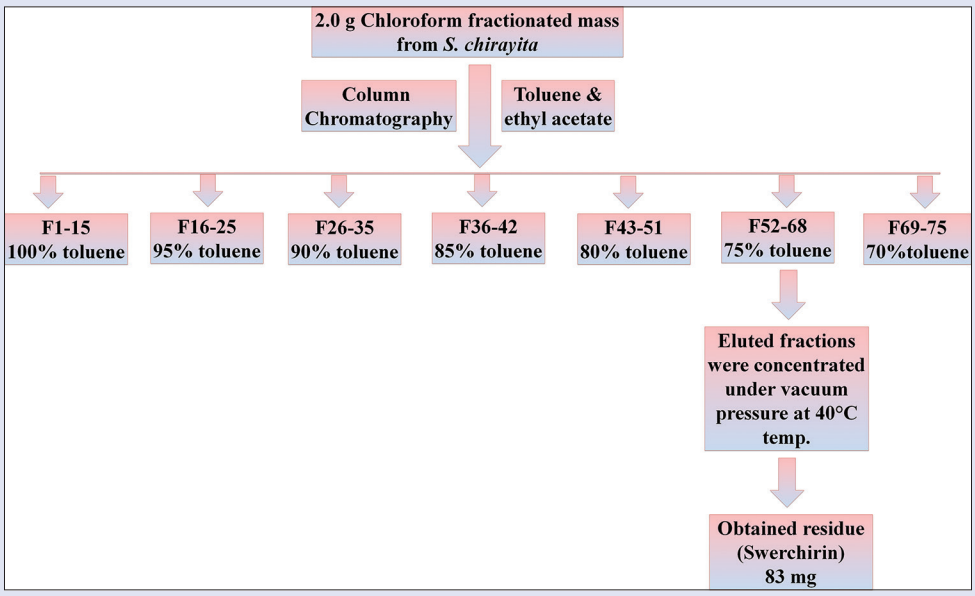

Figure 4: Schematic representation of column chromatography

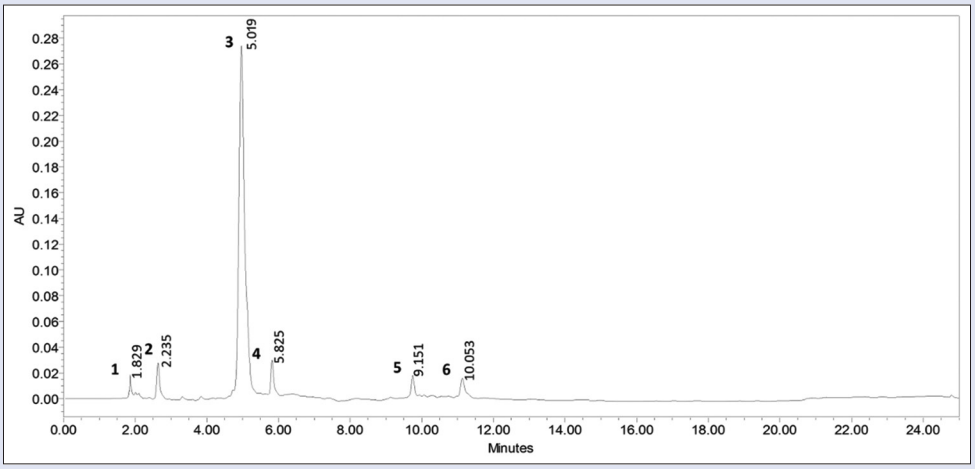

Figure 5: The HPLC profiling of SW. HPTLC: High-performance thin-layer chromatography; SW: Swerchirin

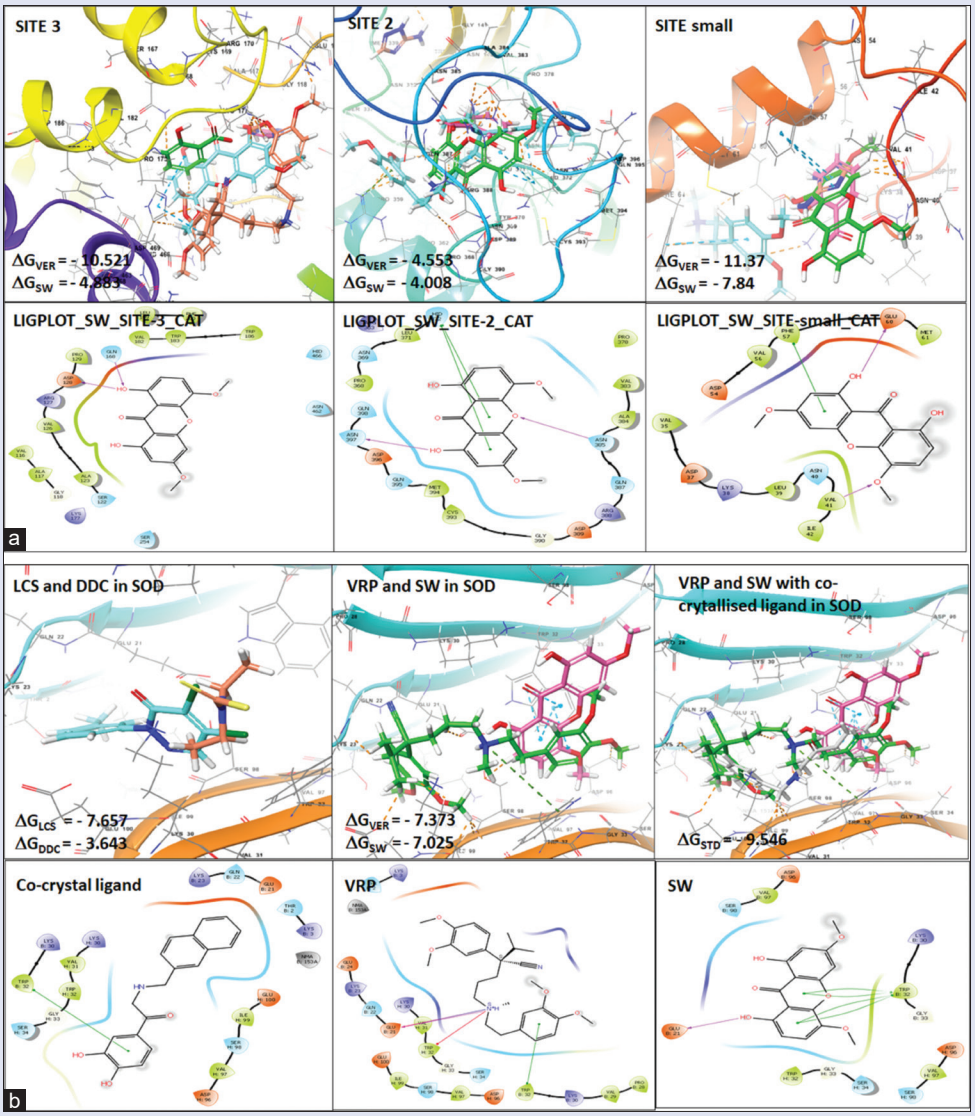

Figure 6: (a) 2D and 3D presentation of SW, 3D presentation of VRP on different active binding sites of CAT (1QQW) protein; (b) 2D and 3D presentation of SW, VRP and co-crystal ligand, 3D presentation of LCS and DDC with SOD (5YTO) protein. 2D: Two-dimensional; 3D: Three-dimensional; SW: Swerchirin; VRP: Verapamil; LCS: 1,4,5-dichloro-2-m-tolylpyridazin-3 (2H)-one; DDC: diethyldithiocarbamic acid

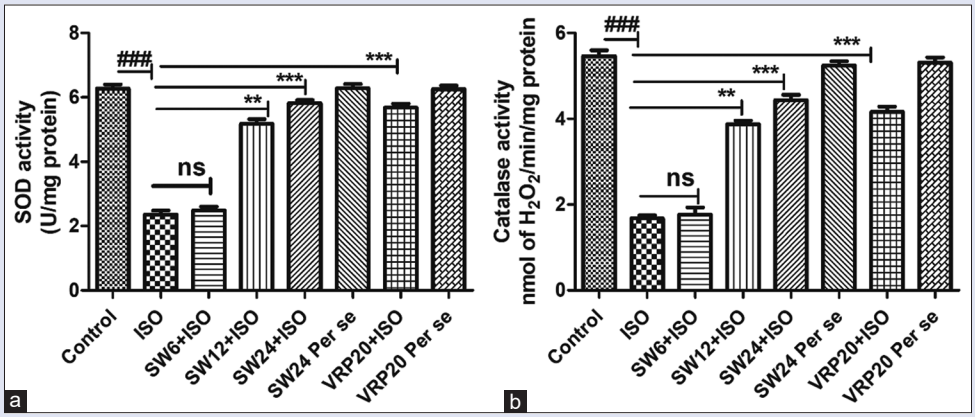

Figure 7: The effect of SW on ISO-induced oxidative stress markers such as (a) SOD activity and (b) CAT activity. The statistical analysis was completed by implementing the One-way ANOVA followed by Tukey's multiple comparison tests to find out the difference between the various groups. The significant value ($P < 0.001$) denoted by ### When compared to control group; the significant value $P < 0.01$ and $P < 0.001$ (significant when compared to ISO) denoted by ** and *** respectively and "ns" indicates $P > 0.05$ (non-significant when compared to ISO). ISO: Isoproterenol; SW: Swerchirin

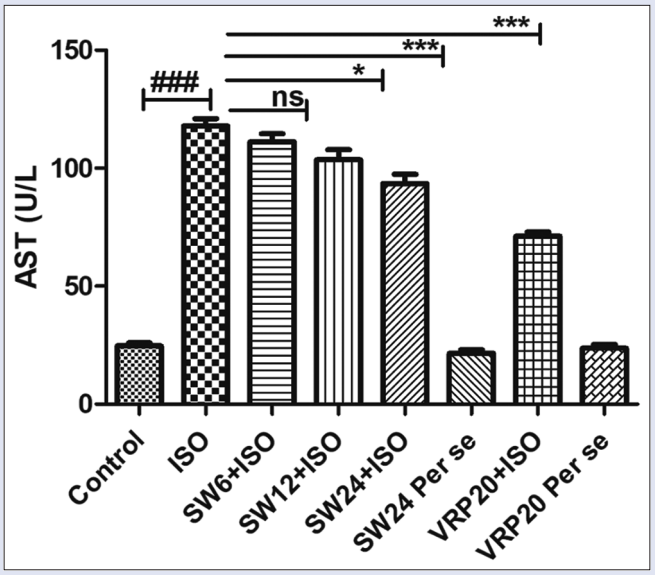

Figure 8: The effect of SW on ISO-induced SGOT or AST. The statistical analysis was completed by implementing the one-way ANOVA followed by Tukey's multiple comparison tests to find out the difference between the various groups. The significant value ($P < 0.001$) denoted by ### when compared to control group; the significant value $P < 0.01$ and $P < 0.001$ (significant when compared to ISO) denoted by ** and *** respectively and "ns" indicates $P > 0.05$ (non-significant when compared to ISO). SW: Swerchirin; ISO: Isoproterenol; SGOT: Serum glutamic-oxaloacetate transaminase; AST: Aspartate transaminase; ANOVA: Analysis of variance

Effect of swerchirin on isoproterenol-induced altered serum glutamic-oxaloacetate

Administration of ISO induced significant myocardial and hepatic tissue damage which was evident by an increased level of AST ($P < 0.001$). When we treated the rats with SW_6, no significant decrease in the level of this injury marker was observed ($P > 0.05$). Treatment with SW_{12}, SW_{24}, and VRP_{20} significantly reversed the activity of AST ($P < 0.05$ for SW_{12} and $P < 0.001$ for SW_{24} and VRP_{20}, respectively) toward normal as shown in Figure 8.

Effect of swerchirin on isoproterenol-induced altered lipid profile

Administration of ISO induced significant alteration in the markers of lipid profile which was evident by the increased level of TG, TC, LDL, VLDL, and reduced level of HDL ($P < 0.001$). When we treated the rats with SW_6, no significant effect on the level of these lipid profile markers was observed ($P > 0.05$). Treatment with SW_{12}, SW_{24}, and VRP_{20} however, significantly reversed the level of TC and TG ($P < 0.01$ for SW_{12} and $P < 0.001$ for SW_{24} and VRP_{20}, respectively), LDL and VLDL ($P < 0.05$ for SW_{12} and $P < 0.01$ for SW_{24} and VRP_{20}, respectively) and HDL ($P < 0.01$ for SW_{12}, SW_{24}, and VRP_{20}, respectively) as shown in Figure 9.

Effect of swerchirin on isoproterenol-induced histopathological damage

Administration of ISO caused significant cardiac damage which was estimated by H and E staining. Analysis of the stained section showed significant cellular degeneration (black arrow), pyknosis vacuolation (yellow arrow), and fibrotic changes (green arrow). When we treated rats with SW_6, no significant change in the extent of cardiac damage was observed. Treatment with SW_{12} showed moderate pyknosis, fatty canges, and cellular disintegration whereas treatment with SW_{24} and VRP_{20} showed no sign of fibrosis, and only mild pyknosis, and cellular disintegration were observed and hence significantly reduced the histological aberration, as shown in Figure 10.

DISCUSSION

Cardiotoxicity is one of the major health care issues and is of major concern for clinicians. Due to the increased production of various ROS, oxidative stress is one of the major confounding issues in the pathogenesis of cardiotoxicity. Oxidative stress is identified by reduced level or activity of SOD, CAT, or increased level of the by-product of oxidative stress i.e., malondialdehyde (MDA).[46] Thus, alteration in the level of these antioxidant enzymes initiates the cascade of the multidirectional inflammatory, apoptotic and fibrotic phase leading to biochemical and histological aberrations in the myocardial tissue. In recent times, natural bioactive products are gaining much attention because of their potent apoptotic, anti-inflammatory, and antioxidant

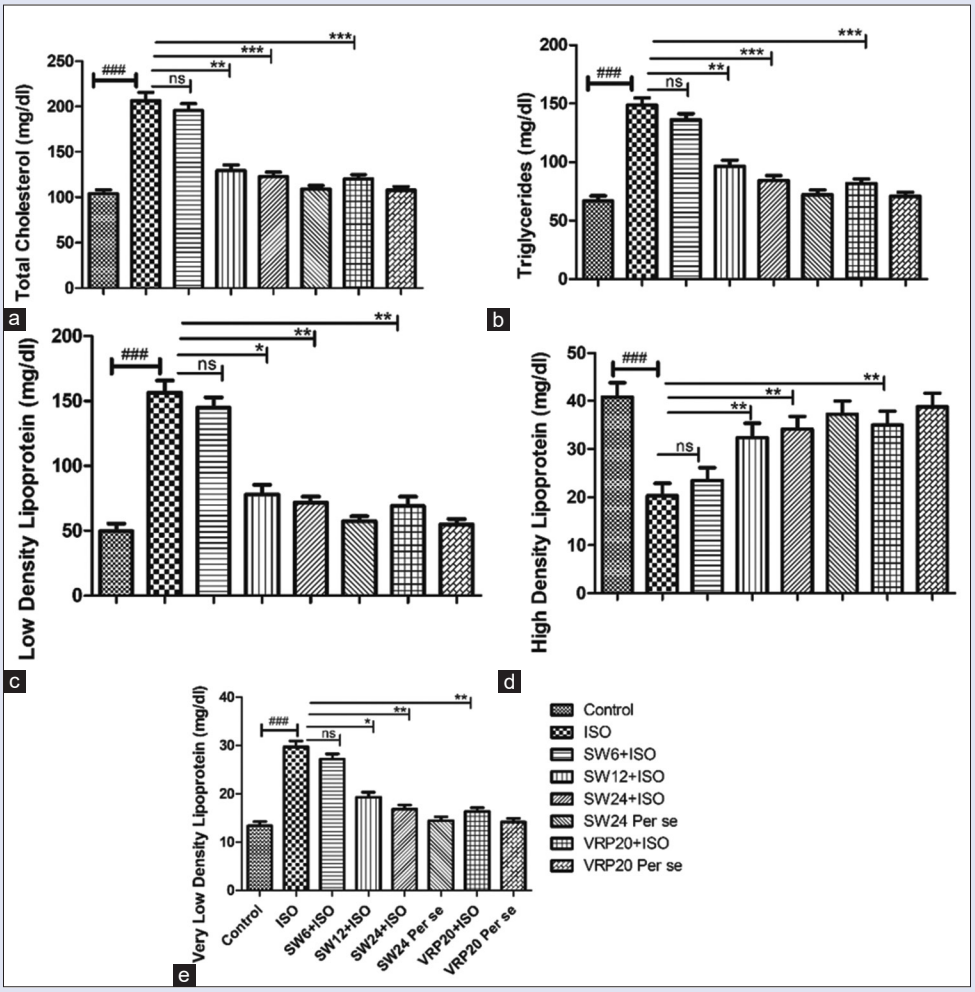

Figure 9: The effect of SW on ISO-induced altered lipid profile (a) TC (b) TG (c) LDL (d) HDL (e) VLDL. The statistical analysis was completed by implementing the one-way ANOVA followed by Tukey's multiple comparison tests to find out the difference between the various groups. The significant value ($P < 0.001$) denoted by **###** when compared to control group; the significant value $P < 0.01$ and $P < 0.001$ (significant when compared to ISO) denoted by ** and *** respectively and "ns" indicates $P > 0.05$ (non-significant when compared to ISO). SW: Swerchirin; ISO: Isoproterenol; TC: Total cholesterol; TGs: Triglyceride; HDL: High-density lipoprotein; VLDL: Very-low-density lipoprotein

properties.[47] Thus, in this study, we have explored the cardioprotective property of isolated SW from *S. chirayita* against ISO-induced cardiotoxicity in male Wistar rats. *S. chirayita* is an endangered Himalayan medicinal plant used traditionally as anti-microbial, anti-leishmanial, CNS-depressant, anti-inflammatory, anti-cancer, anti-viral, and anti-diabetic properties.[48] *S. chirayita* possesses a bitter taste due to the presence of different active metabolites such as amarogentin, SW, swertiamarin, decussatin, swertianin, bellidifolin, isobellidifolin, swertianolin, and mangiferin that are reported to exhibit significant human health benefits.[14]

Thus, in the current study, we have extracted SW and characterized it by HPTLC-MS, NMR spectroscopy, and HPLC. The extracted SW was further used to assess its DPPH free radical and NO scavenging property in an *in vitro* setup. The cardioprotective potency was assessed in the ISO-induced *in vivo* cardiotoxic model. *In vitro,* antioxidant assay i.e., DPPH scavenging capacity was used to evaluate the antioxidant potential of the therapeutically active compound. It is one of the simple, rapid, economical, and extensively used methods to identify the capacity of phytoconstituents to scavenge the free radicals.[49] Although, there is not much evidence on the DPPH antioxidant activity of SW, however, the study by Chen *et al.* 2011 reported that 70% ethanolic extracts of *S. chirayita* possess high DPPH scavenging activity with IC_{50} 267.80 µg/ml.[50] Kumar *et al.* 2011 reported that aqueous extract of *S. chirayita* exhibit IC_{50} 315.83 µg/ml.[51] Furthermore, Kshirsagar *et al.* 2015 conducted the DPPH activity on aqueous, methanolic, and ethanolic extract of *S. chirayita* and reported IC_{50} 551.26 µg/ml, 557.61 µg/ml, and 559.05 µg/ml, respectively.[52] In our study, we found, an IC_{50} value of 109.1 ± 0.852 µg/ml for isolated SW whereas, ascorbic acid exhibited an IC_{50} value of 77.75 ± 2.15 µg/ml for isolated SW. We further performed another *in vitro* study to estimate NO scavenging potential of SW. NO free radical scavenging activity is an exponentially increasing liable method for the determination of antioxidant potential. Previously, Nag *et al.* 2015 reported NO scavenging activity of *S. chirayita* with IC_{50} 279.69 ± 1.98 µg/ml,[53] and Ahirwal *et al.* 2014 reported that methanolic extract of *S. chirayita* exhibit IC_{50} 870.55 ± 0.20 µg/ml.[54] In our study, we found, the IC_{50} value of 278.134 ± 0.852 µg/ml for SW whereas ascorbic acid exhibited an IC_{50} value of 83.15 ± 0.92 µg/ml. Hence, we can conclude that SW extracted from *S. chirayita* possessed significant antioxidants potential and can be a potential cardioprotective agent. Further, to explicate the antioxidant effect of SW, we performed *in silico* study to ascertain its binding affinity towards SOD and CAT. The outcome of the *in silico* showed binding energy of -7.025 Kcal/mol

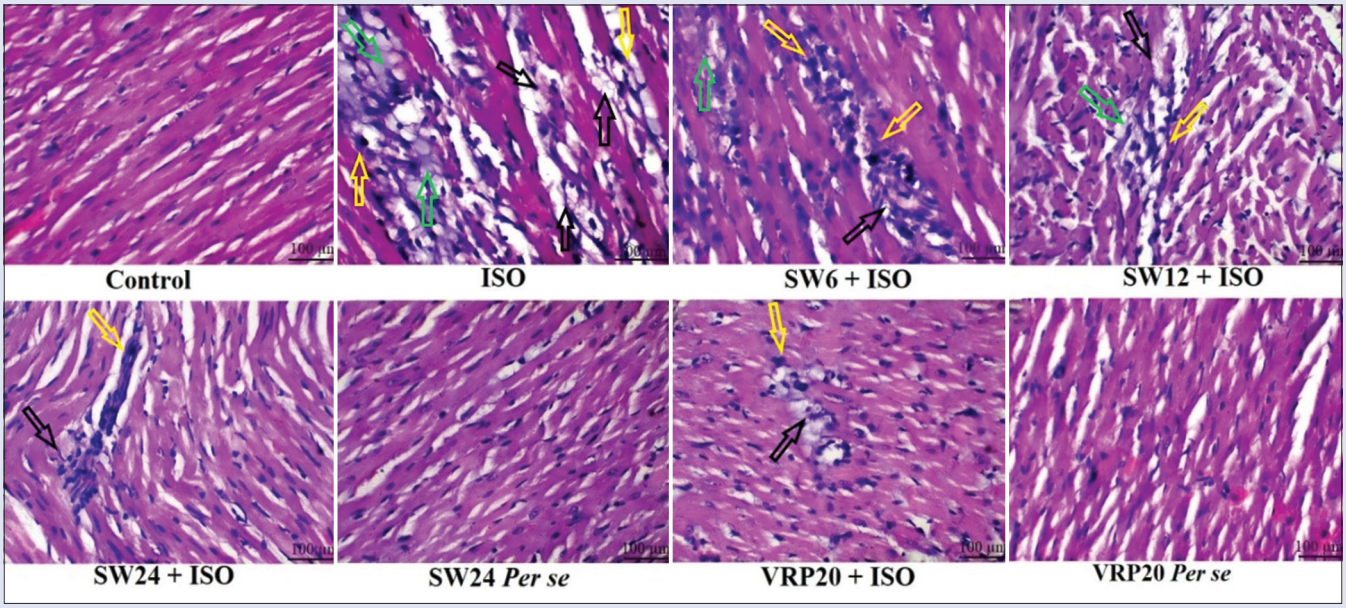

Figure 10: The histopathological damage caused by ISO and the protective effect of SW and VRP. ISO treated group showed significant cellular degeneration (black arrow), pyknosis vacuolation (yellow arrow) and fibrotic changes (green arrow). Treatment with SW_6 failed to reverse the histological abbeartion caused by ISO treatment whereas treatment with SW_{12} and SW_{24} and VRP_{20} showed significant cardioprotection. (Scale bar 100 μm). SW: Swerchirin; ISO: Isoproterenol; VRP: Verapamil

and -7.84 Kcal/mol, respectively for SOD and CAT, thus, confirmed that SW strongly binds with both the antioxidant enzymes and exhibits free radical scavenging property hence responsible for the reversal of these enzymes levels to normal [Figure 6]. To validate *in silico* study, we further performed an *in vivo* study where the antioxidant property of SW was evaluated against ISO-induced oxidative stress. We used ISO which is a β adrenergic agonist and induced significant cardiotoxicity by disruption of redox balance leading to histological aberrations. It is well known that SOD, CAT and other antioxidant molecules maintain the balance of ROS by eliminating the excessively produced ROS and reduction in their level directly manifest cardiotoxicity. During the normal physiological condition, the function of SOD is to convert superoxide free radical into hydrogen peroxide which is further acted upon by CAT and dissociate into H_2O and O_2. GSH further eliminates excess hydrogen peroxide and itself gets converted to GSSG in the presence of GPx.[55] GR convert GSSG into GSH and this cycle continues. However, any disbalance in this cycle results in oxidative stress and cardiotoxicity. In the current study, the administration of ISO significantly decreased the level of SOD and CAT, whereas treatment with SW_{12}, SW_{24} and VRP_{20} efficiently reversed the action of these endogenous antioxidant enzymes towards normal and exhibited cardioprotective activity which was in line with the previous studies[4,56] [Figure 7]. In the clinical practice, AST and lipid profile i.e., TG, TC, LDL, HDL and VLDL are used to correlate the cardiotoxic manifestations. Previously several studies have reported an increase in the level of cardiac injury markers along with the altered lipid profile.[37,38] In our study, when we administered ISO, we also found similar results i.e increased level of AST, TG, TC, LDL VLDL, and reduced level of HDL which is in accordance with the previous reports.[38,57] Administration of SW_{12}, SW_{24} and VRP_{20} effectively reversed their level towards normal, whereas treatment with SW_6 was found to be ineffective [Figures 8 and 9]. Histological aberration is one of the important criteria of cardiac damage. When we administered ISO, we found noticeable damage which was evident by significant cellular degeneration, pyknotic nucleus, and vacuolation. Administration of SW_{12}, $SW_{24,}$ and VRP_{20} effectively reversed the damage toward normal whereas treatment with SW_6 did not exhibit any significant protective effect [Figure 10].

CONCLUSION

Based on the antioxidant property of SW (the principal constituent of the extract of *S. chirayita)* which was exhibited by *in silico, in vitro* and *in vivo* studies performed on ISO-induced cardiotoxicity in Wistar rats and further confirmed by the reversal of histopathological changes to normal, we conclude that SW at the dose of 12 mg/kg and 24 mg/kg showed substantial cardioprotection. This is the initial report of the cardioprotective role of SW, however, a detailed study involving various molecular targets is needed to further strengthen the claim. Additionally, SW should be further explored for various cardiac-specific markers such as lactate dehydrogenase, cardiac troponin T, creatine kinase-MB, B-type natriuretic peptide and this can be considered as the limitation of present work.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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