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Research Article

Evaluation of cardioprotective activity of *Woodfordia fruticosa* flower extracts against doxorubicin induced cardiotoxicity

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Abstract

Woodfordia fruticosa (WF) flower extracts (Petroleum ether- PEWF; Chloroform - CHWF; Ethyl acetate- EAWF; Methanol- MEWF; Hydroalcohol - HAWF) was evaluated for the phytochemical contents using phytochemical screening and TLC fingerprinting that was standardized for phytoconstituents by spectroscopic methods. Phytoconstituents of the extracts were validated using HPLC for specificity by comparing with standard retention time, following which was further screened for *in vitro* DPPH scavenging activity and concurrently for *in vivo* cardioprotective activity against doxorubicin induced cardiotoxicity in rats.

WF flower extracts showed presence of polyphenols, triterpenoids, sterols and cardiac glycosides which was affirmed by TLC. Extracts were spectroscopically standardized for the content of total polyphenols (MEWF> EAWF> HAWF) and flavonoid contents (MEWF> EAWF> HAWF). Standardization performed by comparative retention time with standard phytoconstituents using HPLC revealed the presence of gallic, catechin, ellagic, rutin along with chlorogenic acid and caffeic acid.

Extracts that had significant content of polyphenols and flavonoids reflected comparatively good % DPPH scavenging activity (MEWF - 96.7; EAWF - 95.6; HAWF - 75.5 at 50µg / ml). All the extracts also revealed safe dose margin when subjected to acute oral toxicity. Further, *in vivo* cardioprotective evaluation of MEWF and EAWF extracts in doxorubicin induced cardiotoxicity in rats revealed significant preventive activity ($P<0.05$) against elevation in serum markers as compared to that in doxorubicin treated animals. MEWF extract shown combined potential of inhibition of doxorubicin induced elevation in serum level marker and better % DPPH scavenging activity as compared to EAWF extract.

Keywords: Cardiotoxicity, Doxorubicin, DPPH scavenging, HPLC Standardization, *Woodfordia fruticosa*

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1. Introduction

Woodfordi fruticosa Kurz. (Lythraceae) has its own significance amongst thousands of ethno pharmacologically popular plants in the Indian medicinal system due to its traditional medicinal value, preclinical reports and isolation of bioactive phytoconstituents. Ethno-pharmacological survey revealed the use of *W. fruticosa* flowers to treat disorders of mucus membrane, asthma and cough; extracts were found to contain acrid, alexiritic, analgesic, antihelminthic, antirheumatic and wound healing properties [1]. In addition, *W. fruticosa* flowers are also important ingredients in various Ayurvedic formulations [2, 3].

Various preclinical studies revealed the pharmacological potential of *W. fruticosa* (WF) flower extracts as anti-asthmatic [4], antihyperglycemic [5], hepatoprotective [6, 7], immunomodulatory [8], antiviral [9], antibacterial [10], antifertility [11], antitumor [12], antipyretic and granuloma inhibition [13] activities. The traditional and preclinical claims of WF dried flowers can be ascribed to its important bioactive phytoconstituents, namely, different types of anthraquinones, flavonoids [quercetin-3 having

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galactopyranoside, glucopyranoside, arabinoside, and oxylopyranoside glycosides, in addition to myricetin with galactopyranoside and arbinopyranoside glycosides], saponins, sterols [14, 15, 16], woodfordin A – C, five oligomers of woodfordin E to I and different tannins [17, 18, 19, 20, 21, 22].

What prompted us to investigate this present work is the importance of *W. fruticosa* as an ingredient of Drakshasava *that* has been traditionally claimed for the treatment and prevention of heart disease, preclinically proved as cardioprotective agent and the presence of cardiac glycosides in WF flower extracts. Furthermore, literature survey also reveals a lack of preclinical as well as clinical evaluation for the use of WF flower extracts for the treatment of cardiac disorders, despite the richness of cardioprotective (flavonoids and cardiac glycosides) phytoconstituents. Therefore, the present investigation aimed to evaluate the unexplored potential of WF as a cardioprotective agent.

The occurrence of cardiovascular disease in India has been substantially amplified over the couple of decades [23]. Retrospective observations explained the worsening fact that heart diseases in India are occurring 10 to 15 years ahead than the Western countries. The attributing reasons for this fact are sedentary life style, augmented stress levels, imbalanced diet, abuse of tobacco and alcohol. This problem can be solved by implementation of traditionally safe medicines such as *W. fruticosa* flower extracts. It has been found that WF flower extracts showed the presence of cardiac glycosides which instigated us to evaluate the flower extracts for cardioprotective use.. In the present investigation the standardized extracts are evaluated for antioxidant and cardioprotective potential.

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the standardized extracts are evaluated for antioxidant and cardioprotective potential.

2. Material and methods

Collection plant material and preparation of flower extracts

Dried flowers of *Woodfordia fruticosa* were collected from authentic sources Abbu Miya & Co., Nagpur, Maharashtra and were identified by studying its various morphological and taxonomical features. The prepared herbariums along with collected plant material were identified by Dr. B. D. Gachande, Associate Prof., Botany Department N.E.S, Science College, Nanded, Maharashtra (Specimen S-9/NPC/2011-12).

Flowers of *Woodfordia fruticosa* (WF) were pulverized using a milling machine to obtain coarse powder and was separated on sieves. Coarse powder of plant was exhaustively defatted using petroleum ether (60-80°) (PEWF), extracted successively with chloroform (CHWF), ethyl acetate (EAWF) and methanol (MEWF) using Soxhlet apparatus and was followed by cold maceration with hydro-alcohol (HAWF). All the extracts were collected, filtered, concentrated and stored in tight dessicator.

Chemicals

Doxorubicin Hydrochloride is an injectable commercial product (Fresenius Kabi Oncology Ltd. Solan, India); DPPH from Loba Chem, cardioprotectivity evlaution serum markers from Reckon Diagnostic Pvt. Ltd., Vadodra and Transasia Biomedicals Ltd., Solan were used. The solvents used for extraction and TLC were laboratory grade; and for HPLC fingerprinting analytical grade solvents were used.

Animals

Albino mice (Swiss) of either sex weighing between 20-25 g and albino rats (Wistar strain) of either sex weighing between 200 - 250 g were purchased from Wockhardt Research Centre, Aurangabad, Maharashtra and housed as per standard 12:12 h light/dark cycle with temperature-controlled (24 ± 1˚C) milieu, rodent chow (Lipton, India) and water was provided *ad libitum*. All animal experiments were approved by Institutional Animal Ethics Committee (IAEC) Constituted for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) by Ministry of Environment and Forests, Government of India, New Delhi (Reg No. 1613/PO/a/12/CPCSEA, IAEC approval No. 4-I dt. 10/11/2012).

Instruments

Autoanalyser (ADI DUO), Microplate reader (SPECTROstar Nano), Younglin (S.K) HPLC system with UV detector (Autochro-3000)

Phytochemical screening and standardization of extracts

Phytochemical evaluation of isolated plant extracts were performed using TLC fingerprinting for identification of phytochemical composition. Known derrivatizing agents were implemented for the affirming the presence of phytoconstituents.

Total phenols and total flavonoids

Quantification for total phenols and total flavonoids of the extracts was performed using Folin-Ciocalteu's and flavonoid-$AlCl_3$ reactions. For this determination, gallic acid and rutin were used as standard and the obtained results were expressed as gallic acid equivalent (mg of GA/g) and rutin equivalent (mg of RU/g) of the extracts, respectively [24].

Standardization of extracts for phytoconstituent contents by HPLC

The HPLC system - Younglin (S.K.) with Rheodyne injector (20µl fixed loop), ODS guard column (10 µm, 10 mm X 5 mm ID) followed by Grace 250 C18 reverse-phase column (250 X 4.6 mm, particle size 5µm) and UV detector (Autochro-3000) was employed for chromatographic separation.

Preparation of sample and solution

Stock solutions of 1 mg/ml of all WFF extracts were obtained by dissolving in respective solvents. The standard triterpenoids and steroid markers (oleanolic acid, β-Sitosterol) were dissolved in chloroform to obtain concentration of 100µg/ml. The phenolic markers (quercetin, rutin, catechin, gallic acid and chlorogenic acid)

were dissolved in methanol to obtain 50 µg/ml concentrations.

Chromatographic conditions

The mobile phase was selected by the perusal of literature with separation based modification. The combination of solvents with buffers and acids were used as mobile phase for respective markers [25]**.** The HPLC specification and chromatographic conditions are given in Table 2.

***In Vitro* DPPH radicals scavenging activity**

The absorbance of reaction containing DPPH and sample extract or standard was measured at 517 nm using microplate reader (SpectroStar Nano). Obtained absorbances were computed as % inhibition using formula $[(A_0\text{-}_{A1}) / A_0] \times 100$. % Inhibition was measured for the extract and standard concentration in the range of 10 - 50 µg/ml [26].

Acute oral toxicity

WF flower extracts were subjected to toxicity limit test as per guideline no (423) outlined by the Organization for Economic Co-operation and Development (OECD). Just before treatment, the weight of each mouse was recorded after overnight fasting and were randomly divided into eight groups (n=3). Each group was treated with test extract (p.o.) at dose of 5, 50, 300 and 2000 mg/kg body weight; with two groups per dose. Observation of animals was recorded for 4 h after administering the extract, following which were monitored on daily basis for 14 days for alteration in general behavior and/or other physical activities.

Evaluation of cardioprotective potential against doxorubicin induced cardiotoxicity

Four groups (n = 6) of randomly divided albino wistar rats were treated as follow - Group I served as vehicle control and treated with normal saline (p.o. 5ml/kg); and group II - IV were treated with doxorubicin (DOX) (10mg/kg). Group II received saline, Group III received EAWF and Group IV received MEWF at dose 200mg/kg per day p.o. for 30 days. At the end of 30 days the cardiotoxicity was induced in rats by doxorubicin hydrochloride 10mg/kg I.P. of body weight. After 72 h of doxorubicin injection, blood was collected by retro-orbital puncture under mild anaesthesia, serum was separated and analyzed for lactate dehydrogenase (LDH), creatine kinase (CK), serum creatine kinase isoenzyme (CK-MB) and total protein (TP) using commercial kit of Reckon Diagnostic Pvt. Ltd., Vadodra and Transasia Biomedicals Ltd., Solan, respectively. Analysis was done using autoanalyser.

Statistics

All values are expressed as mean ± SD of six animals. Statistical assessment was done by Dunnet's test preceded by one way analysis of variance, ANOVA with $P < 0.05$ as the significance level.

3. Results and discussion

Preliminary phytochemical screening and standardization

Successive extraction of WF flowers by PE, CH, EA, ME with soxhlet followed by maceration with HA yielded extracts as shown in Table 1. These extracts exhibited the presence of phytochemicals like cardiac glycosides, flavonoids and tannins (Table 1), which were further asserted by TLC (Table 1). Spectrophotometric standardization of extracts revealed MEWF extract contains maximum total phenolics and total flavonoid (Table 1).

Presence of specific phytochemicals like oleanolic acid, caffeic acid, gallic acid, ellagic acid, chlorogenic acid, catechin and rutin were affirmed by means of HPLC fingerprinting in comparison to respective standards (Table 2). HPLC analysis further revealed the presence of gallic acid, rutin, catechin, caffeic acid in EAWF and MEWF (Table 2). This affirms the previous reports on isolated phytoconstituents from WF flowers.

Standardization by means of spectroscopic and or chromatographic techniques is routine and demanding task for the determination of quality of extracts [27]. Polyphenols (tannins and flavonoids) purported antioxidant activity along with the capacity to mitigate oxidative stress-induced tissue damage. Compelling evidence on polyphenols further signifies its ability to reduce the risk of chronic diseases [28].

Table 1: Percentage yield, phytochemical screening and standardization of *Woodfordia fruticosa* flower extracts

Extracts obtained	**PEWF**	**CHWF**	**EAWF**	**MEWF**	**HAWF**
% yield	4.5	4.7	5.6	8.6	7.4
Phytochemical tests/ evlaution/ composition					
Flavonoids	-	-	+	+	+
Alkaloids	-	-	+	+	-
Saponins	-	+	+	+	+
Tannins	-	-	+	+	+
Cardiac Glycosides	-	-	+	+	+
Steroids	+	-	-	+	-
Triterpenoids	+	+	+	+	+
Carbohydrates	+	+	+	+	+
TLC fingerprinting					
Mobile phase	BE:HX (1:1)	TO:EA:ME (0.5:0.5:1.5)	EA:ME (06:04)	CH:ME (1:1)	TO:EA:FA:ME (6.6:1.6:0.4:1.4)
Derivatizing agent	Iodine	5%$FeCl_3$	Iodine	10 % H_2SO_4	Iodine
Rf Values	0.91, 0.82, 0.25, 0.12, 0.04	0.15, 0.29, 0.38, 0.61, 0.95	0.83, 0.88, 0.76, 0.33, 0.28, 0.23, 0.16	0.07, 0.11, 0.19, 0.42, 0.80	0.12, 0.30, 0.52, 0.65, 0.73, 0.82, 0.98
Standardization for content of phytochemicals					
Phenolic Content (mg of GA/g of extract)	ND	ND	639.56±1.30	848.13±0.70	459.96±0.75
Flavonoid Content (mg of RU/g of extract)	ND	ND	83.40±0.754	266.23±0.971	39.56±0.85
% DPPH Scavenging at 50 µg/ml of extracts/ standard					
Std-AA -98.2	30.6	68.8	95.6	96.7	75.5

For phytochemical screening (+): indicate presence of phytoconstituents and (–): absence of phytoconstituents, values of standardization for the content of phytochemical represent the mean ± S. D. of three independent replicates, ND-stands for not determined due to absence of phytoconstituents in extracts, GA: Gallic acid, RU: Rutin, AA: Ascorbic acid, TO: Toluene, EA: Ethyl acetate, ME: Methanol, CH: Chloroform, AC: Acetone, FA: Formic acid, BE: Benzene, HX: Hexane

Table 2: HPLC specifications for phytochemical analysis of *Woodfordia fruticosa* extracts

SN	**Standards**	**Mobile phase *(v/v)***	**Flow rate (ml)**	**Standard *Rt* (Min)**	**Detection (nm)**	**PEWF**	**CHWF**	**EAWF**	**MEWF**	**HAWF**
1	Oleanolic acid	Acetonitrile: water (85:15)	0.9	22	215	ND	ND	ND	22.083	ND
2	Butyric acid	Acetonitrile: water (85:15)	0.7	11.7	215	ND	ND	ND	ND	ND
3	β-Sitosterol	Acetonitrile: water (90:10)	0.9	11.05	220	ND	ND	ND	ND	ND
4	Caffic acid	Acetonitrile: water (90:10)	0.7	4.10	230	ND	ND	4.050	4.016	4.0167
5	Gallic acid + Ellagic acid	Methanol+ Water (50:50) pH-4	07	3.36	280	ND	ND	2.54	2.42	2.56
				4.18		ND	ND	ND	ND	4.083
6	Gallic acid	Methanol+ Water (50:50) pH-3.0 with OPA	0.7	2.48	280	ND	ND	2.54	2.42	2.56
7	Chlorogenic acid		0.7	4.10	280	ND	ND	4.016	ND	3.98
8	Catechin		0.7	5.00	280	ND	ND	5.133	5.133	ND
9	Rutin		0.7	5.95	280	ND	ND	5.983	5.98	5.93
10	Quercetin		0.7	11.05	280	ND	ND	ND	ND	ND

ND: Not detected

Table 3: Effect of *Woodfordia fruticosa* flower extracts on doxorubicin induced alteration in serum markers of rats

Group. No.	Treatment	LDH (IU/L)	CK (IU/L)	CK-MB (IU/L)	Total Protein (g/dl)
01	Vehicle Normal saline (5ml/kg)	119.3±12.931	137.2±16.273	91.7±10.499	7.0±1.253
02	Vehicle + DOX (10mg/kg)	306.8±24.916	445.2±21.965	331.3±25.863	16.1±1.048
03	DOX + EAWF (200mg/kg)	198.5±19.923*	217.8±17.267*	181.7±19.371*	11.3±0.929*
04	DOX + MEWF (200mg/kg)	138.5±16.810*	161.8±17.073*	115.7±14.453*	8.9±0.870*

Values are expressed as Mean±SD at n=6, One way ANOVA followed by Dunnet's test, *P<0.05 compared to the doxorubicin

Free radical scavenging activity

Significant % DPPH scavenging activities was observed in MEWF (96.7%) and EAWF (95.6%) as compared to standard antioxidant ascorbic acid (98.2%) at the concentration of 50 µg/ml.

MEWF exhibited better antioxidant activity than EAWF (Table 1).

Pharmacological studies

WF flower extracts at the dose of 2000 mg/kg of body weight exhibited no mortality in mice and animals were found physically active up to 14 days. Thus LD_{50} cut off was concluded as 2000 mg/kg body weight; safe experimental dose was computed as ≤ 200 mg/kg body weight and was considered as maximum dose for experimental procedure.

Doxorubicin has been commonly used by many researchers to cause cardiotoxicity as one of its chronic side effect [29]. It has been postulated that cardiotoxicity was attributed by increased oxidative stress by the formation of more reactive semiquinone from the doxorubicin after activation by mitochondrial Complex I. Loss of cardiomyocytes along with altered molecular signaling has also been considered for its cardiotoxicity [30, 31 and 32].

Doxorubicin induced cardiotoxicity was preclinically evaluated by quantization of serum markers such as creatine kinase (CK), isoform of creatine kinase expressed in heart muscle (CK - MB), lactate dehydrogenase (LDH) and total protein (TP) [33].

In the present study, cardiotoxicity was induced by acute administration of doxorubicin (10 mg/kg I.P. single dose) [34] at the end of 30 days. Doxorubicin cardiotoxicity was evidenced by significant ($P < 0.001$) elevation in serum level of CK, CK-MB, LDH and total protein in doxorubicin treated Group II as compared to control Group I. Doxorubicin induced elevation in serum level of markers were prevented by both EAWF and MEWF extracts ($P < 0.05$ compared to the doxorubicin treated Group II) at 200 mg/kg dose. The efficacy of WF extracts in preventing doxorubicin induced elevation CK, CK-MB, LDH and total protein might be attributed to the anti-oxidant effects as well to the quantity of phytoconstituents like polyphenols and cardiac glycosides.

MEWF extract showed combined potential of inhibition of doxorubicin induced elevation in serum level of markers and better % DPPH scavenging activity as compared to EAWF extract. However, the precise mechanism based on evaluation of extracts, its bioactivity, guided fractionation and isolation of cardiotoxicity protective phytoconstituents are needed.

Conclusion

The present investigation of WF extracts demonstrated significant protective effect against doxorubicin induced cardiotoxicity. The observed potential of change in biomarker level along with antioxidant effects of WF extract might be contributed by bioactive phytoconstituents and their ability to stabilize free radicals. However, evaluation of mechanism, fractionation and isolation of cardioprotective phytoconstituents are the prospective studies for effective use against cardiotoxicity.

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Conflict of interest

The authors do not have any conflict of interest.

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