Published online in http://ijam.co.in ISSN No: 0976-5921

International Journal of Ayurvedic Medicine, Vol 15 (2), 2024; 513-521

Cardioprotective activity of *Dashanga Agada* in Doxorubicin induced Cardiotoxicity in Mice Cardiomyoblasts (H9c2)

Research Article

Neha M Saunshimath[1], Shrutika S Karoshi[2*], Kishore Bhat[3], Vijay Kumbar[4]

1. PG Scholar, 2. Assistant Professor, Department of Agada Tantra,
KAHER's Shri. B. M. Kankanawadi Ayurveda Mahavidyalaya, Belagavi, Karnataka. India.
3. Consultant Microbiologist, Arihant Hospital, Belagavi, Karnataka, India.
4. Scientist-1, KAHER's Dr. Prabhakar Kore Basic Science Research Centre (BSRC). Belagavi. India.

Abstract

Background: Cancer is still a complicated and difficult condition with serious consequences for the general population. Currently, it poses a major risk to the global health. There are numerous risk factors associated with cancer. They include tobacco, obesity, excessive consumption of packed foods, lack of physical activity, alcohol consumption, etc. Continued prevention, research & early detection are required to improve outcomes for cancer patients. Anticancer medications have been associated with cardiotoxicity, compromising individuals' heart function. Early detection of cardiotoxicity is essential for avoiding irreparable heart damage. Cardio-oncology has arisen as a distinct area of study and therapeutic practice in recent years. Several aspects of drug-induced cardiotoxicity remain unknown. Such toxicities are considered as *garavisha* in Ayurveda. They can be treated by using *Agada* principles. Insect bites can lead to cardiac symptoms, according to both ancient and modern science. *Acharya Vagbhata* has mentioned *hrudpeeda* as one of the *lakshana* of *Vataja Vrischika damsha. Dashanga Agada* (DA) is the formulation mentioned in context of *Keeta visha*. As it is the formulation of choice in insect bites exhibiting cardiac symptoms, it is studied in present research to reduce the cardiotoxicity induced by doxorubicin (Dox). The study was carried out on H9C2 mice cardiomyoblasts. Materials and Methods: Hydroalcoholic extract of Ayurvedic formulation *Dashanga agada* was prepared. H9C2 mice cardiomyoblasts were used to study trial drug *Dashanga Agada* on cardiotoxicity induced by a chemotherapy drug named Doxorubicin. MTT assay, DAPI nuclear staining, DCFH-DA assay for intracellular ROS generation & SOD assay were the four parameters studied on H9C2 cell line. 4 groups were taken for the study. Dashanga agada was studied at different concentrations & different time intervals on the above mentioned parameters. Results: IC_{50} values of DA & Dox for 24hrs were calculated to be 357.15 & 5.48 µg/ml respectively. Dashanga agada was effective in increasing cell viability, improving the nuclear morphology & antioxidant activity when compared to Doxorubicin treated group. Conclusion: Dashanga Agada has cardioprotective effect. Within parallel & pre-treatment groups, parallel treatment gave better results.

Keywords: Cancer, Cardiotoxicity, *Dashanga Agada,* Doxorubicin.

Introduction

One of the most concerned diseases of 20th century, cancer, continues to grow and it has become more common in the 21st. In 2020, India reported an estimated 1.39 million cases of cancer which rose to 1.42 million and 1.46 million in the years 2021 and 2022, respectively. It is regarded as a threat to modernity and the sophisticated socio-cultural pattern that is ruled by Western medicine. (1) Cancer is a type of disease that exhibits abnormal growth & proliferation of abnormal cells, which can lead to death unless it is treated. (2) Lung, breast, rectum, prostate and colon cancers are the most prevalent. (3)

*** Corresponding Author:**
Shrutika S Karoshi
Assistant Professor, Department of Agada Tantra,
KAHER's Shri.B.M.Kankanawadi Ayurveda Mahavidyalaya, Belagavi-590003,
Karnataka, India.
Email Id: shrutikakaroshi05@gmail.com

Scientific databases that were available online were screened between the years 2015 to 2021 for assessing the incidence of cardiotoxicity caused by chemotherapy. One of the most widely used chemotherapy drugs was anthracycline when scrutinized in 66.7% of the research studies. 8.3% cases suffered from cardiac symptoms after undergoing chemotherapy (4). Anthracyclines harm the heart in a cumulative, dose-dependent manner that results in death & destruction of cardiomyocyte, which causes heart failure & LV dysfunction(5). Doxorubicin (Dox), the standard drug of our study also belongs to Anthracycline type of chemotherapeutic agents. Dox is indicated in carcinoma of breast, ovary, thyroid, leukemia, lymphoma etc. Its usage may be restricted due to its primary side effect, cardiotoxicity. Once diagnosed, doxorubicin cardiomyopathy has a bad prognosis & is potentially fatal (6). It is not entirely clear what exactly causes anthracycline cardiotoxicity. The most widely accepted explanation is the oxidative stress-based theory, which involves intramyocardial

generation of ROS (7). Better ways to detect and handle anthracycline-induced cardiotoxicity is becoming increasingly clear, but there are still problems like neutropenia, does-dependent cardiotoxicity.

Plants are naturally occurring antioxidants hence they can be used for reducing cardiotoxicity. They contain phytochemicals like flavonoids, carotenoids & phenols which can scavenge free radicals like lipid peroxide & superoxides.(8) *Agadas* are formulations which accelerate the detoxification of organs and organ systems. The study drug, *Dashanga agada* (DA) is a mixture of ten drugs, nine of which are herbal & one mineral. It is most often prescribed formulation in *Keeta Visha* i.e insect bite. (8)

The purpose of present research was to explore *Dashanga Agada* in Doxorubicin mediated cardiotoxicity when used in distinct treatment settings i.e., pre-treatment and parallel treatment. Many herbal drugs like *vacha, vidanga, shunti* have been studied individually in Dox induced Cardiotoxicity and encouraging results were obtained. We conducted a time-related comparative study of *Dashanga Agada* for better understanding of the manner of action in cardiotoxicity induced by Dox.

Table 1: The ingredients of Dashanga Agada are given below-(9)

Sl.no	Dravya		Scientific name	Family	Part used
1	*Vacha (9)*	10gm of each ingredient was taken	*Acorus calamus* Linn.	Araceae	*Kanda* (Rhizome)
2	*Hingu (10)*		*Ferula narthex* Boiss.	Umbelliferae	*Niryasa* (Gummy resin)
3	*Vidanga (11)*		*Embelia ribes* Burm.	Myrsinaceae	*Phala* (Fruit)
4	*Saindava (12)*		Rock salt	-----	-----
5	*Gaja-pippali (13)*		*Scindapsus officinalis* Schott	Araceae	*Phala* (Fruit)
6	*Patha (14)*		*Cyclea peltata* Hook.	Menispermaceae	*Mula* (Root)
7	*Ativisha (15)*		*Aconitum heterophyllum* Wall.	Ranunculaceae	*Kanda* (Rhizome)
8	*Shunti (16)*		*Zingiber officinale* Rosc.	Scitaminae	*Kanda* (Rhizome)
9	*Maricha (17)*		*Piper nigrum* Linn.	Piperaceae	*Phala* (Fruit)
10	*Pippali (18)*		*Piper longum* Linn.	Piperaceae	*Phala* (Fruit

Materials and Methods

Ingredients of Dashanga agada were procured, authenticated and prepared according to classical reference in KLE's Ayurveda pharmacy (Mfg.Lic.No: AUS/37), Khasbag, Belagavi, Karnataka, India.

Dulbecco`s Modified Eagle Media (DMEM) with low glucose, Antibiotic – Antimycotic (100X) solution, and Fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, MA, USA. MTT (3-(4,5-dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide) and DAPI (4′,6-diamidino-2-phenylindole) were purchased from HiMedia Laboratories, India. Dimethyl sulfoxide (DMSO) and paraformaldehyde were procured from Qualigens, Mumbai, India.

Method

Preparation of Dashanga Agada

- All the above-mentioned ingredients of *Dashanga Agada* were taken individually and fine powder (120 mesh) was made.
- Shodhana of *hingu* was done by frying it in *ghrita*(ghee).
- Fine powders of individual drugs were weighed and taken in equal quantities.
- All the 10 ingredients were mixed thoroughly and a homogeneous mixture was formed.
- For 10 mins, *Dashanga Agada* was exposed to UV light to prevent microbial growth.
- 100 gms packets of *Dashanga Agada* were packed and stored in an airtight container.

Preparation of Hydroalcoholic extract of Dashanga Agada

Dashanga agada was prepared by mixing fine powders (120 mesh) of above mentioned ingredients in equal quantity. 5gms of DA was added to a flask containing 80ml distilled water and 20ml ethanol. After shaking the solution on a flask shaker for 6hrs, it was kept undisturbed for following 8hrs.The obtained filtrate was kept on water bath and extract was collected for further study.

Maintenance & treatment of H9C2 cell line (19)

H9C2 mice cardiomyoblasts were obtained from National Centre for Cell Science, Pune, India. After receiving the cell lines, they were kept for overnight incubation to check for their toxicity in Maratha Mandal's N.G.H. Institute of Dental Sciences & Research Centre, Belagavi, Karnataka, India. Once it was confirmed that the cells were non-toxic, some cells were stored for performing further assays. Remaining cells were used to carry out MTT Assay. 4 groups were formed in the experiment.

1. H9C2 normal cells (Control)
2. Cells treated with doxorubicin subjected to stress induction for a period of 24 hours.
3. 24hrs of pretreatment of cells with *Dashanga Agada* followed by Doxorubicin treatment (Pre).
4. *Dashanga Agada* and doxorubicin treatment at the same time (Parallel).

Analysis of *Dashanga Agada*

Analysis and Authentication - Quality analysis and authentication of raw drugs & *Dashanga Agada* was done according to API standards in AYUSH approved Drug Testing Laboratory for ASU Drugs at KAHER's Shri. B.M.K Ayurveda Mahavidyalaya, Belagavi (Batch No.30352, Expiry Date- May 2024)

Organoleptic characters – Below characters were recorded by using sensory organs.

Table 2: Organoleptic Properties

Sr. No	Organoleptic test	Results
1	Part	Fine Powder
2	Color	Brown
3	Taste	Pungent
4	Odour	Characteristic

Table 3: Physicochemical Analysis

Sr. No	Physicochemical standards	Results
1	Loss on drying	4.095%
2	Ash Value	13.760%
3	Acid Insoluble ash	0.943%
4	Water soluble extractive	34.237%
5	Alcohol soluble extractive	23.552%
6	pH	4.52%

Table 4: Values based on reference article – (19)

Sr. No	Parameters	Results
1	Loss on Drying at 110°C (% w/w)	10.57
2	Total Ash (% w/w)	8
3	Acid Insoluble Ash (% w/w)	2.45
4	Water Soluble Extractive (%w/w)	23.85
5	Alcohol Soluble Extractive (%w/w)	15.87
6	pH at 5% aqueous solution (% w/v)	4.58

Phytochemical Analysis – Water Extract

The water extract of Dashanga agada was positive for phytochemicals carbohydrates, reducing sugars, saponins, flavonoids, alkaloids and tannins.

Phytochemical Analysis – Alcohol Extract

The alcohol extract of Dashanga agada was positive for phytochemicals carbohydrates, reducing sugars, steroids, cardiac glycosides, alkaloids and tannins.

Test for specified microorganisms

All the microorganisms tested i.e. *E coli, S aureus, P aeruginosa and S abony* were absent.

Tabel 5: Microbial limit test

Sr. No		Limits	Results
1	Total Bacterial count	30-300 cfu/ml	16 cfu/ml
2	Total Fungal count	10-100 cfu/ml	04 cfu/ml

Table 6: Thin Layer Chromatography of Dashanga Agada

Extract	Solvent	Rf values
5g DA+ 20ml ethanol	Toluene: Ethyl Acetate= 7ml: 3ml	Short wave-0.26,0.3,0.33,0.36,0.46,0.512,0.612,0.6,0.77,0.88,0.925 Long wave 0.2,0.23,0.3,0.35,0.425,0.512,0.52,0.58,0.70.8,0.85, 0.9,0.96

In previous study Rf values that were identified are as follows-

- Short waves – 10 values
- Long waves – 11 values

MTT Cell Viability Assay

Cells were seeded on to 96-well micro plates & incubated at 37°C for one night using 5% CO_2 & 95% humidity. 10,000 cells were added in each well by calculating them by trypan blue assay(20). For calculation of IC_{50} value, both DA and Dox were used in increasing concentrations ranging from 3.125 to 100 µg/ml and they were studied for 24 & 48 hrs individually**.** After two PBS washes, viable cells were stained with MTT staining solution at a concentration of 5 mg/ml for three hrs and plate was incubated at 37°C.[2]After 3 hours of solubilizing formazan, the solvent in each well was substituted with100 µL of DMSO & absorbance at 570 nm was calculated with a spectrophotometer. (21) The study was carried out at 24h & 48h separately to calculate the IC50 dose of both Dox & DA. To calculate Percent cell viability formula used was = 100 × (absorbance of treatment/absorbance of control). Using Graph Pad Prism 8.0.2, we calculated the IC50 of *Dashanga Agada* and Doxorubicin(20).

DAPI Nuclear Staining (22)

By using DAPI Fluorescent dye, nuclear morphological changes of cells that were treated with Dox & DA were visualized. H9C2 cells were cultured and fixed. In the staining solution (10 mM Tris-Cl, pH 7.4; 10 mM EDTA, pH 8 and 100 mM NaCl), 50 ng/ml DAPI was made available and H9C2 cells were incubated for fifteen minutes in a dark room, at ambient temperature. Cells were washed with PBS. Under the fluorescent microscope, cells were visualized with the help of DAPI filter at 100X, along with 372 & 456nm wave length of excitation & emission.

Superoxide Dismutase Assay

The activity of superoxide dismutase (SOD) for DA & Dox was measured using the Beauchamp & Fridovich technique(23). Each experimental group received 50 lg/ml of total cell protein and reaction mixture containing 100 mM potassium phosphate buffer-pH 7.8, 100 mM EDTA pH 8.0, 65 mM L-methionine, 750 mM NBT, and 2 mM riboflavin. After 30 minutes of exposure to direct light, the samples were placed in the dark in the tubes to stop the reaction & at 560 nm absorbance was measured. The activity was measured in units of per milligram of cellular protein.

DCFH-DA Assay for intracellular ROS (24)

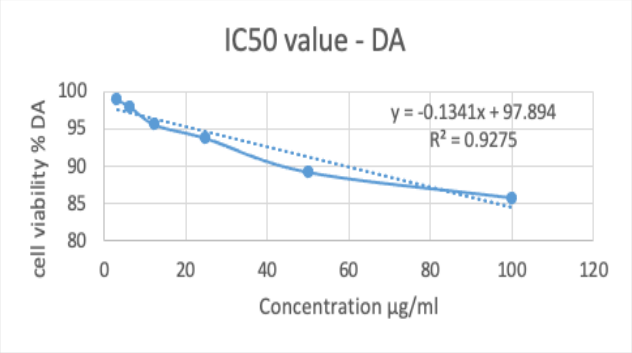
DCFH-DA stain has been used to measure intracellular reactive oxygen species generation. In a 6-well plate, H9C2 cells were cultured (1×106 cells/well) at different concentrations of both Dox (5.5µg/ml) and DA (12.5, 6.25, 3.125 µg/ml) & then placed in an incubator filled with CO_2 for 24 hours. After 24 hours, 1 ml of cells were treated using 100ml of DCFH-DA for a period of 10 minutes at 37° C. For measuring intensity of fluorescence, the cells were visualised under FITC filter with 100 magnifications. 490 and 520 nm were excitation and emission wavelengths, respectively. Intensity of fluorescence is directly proportional to amount of ROS generated (25). The outcomes were expressed as a percentage rise in intensity of fluorescence.

Statistical Analysis

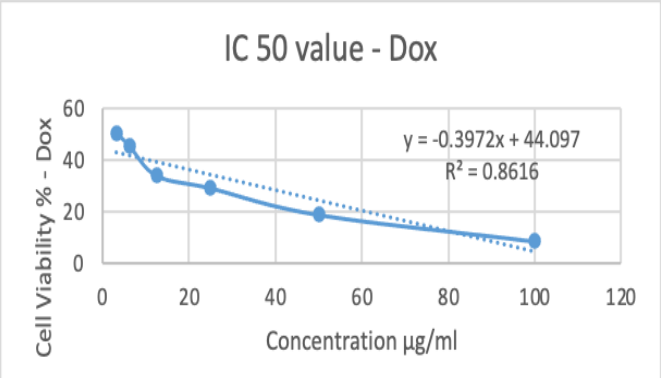
The study was performed in triplicates & findings were presented as standard error ± mean (SEM). Data was statistically evaluated using version 5 of graph pad prism. For MTT assay 24 h incubation the 2way analysis of variance followed by Sidak's multiple comparison test was used. 2way analysis of variance, followed by Bonferroni's multiple comparisons test was applied for 48hrs incubation. For expressing cell viability between Dox pre & parallel, % of intensity of fluorescence & antioxidant capacity, multiple comparisons test using Tukey's two-way ANOVA was used.

IC_{50} VALUE -

Graph 1- IC_{50} value of DA for 24 hrs



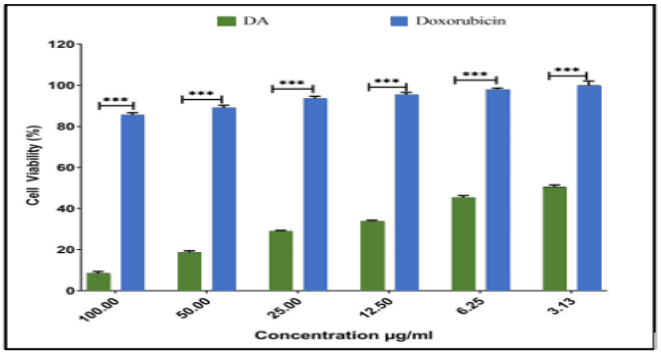
Graph 2 - IC_{50} value of Dox for 24hrs



IC_{50} values for DA & Dox were calculated to be 357.15 µg/ml & 5.48 µg/ml respectively.

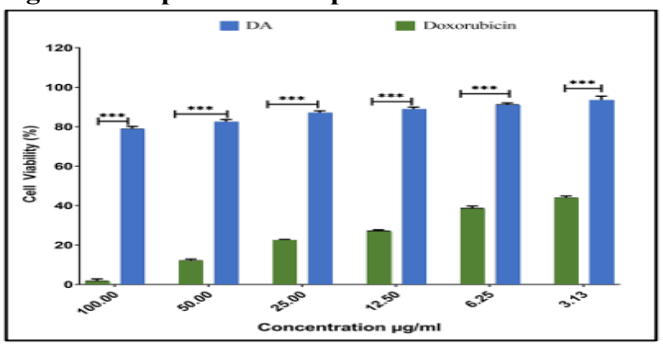
MTT Assay for cell viability

Graph 3: MTT assay for Dox Vs DA from 100 to 3.13µg/ml concentration for 24 hrs, the differences in significance specified as ***p<0.001 between DA & Dox



MTT Assay was performed to calculate the IC_{50} value & cytotoxicity at different concentrations of both standard drug and trial drug. Above graph denotes that with decrease in concentration of both control drug and trial drug, cell viability was increasing. At higher concentrations cell viability of DA & Dox were calculated to be around 80-85% and 15%-30% respectively. At lowest concentration the cell viability was highest in both DA & Dox group i.e., 98% and 50% respectively. IC_{50} value of DA and Dox were calculated to be 357.15 µg/ml & 5.48 µg/m respectively. Lesser concentration of trail drug has shown less cytotoxicity.

Graph 4: MTT assay for Dox Vs DA from 100 to 3.13µg/ml concentration for 48 hrs, the differences in significance specified as ***p<0.001 between DA & Dox



Above graph represents 48hrs treatment of MTT assay. Cell viability was significantly more in DA group in all the concentrations. At lowest conc. of 3.13 µg/ml the viability of cells was observed around 90%. Whereas in Dox group the cell viability was markedly reduced. At higher concentrations of Dox, the cell viability was around 10-15% only. It means that the drug is highly toxic to cells at higher concentrations. At lowest dose of 3.13 µg/ml cell viability was comparatively higher i.e., approximately 50%. IC_{50} value of DA & Dox in 48hrs treated group was calculated to be 277µg/ml & 3.71 µg/ml respectively.

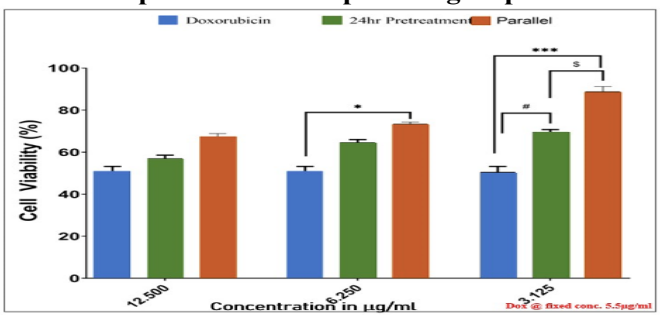
Based on the above outcomes, nontoxic doses of both DA and Dox were selected for further studies. It was observed that 24 hrs MTT assay had better cell viability than 48hrs. Hence it was chosen for upcoming procedures. In all studies, the IC_{50} dose of 5.5 µg/ml Dox was employed to exert stress for 24 hours. 3 concentrations of DA were chosen for further study as their % of cell viability were above 95%.

Published online in http://ijam.co.in ISSN No: 0976-5921

International Journal of Ayurvedic Medicine, Vol 15 (2), 2024; 513-521

Concentrations selected for further study were 12.50, 6.25 & 3.125 µg/ml respectively.

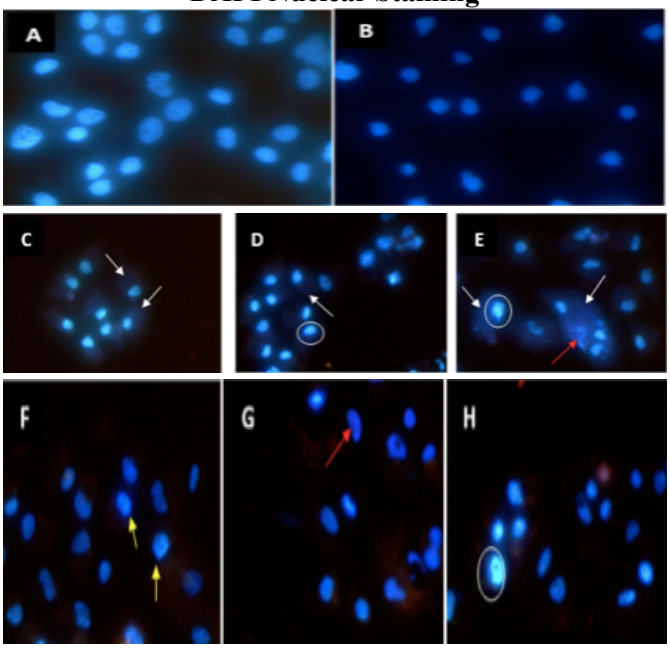
Graph 5: Invitro cytotoxic effect of DA & Dox on H9C2 cell line for 24hrs- Dox, Pre & Parallel groups, The difference in significance specified as *p< 0.05 between Dox & parallel group, #p<0.05 between Dox & 24h pretreatment, $p<0.05 between 24h pretreatment & parallel group



Graph representing % cell viability of Dox, pre & parallel groups

At lowest concentration of 3.12 µg/ml, in pretreatment group cell viability was 70%. Whereas in parallel treatment group 89% cell viability was observed. From the above data when we compare within the 3 groups, it can be concluded that parallel treatment is better than pretreatment group. While comparing in between the groups highest % of cell viability was observed with 3.12 µg/ml dose i.e., the least dose of DA. Hence from above graph it can be concluded that by treating cells with 5.5 µg/ml of Dox and 3.12 µg/ml of DA simultaneously maximum cell viability can be achieved.

DAPI Nuclear Staining



(A)Untreated cells (Normal)
(B) Dox Treated cells
(C) Pre-24 DA 3.125 + Dox 5.5
(D) Pre-24 DA 6.25 + Dox 5.5
(E) Pre-24 DA 12.5 + Dox 5.5
(F) Parallel-DA 3.12 + Dox 5.5
(G) Parallel-DA 6.25 + Dox 5.5
(H) Parallel -DA 12.5 + Dox 5.5

-White arrow marks represent membrane blebbing.

-White circles denote chromatin condensation.

-Red arrow denotes nuclear fragmentation

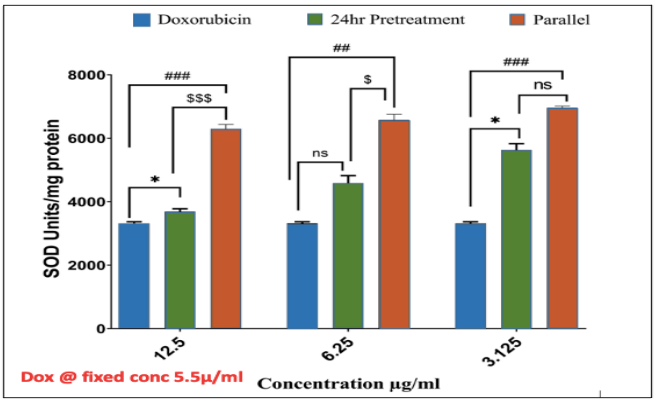
-Yellow arrow represents apoptotic bodies.

(A)Untreated cells (Normal) – The cell had intact nucleus. No nuclear shrinkage was observed. Uniform DAPI Staining was seen throughout the cell.
(B) Dox Treated cells- Condensed chromosome with intense DAPI Staining was observed.
(C) Pretreatment group 24 DA 3.125 + Dox 5.5 – There was nuclear shrinkage. DNA fragmentation seen. The bright florescence represents destruction of nucleus. Apoptotic bodies were visible.
(D) Pretreatment group 24 DA 6.25 + Dox 5.5 – Membrane blebbing and nuclear condensation were visible.
(E) Pretreatment group 24 DA 12.5 + Dox 5.5 – Red arrow denotes nuclear fragmentation. Chromatin condensation was observed.
(F) Parallel treatment group DA 3.12 + Dox 5.5 – Condensed chromosome was visible. Apoptotic bodies were observed.
(G) Parallel treatment DA 6.25 + Dox 5.5 – Nuclear fragmentation was seen.
(H) Parallel treatment DA 12.5 + Dox 5.5 - condensed chromatin was represented by darker shadow.

From the above images it was observed that the cellular changes were more in Dox treated group compared to pre and parallel group. While comparing with pre and parallel groups, less cellular changes were observed in parallel group. So it can be inferred that parallel treatment was better compared to pretreatment group.

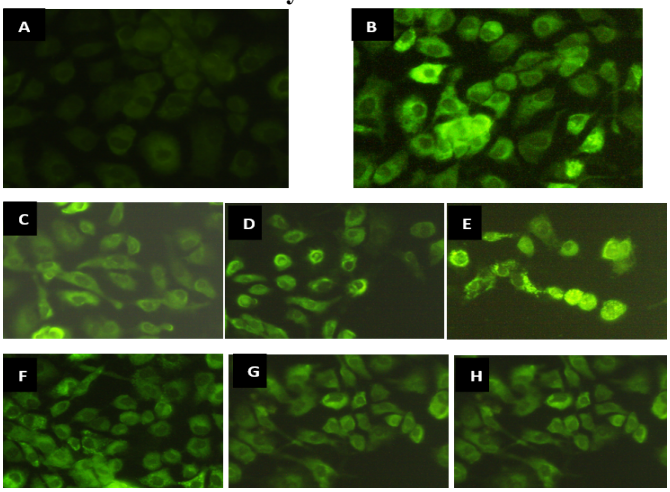
Superoxide Dismutase Assay

Graph 6: SOD activity measured in units/mg protein, the difference in significance specified as *p<0.05 between Dox & 24h pretreatment, ###p<0.001 between Dox & Parallel treatment, $ p<0.05 between 24h pretreatment & parallel group



Superoxide dismutase activity of Dox, pre and parallel was calculated at different concentrations. SOD activity was highest in parallel treated group at 3.12µg/ml concentration. In comparison with dox, parallel treatment group is markedly significant at all 3 concentrations. It means DA is showing better antioxidant production. With decrease in concentration of DA, SOD activity was increasing. The results of pre-treatment are comparable to Dox treated group. When compared within pre & parallel treatment, parallel treatment was better. Maximum amount of antioxidant production was observed in parallel treated group.

DCFH-DA assay for intracellular ROS



H9C2 cells were used to test DA's impact on intracellular ROS production by DCFH-DA labelling. Spectrofluorometer was used to detect the % of ROS generation.

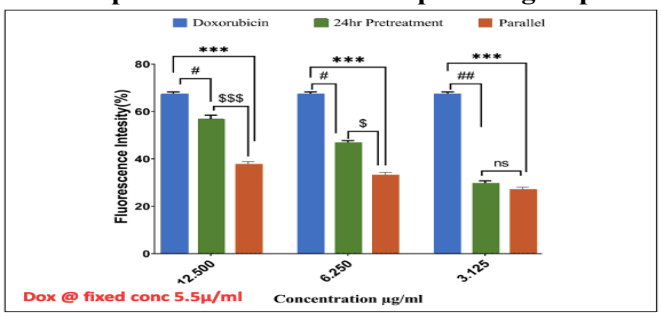
(A)This microscopic photograph visualizes untreated H9C2 cells. Weak fluorescence was observed in these cells.

(B)The image shows Dox treated cells. There is comparatively very high fluorescence in the Dox treated cells. It represents increased reactive oxygen species.

(C), (D), (E) represents the 24hrs pretreatment group with different concentrations of DA (3.12, 6.25 & 12.5 µg/ml) f/b Dox (5.5µg/ml).H9C2 cells show deep intensity of fluorescence.

(F), (G), (H) represents parallel group with different concentrations of Dox (5.5µg/ml) and DA (3.12, 6.25 & 12.5 µg/ml).

Graph 7- Analysis of reactive oxygen species expressed in terms of % intensity of fluorescence. The difference in significance expressed as #p<0.05 between Dox & 24h pretreatment, $$$p<0.001 between 24h pretreatment & parallel group, ***p<0.001 between Dox & parallel group



Dox treated cells had an intensity of 66.2% which means the ROS generation is very high. At higher concentrations, fluorescence intensity was high in all the groups. At lowest concentration of 3.12 µg/ml pre and parallel group did not show significant difference. The green fluorescence of ROS is less in parallel as compared to pre. % of intensity in pre group was 30 and in parallel group it was 26. When we compared in between the group at lowest concentration of 3.12 µg/ml, the ROS generation is less, which mean cellular changes are less when given in this dose compared to other groups. Also parallel treatment is better than pre.

Discussion

Table 7: Activity profile of ingredients of *Dashanga Agada*

Sr.No	Ingredients	Activity
1	*Vacha (9)*	Sedative, analgesic, hypotensive, anticonvulsant, Antioxidant
2	*Hingu (10)*	Anticarcinogenic, anti-inflammatory, CNS Stimulant
3	*Vidanga (11)*	Antibiotic, anti-inflammatory, anticancer, Immunostimulant, antipyretic.
4	*Saindhav (12)*	Antioxidant
5	*Gaja-pippali (13)*	Hypoglycemic, antiprotozoal.
6	*Patha (14)*	muscle relaxant, antitumor, antibacterial
7	*Ativisha (15)*	Hypotensive, antipyretic, analgesic
8	*Shunti (16)*	anti-inflammatory, Antitumoral, analgesic
9	*Maricha (17)*	antioxidant, sedative, muscle relaxant, anti-inflammatory
10	*Pippali (18)*	Anti-inflammatory, antiulcerogenic, Immunostimulatory.

1. Antioxidant Activity-

- The study reported that highest level ($p<0.05$) of antioxidant activity was observed in methanolic extract of *Acorus calamus*. A great source of antioxidants may be the flavonoids and phenols derived from it. (26)
- Extract/essential oils of *Embelica ribes* showed cardioprotective, antioxidant along with other pharmacological actions.(27)
- It has been observed that the *Scindapsus officinalis* fruit's ethyl acetate and methanol extract has anticancer properties. The antioxidant action of the methanolic extract was seen.(28)
- According to this research, *Zingiber officinale* has potent antioxidant activity.(29)

2. Anticancer activity –

- These findings suggest that orally administering *Acorus calamus* extract may have antitumor & anti-cancer effects.(30)
- It has been proposed in the study that ferutinin has a dose-dependent & cell-specific cytotoxic impact on breast cancer cells.(31)
- This research proved that *cissampelos* pariera has considerable anti-tumor properties both in vivo & in vitro.(32)

3. Cardio protective activity-

(a) Pre-treatment of aqueous extract of *Embelica ribes* showed cardioprotective properties in rats by reducing myocardial damage and boosting antioxidant defense against isoproterenol-induced infarction of the myocardium.(33)

4. Antitumor Activity-

(a) High salt exposures appear to prevent the formation of tumors through enhancing anti-tumor immunity & altering the activity of suppressor cells that are myeloid derived.(34)

(b) This research proved that *Cissampelos pariera* has considerable anti-tumor properties both in vivo & in vitro. It has been effective in increasing antioxidant activity as well. (35)

5. Cytotoxic activity-

- When compared to paclitaxel therapy alone, the combination of piperine & paclitaxel had more effective antiproliferative & cytotoxic effect. (36)

Discussion on MTT Assay

The MTT assay involves treating cells for assessing their impact on cell viability. It is used to determine the cause of cell death due to cytotoxic substances. According to the results of MTT assay, 24 hrs treatment resulted in greater cell viability than 48 hrs treatment. This suggests that exposure to both the drugs for shorter periods of time may have less impact on viability of cells. IC_{50} Values of DA and Dox were calculated to be 357.15 & 5.48 µg/ml respectively. These values represent the concentrations of DA & Dox required to inhibit cell viability by 50 percent. To investigate concentration-dependent inhibition of DA, various concentrations of *Dashanga Agada* were used, and it was observed that lower concentrations showed higher cell viability. This indicates a possible dose-dependent effect, with lower DA concentrations exerting less cytotoxicity. It is observed that DA is comparatively very less toxic to cells than Dox. Additionally, the MTT assay was performed for both pre & parallel treatment groups. It was observed that both pre & parallel treatment were effective. Both groups demonstrated efficacy in inhibiting cell viability, but the parallel group gave better results. DA counteracted the action of Dox. Through the results we can assess the cardioprotective activity of *dashanga agada* where parallel treatment acted better than pre-treatment.

Discussion on DAPI Nuclear Staining

The number of nuclei and overall cell morphology were assessed using DAPI staining. The principle behind DAPI nuclear staining is based on its ability to bind to the minor groove of double-stranded DNA. Nuclei within the cells & tissues may be specifically seen due to the fluorescence that DAPI emits, which produces a characteristic blue signal. Findings of the study indicate that both pre & parallel treatment group showed better outcomes in terms of nuclear changes compared to Dox treated group. Observed nuclear changes like DNA fragmentation, nuclear shrinkage & chromatin condensation indicate changes in cellular physiology and may also point to stress/damage to the cells. Reduced nuclear changes in the pre & parallel treatment groups might indicate that the intervention is protective. DA alleviated the side effects seen in the doxorubicin group. This protective effect might be linked to a variety of reasons, including concentration, mechanism of action or treatment timing of the drug itself. Comparatively cellular & nuclear changes were less in parallel treatment group than pretreatment.

Discussion on Superoxide Dismutase Assay

SOD assay is a widely used biochemical method for measuring the action of enzyme called superoxide dismutase in biological specimens.The underlying idea of the SOD assay is SOD's capacity to prevent superoxide radicals from reducing nitroblue tetrazolium (NBT). According to the level of SOD activity in a specimen, the NBT reduction decreases. Drugs in DA contain phytochemicals like flavonoids, carotenoids & phenols which can scavenge free radicals like lipid peroxide & superoxides. Results indicated that SOD levels were lower in the doxorubicin-treated group, suggesting a decrease in antioxidant capacity. However, after treating the cells with a certain concentration of *Dashanga agada*, in both the pre and parallel treatment groups, SOD levels were higher. DA is seen to increase the antioxidant activity which is useful in reversing the Dox pathology. It is also beneficial for cellular health. Additionally, it is mentioned that among the three concentrations tested, the concentration of 3.12 µg/ml was comparatively better, particularly in the parallel treatment group.

Discussion on DCFH-DA Assay for intracellular ROS

The DCFH-DA test is a widely used method for quantifying intracellular ROS levels. Our findings revealed that doxorubicin-treated cells exhibited high green fluorescence, indicating significant ROS production and cellular damage. These findings correspond with prior research indicating that doxorubicin might cause oxidative damage and produce ROS. In contrast, *dashanga agada*-treated cells demonstrated reduced fluorescence intensity, suggesting a potential protective effect against ROS-induced damage. The effect of different concentrations of DA was studied & discovered that 3.12 g/ml was the most effective concentration for lowering ROS production and cellular damage. The results in the parallel group were considerably better. *Vacha, vidanga, gajapippali* and *shunti* have been studied to have antioxidant property. These drugs might have helped in reducing the ROS levels

Conclusion

Dashanga agada is effective in reducing the cardiotoxicity induced by doxorubicin. It has cardioprotective activity. Within pre and parallel treated groups, parallel group was more effective at 3.12µg/ml concentration. Null hypothesis is accepted.

References

1. Balachandran P, Govindarajan R. Cancer--an ayurvedic perspective. Pharmacol Res. 2005 Jan [cited on 2023 April 3] ;51(1):19-30. doi: 10.1016/j.phrs.2004.04.010. PMID: 15519531. Available from https://pubmed.ncbi.nlm.nih.gov/15519531/
2. Accessed on 2023 April 3 - https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2022.html#

3. Accessed on 2023 April 3- https://www.who.int/news-room/fact-sheets/detail/cancer
4. Lima MAC, Brito HRA, Mitidieri GG, de Souza EP, Sobral ACG, Melo HHMA et al. Cardiotoxicity in cancer patients treated with chemotherapy: A systematic review. Int J Health Sci (Qassim) [Internet]. 2022 Nov-Dec [cited 2023 May 15]; 16(6):39-46. PMID: 36475028; PMCID: PMC9682875. Available from https://pubmed.ncbi.nlm.nih.gov/36475028/
5. Henriksen PA. Anthracycline cardiotoxicity: an update on mechanisms, monitoring and prevention. Heart. 2018 Jun [cited on 2023 May 15]; 104(12):971-977. doi: 10.1136/heartjnl-2017-312103. Epub 2017 Dec 7. PMID: 29217634. Available from https://pubmed.ncbi.nlm.nih.gov/29217634/
6. Jordan MA. Mechanism of action of antitumor drugs that interact with microtubules and tubulin. Current Medicinal Chemistry-Anti-Cancer Agents [Internet]. 2002 Jan 1[Cited on 2023 April 17]; 2(1):1-7. Available from https://pubmed.ncbi.nlm.nih.gov/12678749/
7. Šimůnek T, Štěrba M, Popelová O, Adamcová M, Hrdina R, Geršl V. Anthracycline-induced cardiotoxicity: Overview of studies examining the roles of oxidative stress and free cellular iron. Pharmacological Reports [Internet]. 2009 Jan-Feb [cited on April 2023]; 61(1):154-171,ISSN1734-1140. Available from -https://doi.org/10.1016/S1734-1140(09)70018-0.
8. Xu, D. P., Li, Y., Meng, X., Zhou, T., Zhou, Y., Zheng, J., Zhang, J. J., & Li, H. B. (2017). Natural Antioxidants in Foods and Medicinal Plants: Extraction, Assessment and Resources. *International journal of molecular sciences*, *18*(1), 96. https://doi.org/10.3390/ijms18010096
9. Paradkar HS. Acharya Vagbhata, Astang Hrudaya, Varanasi: Chaukhambha Surabharati Prakashana; K.R.S. Chapter 37, Uttaratantra, Shlok 11-17. 2017.p 916.
10. Paradkar HS. AcharyaVagbhata, Astanga Hrudaya, Varanasi: Chaukhambha Surabharati Prakashana; chapter 37, Uttara tantra, shloka 27-28; 2010. p.1682
11. Sharma PC, Yelne MB, Dennis TJ. Database on Medicinal Plants used in Ayurveda (Reprint), Vol I. CCRAS: 2002; Pg 469-472.
12. Levekar GS et al, Database on Medicinal Plants used in Ayurveda & Siddha, CCRAS, Vol VIII, 2007; Pg 125-132.
13. Levekar GS et al, Database on Medicinal Plants used in Ayurveda & Siddha, (Reprint), Vol V, CCRAS, 2008, 478-482p.
14. Kamat SD, Dhanvantari Nighantu, Chapter no 2, Delhi, Chaukhamba Publication,2002; Page no-134, 135.
15. Sharma PC, Yelne MB, Dennis TJ. Database on Medicinal Plants used in Ayurveda, Vol IV, CCRAS, Pg 182-186.
16. Sharma PC, Yelne MB, Dennis TJ. Database on Medicinal Plants used in Ayurveda, (Reprint), Vol II, CCRAS, 2005, Pg 438-441.
17. Billore KV, Yelne MB, Dennis TJ, Chaudhari BG. Database on Medicinal Plants used in Ayurveda, Vol VII, CCRAS, 2008, Pg 38-42.
18. Levekar GS et al, Database on Medicinal Plants used in Ayurveda & Siddha, (Reprint) Vol V, CCRAS, 2008, (b)Pg 315-323
19. Levekar GS et al, Database on Medicinal Plants used in Ayurveda & Siddha, (Reprint) Vol V, CCRAS, 2008, (c) Pg 187-192
20. Sharma PC, Yelne MB, Dennis TJ. Database on Medicinal Plants used in Ayurveda, (Reprint), Vol III, CCRAS, 2005, Pg 472-477.
21. IOSR Journal Of Pharmacy (e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219 Www.Iosrphr.Org Volume 3, Issue 6 (July 2013), Pp 20-25 20 Physicochemical Analysis of Dashanga Agada – An Ayurvedic Formulation
22. Segeritz CP, Vallier L. Cell Culture: Growing Cells as Model Systems In Vitro. Basic Science Methods for Clinical Researchers [Internet]. 2017 Apr 7 [cited on 2023 Feb 5]:151–72. doi: 10.1016/B978-0-12-803077-6.00009-6. Epub 2017 Apr 7. PMCID: PMC7149418. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7149418/
23. Lin X, Bai D, Wei Z, Zhang Y, Huang Y, Deng H, *et al*. Curcumin attenuates oxidative stress in RAW264.7 cells by increasing the activity of antioxidant enzymes and activating the Nrf2-Keap1 pathway. PLoS One[Internet]. 2019 May 21 [cited on 2023 Feb 9];14(5):e0216711. doi: 10.1371/journal.pone.0216711. PMID: 31112588; PMCID: PMC6528975. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6528975/
24. Jain A, Rani V. Mode of treatment governs curcumin response on doxorubicin-induced toxicity in cardiomyoblasts. Molecular and cellular biochemistry [Internet]. 2018 May [cited on 2022 July 16]; 442(1-2):81–96. Available from-https://doi.org/10.1007/s11010-017-3195-6
25. Hattiholi A, Tendulkar S, Kumbar V, Rao M, Kugaji M, Muddapur U, *et al*. Evaluation of Anti-cancer Activities of Cranberries Juice Concentrate in Osteosarcoma Cell Lines (MG-63). Indian J of Pharmaceutical Education and Research [Internet]. 2022 Sept [cited on 2023 March 15]; 56(4):1141-9. Available from- https://www.researchgate.net/publication/363851821_Evaluation_of_Anti-cancer_Activities_of_Cranberries_Juice_Concentrate_in_Osteosarcoma_Cell_Lines_MG-63
26. Beauchamp C & Fridovich I. Super oxide dismutase: Improved assays and assay applicable to acrylamide gels. Analytical Biochemistry [Internet]. 1971[cited on 2023 March 17]; 44: 276–287.Available from http://dx.doi.org/10.1016/0003-2697(71)90370-8
27. Annamalai G, Kathiresan S, Kannappan N.[6]-Shogaol, a dietary phenolic compound, induces oxidative stress mediated mitochondrial dependant apoptosis through activation of proapoptotic factors

in Hep-2 cells.Biomedicine & Pharmacotherapy[Internet].2016 Aug [Cited on 2023 March 25];82: 226-236. Available from -https://doi.org/10.1016/j.biopha.2016.04.044.

28. Jesudason EP, Masilamoni JG, Jebaraj CE, Paul DS, Jayakumar R. Efficacy of DL-α lipoic acid against systemic inflammation-induced mice: antioxidant defense system. Mol Cell Biochem [Internet].2018 April 10[Cited on March 24]; 313: 113–123. Available from-https://doi.org/10.1007/s11010-008-9748-y
29. Li KS and Wah CS. Antioxidant and antibacterial activity of Acorus calamus .L leaf and rhizome extracts. Jurnal Gizi Klinik Indonesia [Internet]. 2017 Apr 28 [cited on 2023 Apr 1];13(**4**):144. Avaliable from https://doi.org/10.22146/ijcn.17937
30. Sharma V, Gautam DNS, Radu AF, Behl T, Bungau SG, Vesa CM. Reviewing the Traditional/Modern Uses, Phytochemistry, Essential Oils/Extracts and Pharmacology of *Embelia ribes* Burm. Antioxidants (Basel) [Internet]. 2022 Jul 13[Cited on 2023 Apr 1];11(7):1359. doi: 10.3390/antiox11071359. PMID: 35883850; PMCID: PMC9311956.Available from https://pubmed.ncbi.nlm.nih.gov/35883850/
31. Chou CK, Liu W, Hong YJ, Dahms HU, Chiu CH, Chang WT, *et al*. Ethyl Acetate Extract of *Scindapsus* cf. *hederaceus* Exerts the Inhibitory Bioactivity on Human Non-Small Cell Lung Cancer Cells through Modulating ER Stress. Int J Mol Sci [Internet]. 2018 Jun 21[Cited on 2023 Apr 3]; 19(7):1832. doi: 10.3390/ijms19071832. PMID: 29933620; PMCID: PMC6073426. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6073426/
32. Ghasemzadeh A, Jaafar HZ, Rahmat A. Antioxidant activities, total phenolics and flavonoids content in two varieties of Malaysia young ginger (Zingiber officinale Roscoe). Molecules [Internet]. 2010 Jun 14[cited on 2023 Apr 3];15(6):4324-33. doi: 10.3390/molecules15064324. PMID: 20657444; PMCID: PMC6264263. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6264263/
33. Unnikrishnan MC, Kuttan R, Tumour reducing and anticarcinogenic activity of selected spices. Cancer Letters [Internet]. 1990 May 15[cited on 2023 Apr 5]; 51(1): 85-89. Available from https://doi.org/10.1016/0304-3835(90)90235-P.
34. Naji Reyhani Garmroudi S, Karimi E, Oskoueian E, Homayouni-Tabrizi M, Iranshahi M. Ferutinin: A phytoestrogen from ferula and its anticancer, antioxidant, and toxicity properties. J Biochem Mol Toxicol[Internet]. 2021 Apr [Cited on 2023 Apr 6];35(4):e22713. doi: 10.1002/jbt.22713. Epub 2021 Jan 27. PMID: 33501774. Available from https://pubmed.ncbi.nlm.nih.gov/33501774/
35. Thavamani BS, Mathew M, Dhanabal SP. Anticancer activity of cissampelos pareira against dalton's lymphoma ascites bearing mice. Pharmacogn Mag[Internet]. 2014 Jul[Cited on 2023 Apr 6];10(39):200-6. doi: 10.4103/0973-1296.137356. PMID: 25210304; PMCID: PMC4159910. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4159910/
36. Bhandari U, Ansari MN, Islam F. Cardioprotective effect of aqueous extract of Embelia ribes Burm fruits against isoproterenol-induced myocardial infarction in albino rats. Indian J Exp Biol [Internet]. 2008 Jan [Cited on 2023 Apr 7];46(1):35-40. PMID: 18697569. Available from https://pubmed.ncbi.nlm.nih.gov/18697569/
37. Willebrand R, Hamad I, Van Zeebroeck L, Kiss M, Bruderek K, Geuzens A,et al. (2019) High Salt Inhibits Tumor Growth by Enhancing Anti-tumor Immunity. Front. Immunol [Internet]. 2019 Jun 4[Cited on 2023 Apr 8];10: 1141. doi: 10.3389/fimmu.2019.01141. Available from- https://www.frontiersin.org/articles/10.3389/fimmu.2019.01141/full
38. Thavamani BS, Mathew M, Dhanabal SP. Anticancer activity of cissampelos pareira against dalton's lymphoma ascites bearing mice. Pharmacogn Mag [Internet]. 2014 Jul [Cited on 2023 Apr 8];10(39):200-6. doi: 10.4103/0973-1296.137356. PMID: 25210304; PMCID: PMC4159910.Available from - https://pubmed.ncbi.nlm.nih.gov/25210304/
39. Koechli OR, Sevin BU, Perras JP, Chou TC, Angioli R, Steren A, *et al* . Characteristics of the combination paclitaxel plus doxorubicin in breast cancer cell lines analyzed with the ATP-cell viability assay. Breast Cancer Res Treat [Internet]. 1993 Oct [Cited on 2023 Apr 10] ;28(1):21-7. doi: 10.1007/BF00666352. PMID: 7907234.Available from - https://pubmed.ncbi.nlm.nih.gov/7907234/
40. Rao GP. Sharangadhar Samhita. 1st ed.Chaukhambha publications; 2013[cited on 2023 Apr 12]. Chapter 4.p.17.
41. Sharma RK, Dash VB. Charaka Samhita. (Reprint). Chaukhambha publications; 2022[cited on 2023 Apr 24]. Vol 1. Chapter 1. Verse 134.p. 61.

*****