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In vitro Antioxidant and Cardio- protective effect of Borassusflabellifer L mesocarp extract against doxorubicin induced toxicity in H9c2 cardiomyocyte cell line

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ABSTRACT
Cardiotoxicity is one of the most critical complications of chemotherapy is cardiotoxicity, which drastically increases mortality and morbidity rates. The term "toxicity that affects the heart' is commonly used to refer to cardiotoxicity. Doxorubicin is an anticancer drug used in cancer therapy but produces reactive oxygen species(ROS) that are toxic to heart cells. Borassusflabellifer belonging to the family Arecaceae commonly known as Palmyra palm is a native of tropical Africa but cultivated throughout India. Traditionally B. flabelliferis used to treat variety of diseases and has ethnomedicinal claims. The purpose of the study was to determine the cardioprotective activity of ethanolic extract of Borassusflabellifer L. mesocarp against the heart induced by doxorubicin. B. flabellifer mesocarp ethanol extract was obtained by maceration followed by phytochemical studies..Free radical scavenging activity were assessed using DPPH assay. The cells were incubated with different concentration of extract ofBorassusflabellifer mesocarp for 24hrs.Cell viability was determined by using MTT assay, respectively.Phytochemical screening revealed the presence of alkaloids, carbohydrates, flavnoids, phenols, tannins, saponins, glycosides, and terpenoids. The ethanolic extract of Borassusflabellifer exhibits antioxidant activity. Doxorubicin markedly reduced cell viability, and this was accompanied by a rise in ROS generation. The pre-treatment with Borassusflabellifer leaf ethanolic extracts improved cell viability and inhibited the production of reactive oxygen species. According to the results of this investigation, the mesocarp extract of Borassusflabellifer showed protection against cardiomyocyte damage caused by oxidative stress. Significant antioxidant and cardioprotective activity is present in the ethanolic extract of the mesocarp of Borassusflabellifer.

Keywords : Borassusflabellifer, DPPH, ROS, doxorubicin, cardiomyocyte damage

I. INTRODUCTION

Cardiovascular Diseases are the leading cause of deaths around the world as reported by World Health Organization recently, where, cardiotoxicity has become very critical for scientific community in past two decades. Adriamycin, often known as doxorubicin, is an anthracycline antibiotic that has been used for the treatment of several malignancies for more than 30 years. In a wide range of malignant conditions, including Hodgkin's and non-Hodgkin's lymphomas, osteosarcoma, Kaposi's sarcoma, soft tissue sarcoma, and breast and esophageal carcinomas, it acts quite well.Due to its unwanted, severe, dose-dependent cardiotoxic side effects, which usually result in congestive heart failure, doxorubicin's clinical usage is restricted.

Reactive oxygen species, or ROS, are a major contributor to oxidative damage to a range of biological constituents. Oxidative stress results in permanent DNA damage, altered cardiac energy levels, and increased toxicity in cardio-myocytes[(1)]. By knowing the free radical mechanism of anthracycline-induced cardiotoxicity, one may determine that peroxynitrites, a reactive oxidant primarily implicated in doxorubicin cardiotoxicity, are formed by the fast interaction of nitric oxide and superoxide. Although overexpressing endogenous antioxidants in human hearts is not yet possible, other approaches have been tried in an effort to strengthen the antioxidant defense system in anthracycline-using patients[(2)]. The use of antioxidants to prevent DOX-induced cardiomyopathy is becoming more and more intriguing, since they show great promise in animal models of anthracycline-induced cardiotoxicity. Plant compounds with protective or illness-preventive qualities are known as phytochemicals.

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Borassusflabellifer L, belongs to family Arecaceae, commonly known as Palmyra palm is a native of tropical Africa but cultivated throughout India[3]. The phytochemicals like alkaloids, flavonoids, terpenes, glycosides, saponins, phenolic, tannin, steroids, and sterols are found in palmyra fruit. These compounds are well-known for their carditonic properties, antimicrobial properties, Flavanoids and tannin are phenolic compounds that act as primary antioxidants and have antimicrobial, anti-inflammatory, anti-allergic, anticancer, anti-neoplastic, and anti-neoplastic activity, as well as the ability to treat intestinal disorders. Saponins are also therapeutically important due to their hypolipidemic and anticancer activity. Saponins are also required for cardiac output activity[4].

The mesocarp of Borassusflabellifer is chosen for the present study as its preliminary phytochemical screening revealed the presence of saponins, phenolic compounds and flavonoids. Further, the leaf extract exhibited a greater in vitro antioxidant activity. In view of the manifold effects that have been reported in literature for this plant, and due to the presence of the above active principles in the leaf it is hypothesized that the ameliorative effect of the mesocarp extract of Boraasusflabellifer in countering the cardiotoxic effects caused by Doxorubicin could be explored[5].

II. MATERIALS AND METHODS:

2.1 Collection and preparation of plant extract:

The fresh mesocarp of Borassusflabellifer (L.)was collected from Arayoor, Thiruvananthapuram, Kerala, India during the month of January.2023.. The plant was authenticated by Mr.Rogimon P.Thomas, Professor & HOD, Department of Botany, CMS college, Kottayam The dried material was powdered and passed through a 10-mesh sieve. The powdered plant material was then extracted with ethanol by extraction method using maceration. At the completion of the extraction procedure, the solvent was removed by means of rotary evaporator, yielding the extracted compound.

2.2 Phytochemical Screening of Plant extract

The potential pharmacological action of a plant can be predicted by identifying the phytoconstituents present in the plant material[6]. The Borassusflabellifer mesocarp extract was subjected to phytochemistry in order to determine whether or not it contained several phytochemical classes, including glycosides, alkaloids, tannins, saponins, polysaccharides, flavonoids, phenols, and triterpenoids[7].

2.3 Total phenol determination

The total phenolic content was determined using the method of Olufunmiso et al[8].A volume of 2ml of each extracts orstandard was mixed with 1 ml of Folin Ciocalteau reagent(previously diluted with distilled water 1:10 v/v) and 1 ml(7.5g/l) of sodium carbonate. The mixture was allowed tostand for 15 min under room temperature. The colour developed was read at 765 nm using UV/visiblespectrophotometer. The total phenolic content wascalculated from the standard graph of gallic acid and the results were expressed as gallic acid equivalent(mg/100mg).

2.4 Total flavonoids determination

The total flavonoid content was determined using themethod of Olufunmisoet al[8].1ml of 2% AlCl3 solution was added to 3ml of extract or standardandallowed tostandfor15 min at room temperature; the absorbance of the reactionmixture was measured at 420 nm using UV/visible spectrophotometer. The content of flavonoids was calculated using standard graph of quercetin and the results wereexpressed as quercetin equivalent (mg/100mg).

2.5 Determination of DPPH Assay

The DPPH test was carried out in accordance with the earlier description [9]. To sum up, three milliliters of different concentrations of Borassusflabellifer mesocarp extract were added to the DPPH solution and diluted to three milliliters using methanol. After that, this solution was left to incubate for 40 minutes in the dark. The absorbance of the designated solution at 517 nm was measured using a spectrophotometer. Each study was conducted three times, and the percentage of inhibition in vitamin C equivalents was determined. This formula was used to determine the % DPPH scavenging potential:

$$\% \text{ Inhibition} = \frac{A(\text{blank}) - B(\text{sample})}{A(\text{blank})} \times 100 \quad (1)$$

A(blank)

2.6 In vitro Cardio protective study using H9c2 (Rat cardiomyocyte) cell line:

Cell culture:

Normal H9c2 (rat cardiomyocyte) cell line was obtained from National Centre for Cell Sciences (NCCS), Pune. The cells were cultured in

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Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (100µg/ml) in a humidified atmosphere of 50µg/ml at 37^0C in 5% CO_2 and subcultured when they reached 70% confluence.

Cell viability (MTT) assay:

The cell viability was determined by using MTT assay as described procedure of Mosmann, 1983. Cells (1×10^5/well) were plated in 24-well plates and incubated in 37^0C with 5% CO_2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 2hrs. After 2 hrs, 5µM Doxorubicin was added in all the wells and incubated for 24 hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells. The absorbance at 570nm was measured with UV-Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC50) was determined graphically[7]. The % cell viability was calculated using the following formula:

% Cell viability = A_{570} of treated cells / A_{570} of control cells × 100 (2)

III. RESULTS

The crude extracts so obtained after each of the successivemaceration extraction process were concentrated on waterbath by evaporation the solvents completely to obtain theactualyieldofextraction.

The percentage yield was found to be 5.0216% w/w

3.1 Phytochemical Analysis

The presence of saponins, phenols, flavonoids, carbohydrates, alkaloids and glycosides was confirmed by phytochemical analysis by visually monitoring the extract's pre-specified color shift and precipitate development, while tanninswas not detected (Table 1).

Table1. Phytochemical analysis of Borassusflabellifer L ethanolic mesocarp extract.

Tests	Observation	Results
Alkaloid	PPT	+
Phenols	Lightpurplecolour	++
Tannins	No colour	-
Glycosides	Yellow orange colour	+
Saponins	1cmfroth	+
carbohydrates	Reddish brown PPT	+
Flavonoid	Lightyellowcolour	+

PPT=Precipitate formation, froth=Presence of froth in test tube.+=present,−=absent

3.2 Total phenolic and flavanoid content determination

The determination of the total phenolic content, expressed as mg gallic acid equivalents and per 100 mg dry weight ofsample. The total flavonoids content of the extracts wasexpressed as percentage of quercetin equivalent per 100 mgdry weight of sample

From the calibration curve of gallic acid the phenolic content of ethanolic extract of B. flabellifer were found to be 104.00µg/mland from the calibration curve of quercetin the flavonoid content of ethanolic extract of B. flabellifer found to be 98 µg/ml.

3.3 DPPH Assay

In the DPPH assay, the mesocarp extract of Borassusflabellifer (ethanolic) exhibited significantantioxidantactivityata200µg/mLconcentrationofapproximatelyascorbic acid

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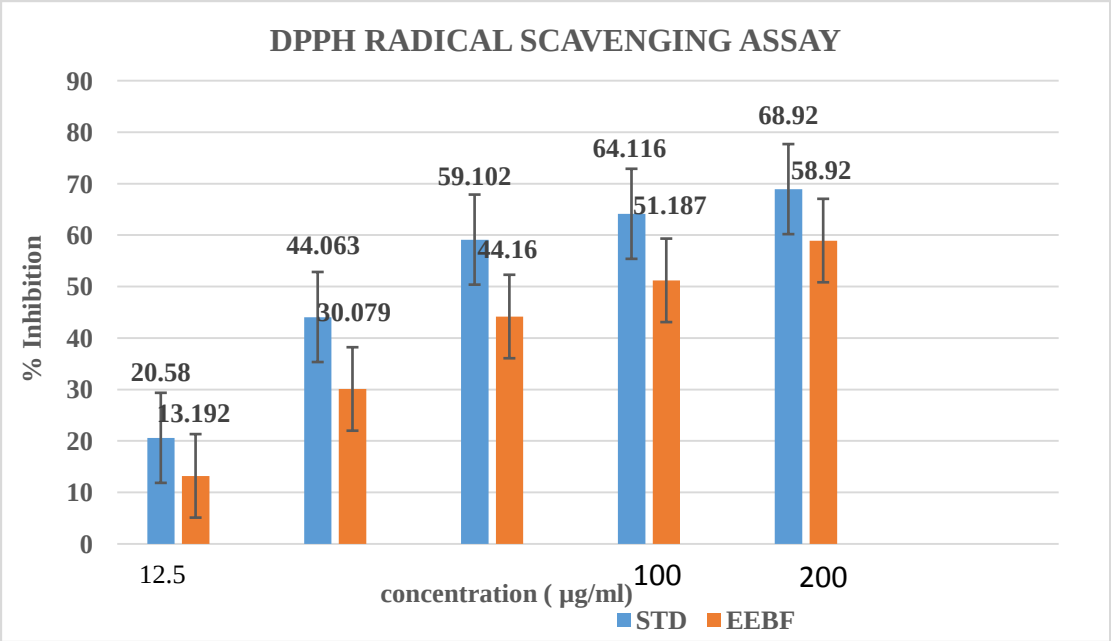

Fig 1. AntioxidantpotentialofBorassusflabelliferconcerningascorbicacidbyDPPHassay
IC50 value of Borassusflabellifer L. mesocarp extract = 94.11 µg/mL

3.4 In vitro cardio protective study using H9c2 cell line

Cell viability (MTT) Assay:

The cytotoxic effect of various concentration of Doxorubicin was evaluated by using the MTT assay. The formation of formazan was measured at 500-600nm. The figure 3 and 5(B) showed that the cytotoxic effect of doxorubicin-induced cardiotoxicity on H9c2 cell line after 24hrs. In doxorubicin treated cell line significantly decreased the cell viability in a dose-dependent manner and it shows increased cell death because of its excessive ROS production activity. The IC_{50} value was calculated as 4.38µg/ml using y = m

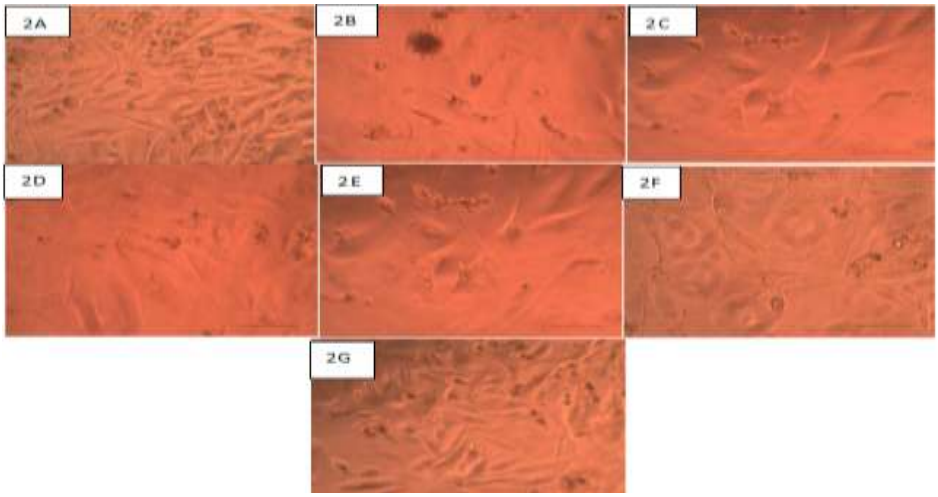

Fig 2 : Control cell(2A), Doxorubicin(2B), doxorubicin + 1.5µg/mL EEBF(2C), doxorubicin+ 3.1µg/mL EEBF
(2D), doxorubicin + 6.25µg/mL EEBF(2E), doxorubicin + 12.5µg/mL EEBF(2F), doxorubicin +25 µg/mL EEBF (2G) Percentage viability of extract at concentration of 25µg/mL was found to be 82.02%

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IV. DISCUSSIONS

The crude extracts so obtained after each of the successivemaceration extraction process were concentrated on waterbath by evaporation the solvents completely to obtain theactualyieldofextraction. Phytochemical screening of ethanolic mesocarp extract of Borassusflabellifer showed the presence of flavanoids, phenols, alakaloids, carbohydrates, saponins, glycosides. Total phenolic and flavanoidcontent was found to be 104.00µg/mL and 98µg/mL.All these phytochemicals are well known for their antioxidant. Antiinflammatory properties.

Antioxidants are the substance which is used to delay or prevent the oxidation of the substrate. It may help the body to protect itself against various types of oxidative damage caused by reactive oxygen species, which are linked to a variety of diseases including cancer, diabetes, arthritis, and acceleration of the aging process[10].Free radicals and other reactive oxygen species are considered as an important causative factor for various diseases like neurodegenerative diseases, cancer, and cardiovascular diseases[11,12].Antioxidants function by scavenging initial radicals, reducing oxygen concentration, preventing singlet oxygen from forming, binding metal ion catalysts, breaking chains to stop further hydrogen abstraction from substances, and decomposing primary products into non-radical compounds.

The oxidative damages caused by ROS on lipids, proteins and nucleic acids may trigger various chronic diseases, such as coronary heart diseases, atherosclerosis, cancer, and aging[13]. The health-promoting effect of antioxidants from plant materials is thought to arise from their protective effects by counteracting ROS(11). Assessing the antioxidant capacity of foods, botanicals and other nutritional antioxidant supplements. Plants are expressed their significant amount of antioxidants to prevent the oxidative stress caused by photons and oxygen and they represent a potential source of new compounds with antioxidant activity.

Many medicinal plants possessed large amounts of antioxidants such as polyphenols (phenolic acids, flavonoids, and anthocyanin) which can play an important role in adsorbing and neutralizing free radicals[14,15]. There is increasing interest in natural antioxidant products for use as medicines and food additives. Many studies have shown that antioxidants present in plants at a higher level and these biomolecules exert a potential protective effect against oxidative damage.

Assessing the antioxidant impact of plant extracts is commonly done using DPPH radical scavenging activity. An established stable radical and radical scavenger is DPPH. One can determine the radical character of a chemical reaction by measuring how quickly it decreases when DPPH is added. a prominent 520 nm-centered absorption band. When the DPPH radical is neutralized, it turns light yellow or colorless from its deep violet state in solution. The ability of antioxidant chemicals to lose hydrogen and the structural conformation of these components determine the extracts' capacity to scavenge free radicals. One characteristic of a radical scavenger is the DPPH radical's capacity to interact with hydrogen (H). Methanol is used to create a DPPH radicals solution.

From the results of DPPH assay showed that the ethanolic extract of Borassusflabellifer mesocarp possess the potent antioxidant effect.

With the majority of the molecular and functional characteristics of adult cardiomyocytes, the H9c2 (rat cardiomyocyte) cell line, which was generated from the rat heart ventricle of the embryo, was extensively employed as an in-vitro cellular cardiac model. Heart failure is a significant risk factor in cardiac hypertrophy. The advantage of using the H9c2 cell line is that it is a substitute for animals and can replicate the hypertrophic responses precisely[16.17]. These results therefore contribute to the selection of the H9c2 cell line as a model for the investigation of the in-vitro cardioprotective activity of Borassusflabellifer mesocarp extract.

The MTT assay is the most commonly used method for determining the viability of the cells. It is a colorimetric assay for assessing cell metabolic activity. The viable cell contains NAD(P)H - dependent oxidoreductase enzymes which reduce the MTT reagent to formazan, an insoluble crystalline product with a deep purple color. Formazan crystals are dissolved using a solubilizing solution and absorbance was measured at 500-600nm. When the cells die they lose their ability to convert MTT into formazan. If more cells are inviable they show thick color formazan formation. The color formation serves as a convenient marker of the viable cells.

Doxorubicin has several mechanisms for cardiotoxic effects, but an excessive generation of free radicals (ROS) is the major mechanism behind drug-induced toxicity[18]. Doxorubicin has Quinone moiety in their molecule which is converted

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enzymatically or non-enzymatically by cytochrome P450 into its semiquinone moiety by the acquisition of electrons. This semiquinone form is then oxidized by molecular oxygen to yield free radicals which are leading to cardiotoxicity. Doxorubicin has reduced the half percentage of cell viability on H9c2 cell line at the concentration of 100µMl after 24hrs. So the Doxorubicin has a cardiotoxic effect in a dose-dependent manner. The pretreatment with Borassusflabellifer mesocarp extracts (ethanol) showed significant increased in cell viability and reduced the cell death.

V. CONCLUSION

In this study concluded that ethanolic extract of Borassusflabellifer L. mesocarp possess significant antioxidant and cardioprotective activity. Further study is needed for the evaluation of the mechanism of action of plant extract.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation**.**

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