

# Advanced GC–MS Characterization of Bioactive Constituents in *Punica granatum* Leaves and Their Therapeutic Antioxidant Potential Against Cardiac and Hepatic Toxicity in Rats

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## ABSTRACT

The study was mainly designed to evaluate the antioxidant properties of *Punica granatum* dried leaf extract. Experimental plants extract phytochemical screening was done and has shown the presence of pyrogallol in the leaf and exhibited potential in vitro antioxidant property. The cardioprotective and hepatoprotective role of the plant was investigated by measuring level of serum and, cardiac enzymes with drug induced stressed animals. On observation the levels of AST, ALT, LDH and CK tends to be normal in *Punica granatum* dried leaf extract treated animals rather than non-treated animals, where both animal groups were induced toxicities with doxorubicin and Isoproterenol. Further, Macro and histopathological studies suggested the conservation of healthy cardiac architecture. Thus, we have concluded that *Punica granatum* dried leaf extract also could be a new addition as cost effective herbal antioxidant.

**Key word:** Doxorubicin, Isoproterenol, cardiotoxicity, hepatotoxicity and herbal antioxidant

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## 1. INTRODUCTION

The development of new drugs is often hindered by cytotoxicity, a major contributor to attrition during both preclinical and clinical stages. This toxicity can manifest in various forms, including damage to the liver, heart, and other organs. Certain medications, such as those used in chemotherapy, are known to induce cytotoxicity [1].

Prolonged use of neurological and psychiatric medications can also lead to cardiotoxicity due to the accumulation of toxins in the body [2]. In recent decades, approximately 10% of drugs have been withdrawn from the market due to safety concerns, primarily related to cardiovascular health, as well as liver and kidney damage.

When assessing the cardiac safety of new drugs, the risk of long QT syndrome is a critical consideration. This evaluation is a standard part of the preclinical testing process [3]. The identification and use of cardiac biomarkers have become increasingly sophisticated.

Previously, only a limited number of biomarkers were used to assess cardiac health like AST, LDH and creatine kinase (CK) [4].

Advances in biomedical research have led to the discovery of cardio-specific enzymes, including LDH1 and CK-MB. The development of automated immunoassays enabled the measurement of CK-MB and myoglobin in mass, rather than activity [5]. Today, cardiac troponins are widely recognized as the gold standard for diagnosing acute myocardial injury due to their high sensitivity and specificity.

Certain medications, including traditional chemotherapy agents and newer targeted therapies, can cause cardiotoxicity, leading to cardiac muscle dysfunction and potentially heart failure. Affected patients often have no prior history of cardiovascular disease. Common culprits include anthracyclines, taxanes, and monoclonal antibodies, as well as certain antiretroviral, antidiabetic, and illicit drugs [6-8].

Doxorubicin, a chemotherapy agent, can cause cardiotoxicity, typically within 72 hours of the

## Advanced GC–MS Characterization of Bioactive Constituents in *Punica granatum* Leaves and Their Therapeutic Antioxidant Potential Against Cardiac and Hepatic Toxicity in Rats.

initial treatment cycle. Potential side effects include heart attack, chest pain, pulmonary oedema, ECG changes, arrhythmia, and cardiac arrest. Due to these risks, doxorubicin is often contraindicated for patients undergoing hyperbaric oxygen therapy.

If patients experience decreased left ventricular ejection fraction, doxorubicin treatment should be discontinued. An endomyocardial biopsy can diagnose doxorubicin-induced cardiomyopathy, characterized by cytoplasmic vacuolization and myofibril loss. Unfortunately, there is no specific treatment for doxorubicin-induced cardiomyopathy<sup>[9]</sup>.

Isoproterenol can cause cardiac hypoxia by inducing oxygen supply deficiency, leading to cardiac necrosis. It also triggers toxicological changes by converting oxidative stress into cardiac tissue damage, reducing antioxidant enzymes. This results in lipid peroxidation, cellular leakage, and cardiac cell enlargement. Phospholipase activation causes ST-segment elevation, leading to acute cardiac injury, inflammation, and myocardial ischemia. Signalling pathways like MAPK (p38) and NFκB promote cellular death, ultimately leading to cardiac fibrosis with chronic isoproterenol use<sup>[10]</sup>.

Phytochemicals offer a rich source of pharmacophores that can be used to develop novel, safe, and effective cardioprotective and hepatoprotective agents. These agents can help combat drug-induced cardiotoxicity and hepatotoxicity.

In this study, we evaluated the antioxidant properties of the ethanolic extract of *P. granatum* leaves using vitamin E and ascorbic acid as reference standards<sup>[11]</sup>.

We then investigated the protective effects of this extract against doxorubicin and isoproterenol-induced cardiotoxicity and hepatotoxicity in Wistar Albino rats.

Our results showed that the ethanolic extract of *P. granatum* leaves reduced the activity of lactate dehydrogenase (LDH) and creatine kinase (CK-MB) enzymes, indicating its potential to mitigate cardiotoxicity<sup>[12]</sup>. The extract also exhibited antioxidant properties, as evidenced by increased levels of tissue glutathione, catalase, and superoxide dismutase.

Overall, our findings suggest that the ethanolic extract of *P. granatum* leaves has a noticeable protective effect against doxorubicin and isoproterenol-induced toxicity, which is likely due to its antioxidant properties.

Current compliances suggested that *P. granatum* from the ethanolic extract has shown noticeable protective role on DOX and ISP induced toxicity (cardio and hepatotoxicity) occurs due to oxidative stress.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Plant material collection. Fresh leaves of *Punica granatum* were collected from the local area of Devanahalli, Bangalore.

- *Experimental Animals.* Healthy Wistar albino rats (150-220g) were sourced from Vaarunya Biolabs Private Limited. The animals were housed in a well-ventilated facility at Mallige College of Pharmacy, with access to standard laboratory diet and water. The rats were allowed to acclimate to the environment (temperature: 20-25°C, 12-hour light/dark cycle, and 50-70% relative humidity) for one week before experimentation. The protocol MCP094/2020-21 of the experiment was approved by the institutional Animal Committee (IAEC), and all experiments was conducted as per the CPCSEA guidelines.
- *Histopathological examination.* The dissected heart was examined, preserved in 10% formalin, and processed at Vaarunya Biolabs Private Limited, Bangalore-560074, Karnataka.
- *Antioxidant activity assessment.* 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used to evaluate the free radical scavenging activity of *Punica granatum* dried leaf extracts. The DPPH assay measured the antioxidant property of the extracts. In this method, 0.1mL of extract was added to 3.3mL of methanol DPPH solution (0.1mM). After 30 minutes of incubation, the absorbance of the extract was measured at 517nm using a spectrophotometer. Ascorbic acid served as the standard control, methanol as the blank, and DPPH solution with methanol as the control.
- *Phytochemical profiling.* The phytochemical composition of the *P. granatum* dried leaf ethanolic extract was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). This technique identified the presence of various bioactive compounds and alkaloids.

# Advanced GC–MS Characterization of Bioactive Constituents in *Punica granatum* Leaves and Their Therapeutic Antioxidant Potential Against Cardiac and Hepatic Toxicity in Rats.

**Methodology:** The GC-MS analysis was performed using a database from WILEY and FAME Library. The chromatograms obtained from the analysis provided information on the peak areas, molecular weights, and structures of the compounds present in the extract.

**Results:** The GC-MS chromatogram of the *P. granatum* dried leaf extract revealed 26 distinct peaks, indicating the presence of 26 major phytochemical constituents. These findings suggest that *P. granatum* is a rich source of bioactive compounds with potential therapeutic applications.

## 2.2 Methods

### 2.2.1 Extract preparation

Fresh leaves from the plant were carefully removed, washed with distilled water to remove dirt, and dried in the shade. The dried leaves were then powdered using an electric blender. The powdered material (50g) was mixed with 500ml of ethanol and subjected to Soxhlet extraction for 8 hours. The resulting extract was evaporated to dryness at a controlled temperature (35-40°C) and stored in airtight containers in the refrigerator. [13].

### 2.2.2 Phytochemical evaluation:

*Initial laboratory tests for preliminary phytochemical screening*

Phytochemical studies in various fragments of extract of *P. granatum*:

#### a. Test for Proteins:

To detect proteins, 2ml of the extract was mixed with 1ml of 40% NaOH solution and 1-2 drops of 1% CuSO<sub>4</sub> solution. The appearance of a violet color indicated the presence of peptide linkages.



(a)

**Figure 1. (a) Fresh *P. granatum* Leaves. (b), Dried *P. granatum* dried Leaves**

#### Test for cholesterol:

A dry test tube was filled with 2ml of the extract and 2ml of chloroform. Then, 2-3 drops of concentrated sulfuric acid and 2 drops of acetic anhydride were added. The color change from red to blue-green indicated the presence of cholesterol.

#### c. Test for Amino Acids:

2ml of the sample was mixed with 2ml of ninhydrin reagent and heated in a water bath for 20 minutes. The appearance of a purple color confirmed the presence of amino acids.

#### Test for Carbohydrates:

2ml of the extract was mixed with 2 drops of Molisch's reagent and shaken well. Then, 2ml of concentrated sulfuric acid was carefully added to the sides of the test tube. A reddish-violet color indicated the presence of carbohydrates.

#### e. Test for Flavonoids:

A portion of the aqueous filtrate of the dried leaf ethanolic extract was mixed with 5ml of dilute ammonia solution. The addition of ammonia confirmed the presence of flavonoids, which disappeared upon standing.

#### f. Test for Terpenoids:

5ml of the extract was mixed with 3ml of concentrated sulfuric acid and 2ml of chloroform. The formation of a reddish-brown coloration at the interface indicated a positive result for terpenoids.

#### Test for Alkaloids:

1% HCl was added to the extract, followed by 6 drops of Dragendorff's reagent and Mayer's reagent. The formation of an organic precipitate confirmed the presence of alkaloids.

#### Test for Cardiac Glycosides:

2ml of glacial acetic acid containing 1 drop of FeCl<sub>3</sub> solution was added to 5ml of the extract. The mixture was then layered with 1ml of concentrated sulfuric acid. The appearance of brown rings at the interface indicated the presence of deoxy sugar, characteristic of cardenolides. Additional color changes, such as

## Advanced GC–MS Characterization of Bioactive Constituents in *Punica granatum* Leaves and Their Therapeutic Antioxidant Potential Against Cardiac and Hepatic Toxicity in Rats.

a violet ring below the brown ring or a greenish ring in the acetic acid layer, further supported the presence of cardiac glycosides.

### i. Test for Steroids:

0.5g of the ethanolic extract was mixed with 2ml of acetic anhydride and 2ml of concentrated sulfuric acid. A color change from violet to blue or green indicated the presence of steroids.

### j. Test for Saponins:

The extract was vigorously shaken with 20ml of distilled water in a graduated cylinder for 15 minutes. The formation of a 1cm foam layer confirmed the presence of saponins.

### k. Test for Tannins:

A few drops of 1% lead acetate were added to 5ml of the extract. The formation of a yellow precipitate indicated the presence of tannins.

### GC-MS Analysis for Pharmacophore Identification:

To confirm the presence of potent pharmacophores, a Gas Chromatography-Mass Spectrometry (GC-MS) analysis was conducted on the ethanolic dried leaf extract of *P. granatum*. The analysis was performed using a Clarus SQ 8C Gas Chromatograph-Mass Spectrometer.

### Data Interpretation:

The National Institute of Standards and Technology (NIST14) database was used as a reference standard for data interpretation. The mass spectrum obtained from the GC-MS analysis was carefully interpreted to identify the bioactive compounds present in the extract.

### 2.2.3. In vitro antioxidant assay

The antioxidant activity of the dried leaf ethanolic extracts was evaluated using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. The study employed a freshly prepared DPPH solution, which was added to the extract in serial dilutions ranging from 3.12 µg/ml to 100 µg/ml.

The total volume of each test tube was adjusted to 5 ml, and the mixture was incubated for 30 minutes. The absorbance was then measured at 517 nm using a spectrophotometer. Ascorbic acid and Butylated hydroxytoluene (BHT)

served as standard references. A control sample containing no extract was also analyzed, and methanol was used as a blank solution [14].

### 2.2.4. Animal experimentation

To determine the oral acute toxicity of *P. granatum* dried leaf extract, we conducted a study in accordance with OECD guidelines 425, as no previous toxicity studies were available. The study used Wistar albino rats to select the dose for the extract.

#### ● Acute Toxicity Test

Five animals were used for the test. After overnight fasting, the animals received a single oral dose of 2000 mg/kg body weight of the *P. granatum* dried leaf ethanolic extract (EELPG). Food was withheld for 3-4 hours after administration. The animals were observed individually at regular intervals for 14 days, with special attention given during the first 4 hours and 24 hours.

#### ● Observations and Dose Selection

The animals were monitored for morphological behaviour, general toxic signs, and mortality. Based on the results, one-fourth of the LD50 (>2000) was selected for further study [15]. Consequently, doses of 200 mg/kg (low dose) and 400 mg/kg (high dose) were chosen to evaluate the cardioprotective activity of the extract.

### Study design with animal:

Wistar Albino rats were randomly divided into four groups, each consisting of six animals. The groups were designed to investigate the protective effects of *P. granatum* dried leaves against isoproterenol-induced cardiotoxicity.

*To determine the effect of P. granatum dried leaves against Isoproterenol induced cardiotoxicity*

- I. Normal control group: Animals received 0.9% NaCl (normal saline solution) orally.
- II. Positive control group: Animals were administered isoproterenol (200mg/kg) subcutaneously in two divided doses on the 28th and 29th days of treatment [16].
- III. *P. granatum* dried leaves extract control group: Animals received *P. granatum* leaves extract (200mg/kg) orally for 28 days.
- IV. *P. granatum* dried leaves extract + Isoproterenol Group: Animals were administered *P. granatum* dried leaves extract (400mg/kg) orally for 28 days, along with isoproterenol (200mg/kg)

## Advanced GC–MS Characterization of Bioactive Constituents in Punica granatum Leaves and Their Therapeutic Antioxidant Potential Against Cardiac and Hepatic Toxicity in Rats.

subcutaneously in two divided doses on the 28th and 29th days of treatment.

### A. To determine the effect of *P. granatum* dried leaves against Isoproterenol induced cardiotoxicity

- I. Normal control group: Animals received 0.9% NaCl (normal saline solution) orally.
- II. Positive control group: Doxorubicin (10mg/kg) at *i.p.* (intraperitoneal) route on 28<sup>th</sup> and 29<sup>th</sup> day of treatment<sup>[17]</sup>.
- III. *P. granatum* dried leaves extract control group: *P. granatum* leaves extract 200mg/kg, at *p.o* dose was given for 28days.
- IV. *P. granatum* dried leaves extract pretreated with ISO: *P. granatum* dried leaves extract 400mg/kg, *p.o* was administered for 28 days along with doxorubicin (10mg/kg) at *i.p.* route on 28<sup>th</sup> and 29<sup>th</sup> day of treatment protocol.

### Evaluating biomarkers associated with cardiotoxicity and hepatotoxicities:

- **Serum lipid profile**  
Blood samples were collected from the retro-orbital plexus on the 30th day, 24 hours after treatment. Serum was separated by centrifugation at 10,000 rpm for 15 minutes. The levels of hepatic biomarkers, including alkaline phosphatase (ALP) and aspartate transaminase (AST), were estimated using biochemical assays and colorimetric processes<sup>[18]</sup>.
- **Cardiac enzymes**  
In cardiac tissue homogenate samples, lactate dehydrogenase (LDH) levels were determined using enzyme-linked immunosorbent assay (ELISA). Creatine kinase-MB (CK-MB) levels were also determined according to the manufacturer's instructions.

### Histopathological examination:

Heart tissue samples were fixed in 10% neutral buffered formalin for 24 hours. The samples were then dehydrated using a series of ethanol concentrations (70%, 80%, 90%, and 100%), cleared with xylene, and embedded in paraffin wax. Sections (5 µm) were cut from the prepared blocks and stained with haematoxylin and eosin. A light microscope was used to examine the tissue sections.

### Heart weight index (HWI):

After blood collection, rats were sacrificed by cervical dislocation. The heart was then dissected, washed with ice-cold saline, blotted

dry with filter paper, and weighed. The heart weight index (HWI) was calculated using the formula:

$$\text{Heart weight index (HWI)} = \frac{\text{Heart weight (HW)}}{\text{Body weight (BW)}}$$

The results are presented in Table 6 and graphically illustrated.

Myocardial tissue samples were immediately fixed in 10% buffered neutral formalin solution and processed for histopathological studies.

## 3 RESULTS AND DISCUSSION

### 3.1. Determination of *pharmacophores* in the ethanolic dried leaf extract of *P. granatum*

Phytochemical analysis of the ethanolic dried leaf extract of *P. granatum* revealed the presence of alkaloids, flavonoids, saponins, tannins, and phenolic compounds (Table 1). These findings were further confirmed by Gas Chromatography-Mass Spectrometry (GC-MS) analysis, which identified a total of 26 phytochemicals in the extract (Figure 2 and Table 2).

Notably, the GC-MS analysis detected a strong presence of 1,2,3-Benzenetriol (pyrogallol) at a retention time of 8.11. Pyrogallol has been previously recognized for its antioxidant properties, highlighting the potential benefits of *P. granatum* leaf extract.

**Table 1: Chemical constituents of *P. granatum* dried leaves extract:**

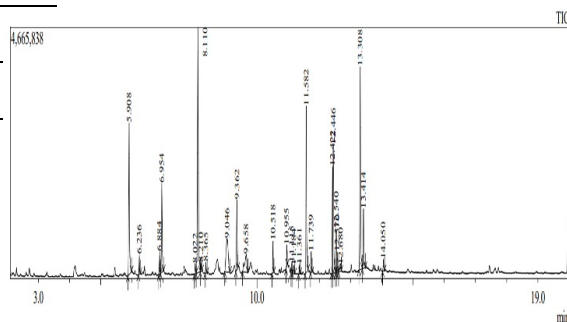
Sl. No.	Particulars	Results
1.	Flavonoids	+
2.	Alkaloids	+
3.	Tannins	+
4.	Phenols	+
5.	Saponins	+
6.	Steroids	-

## Advanced GC–MS Characterization of Bioactive Constituents in *Punica granatum* Leaves and Their Therapeutic Antioxidant Potential Against Cardiac and Hepatic Toxicity in Rats.

7.	Glycosides	+
8.	Carbohydrates	+

Note: Present (+), Absent (-); and each testing done on triplicate.

**Figure 2.** Spectrometric representation for the ethanolic dried leaf extract of *P. granatum* on GC–MS analysis



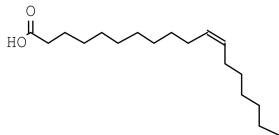
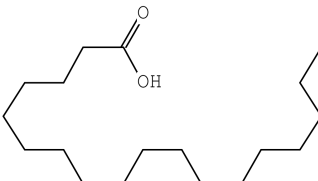
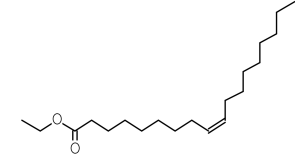
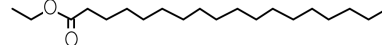
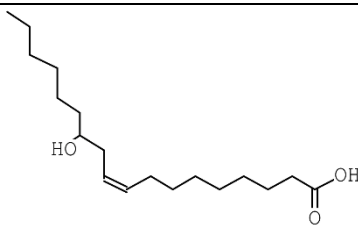
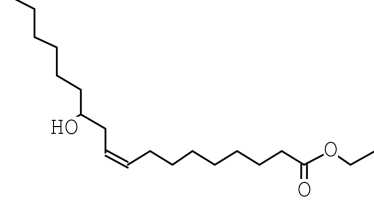
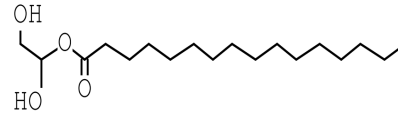
**Table 2.** Pharmacophores identification in the dried leaf of ethanolic extract of *P. granatum* on GC-MS studies.

Peak	R. Time	Name of the compound	Molecular formula	Molecular weight	Peak Area %	Structure of compounds
1	5.908	Maltol	C <sub>6</sub> H <sub>6</sub> O <sub>3</sub>	126	10.70	
2	6.236	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	C <sub>6</sub> H <sub>8</sub> O <sub>4</sub>	144	1.01	
3	6.884	Benzofuran, 2,3-dihydro-	C <sub>8</sub> H <sub>8</sub> O	120	1.28	
4	6.954	5-Hydroxymethylfurfural	C <sub>6</sub> H <sub>6</sub> O <sub>3</sub>	126	5.64	
5	8.022	8-Methylnonanoic acid	C <sub>10</sub> H <sub>20</sub> O <sub>2</sub>	172	0.60	
6	8.110	1,2,3-Benzenetriol	C <sub>6</sub> H <sub>6</sub> O <sub>3</sub>	126	18.77	
7	8.210	2-Furanmethanol, tetrahydro-	C <sub>5</sub> H <sub>10</sub> O <sub>2</sub>	102	0.63	

**Advanced GC–MS Characterization of Bioactive Constituents in Punica granatum Leaves and Their Therapeutic Antioxidant Potential Against Cardiac and Hepatic Toxicity in Rats.**

8	8.365	2,4,7,9-Tetramethyl-5-decyn-4,7-diol	C <sub>14</sub> H <sub>26</sub> O <sub>2</sub>	226	0.65	
9	9.046	beta-D-Glucopyranose, 1,6-anhydro-	C <sub>6</sub> H <sub>10</sub> O <sub>5</sub>	162	6.45	
10	9.362	Dodecanoic acid	C <sub>12</sub> H <sub>24</sub> O <sub>2</sub>	200	4.21	
11	9.658	1,5-Anhydro-d-mannitol	C <sub>6</sub> H <sub>12</sub> O <sub>5</sub>	164	3.31	
12	10.518	Tetra decanoic acid	C <sub>14</sub> H <sub>28</sub> O <sub>2</sub>	228	1.49	
13	10.955	Neophytadiene	C <sub>20</sub> H <sub>38</sub>	278	0.88	
14	11.115	1,2-Benzenedicarboxylic acid, bis (2-methyl)	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	278	0.39	
15	11.184	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	C <sub>20</sub> H <sub>40</sub> O	296	0.38	
16	11.361	1,2-Benzenedicarboxylic acid, bis (2-methyl)	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	278	0.36	
17	11.582	n-Hexadecenoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256	9.62	
18	11.739	Hexadecenoic acid, ethyl ester	C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>	284	0.88	
19	12.422	10E,12Z-Octadecadienoic acid	C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>	280	5.96	

**Advanced GC–MS Characterization of Bioactive Constituents in Punica granatum Leaves and Their Therapeutic Antioxidant Potential Against Cardiac and Hepatic Toxicity in Rats.**

20	12.446	cis-Vaccenic acid	C18H34O2	282	6.80	
21	12.540	Octadecanoic acid	C18H36O2	284	2.65	
22	12.570	Ethyl Oleate	C20H38O2	310	1.05	
23	12.680	Octadecanoic acid, ethyl ester	C20H40O2	312	0.63	
24	13.308	Ricinoleic acid	C18H34O3	298	11.83	
25	13.414	9-Octadecenoic acid, 12-hydroxy-, ethyl ester	C20H38O3	326	3.26	
26	14.050	Hexadecenoic acid, 2-hydroxy-1-(hydroxy	C19H38O4	330	0.59	

**3.2. DPPH assay:**

The in vitro DPPH free radical scavenging assay revealed that the *P. granatum* ethanolic leaf extract possesses significant antioxidant activity, albeit lower than commercial ascorbic acid. Nevertheless, the extract exhibits potential as a natural antioxidant alternative.

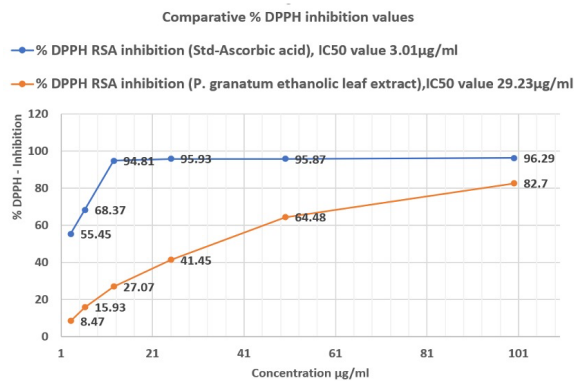
**Table 3. Comparative % of DPPH inhibition value**

Sample concentration (µg/ml)	% DPPH RSA inhibition (Std-Ascorbic acid) with IC50 3.01µg/ml	%DPPH RSA inhibition ( <i>P. granatum</i> ethanolic leaf extract) with IC50 29.23 µg/ml
3.12	55.45	8.47
6.25	68.37	15.93
12.5	94.81	27.07
25	95.93	41.45
50	95.87	64.48

# Advanced GC–MS Characterization of Bioactive Constituents in Punica granatum Leaves and Their Therapeutic Antioxidant Potential Against Cardiac and Hepatic Toxicity in Rats.

100	96.29	82.7
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**Figure 3. Comparative % of DPPH inhibition graphical representation with IC50 values**



### 3.3 Evaluating projective effect of extract on drug (doxorubicin and Isoproterenol) induced rat:

#### Serum and cardiac enzyme profiling:

The serum enzymes alanine transaminase (ALT) and aspartate transaminase (AST) were significantly elevated in the drug-induced positive control group. In contrast, treatment with the *P. granatum* ethanolic leaf extract resulted in a decrease in enzyme levels, with higher doses approaching those of the normal control group (Table 4 and Figure 4). These findings suggest that the extract possesses hepatoprotective properties.

Cardiac enzyme profiling revealed that the *P. granatum* ethanolic leaf extract exhibited potent antioxidant activity by normalizing cardiac enzyme values in rats treated with doxorubicin and isoproterenol (Table 5 and Figure 5).

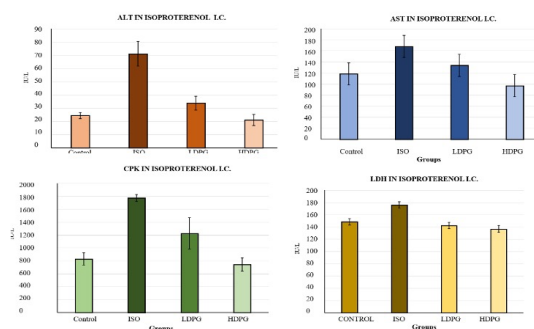
**Table 4: effect of *P. granatum* dried leaves extract on cardiac marker enzyme in the serum of healthy control, Isoproterenol induced cardiotoxicity and rats with oxidative stress.**

Group	ALT (IU/L)	AST (IU/L)	CPK (IU/L)	LDH (IU/L)
I. Healthy control	24.34 ± 2.33	118 ± 6.65	828.67 ± 95.25	148 ± 5.13
II. ISO	71 ± 9.30 <sup>##</sup>	167 ± 8.95 <sup>##</sup>	1771 ± 53.86 <sup>##</sup>	175.34 ± 5.24 <sup>#</sup>
III. PGLE 200mg + ISO	33.67 ± 5.24 <sup>*</sup>	133 ± 6.67 <sup>*</sup>	1224 ± 242.1	142 ± 5.13 <sup>**</sup>
IV. PGLE 400 mg + ISO	21 ± 4.16 <sup>**</sup>	96.67 ± 8.01 <sup>**</sup>	742.34 ± 102.24 <sup>**</sup>	136.34 ± 5.54 <sup>**</sup>

N=6, Values are expressed as Mean ± SEM and analysed by One way ANOVA test, where \*P<0.05, \*\*P<0.01 in comparison with ISO

only group, #P<0.05, ##P<0.01 in comparison with healthy control group.

**Figure 4. Effect of *P. granatum* dried leaves extract in serum and cardiac marker enzyme ALT/AST/CPK/ LDH for normal, positive control-Isoproterenol induced oxidative stress and cardiotoxicity in rats.**



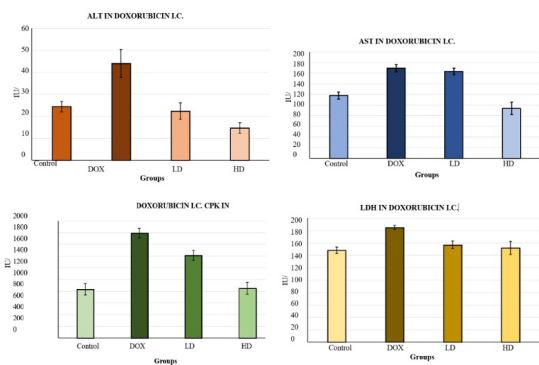
**Table 5: Effect of *P. granatum* dried leaves extract on cardiac marker enzyme in the serum of healthy control, doxorubicin induced cardiotoxicity and rats with oxidative stress.**

Group	ALT (IU/L)	AST (IU/L)	CPK (IU/L)	LDH (IU/L)
I. Healthy control	24.34 ± 2.34	118 ± 6.65	828.67 ± 95.25	148 ± 5.13
II. DOX	44 ± 6.43 <sup>#</sup>	169 ± 6.74 <sup>##</sup>	1786.67 ± 79.53 <sup>##</sup>	184.67 ± 3.48 <sup>##</sup>
III. PGLE 200mg + DOX	22.34 ± 3.75 <sup>*</sup>	163.4 ± 6.01	1410.34 ± 81.20 <sup>*</sup>	156.67 ± 6.22 <sup>*</sup>
IV. PGLE 400 mg + DOX	14.67 ± 2.40 <sup>**</sup>	94 ± 11.67 <sup>**</sup>	845.34 ± 99.92 <sup>**</sup>	151.6 ± 10.52 <sup>*</sup>

N=6, Values are expressed as Mean ± SEM and analysed by One way ANOVA test, where, \*P<0.05, \*\*P<0.01 in comparison with DOX only group, #P<0.05, ##P<0.01 in comparison with healthy control group.

**Figure 5: Effect of *P. granatum* dried leaves extract on serum and cardiac marker enzyme ALT/AST/CPK/LDH in serum control and Doxorubicin induced cardiotoxicity and oxidative stress in rats.**

## Advanced GC–MS Characterization of Bioactive Constituents in Punica granatum Leaves and Their Therapeutic Antioxidant Potential Against Cardiac and Hepatic Toxicity in Rats.



### Estimation of Heart weight Index (HWI)

Group	HWI (mg/g)
1. Healthy control group	3.05 ± 0.09
2. ISO	4.59 ± 0.04 <sup>###</sup>
3. PGLE 200mg + ISO	3.77 ± .04 <sup>**</sup>
4. PGLE 400 mg +ISO	2.46 ± 0.17 <sup>**</sup>

The HWI was calculated using the established method. Results indicated that rats administered doxorubicin and isoproterenol exhibited elevated HWI values compared to the normal group (Table 6 and Figure 7). This increase may be due to oxidative stress-induced inflammation, oedema, and myonecrosis in the heart. Treatment with *Punica granatum* leaves extract effectively maintained HWI within a normal range, suggesting its potential to mitigate drug-induced cardiotoxicity through antioxidant mechanisms.

**Table 6: Effect of *Punica granatum* leaves extract on Heart weight Index (HWI) in the healthy control, Doxorubicin/Isoproterenol-induced cardiotoxicity and rats with oxidative stress.**

Group	HWI (mg/g)
1. Healthy control group	3.05 ± 0.09
2. ISO	4.59 ± 0.04 <sup>###</sup>
3. PGLE 200mg + ISO	3.77 ± .04 <sup>**</sup>
4. PGLE 400 mg +ISO	2.46 ± 0.17 <sup>**</sup>

1. Healthy control	3.47 ± 0.037
2. DOX	4.55 ± 0.07 <sup>###</sup>
3. PGLE 200mg + DOX	3.52 ± 0.005 <sup>***</sup>
4. PGLE 400mg + DOX	2.71 ± 0.037 <sup>***</sup>

N=6, Values are expressed as Mean ± SEM and analysed by One way ANOVA test, where, \*P<0.05, \*\*P<0.01 in comparison with ISO only group, #P<0.05, ##P<0.01 in comparison with healthy control group.

**Table 7: Effect of *P. granatum* dried leaves extract on Heart architecture of healthy control, ISO (Isoproterenol) induced cardiotoxicity and rats with oxidative stress groups.**

Treat ment	Inflam mation	Myone crosis	Ed em a
I. Healt hy contr ol	-	-	-
II. ISO	+++	+++	++ +
III. PGLE 200m g + ISO	++	++	+
IV. PGLE 400 mg + ISO	+	+	+

Nil (-), Mild (+), Moderate (++) , Severe (+++)

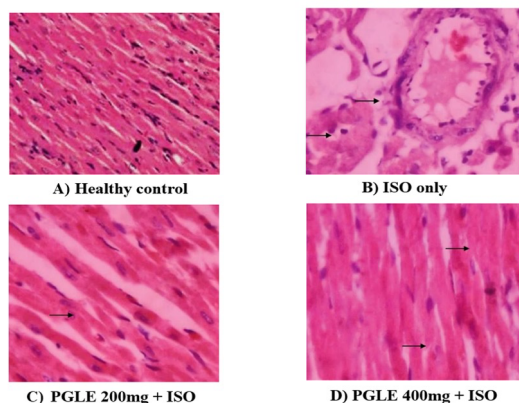
**Table 8: Effect of *P. granatum* dried leaves extract on Heart architecture of healthy control, Doxorubicin (DOX) induced cardiotoxicity and rats with oxidative stress groups.**

## Advanced GC–MS Characterization of Bioactive Constituents in *Punica granatum* Leaves and Their Therapeutic Antioxidant Potential Against Cardiac and Hepatic Toxicity in Rats.

Treat ment	Inflam mation	Myone crosis	Ed em a
I. Healt hy contr ol	-	-	-
II. DOX	+++	+++	++ +
III. PGLE 200m g + ISO	++	++	+
IV. PGLE 400 mg + ISO	+	+	-

Nil (-), Mild (+), Moderate (++), Severe (+++)

**Figure 6: Photographs of Histopathological findings of ISO treated group**

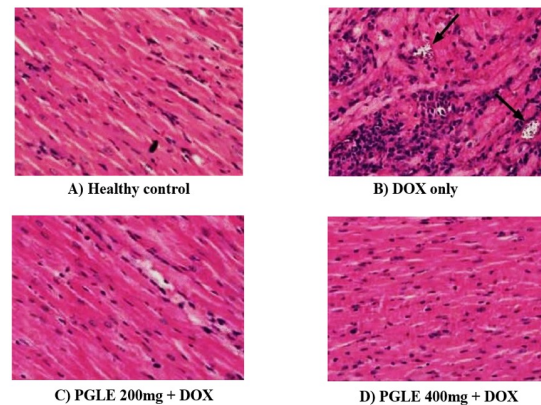


### Histopathological examination of heart tissues

In the normal groups on histopathological examination, we noticed usual striation structure in myocardium with a branched like presence, as depicted in Figure 6A & 7A, while on administration of ISO or DOX alone caused disruption on normal myocardium attributes, which may result from oxidative damage by inflammation followed by oedema and then ultimately necrosis (Figure 6B & 7B). Now the reversal of damaged structural disruption also was noticed upon treatment of *Punica granatum* leaves extract. (Figure 6C, 6D & 7C,7D). This

indicated reduction of oxidative stress in ISO/DOX induced cardiotoxic occurrence on application of *Punica granatum* leaves extract.

**Figure 7: Photographs of Histopathological findings of DOX treated group**



### 4.DISCUSSION

The plant sources of *Punica granatum* as fruit are already reported for antioxidant and cardioprotective activities [19]. Here, we used *Punica granatum* leaf ethanolic extract first time to examine its protective response on drug induced (doxorubicin and Isoproterenol) hepatotoxicity and cardiotoxicity on rat model.

ISO, Isoproterenol as non-selective and potent  $\beta$ -adrenergic receptor agonist produce myocardial contractility, but on prolong administration at higher doses (>200 mg/kg in rat) causes myocardial injury, hypoxia, and subsequently primes to necrosis in myocardium.

Dysregulation of contractile function parameters leads to activation of several cardio-specific enzymes like LDH and CK-MB isoenzymes in the myocardium tissue sample which indicates the pathological characteristics of a heart, and this may be used as diagnostic biomarker enzymes [20].

In this current study, rats treated with *P. granatum* leaf ethanolic extract exhibited significant reduction in CK-MB and iso-enzyme LDH, finally could allowed in controlling pro-inflammatory/ inflammatory cytokines status or reviving heart injury through its antioxidant potentiality, with inhibition of lipid peroxidation and reduced permeability of myocardial membrane. The ethanolic extract of *P. granatum* leaf was found to be safe in rats, with no observed toxicity in terms of mortality, internal organ damage, or behavioural changes (LD50 > 5000 mg/kg). The

## Advanced GC–MS Characterization of Bioactive Constituents in *Punica granatum* Leaves and Their Therapeutic Antioxidant Potential Against Cardiac and Hepatic Toxicity in Rats.

USFDA has categorized the extract as safe, with a No-Observed-Adverse-Effect Level (NOAEL) of 1200 mg/kg/day<sup>[21]</sup>.

The study also explores the antioxidant potential of an ethanolic extract of *Punica granatum* leaves in a cardiotoxicity which is doxorubicin-induced. Cardiomyopathy is also induced by doxorubicin in various species of rodents by increasing oxidative stress in myocardial tissue and the circulatory system. This study demonstrated marked cardiotoxicity induced by doxorubicin, as evidenced by the increase in LDH and CK-MB enzymes. Ethanolic extract of *P. granatum* leaves treatment significantly prevented increase in these enzymes and also reversed the doxorubicin-induced myocardial damage. Lactate dehydrogenase (LDH) and creatine kinase MB (CK-MB) enzymes levels in the blood of myocardium reflected the extent of myocardial damage. This was further supported by changes in the histopathology of heart, including loss of striation, fibrosis, apoptosis and inflammatory cell infiltration<sup>[22]</sup>.

Along with developing Isoproterenol and doxorubicin induced cardiotoxicity in rat, further metabolic dysregulation also been seen in rat liver with increasing oxidative stress by the elevated LDH in cardiac system as well as in body. This could directly impact on liver functionality. Increased levels of cardiac tissue bio-marker enzyme due to myocardial infarction, it could trait to the damaged of the functional integrity of a liver because these enzymatic activities are interconnected by cytoplasmic composition of the body system. Released of one tissue damaging enzymes or other cytokines into circulation after cellular injuries coincides vital organ damage<sup>[23]</sup>. Balancing the values near the normalcy the administration of *P. granatum* in the rats treated with drugs have prevented the leakage of overall circulatory enzyme, when compared with control rats, which in turn minimized the isoproterenol induced liver injury. AST and ALT predominantly present in the liver when compared to other sites, and consequently changes in their concentration reflects the hepatic injury more specifically<sup>[24]</sup>.

Further, both Isoproterenol and doxorubicin have also resulted in a significant reduction in heart weight with seriously impacting the ratio of heart and body weight, which leads to depletion of myofibrils function and necrosis of myocardium<sup>[25]</sup>. Body weight reduction is also associated with constant dysregulation in

circulatory and metabolic system in body on long term administration of ISP and DOX in animals, are measured as multifactorial. Treatment with *P. granatum* ethanolic leaf extract mitigated these myocardial necrosis as well as improved pathological enzymatic profile in experimental rats.

### 5. CONCLUSION

In the present study, *P. granatum* ethanolic leaf extract (200 mg/kg) is proven to have antioxidant potentiality against doxorubicin and Isoproterenol induced cardiotoxicity and hepatotoxicity in experimental rats on the specified long-term administration of respective drugs. We used Isoproterenol to investigate potential protective strategies against doxorubicin-induced cardiotoxicity. This study also suggested that reduction in oxidative stress had influence in managing stress at mitochondrial malfunction. Finally, it helps in protecting myocardium activities and hepatic integrity. With this satisfactory pathological and bio-enzymatic observation on experimental animals, there is a further prospect of conducting study in detail about therapeutic novelty *P. granatum* ethanolic leaf extract at molecular and genetic level.

### References: -

1. Fung M et al. Evaluation of the characteristics of safety withdrawal of prescription drugs from worldwide pharmaceutical markets-1960-1999. *Drug Information Journal*. 2001;35(1):293-317.
2. McGowan JV et al. Anthracycline chemotherapy and cardiotoxicity. *Cardiovascular Drugs and Therapy*. 2017;31(1):63-75.
3. Braam SR et al. Prediction of drug-induced cardiotoxicity using human embryonic stem cell-derived cardiomyocytes. *Stem Cell Research*. 2010;4(2):107-116.
4. Varga ZV et al. Drug-induced mitochondrial dysfunction and cardiotoxicity. *American Journal of Physiology. Heart and Circulatory Physiology*. 2015;309(9):H1453-H1467.
5. Lipshultz SE et al. Managing chemotherapy-related cardiotoxicity in survivors of childhood cancers. *Paediatric Drugs*. 2014;16(5):373-389.

## Advanced GC–MS Characterization of Bioactive Constituents in *Punica granatum* Leaves and Their Therapeutic Antioxidant Potential Against Cardiac and Hepatic Toxicity in Rats.

6. Jain D et al. Cardiotoxicity of cancer chemotherapy: Identification, prevention and treatment. *Annals of Translational Medicine*. 2017;5(17):348-360.
7. Madonna R. Early diagnosis and prediction of anticancer drug-induced cardiotoxicity: From cardiac imaging to “Omics” technologies. *Revista Española de Cardiología (English Edition)*. 2017;70(7):576-582.
8. Sadurska E. Current views on anthracycline cardiotoxicity in childhood cancer survivors. *Pediatric Cardiology*. 2015;36(6):1112-1119.
9. Spalato M, Italiano A. The safety of current pharmacotherapeutic strategies for osteosarcoma. *Expert Opin Drug Saf*. 2021 Apr;20(4):427-438.
10. Guo, M.; Sun, D.; Fan, Z.; Yuan, Y.; Shao, M.; Hou, J.; Zhu, Y.; Wei, R.; Zhu, Y.; Qian, J.; et al. Targeting MK2 Is a Novel Approach to Interfere in Multiple Myeloma. *Front. Oncol*. 2019, 9, 722.
11. Afaq F, Saleem M, Krueger C G, Reed J D, Mukhtar H (2005) Anthocyanin-and hydrolyzable tannin-rich pomegranate Fruit extract modulates MAPK and NF-KB pathways and inhibits skin tumorigenesis in CD-1 mice. *International Journal of Cancer* 113(3) : 423-33.
12. Zahin M, Aqil F, Ahmad I (2010) Broad spectrum antimutagenic activity of antioxidant active fraction of *Punica granatum* L. peel extracts. *Mutation Research* 703(2) : 99-107.
13. Hewawasam, K. A. P. W. Jayatilaka, L. K. B. Mudduwa, and C. Pathirana, “Ameliorative effects of *Asparagus falcatus* (L.) and *Vetiveria zizanioides* (L.) Nash on carbon tetrachloride induced oxidative stress in mice,” *Indian Journal of Traditional Knowledge*, vol. 16, no. Suppl 3, pp. 417–424, 2017.
14. Ahmed, M, Saeed, F, et al, “Evaluation of Insecticidal and Antioxidant activity of selected Medicinal plants” *Journal of Pharmacognosy & Phytochemistry*, 2013, 2(3), 153-158.
15. R.M, Patel, N.J, “In vitro antioxidant activity of coumarin compounds by DPPH superoxide and nitric oxide free radical scavenging methods” *Journal of advanced pharmacy education & research*, vol-1 2011, 52-68.
16. Organization for Economic Cooperation and Development (OECD) OECD Guidelines for Testing of Chemicals [Internet]. 2006 Adopted 2006 Mar 23, cited 2009 Mar 22 France OECD Publishing:1–27 Section 4, Health Effects: Test No. 425: Acute Oral Toxicity: Up-and-Down Procedure; 2006.
17. Alderman, E. L., and Harrison, D. C. (1971). Myocardial hypertrophy resulting from low dosage isoproterenol administration in rats. *Proc Soc Exp Biol Med* 136, 268–70.
18. Karuna Priyachitra, Archana Pramod Shaha, Urvashi Jain, Nitin B Ghiware, Imran Ataurrahman Sheikh, et. al. Synergistic Hepatoprotective Effect of Methanolic Extracts of *Urtica Dioica* LINN. Leaves and Silymarin CCL4-Induced Hepatic Damage in HEPG2 Cells. *Journal of Advanced Zoology* ISSN: 0253-7214 Volume 44 Issue S-2 Year 2023 Page 4133:4145
19. Mohammad Hassanpour Fard, Arvindkumar E Ghule, Subhash L Bodhankar, Madhurima Dikshit “Cardioprotective effect of whole fruit extract of pomegranate on doxorubicin-induced toxicity in rat”, *PharmBiol*. 2011 Apr;49(4):377-82.
20. National Research Council (NRC) Toxicity Testing for Assessing Environmental Agents. National Academics Press; Washington, DC, USA: 2006. Interim Report. [Google Scholar].
21. Rathore N, John S, Kale M, Bhatnagar D (1998) Lipid peroxidation and antioxidant enzymes in isoproterenol induced oxidative stress in rat tissues. *Pharmacological Research* 38 (4): 297–303.
22. W. Fisher, F. Salloum, A. Das, H. Hyder, R.C. Kukreja, Phosphodiesterase-5 inhibition with sildenafil attenuates cardiomyocyte apoptosis and left ventricular dysfunction in a chronic model of doxorubicin cardiotoxicity, *Am. Heart Assoc*, 111 (2005), pp. 1601-1610.
23. Vikas S. Warpe, Vishal R. Mali, Arulmozhi S, Subhash L. Bodhankar, Kakasaheb R. Mahadik, Cardioprotective effect of ellagic acid on doxorubicin induced cardiotoxicity in wistar rats, *PharmBiol*. 2015, Apr; 5 (1): 1-8.
24. Waleska Dornas and Detlef Schuppan, Mitochondrial oxidative injury: a key player in

**Advanced GC–MS Characterization of Bioactive Constituents in Punica granatum Leaves and Their Therapeutic Antioxidant Potential Against Cardiac and Hepatic Toxicity in Rats.**

nonalcoholic fatty liver disease, *Comp Biochem Physiol A Mol Integr Physiol* 142: 102–110, 2020.

25. Leah E. Weinberg and Pawan K. Singal. 1987. Refractory heart failure and age-related differences in adriamycin-induced myocardial changes in rats. *Canadian Journal of Physiology and Pharmacology*. 65(9): 1957-1965.