Chapter 3

PHYTOCHEMICAL SCREENING AND ANTIOXIDANT EVALUATION OF AMORPHOPHALLUS CAMPANULATUS (ROXB.) BL. TUBER
Chapter 3 (A)

Phytochemical screening and *in vitro* antioxidant evaluation of *Amorphophallus campanulatus* (Roxb.) Bl. tuber
3A.1. INTRODUCTION

Plants are playing an increasing role in human health. It was estimated that about 80% of all the world’s medicines are originally derived from plant sources, especially those found in tropical regions. Plants are some of the most attractive sources of new drugs and have been shown to produce promising results in the treatment of a number of disorders. Plants produce an amazing array of organic chemicals with an enormous diversity of structural types. Many of these phytochemicals are essential for plant growth and development and are widely used by humans due to its health benefits. Phytochemicals, by acting individually or synergistically, helps to reduce the risk for a variety of chronic and inflammatory conditions. These include atherosclerosis and stroke, myocardial infarction, certain types of cancers, diabetes mellitus, allergy, asthma, arthritis, Crohn’s disease, multiple sclerosis, Alzheimer’s disease, osteoporosis, psoriasis, septic shock, AIDS, menopausal symptoms, and neurodegeneration (Rouhi, 2003; Aggarwal et al., 2004; Cseke et al., 2006).

Amorphophallus campanulatus (Roxb.) Blume is a tuber crop of south East Asian origin. It is a perennial herb commonly known as elephant foot yam. A. campanulatus tubers are traditionally used in inflammations, colic, piles, hemorrhoids, liver diseases and tumors (Nair, 1993). It is largely cultivated throughout the plains of India for using its corm (bulb) as food (Das et al., 2009a). Food plants and culinary herbs and spices are known to contain myriad phytochemicals with medicinal properties (Aggarwal et al., 2004). Many of these phytochemicals act as antioxidants, preventing cellular destruction and abnormalities. In addition to the edible plants, many other plant species have also
been investigated in the search for novel antioxidants (Chu et al., 2000). Because there is still a demand to find more information concerning the antioxidant potential of plant species as they are safe and also bioactive. Therefore, in recent years, considerable attention has been directed towards the identification of plants with antioxidant ability. Literature survey reveals that no authentic work has been done so far on antioxidant activity of *A. campanulatus* tuber, though ethnic people use it widely to cure different types of diseases. Thus, the present investigation was undertaken to identify the phytochemical constituents and the *in vitro* antioxidant evaluation of *Amorphophallus campanulatus* tuber extracts.

### 3A.2. MATERIALS AND METHODS

#### 3A.2.1. Chemicals

Quercetin was purchased from Sisco Research Laboratories (SRL), Mumbai, India. Ascorbic acid was obtained from Merck, Mumbai, India. Silymarin and 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) were procured from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were of analytical grade.

#### 3A.2.2. Preparation of plant extracts

*A. campanulatus* tubers were cleaned, chopped, shade-dried and powdered. A 50 g of dried powder was Soxhlet extracted with 400 mL of n-hexane and followed by methanol for 48 h. Then the extracts (n-hexane and methanol) were collected and the solvents evaporated under vacuum in a rotary evaporator with an approximate yield of 0.45% (w/w) and 9.3% (w/w) respectively. The steps were repeated with a new set of dried powder and solvents until the required quantity was achieved. The concentrate was suspended in DMSO for *in vitro* studies.
3A.2.3. Preliminary phytochemical screening

Preliminary phytochemical screening of n-hexane (ACHE) and methanolic extracts (ACME) of *A. campanulatus* tuber were carried out for the detection of phytoconstituents using standard conventional protocols (Kokate et al., 2008; Evans and Trease, 2002; Khandelwal, 1995). Both the extracts were subjected for the phytochemical screening assays of Alkaloids, Flavonoids, Phenolic compounds and Tannins, Glycosides, Steroids, Saponins, Fixed oils and Fats, Carbohydrates and Protein and Amino acids.

(Detailed protocols are given in chapter 2, section 2.2.2. Preliminary phytochemical screening)

3A.2.4. Evaluation of *in vitro* antioxidant activity

The *in vitro* antioxidant activity of n-hexane extract and methanolic extract of *A. campanulatus* tuber were measured by the following assays.

3A.2.4.1. Determination of total phenolic compounds in the extracts

The amount of total phenolic compounds in the extracts was determined using the Folin-Ciocalteu method (Yu et al., 2002). A calibration curve of gallic acid was prepared and the results were expressed as mg GAE (gallic acid equivalents)/g dry extract.

3A.2.4.2. Determination of total flavonoid content in the extracts

The total flavonoid content of ACHE and ACME was determined spectrophotometrically by the method described by Quettier-Deleu et al. (2000). It was determined using a standard curve with quercetin and expressed as milligrams of quercetin equivalents (QE/g of dry extract).
3A.2.4.3. Evaluation of total antioxidant capacity

The total antioxidant capacity of the extracts was determined according to the method of Jayaprakasha et al. (2004). Ascorbic acid was used as standard and the total antioxidant capacity was expressed as the equivalent of ascorbic acid per gram of extract.

3A.2.4.4. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The antioxidant activity of ACHE and ACME were measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. The reduction capability of DPPH radicals is determined by the decrease in its absorbance at 517 nm (Aquino et al., 2001). Ascorbic acid was used as standard control.

3A.2.4.5. Assay of hydroxyl radical-scavenging activity

The inhibitory effect of the extracts to prevent the degradation of deoxyribose by Fe$^{3+}$ ions in presence of H$_2$O$_2$-EDTA-ascorbate was determined in hydroxyl radical scavenging assay (Ohkawa et al., 1979). The reference standard used was quercetin.

3A.2.4.6. Determination of reducing power

The antioxidant activity of ACHE and ACME was also manifested through their reducing power. In this assay, the Fe$^{3+}$ → Fe$^{2+}$ transformation was established as reducing capacity (Oyaizu, 1986). Ascorbic acid was used as a standard antioxidant compound.

All the tests were performed in triplicate and the results were expressed with the mean values.
(Detailed protocols are described in chapter 2, section 2.2.3. In vitro antioxidant assays)

3A.2.5. Liquid chromatography-mass spectrometry (LC-MS) Analysis

The phytochemical profiling of the methanolic extract of A. campanulatus tuber was carried out using LC-MS 2010A instrument (Shimadzu, Kyoto, Japan). 10 µl of the filtered sample was injected to the manual injector using a Microsyringe (1-20µl, Shimadzu). The mobile phase used was acetonitrile: 0.1% OPA in methanol (70:30) in an isocratic mode. The column and pump used were Reverse Phase C-18 (25 cm X2.5 mm) (phenomenex) and SPD 10 AVP-RD respectively. The separated compounds were then ionized using Electrospray Ionisation method (ESI). The flow rate was maintained to 1.6 ml/min with a temperature of 25°C and spectral data were collected at 315 nm. Mass analysis was performed in the range 50-800 m/z, under both positive and negative ion mode. The class VP integration software was used for the data analysis. The constituents of the extracts were identified by referring the LC-MS library, Metwin 2009 (version 2.1).

3A.3. RESULTS

3A.3.1. Preliminary phytochemical screening

The n–hexane and methanolic extracts of A. campanulatus tuber subjected for preliminary phytochemical screening revealed the presence of various phytochemical constituents as depicted in table 3.1.
Table 3.1. Phytochemical screening of *A. campanulatus* tuber

<table>
<thead>
<tr>
<th>Constituents</th>
<th>n-hexane extract</th>
<th>Methanolic extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fixed oils and fats</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Proteins and amino acids</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

“+” indicates the presence of constituents

“-” indicates the absence of constituents

3A.3.2. *In vitro* antioxidant activity

(A). *Phenolic contents, flavonoid contents and total antioxidant activity*

The results are summarized in table 3.2. ACME had a higher quantity of total phenolics (23.0 ± 2.3 mg GAE/g dry extract) than ACHE (5.7 ± 1.2 mg GAE/g dry extract). ACME, which showed a high total antioxidant activity (90.0 ± 2.9 mg ascorbic acid/g dry extract), also had a great quantity of flavonoids (5.2 ± 0.8 mg QE/g dry extract). Whereas, the total antioxidant activity (35.0 ± 4.7 mg ascorbic acid /g dry extract) and flavonoid content (0.53 ± 0.2 mg QE/g dry extract) were comparatively low in ACHE than the methanolic extract.
(B). *DPPH radical scavenging activity*

The DPPH radical scavenging activity of extracts and standard exhibited a concentration dependent reaction trend. The IC$_{50}$ values of ascorbic acid, ACME and ACHE were 4.2, 52.4 and 1470.5 µg/mL respectively (Table 3.2).

(C). *Hydroxyl radical scavenging activity*

ACME exhibited a better hydroxyl radical scavenging activity than ACHE. Extracts and quercetin, the standard antioxidant, scavenged hydroxyl radicals in a concentration dependent manner and the estimated IC$_{50}$ values of ACHE, ACME and quercetin were 29.2, 23.4 and 20.8 µg/mL respectively (Table 3.2).

(D). *Reducing power*

Ascorbic acid used as reference compound exhibited a superior reducing power at all concentrations, compared with ACHE and ACME (Figure 3.1). At 0.50 mg/mL, the absorbencies of ascorbic acid, ACHE and ACME (at 700 nm) were 1.98, 0.081 and 0.132 respectively. These values reflect the following reducing capability: ascorbic acid > ACME > ACHE.

Table 3.2. *In vitro* antioxidant activity of the n-hexane and methanolic extracts of *A. campanulatus* tuber

<table>
<thead>
<tr>
<th><em>A. campanulatus</em> extracts/Standard</th>
<th>Phenolic contents (mg GAE/g dry extract)</th>
<th>Flavonoid contents (mg QE/g dry extract)</th>
<th>Total antioxidant activity (mg Ascorbic acid /g dry extract)</th>
<th>DPPH radical scavenging activity IC$_{50}$ (µg /ml)</th>
<th>Hydroxyl radical scavenging activity IC$_{50}$ (µg /ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-hexane extract (ACHE)</td>
<td>5.7 ± 1.2</td>
<td>0.53 ± 0.2</td>
<td>35 ± 4.7</td>
<td>1470.5</td>
<td>29.2</td>
</tr>
<tr>
<td>Methanolic extract (ACME)</td>
<td>23 ± 2.3</td>
<td>5.2 ± 0.8</td>
<td>90 ± 2.9</td>
<td>52.4</td>
<td>23.4</td>
</tr>
<tr>
<td>Quercetin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.2</td>
<td>20.8</td>
</tr>
</tbody>
</table>
Figure 3.1. Reducing power of *A. campanulatus* tuber n-hexane and methanolic extracts compared with standard antioxidant ascorbic acid (Ascorbic acid is diluted 1:10).

3A.3.3. LC-MS analysis of ACME

The Phytochemical profiling of ACME, carried out by LC-MS analysis, revealed the presence of eight major phytochemicals with proven antioxidant/ cytotoxic/anticancer properties viz., Cinnamaldehyde, Ferulic acid, Retinol, Quercetin, Quercetagetin, 1-Caffeoyl-β-D-glucose, Triacontanol and Asiatic acid. The mass spectrum of ACME by LC-MS is given in figure 3.2. The list of pharmacologically active phytochemicals is depicted in table 3.3 and their respective chemical structures are given in figure 3.3.
Table 3.3. List of major antioxidant/cytotoxic/anticancer compounds identified in ACME by LC-MS analysis

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Name of the compounds</th>
<th>Library sequence No.</th>
<th>Molecular Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cinnamaldehyde</td>
<td>MTW/UM/2.1/3564/09</td>
<td>132.16</td>
</tr>
<tr>
<td>2</td>
<td>Ferulic acid</td>
<td>MTW/UM/2.1/8796/09</td>
<td>194.19</td>
</tr>
<tr>
<td>3</td>
<td>Retinol</td>
<td>MTW/UM/2.1/9867/09</td>
<td>286.44</td>
</tr>
<tr>
<td>4</td>
<td>Quercetin</td>
<td>MTW/UM/2.1/9998/09</td>
<td>302.23</td>
</tr>
<tr>
<td>5</td>
<td>Quercetagetin</td>
<td>MTW/UM/2.1/4652/09</td>
<td>318.25</td>
</tr>
<tr>
<td>6</td>
<td>1-Caffeoyl-β-D-glucose</td>
<td>MTW/UM/2.1/8900/09</td>
<td>342.30</td>
</tr>
<tr>
<td>7</td>
<td>Triacontanol</td>
<td>MTW/UM/2.1/5636/09</td>
<td>438.83</td>
</tr>
<tr>
<td>8</td>
<td>Asiatic acid</td>
<td>MTW/UM/2.1/6023/09</td>
<td>488.71</td>
</tr>
</tbody>
</table>

Figure 3.2. Mass spectrum of ACME by LC-MS analysis

(A). Mass spectrum of positive ionization. (B). Mass spectrum of negative ionization.
<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Cinnamaldehyde" /></td>
<td>Cinnamaldehyde</td>
</tr>
<tr>
<td><img src="image" alt="Ferulic acid" /></td>
<td>Ferulic acid</td>
</tr>
<tr>
<td><img src="image" alt="Retinol" /></td>
<td>Retinol</td>
</tr>
<tr>
<td><img src="image" alt="Quercetin" /></td>
<td>Quercetin</td>
</tr>
<tr>
<td><img src="image" alt="Quercetagetin" /></td>
<td>Quercetagetin</td>
</tr>
<tr>
<td><img src="image" alt="1-Caffeoyl-β-D-glucose" /></td>
<td>1-Caffeoyl-β-D-glucose</td>
</tr>
<tr>
<td><img src="image" alt="Triacontanol" /></td>
<td>Triacontanol</td>
</tr>
<tr>
<td><img src="image" alt="Asiatic acid" /></td>
<td>Asiatic acid</td>
</tr>
</tbody>
</table>

(Chemspeaker, 2013)

**Figure 3.3.** Chemical structures of the antioxidant/cytotoxic/anticancer compounds identified in ACME
3A.4. DISCUSSION

Approximately 80% of the world populations depend exclusively on plants for their health and healing. Much of the health benefits of medicinal plants, spices, fruits and vegetables have been attributed to phytochemicals, which are the potential sources of natural antioxidants, e.g., carotenoids, flavonoids, isoflavonoids, alkaloids, tannins, phenolic diterpenes and phenolic acids (Kim et al., 2011a). Thousands of phytochemicals have been identified in fruits and vegetables, yet there are still many that have not been identified. Different phytochemicals have been found to possess a range of activities, which may help in protecting against chronic diseases (Boyer and Liu, 2004). In the present investigation, the identified class of phytochemicals such as flavonoids, alkaloids, tannins, phenolic compounds, saponins and glycosides indicates that the methanolic extract of *A. campanulatus* tuber is a rich source of natural antioxidants. Thus it is hypothesized that the extract may capable to inhibit the oxidative damage, cancer cell proliferation, regulate inflammatory and immune response, and protect against lipid oxidation.

Antioxidants are micronutrients that have gained importance in recent years due to their ability to neutralize free radicals. Antioxidants have been reported to prevent oxidative damage caused by free radical; they can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also by acting as oxygen scavengers (Buyukkuroglu et al., 2001). In the present study, the *in vitro* antioxidant activity of the n-hexane and methanolic extracts of *A. campanulatus* tuber was evident from DPPH radical scavenging assay, hydroxyl radical scavenging assay and reducing power assay.
DPPH is a stable, nitrogen-centered free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH produces a violet color in methanol solution and when it encounters antioxidants (proton donors), it gets reduced to a yellow colored product, diphenylpicryl hydrazine (Soares et al., 1997). Among the two extracts of *A. campanulatus* tuber, ACME showed definite DPPH radical scavenging activity in comparison with quercetin and thus indicate that it possess potent proton donating ability and could serve as free radical inhibitor or scavenger.

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells including DNA, lipids and proteins. Hydroxyl radical has the capacity to join nucleotides in DNA and can cause strand breakage which contributes to carcinogenesis, mutagenesis and cytotoxicity. Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity (Shukla et al., 2012). The ability of *A. campanulatus* tuber extracts to quench the hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation. The potent hydroxyl radical scavenging activity exhibited by ACME can also be correlated with its active hydrogen donating ability.

The reducing capacities of the extracts were investigated by $\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$ transformation. Presence of reductones causes the reduction of the $\text{Fe}^{3+}$/ferricyanide complex to the $\text{Fe}^{2+}$ form. In the present study, the total reducing activity exhibited by ACME was comparatively higher than that of ACHE. The reducing properties are generally associated with the presence of reductones which have been shown to
exert antioxidant action by breaking the free radical chain reaction donating a hydrogen atom. Thus, the reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. Reductones can react with the free radicals and convert them into more stable products. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation (Meir et al., 1995).

Likewise, ACME had a higher quantity of total phenolics and flavonoids compared to ACHE. It has been reported that flavonoids and phenolics contribute towards the potent antioxidant activity of the plants. Phenolic compounds function as high-level antioxidants because they possess the ability to absorb and neutralize free radicals as well as quench reactive oxygen species. Flavonoids, one of the most diverse and widespread groups of natural compounds, are also probably the most natural phenolics capable of exhibiting in vitro and in vivo antioxidant activities (Sancheti et al., 2013). Therefore, the noteworthy antioxidant effect of ACME possibly may be due to the occurrence of phytoconstituents determined in the phytochemical screening, which exclusively or in synergism demonstrate beneficial effects.

The LC-MS analysis for the phytochemical profiling of the methanolic extract of A. campanulatus tuber revealed the presence of eight major phytochemicals with proven antioxidant/cytotoxic/anticancer properties viz., Cinnamaldehyde, Ferulic acid, Retinol, Quercetin, Quercetagetin, 1-Caffeoyl-β-D-glucose, Triacontanol and Asiatic acid. Among these phytochemicals Cinnamaldehyde (Gowder and Devaraj, 2006), Ferulic acid (Kim et al., 2011b), Quercetin (Li et al., 2013), Quercetagetin (Gong et al., 2012), 1-Caffeoyl-β-D-
glucose (Du et al., 2006), Triacontanol (Ramanarayan et al., 2000) and Asiatic acid (Ma et al., 2009) are well-known for its antioxidant activities.

In addition to the reported antioxidant activities, Cinnamaldehyde, Ferulic acid, Retinol, Quercetin and Asiatic acid are known for its cytotoxic and/or anticancer effects. Lin et al. (2013) reported that cinnamaldehyde is an apoptotic inducer that acts on the mitochondrial death pathway in human hepatoma PLC/PRF/5 cells. Whereas ferulic acid, a phenolic compound, showed antiproliferative effect against colon cancer cell lines (Janicke et al., 2011). Besides, Delage et al. (2005) reported that retinol also known as vitamin A, exhibited a protective role against disturbances associated with hyperlipidic diet exposure in 1,2-dimethylhydrazine (DMH) induced colon carcinogenesis. Quercetin, the ubiquitous bioactive flavonoid, has also been shown to induce growth inhibition in colon cancer cells. Kim et al. (2010) reported that quercetin induces apoptosis via AMP-activated protein kinase (AMPK) activation and p53-dependent apoptotic cell death in HT-29 colon cancer cells. Asiatic acid, a triterpene, is known to be cytotoxic to several tumor cell lines and can induce apoptosis in colon cancer RKO cells (Cho et al., 2006).

Taken together, the result of LC-MS analysis of ACME indicates that it is a potential source of phytochemicals with antioxidant and anticancer properties. However, further studies are required to establish its in vivo antioxidant and anticancer activities particularly its chemopreventive efficacy against colon and liver cancer. In view of this, we have evaluated the chemopreventive potential of ACME against DMH induced colon carcinogenesis and N – Nitrosodiethylamine (NDEA)
induced hepatocellular carcinoma in rats and their results are described in the subsequent chapters.

In conclusion, the results obtained in the current study demonstrated that ACME contained higher levels of total phenolic compounds and was capable of inhibiting, quenching free radicals to terminate the radical chain reaction, and acting as a reducing agent. More precisely, the *in vitro* antioxidant activity of ACME might be attributed to the synergistic effect of the identified antioxidant phytochemicals. The biological activities of ACME should not be exclusively explained based on the effects of the major compounds because it may also include the response to other bioactive compounds present in smaller concentrations. Interaction between compounds (synergistic effect) present in the extracts can also not be excluded at this point. This study also supports the contention that traditional medicines remain a valuable source in the potential discovery of natural product pharmaceuticals. The significant antioxidant activity of the methanolic extract of *A. campanulatus* tuber provides a scientific validation for the traditional use of this tuber as an accessible source of phytochemicals with consequent health benefits. Among the two extracts of *A. campanulatus* tuber investigated for antioxidant efficacy, ACME revealed a promising *in vitro* antioxidant activity and hence it was chosen for *in vivo* studies.
Chapter 3 (B)

*In vivo* antioxidant evaluation of *Amorphophallus campanulatus* (Roxb.) Bl. tuber against thioacetamide induced oxidative stress in rats
3B.1. INTRODUCTION

Reactive oxygen species (ROS) are generated from normal metabolic processes or produced after stimulation from exogenous factors and/or agents, such as ultraviolet light, ionizing radiation, and chemical reactions. These reactive species are capable of damaging a wide range of essential biomolecules. Oxidative stress is caused by a disturbance between the antioxidant defense mechanisms and the level of ROS, and has been associated with many pathological disorders such as atherosclerosis, diabetes and cancer. Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. They exert their effects by scavenging free radicals, activating a battery of detoxifying proteins, or preventing the generation of free radicals (Chen et al., 2009; Erukainure et al., 2011). Various endogenous antioxidant defense mechanisms play an important role in the elimination of ROS and lipid peroxides and therefore protect the cells against its toxic effects (Halliwell et al., 1992).

The antioxidant defense can be further strengthened with a diet rich in antioxidants. Recently, interest in finding naturally occurring antioxidants has increased considerably to replace synthetic antioxidants, which are being restricted due to their side effects (Patel et al., 2011; Ravikumar and Gnanadesigan, 2011). The growing interest in the substitution of synthetic food antioxidants by natural antioxidants and the health implications of antioxidants in nutraceuticals has hastened the research on vegetable sources and the screening of raw materials for identifying antioxidants. (Sreelatha et al., 2012).

This chapter deals with the in vivo antioxidant evaluation of *Amorphophallus campanulatus* tuber in preventive and curative models. As the methanolic extract of
A. campanulatus tuber exhibited a promising in vitro antioxidant activity, it was further evaluated for its antioxidant potential against thioacetamide (TAA) induced oxidative stress in rats.

3B.2. MATERIALS AND METHODS

3B.2.1. Chemicals

Thioacetamide was purchased from Loba Cheme, Mumbai, India. Assay kits for serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and lactate dehydrogenase were purchased from Agappe Diagnostic, India. All other chemicals were of analytical grade.

3B.2.2. Animals and diets

Male Wistar rats were used in this study. The animals were housed in polypropylene cages and given standard rat chow (Sai Feeds, Bangalore, India) and drinking water ad libitum. The animals were maintained at a controlled condition of temperature of 26–28 °C with a 12 h light: 12 h dark cycle. Animal studies were followed according to Institutional Animal Ethics Committee (IAEC) regulations approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (Reg. No. B 2442009/6) and conducted humanely.

3B.2.3. Preparation of plant extract

A. campanulatus tubers were powdered and subjected to Soxhlet extraction with methanol and concentrated under reduced pressure using a rotary evaporator. The yield of methanolic extract was 9.3% (w/w). Extract was suspended in 5% Tween 80 to respective dosages for in vivo studies and stored at -20 °C.
3B.2.4. Preparation of doses and treatments

Thioacetamide suspended in normal saline was administered (100 mg/kg body weight) subcutaneously to induce the oxidative stress in rats (Ahmad et al., 1999). Silymarin at an oral dose of 100 mg/kg body weight was used as standard control in the experiment (Shyamal et al., 2010). Two different doses (125 and 250 mg/kg) of ACME suspended in 5% Tween 80 were also prepared for oral administration to the animals. The acute toxicity study reported by Hurkadale et al., (2012) proves that the methanolic extract of *A. campanulatus* tuber was safe up to a dose of 3000 mg/kg, b.w.

3B.2.5. Experimental design of pre-treatment evaluation (Roy et al., 2006)

Male Wistar rats weighing 156 ± 5.8 gm (Mean ± S.D, n = 30) were used in this study. Rats were divided into five groups with six rats in each group and that were treated as follows:

- **Group I** : Control rats (vehicle only)
- **Group II** : Thioacetamide control (100 mg/kg, s.c.)
- **Group III** : Thioacetamide (as in group II) + Silymarin (100 mg/kg, p.o.)
- **Group IV** : Thioacetamide (as in group II) + ACME (125 mg/kg, p.o.)
- **Group V** : Thioacetamide (as in group II) + ACME (250 mg/kg, p.o.)

All the groups except group I received a single dose of thioacetamide (100 mg/kg; s.c) suspended in normal saline on 9th day of the experiment. Nine days before the thioacetamide challenge, group III, IV and V rats received 100 mg/kg silymarin, 125 mg/kg ACME and 250 mg/kg ACME respectively. Group I animals treated as vehicle control received 5% Tween 80 and normal saline instead of drug
and thioacetamide respectively. All the animals were sacrificed 24 h after thioacetamide administration.

3B.2.6. Experimental design of post-treatment evaluation (Ahmad et al., 2002)

Male Wistar rats weighing 155 ± 7.5 gm (Mean ± S.D, n = 30) were used in this study. Rats were divided into five groups with six rats in each group and that were treated as follows:

- **Group I**: Control rats (vehicle only)
- **Group II**: Thioacetamide control (100 mg/kg, s.c.)
- **Group III**: Thioacetamide (as in group II) + Silymarin (100 mg/kg, p.o.)
- **Group IV**: Thioacetamide (as in group II) + ACME (125 mg/kg, p.o.)
- **Group V**: Thioacetamide (as in group II) + ACME (250 mg/kg, p.o.)

All the groups except group I received a single dose of thioacetamide (100 mg/kg; s.c) on 1st day of the experiment. Groups III–V received silymarin and ACME 2, 24 and 48 h after thioacetamide challenge. Group I animals treated as vehicle control received 5% Tween 80 and normal saline instead of drug and thioacetamide respectively. All the animals were sacrificed 72 h after thioacetamide administration.

3B.2.7. Serum enzyme analysis

Hepatotoxicity was assessed by quantifying the serum levels of AST (EC 2.6.1.1), ALT (EC 2.6.1.2), ALP (EC 3.1.3.1) and LDH (EC 1.1.1.27) by kinetic method using the kit of Agappe Diagnostic Ltd., India. Activities of these serum enzymes were measured using semi autoanalyzer (RMS, India).
3B.2.8. Tissue analysis

Liver and kidney were excised, washed thoroughly in ice-cold saline to remove the blood. Ten percent of homogenate was prepared in 0.1M Tris HCl buffer (pH – 7.4) (Ilavarasan et al., 2003). The homogenate was centrifuged at 3000 rpm for 20 min at 4°C and the supernatant was used for the estimation of reduced glutathione (GSH), glutathione-S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), lipid peroxidation (Thiobarbituric Acid Reactive Substances – TBARS) and total protein.

GSH levels in tissues were determined based on the formation of a yellow colored complex with DTNB (Ellman, 1959). GST (EC 2.5.1.18) activity was determined from the rate of increase in conjugate formation between reduced glutathione and CDNB (Habig et al., 1974). GR (EC 1.6.4.2) activity was assayed at 37 °C and 340 nm by following the oxidation of NADPH by GSSG (Carlberg and Mannervik, 1985). GPx (EC 1.11.1.9) activity was determined by measuring the decrease in GSH content after incubating the sample in the presence of H_2O_2 and NaN_3 (Rotruck et al., 1973). CAT (EC 1.11.1.6) activity was determined from the rate of decomposition of H_2O_2 (Beers and Sizer, 1952). The level of lipid peroxidation was measured as malondialdehyde (MDA), a thiobarbituric acid reacting substance (TBARS), using 1’1’3’3’ tetramethoxypropane as standard (Niehuis and Samuelsson, 1968). Protein contents of the tissues were determined using bovine serum albumin (BSA) as the standard (Lowry et al., 1951).

(Detailed procedures are explained under chapter 2, section 2.2.13. Procedures for in vivo antioxidant assays)
Percent protection was calculated using the formula,

\[
\text{Percentage protection} = \frac{\text{Toxic control} - \text{Extract treated}}{\text{Toxic control} - \text{Normal control}} \times 100
\]

3B.2.9. Histopathological studies

Small pieces of liver tissues fixed in 10% neutral buffered formalin were processed for embedding in paraffin. Sections of 5–6 µm were taken and stained with hematoxylin and eosin and examined for histopathological changes under the microscope (Motic AE 21, Germany). The microphotographs were taken using Moticam 1000 camera at original magnification of 100×.

Liver sections were graded numerically to assess the degree of histological features in acute hepatic injury. Centrilobular necrosis is the necrosis around the central vein characterized by prominent ballooning, swollen granular cytoplasm with fading nuclei. Bridging hepatic necrosis is a form of confluent necrosis of liver cells linking central veins to portal tracts or portal tracts to one another (Lefkowitch, 1996). A combined score of centrilobular necrosis, bridging hepatic necrosis and lymphocyte infiltration was given a maximum value of 6 and descriptive modifiers such as mild, moderate, and severe was applied to activity and staging. The parameters were graded from score 0 to 6, with 0 indicating no abnormality, 1 to 2 indicating mild injury, 3 to 4 indicating moderate injury and 5 to 6 representing severe liver injury.

3B.2.10. Statistical analysis

Results are expressed as mean ± standard deviation (SD). All statistical comparisons were made by means of one-way ANOVA test followed by Tukey’s post hoc analysis and \( p \)-values less than or equal to 0.05 were considered significant.
3B.3. RESULTS

3B.3.3. Pre-treatment evaluation

3B.3.3.1. Effect of TAA and ACME on serum marker enzymes

The results are graphically depicted in figure 3.4 (A, B, C and D). The serum levels of AST, ALT, ALP and LDH in group II were significantly \( p \leq 0.05 \) elevated by the administration of a single dose of TAA, when compared to normal control. Treatment with ACME at a dose of 125 and 250 mg/kg showed a significant decrease \( p \leq 0.05 \) of AST, ALT, ALP and LDH. Standard control drug, Silymarin at a dose of 100 mg/kg also prevented the elevation of serum enzymes. Treatment with 250 mg/kg of ACME and 100 mg/kg of Silymarin exhibited a protection of 81.7 and 77.5% in AST levels, 43.0 and 29.3% in ALT levels, 38.0 and 35.2% in ALP levels and 72.5 and 65.5% in LDH levels respectively. The preventive effect of the extract in decreasing the elevated levels of serum enzymes was in a dose dependent manner.

![Figure 3.4](image-url)

Figure 3.4. Effects of ACME and Silymarin pre-treatment on changes in serum enzyme levels of rats intoxicated with TAA. (A). Aspartate aminotransferase

N - Normal control, T - Thioacetamide control, S - Silymarin, D1 - ACME (125 mg/kg), D2 - ACME (250 mg/kg).

Values are mean ± S.D, error bar indicating the standard deviation, \( n = 6 \) animals. \( \dagger p \leq 0.05 \) versus normal control. \( * p \leq 0.05 \) versus thioacetamide control.
Figure 3.4. (Cont.) Effects of ACME and Silymarin pre-treatment on changes in serum enzyme levels of rats intoxicated with TAA.

(B). Alanine aminotransferase (C). Alkaline phosphatase

N - Normal control, T - Thioacetamide control, S - Silymarin, D1 - ACME (125 mg/kg), D2 - ACME (250 mg/kg).

Values are mean ± S.D, error bar indicating the standard deviation, n = 6 animals. † p ≤ 0.05 versus normal control. *p ≤ 0.05 versus thioacetamide control.
Figure 3.4. (Cont.) Effects of ACME and Silymarin pre-treatment on changes in serum enzyme levels of rats intoxicated with TAA. (D). Lactate dehydrogenase.

N - Normal control, T - Thioacetamide control, S - Silymarin, D1 - ACME (125 mg/kg), D2 - ACME (250 mg/kg).

Values are mean ± S.D, error bar indicating the standard deviation, n = 6 animals. † p ≤ 0.05 versus normal control. * p ≤ 0.05 versus thioacetamide control.

3B.3.3.2. Effect of ACME on TAA induced changes in the antioxidant status of hepatic and renal tissues

The protective effects of ACME against TAA induced changes in the liver and kidney antioxidant status are shown in table 3.4 and 3.5 respectively.

(A). Reduced glutathione (GSH) level

In the pre-treatment groups, a significantly (p ≤ 0.05) lower levels of GSH were observed in rats administered alone with TAA. Treatment with ACME exhibited significant increase (p ≤ 0.05) in both hepatic and renal glutathione levels. In liver and kidney, 250 mg/kg of ACME showed a protection of 84.6 and 83.6%
respectively. Silymarin treated rats also prevented the lowering of GSH and the percentage of protection was 63.5 and 63.9 respectively for liver and kidney.

(B). Glutathione - S - transferase (GST) activity

When compared to normal control the GST activity of liver and kidney tissues were significantly ($p \leq 0.05$) reduced in TAA intoxicated rats. ACME dose dependently increased ($p \leq 0.05$) the activity of GST in both the hepatic and renal tissues. Treatment with 250 mg/kg ACME exhibited a significant increase i.e., 79.4 and 88.6%, respectively in hepatic and renal tissues. In addition, silymarin treated rats also prevented the TAA induced decrease in GST activity by 69.2 and 70.9% in hepatic and renal tissues respectively.

(C). Glutathione reductase (GR) activity

GR activity was significantly decreased ($p \leq 0.05$) in TAA treated animals when compared to the normal control groups. A significant increase ($p \leq 0.05$) in the level of GR was observed in ACME (125 and 250 mg/kg) and silymarin (100 mg/kg) treated rats intoxicated with TAA. Both hepatic and renal tissues showed the same pattern of GR activity in all groups treated with ACME and silymarin. The percentage of protection in liver and kidney tissues were 78.9 and 75.9 respectively for 250 mg/kg of ACME. Silymarin restored the GR activity by 67.1% in liver and 54.8% in kidney.

(D). Glutathione peroxidase (GPx) activity

Activities of hepatic and renal GPx were significantly ($p \leq 0.05$) lowered in TAA treated rats. ACME dose dependently prevented the lowering of GPx in both the organs compared to TAA alone treated groups. In liver and kidney, 250 mg/kg of ACME showed a protection of 80.7 and 80.6% respectively. Silymarin treated rats also prevented the lowering of GPx by 78.3% in hepatic and 78.6% in renal tissues.
(E). Catalase (CAT) activity

Animals injected with TAA alone showed significant ($p \leq 0.05$) reduction in hepatic and renal CAT activity. ACME dose dependently increased the activity of CAT in both hepatic and renal tissues. Treatment with 250 mg/kg of ACME exhibited significant increase i.e., 75.8 and 78.6%, respectively in liver and kidney. In addition, silymarin treated rats also prevented ($p \leq 0.05$) the TAA induced decrease in CAT activity by 68.7 and 82.2% in hepatic and renal tissues respectively.

(F). Lipid peroxidation (MDA) level

A significant increase ($p \leq 0.05$) in tissue MDA level was observed in TAA alone treated rats. However, TAA induced elevation of MDA concentration were lowered ($p \leq 0.05$) by 78.5% in hepatic and renal tissues of rats treated with ACME at a dose of 250 mg/kg. Silymarin also showed a protection ($p \leq 0.05$) of 54.8% in liver and 59.3% in kidney.

3B.3.3.3. Histopathological analysis

Normal architecture of the liver (Figure 3.5.A) was completely lost in rats treated with TAA (Figure 3.3.B) with the appearance of centrilobular necrosis, bridging hepatic necrosis and lymphocyte infiltration with a score of $5.3 \pm 0.5$ (mean ± S.D.; $n=3$). The animals administered with silymarin and ACME at 100 and 250 mg/kg showed a significant ($p \leq 0.05$) protection from TAA induced liver damage as evident from hepatic architectural pattern with mild to moderate hepatitis with scores $3.3 \pm 0.5$; $3.0 \pm 1.0$; and $2.6 \pm 0.5$ (mean ± S.D.; $n=3$; $p \leq 0.05$), respectively (Figure 3.5.C–E).
Table 3.4. Pre-treatment (Protective effects) of ACME against TAA induced changes in the liver antioxidant status

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>TAA (100mg/kg)</th>
<th>Silymarin (100mg/kg) +TAA</th>
<th>ACME (125mg/kg) +TAA</th>
<th>ACME (250mg/kg) +TAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH&lt;sup&gt;1&lt;/sup&gt;</td>
<td>24.8 ± 0.4</td>
<td>15.2 ± 0.3&lt;sup&gt;†&lt;/sup&gt;</td>
<td>21.3 ± 0.5*</td>
<td>18.2 ± 0.3*</td>
<td>23.3 ± 0.4*</td>
</tr>
<tr>
<td>GST&lt;sup&gt;2&lt;/sup&gt;</td>
<td>74.8 ± 0.4</td>
<td>37.3 ± 0.4&lt;sup&gt;†&lt;/sup&gt;</td>
<td>63.3 ± 0.3*</td>
<td>59.3 ± 0.5*</td>
<td>67.1 ± 0.2*</td>
</tr>
<tr>
<td>GR&lt;sup&gt;3&lt;/sup&gt;</td>
<td>19.8 ± 0.7</td>
<td>7.7 ± 0.5&lt;sup&gt;†&lt;/sup&gt;</td>
<td>15.8 ± 0.3*</td>
<td>10.6 ± 0.3*</td>
<td>17.2 ± 0.3*</td>
</tr>
<tr>
<td>GPx&lt;sup&gt;4&lt;/sup&gt;</td>
<td>298.4 ± 7.2</td>
<td>174.2 ± 8.9&lt;sup&gt;†&lt;/sup&gt;</td>
<td>271.6 ± 7.4*</td>
<td>209.7 ± 9.0*</td>
<td>274.5 ± 6.3*</td>
</tr>
<tr>
<td>CAT&lt;sup&gt;5&lt;/sup&gt;</td>
<td>51.5 ± 2.5</td>
<td>35.4 ± 1.4&lt;sup&gt;†&lt;/sup&gt;</td>
<td>46.5 ± 1.9*</td>
<td>39.1 ± 1.0*</td>
<td>47.6 ± 1.2*</td>
</tr>
<tr>
<td>MDA&lt;sup&gt;6&lt;/sup&gt;</td>
<td>45.6 ± 0.5</td>
<td>75.7 ± 1.1&lt;sup&gt;†&lt;/sup&gt;</td>
<td>59.2 ± 0.8*</td>
<td>63.3 ± 1.0*</td>
<td>52.0 ± 0.2*</td>
</tr>
</tbody>
</table>

Table 3.5. Pre-treatment (Protective effects) effects of ACME against TAA induced changes in the kidney antioxidant status

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>TAA (100mg/kg)</th>
<th>Silymarin (100mg/kg) +TAA</th>
<th>ACME (125mg/kg) +TAA</th>
<th>ACME (250mg/kg) +TAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH&lt;sup&gt;1&lt;/sup&gt;</td>
<td>18.2 ± 0.4</td>
<td>8.1 ± 0.2&lt;sup&gt;†&lt;/sup&gt;</td>
<td>14.7 ± 0.5*</td>
<td>11.1 ± 0.5*</td>
<td>16.1 ± 0.5*</td>
</tr>
<tr>
<td>GST&lt;sup&gt;2&lt;/sup&gt;</td>
<td>49.8 ± 0.8</td>
<td>27.4 ± 0.4&lt;sup&gt;†&lt;/sup&gt;</td>
<td>43.3 ± 0.3*</td>
<td>39.2 ± 0.4*</td>
<td>47.2 ± 0.3*</td>
</tr>
<tr>
<td>GR&lt;sup&gt;3&lt;/sup&gt;</td>
<td>16.7 ± 0.3</td>
<td>6.3 ± 0.4&lt;sup&gt;†&lt;/sup&gt;</td>
<td>12.0 ± 0.5*</td>
<td>9.2 ± 0.4*</td>
<td>14.2 ± 0.3*</td>
</tr>
<tr>
<td>GPx&lt;sup&gt;4&lt;/sup&gt;</td>
<td>285.7 ± 6.7</td>
<td>160.3 ± 8.1&lt;sup&gt;†&lt;/sup&gt;</td>
<td>258.9 ± 5.3*</td>
<td>197.6 ± 8.9*</td>
<td>261.5 ± 5.8*</td>
</tr>
<tr>
<td>CAT&lt;sup&gt;5&lt;/sup&gt;</td>
<td>58.8 ± 1.2</td>
<td>47.1 ± 1.7&lt;sup&gt;†&lt;/sup&gt;</td>
<td>56.8 ± 1.9*</td>
<td>49.3 ± 1.5*</td>
<td>56.3 ± 0.5*</td>
</tr>
<tr>
<td>MDA&lt;sup&gt;6&lt;/sup&gt;</td>
<td>42.4 ± 0.4</td>
<td>72.3 ± 0.7&lt;sup&gt;†&lt;/sup&gt;</td>
<td>54.6 ± 0.3*</td>
<td>59.3 ± 0.5*</td>
<td>49.4 ± 0.6*</td>
</tr>
</tbody>
</table>

<sup>1</sup>(nmol/mg protein); <sup>2</sup>(µmol CDNB-GSH conjugate formed/min/mg protein); <sup>3</sup>(nmol of GSSG utilized/min/mg protein); <sup>4</sup>(nmol of GSH oxidized/min/mg protein); <sup>5</sup>(U/mg protein); <sup>6</sup>(nmol/g tissue).

Values are the mean ± S.D from 6 rats in each group. Statistical significance: p ≤ 0.05. † TAA group differs significantly from normal control group. * Silymarin (100mg/kg) + TAA, ACME–125 mg/kg + TAA and ACME–250 mg/kg + TAA groups differ significantly from TAA alone treated group.
Figure 3.5. Histopathological analysis of hepatic tissues in pre-treated group of animals. (hematoxylin and eosin, 100×)

(A) Normal control; (B) TAA control, (100 mg/kg s.c.); (C) Silymarin (100 mg/kg) + TAA; (D) ACME (125 mg/kg) + TAA; (E) ACME (250 mg/kg) + TAA.
3B.3.4. Post-treatment evaluation

3B.3.4.1. Effect of TAA and ACME on serum marker enzymes

Graphical representations of the results are shown in figure 3.5 (A, B, C and D). When compared to normal control, TAA treated animals showed significant ($p \leq 0.05$) elevation of serum enzymes such as AST, ALT, ALP and LDH. Post treatment with ACME (125 and 250 mg/kg) and silymarin (100 mg/kg) showed a significant decrease ($p \leq 0.05$) of serum enzymes, compared to TAA control. 250 mg/kg of ACME exhibited a reversal of 94.1, 58.6, 79.3 and 87.4% for AST, ALT, ALP and LDH respectively. Silymarin also showed a remarkable ($p \leq 0.05$) restoration of 84.0, 47.8, 64.4 and 88.1% for AST, ALT, ALP and LDH respectively towards TAA intoxication.

![Graph (A)](image_url)

Figure 3.6. Effects of ACME and Silymarin post-treatment on changes in serum enzyme levels of rats intoxicated with TAA. (A). Aspartate aminotransferase

N - Normal control, T - Thioacetamide control, S - Silymarin, D1 - ACME (125 mg/kg), D2 - ACME (250 mg/kg).

Values are mean ± S.D, error bar indicating the standard deviation, n = 6 animals. † $p \leq 0.05$ versus normal control. *$p \leq 0.05$ versus thioacetamide control.
Figure 3.6. (Cont.) Effects of ACME and Silymarin post-treatment on changes in serum enzyme levels of rats intoxicated with TAA. 

(B). Alanine aminotransferase (C). Alkaline phosphatase

N - Normal control, T - Thioacetamide control, S - Silymarin, D1 - ACME (125 mg/kg), D2 - ACME (250 mg/kg).

Values are mean ± S.D, error bar indicating the standard deviation, n = 6 animals. †p ≤ 0.05 versus normal control. *p ≤ 0.05 versus thioacetamide control.
Figure 3.6. (Cont.) Effects of ACME and Silymarin post-treatment on changes in serum enzyme levels of rats intoxicated with TAA. (D). Lactate dehydrogenase.

N - Normal control, T - Thioacetamide control, S - Silymarin, D1 - ACME (125 mg/kg), D2 - ACME (250 mg/kg).

Values are mean ± S.D, error bar indicating the standard deviation, n = 6 animals. † p ≤ 0.05 versus normal control. *p ≤ 0.05 versus thioacetamide control.

3B.3.4.2. Effect of ACME on TAA induced changes in the antioxidant status of hepatic and renal tissues

The curative effects of ACME against TAA induced changes in the liver and kidney antioxidant status are shown in table 3.6 and 3.7 respectively.

(A). Reduced glutathione (GSH) level

When compared to TAA alone treated rats, post treatment with ACME (125 and 250 mg/kg) and silymarin significantly (p ≤ 0.05) restored the decreased glutathione levels in liver and kidney. In hepatic tissue, 85.1% reversal in GSH level shown by 250 mg/kg of methanolic extract was comparable with 67.5% exhibited by
100 mg/kg of silymarin. In renal tissue, 250 mg/kg of methanolic extract and 100 mg/kg of silymarin restored the GSH level by 82.5 and 66.8% respectively.

(B). Glutathione - S - transferase (GST) activity

Rats administered with TAA alone showed significant ($p \leq 0.05$) reduction in hepatic and renal GST level. Treatment with ACME and silymarin showed significant reversal ($p \leq 0.05$) of TAA induced decrease in GST activity. Rats treated with 250 mg/kg ACME and 100 mg/kg silymarin restored the decrease of GST levels by 83.6 and 73.9% in liver and 98.7 and 79.2% in kidney respectively.

(C). Glutathione reductase (GR) activity

Glutathione reductase activity was significantly ($p \leq 0.05$) reduced in TAA control group. Treatment with ACME exhibited significant increase ($p \leq 0.05$) in both hepatic and renal GR activity. In liver and kidney, 250 mg/kg of ACME restored the activity of GR by 83.3 and 75.2% respectively. Silymarin treated rats also significantly ($p \leq 0.05$) restored the GR activity by 72.3% in liver and 61.9% in kidney.

(D). Glutathione peroxidase (GPx) activity

Compared to TAA alone treated animals, rats treated with ACME significantly ($p \leq 0.05$) restored the decreased GPx activity in liver and kidney. In hepatic tissue, post treatment with 250 mg/kg of ACME and 100 mg/kg of silymarin exhibited a reversal in GPx activity by 79.4% and 74.5% respectively. In renal tissue, 250 mg/kg of ACME and 100 mg/kg of silymarin reinstated the GPx activity by 78.7 and 73.8% respectively.
(E). Catalase (CAT) activity

Animals injected with TAA alone showed significant ($p \leq 0.05$) reduction in hepatic and renal CAT activity. Treatment with ACME (125 mg/kg and 250 mg/kg) and silymarin (100 mg/kg) markedly ($p \leq 0.05$) restored the TAA induced reduction of CAT activity. Rats treated with 250 mg/kg of ACME and 100 mg/kg of silymarin restored the decrease of CAT levels by 76.1 and 69.0% in the liver and 73.2 and 65.2% in the kidney respectively.

(F). Lipid peroxidation (MDA) level

A significant increase ($p \leq 0.05$) in tissue MDA level was shown in TAA alone treated animals when compared to normal control. ACME and silymarin significantly ($p \leq 0.05$) reversed the elevation of hepatic and renal MDA formation. 250 mg/kg of ACME reinstated the MDA formation by 80.1% in hepatic tissue and 77.6% in renal tissue. Silymarin exhibited 55.1 and 59.7% inhibition in MDA formation in liver and kidney respectively.

3B.3.4.3. Histopathological analysis

In rats treated with TAA, the normal architecture of liver was completely lost with the appearance of centrilobular necrosis, bridging necrosis and lymphocyte infiltration scoring $5.0 \pm 1.0$ (mean $\pm$ S.D.; $n=3$) (Figure 3.6. A and B). Rats treated with silymarin and ACME (125 and 250 mg/kg) after the establishment of toxic injury showed recovery from liver damage with scores $2.3 \pm 0.5$; $1.6 \pm 0.5$ and $1.0 \pm 0.5$ (Mean $\pm$ S.D.; $n=3$; $p \leq 0.05$), respectively (Figure 3.6.C–E).
### Table 3.6. Post-treatment (Curative) effects of ACME against TAA induced changes in the liver antioxidant status

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>TAA (100mg/kg)</th>
<th>Silymarin (100mg/kg) +TAA</th>
<th>ACME (125mg/kg) +TAA</th>
<th>ACME (250mg/kg) +TAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH(^1)</td>
<td>26.1 ± 0.3</td>
<td>14.2 ± 0.4(^1)</td>
<td>22.3 ± 0.5(^*)</td>
<td>18.3 ± 0.5(^*)</td>
<td>24.4 ± 0.3(^*)</td>
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<tr>
<td>GST(^2)</td>
<td>74.2 ± 0.3</td>
<td>39.2 ± 0.4(^1)</td>
<td>65.1 ± 0.3(^*)</td>
<td>59.7 ± 0.3(^*)</td>
<td>68.5 ± 0.4(^*)</td>
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<tr>
<td>GR(^3)</td>
<td>21.0 ± 0.5</td>
<td>9.1 ± 0.6(^1)</td>
<td>17.7 ± 0.5(^*)</td>
<td>13.2 ± 0.3(^*)</td>
<td>19.3 ± 0.5(^*)</td>
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<tr>
<td>GPx(^4)</td>
<td>300.3 ± 7.7</td>
<td>179.8 ± 8.5(^1)</td>
<td>269.6 ± 7.7(^*)</td>
<td>219.3 ± 8.3(^*)</td>
<td>275.5 ± 7.7(^*)</td>
</tr>
<tr>
<td>CAT(^5)</td>
<td>51.9 ± 2.2</td>
<td>36.0 ± 1.5(^1)</td>
<td>47.0 ± 1.8(^*)</td>
<td>39.6 ± 1.2(^*)</td>
<td>48.1 ± 1.3(^*)</td>
</tr>
<tr>
<td>MDA(^6)</td>
<td>45.0 ± 0.1</td>
<td>74.9 ± 0.8(^1)</td>
<td>58.4 ± 0.5(^*)</td>
<td>62.5 ± 0.6(^*)</td>
<td>50.9 ± 0.2(^*)</td>
</tr>
</tbody>
</table>

\(^1\)(nmol/mg protein); \(^2\)(µmol CDNB-GSH conjugate formed/min/mg protein); \(^3\)(nmol of GSSG utilized/min/mg protein); \(^4\)(nmol of GSH oxidized/min/mg protein); \(^5\)(U/mg protein); \(^6\)(nmol/g tissue).

Values are the mean ± S.D from 6 rats in each group. Statistical significance: \(p \leq 0.05\). \(^1\) TAA group differs significantly from normal control group. \(^*\) Silymarin (100mg/kg) + TAA, ACME–125 mg/kg + TAA and ACME–250 mg/kg + TAA groups differ significantly from TAA alone treated group.

### Table 3.7. Post-treatment (Curative) effects of ACME against TAA induced changes in the kidney antioxidant status

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>TAA (100mg/kg)</th>
<th>Silymarin (100mg/kg) +TAA</th>
<th>ACME (125mg/kg) +TAA</th>
<th>ACME (250mg/kg) +TAA</th>
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</thead>
<tbody>
<tr>
<td>GSH(^1)</td>
<td>19.2 ± 0.5</td>
<td>7.6 ± 0.4(^1)</td>
<td>15.3 ± 0.4(^*)</td>
<td>11.3 ± 0.6(^*)</td>
<td>17.2 ± 0.6(^*)</td>
</tr>
<tr>
<td>GST(^2)</td>
<td>49.0 ± 0.2</td>
<td>30.1 ± 0.6(^1)</td>
<td>45.1 ± 0.1(^*)</td>
<td>39.8 ± 0.2(^*)</td>
<td>48.8 ± 0.3(^*)</td>
</tr>
<tr>
<td>GR(^3)</td>
<td>18.8 ± 0.3</td>
<td>7.0 ± 0.5(^1)</td>
<td>14.3 ± 0.4(^*)</td>
<td>8.7 ± 0.4(^*)</td>
<td>15.9 ± 0.3(^*)</td>
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<tr>
<td>GPx(^4)</td>
<td>288.8 ± 7.0</td>
<td>175.0 ± 4.7(^1)</td>
<td>259.1 ± 7.3(^*)</td>
<td>208.4 ± 6.8(^*)</td>
<td>264.6 ± 6.8(^*)</td>
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<tr>
<td>CAT(^5)</td>
<td>58.4 ± 1.4</td>
<td>46.9 ± 1.7(^1)</td>
<td>54.4 ± 1.6(^*)</td>
<td>49.0 ± 1.6(^*)</td>
<td>55.4 ± 1.5(^*)</td>
</tr>
<tr>
<td>MDA(^6)</td>
<td>41.3 ± 0.6</td>
<td>71.6 ± 0.6(^1)</td>
<td>53.5 ± 0.6(^*)</td>
<td>58.4 ± 0.4(^*)</td>
<td>48.1 ± 0.2(^*)</td>
</tr>
</tbody>
</table>

\(^1\)(nmol/mg protein); \(^2\)(µmol CDNB-GSH conjugate formed/min/mg protein); \(^3\)(nmol of GSSG utilized/min/mg protein); \(^4\)(nmol of GSH oxidized/min/mg protein); \(^5\)(U/mg protein); \(^6\)(nmol/g tissue).

Values are the mean ± S.D from 6 rats in each group. Statistical significance: \(p \leq 0.05\). \(^1\) TAA group differs significantly from normal control group. \(^*\) Silymarin (100mg/kg) + TAA, ACME–125 mg/kg + TAA and ACME–250 mg/kg + TAA groups differ significantly from TAA alone treated group.
Figure 3.7. Histopathological changes occurred in rat liver after TAA intoxication and recovery by the treatment with ACME and Silymarin (hematoxylin and eosin, 100×)

(A) Normal control; (B) TAA control, (100 mg/kg s.c.); (C) Silymarin (100 mg/kg) + TAA; (D) ACME (125 mg/kg) + TAA; (E) ACME (250 mg/kg) + TAA.
3B.4. DISCUSSION

The present study demonstrated that the methanolic extract of *A. campanulatus* tuber had therapeutic effects on oxidative stress and liver damage induced by TAA exposure in rats. Thioacetamide, originally used as fungicide, is a centrilobular hepatotoxicant widely used to induce a model of acute and chronic liver injury in rats. TAA induced liver injury is a well established area of considerable pharmacological interest, since reactive oxygen species and free radicals generated in the microsomal drug oxidation participate in the mechanisms of cell death. TAA undergoes a two step bioactivation mediated by microsomal cytochrome P450 2E1 (CYP2E1) to thioacetamide sulfoxide (TASO) and further to thioacetamide S,S-dioxide (TASO$_2$). TASO$_2$, an unstable reactive metabolite, covalently binds to liver macromolecules is responsible for the changes of hepatocytes such as an increase in nuclear volume and enlargement of nucleoli, cell permeability changes, rise in intracellular concentration of Ca++, and effects on mitochondrial activity, which leads to cell death (Gupta and Dixit, 2009; Bautista et al., 2010).

In this study, administration of a single dose of TAA (100 mg/kg body weight) led to hepatic and renal damage, which has been proven by the significant difference in biochemical markers between the TAA control and normal control groups. The increase in the activities of AST, ALT, ALP and LDH in serum of toxic control rats might be due to the increased permeability of plasma membrane or cellular necrosis leading to leakage of the enzymes to the blood stream (Al-Attar, 2011). In pre-treated group of animals, a marked decrease in serum transaminases, ALP and LDH levels demonstrated the preventive effect of ACME in TAA
intoxication. In post-treatment model, administration of ACME at a dose of 250 mg/kg produced a better restoration of serum enzyme levels than 125 mg/kg. It evidently shows the dose dependent curative efficacy of the extract against TAA induced decrease of serum marker enzymes.

Generation of a large amount of ROS due to TAA can overwhelm the antioxidant defense mechanism and damage cellular ingredients such as lipids, proteins and DNA; this in turn can impair cellular structure and function. The intracellular antioxidant system comprises of different free radical scavenging antioxidant enzymes along with some non-enzyme antioxidants like GSH and other thiols. CAT, GST, GPx, and GR constitute the first line of cellular antioxidant defense enzymes. These cellular antioxidants play an important role in the elimination of free radicals. Moreover, there is an equilibrium exists between the production of free radicals the level of antioxidant enzymes under normal conditions. When excess free radicals are produced, this equilibrium is lost and consequently oxidative insult is established (Manna et al., 2007).

Glutathione detoxifies toxic metabolites of drugs, regulates gene expression, apoptosis and transmembrane transport of organic solutes and it is essential to maintain the reduced status of the cell/tissue (Lauterburg, 2002). In the present study, the elevated hepatic and renal GSH level observed in ACME treated rats could explain the dose dependent hepatoprotective action of the extract in both the treatment groups. Our findings also shows that treatment with ACME prior to TAA intoxication aided significantly ($p \leq 0.05$) to enhance the GST activity, a phase II enzyme. This was attributed to the decreased bioactivation of TAA caused by the ACME pre-treatment. In post-treatment rats also the GST level was significantly ($p$
lowered in TAA treated animals and upward reversal was observed after treatment with ACME and silymarin. GST offers protection against lipid peroxidation by promoting the conjugation of toxic electrophiles with GSH (Jakoby, 1988). GR is also essential for the maintenance of GSH levels in vivo (Carlberg and Mannervik, 1985). The significantly (p ≤ 0.05) elevated level of GR activity in the hepatic and renal tissues of pre and post-treatment groups shows the role of ACME to maintain the GSH level in these tissues. These results indicate that the protection afforded by ACME against TAA induced hepatotoxicity may be related to the increased cellular GSH content, the increased GST and GR activity. Further, GPx catalyzes the GSH dependant reduction of H₂O₂ and other peroxides and protects the organism from oxidative damage (Lauterburg, 2002). The significant (p ≤ 0.05) restoration of GPx activity after pre and post-treatment with ACME and silymarin might be due to the antioxidant activity by detoxifying the endogenous metabolic peroxides generated after TAA injury in hepatic and renal tissues.

Catalase is responsible for the breakdown of H₂O₂, an important ROS, formed during the reaction catalyzed by SOD (Ramanathan et al., 2002). Reduced activity of catalase after exposure to TAA in the present finding could be correlated to increased generation of H₂O₂. The pre-treatment and post-treatment of ACME significantly (p ≤ 0.05) aided to maintain the CAT activity near to normal level in both hepatic and renal tissues. This evidently shows the antioxidant property of the extract against oxygen free radicals.

MDA is a major oxidation product of peroxidized polyunsaturated fatty acids and increased MDA content is an important indicator of lipid peroxidation (Freeman and Crapo, 1981). In the present study, treatment of rats with ACME protected the
liver and kidney from the increase of MDA levels in pre and post-treatment groups. This evidently demonstrates the antilipid peroxidative effect of the extract. The increased MDA content might have resulted from an increase of ROS as a result of stress condition in the rats with TAA intoxication.

The hepatoprotective effect of ACME was also evidenced by the histological examination of liver sections. In both pre-treatment and post-treatment experimental model, the recovery towards normalization of histological architecture by ACME treatment was almost similar to that of silymarin. Indeed, there was a remarkable reduction in centrilobular necrosis, bridging hepatic necrosis and lymphocyte infiltration in rats treated with ACME compared to TAA control group. The results of serum biochemical parameters, level of hepatic and renal lipid peroxides, glutathione antioxidant systems, CAT and histopathological studies in the pre-treatment and post-treatment groups together support the dose dependent antioxidant and hepatoprotective activity of ACME.

LC-MS analysis of ACME already revealed the presence of many phytochemicals with potent antioxidant activities (Results are given in chapter 3A, section 3A.3.3. LC-MS analysis of ACME). The reported antioxidant activities of these phytochemicals were established by various in vitro and/or in vivo studies. It includes Cinnamaldehyde (Gowder and Devaraj, 2006), Ferulic acid (Kim et al., 2011b), Quercetin (Li et al., 2013), Quercetagetin (Gong et al., 2012), 1-Caffeoyl-β-D-glucose (Du et al., 2006), Triacontanol (Ramanarayan et al., 2000) and Asiatic acid (Ma et al., 2009). Hence, in the present study, the antioxidant and hepatoprotective activity exhibited by ACME might be attributed to the presence of the identified class of phytochemicals. The possible mechanism behind the
antioxidant and hepatoprotective property of ACME may be associated with stimulation of antioxidant defense system against the free radicals generated by TAA or by the inhibition of cytochrome-P450 enzyme system responsible for the generation of the toxic free radicals from these chemicals. The results do suggest that *A. campanulatus* tuber might ameliorate oxidative damage induced by free radicals and this can be employed as main ingredient in medical food/nutraceuticals for disorders due to oxidative stress. Nevertheless, the present study supports the traditional use of *A. campanulatus* tuber as liver tonic (Das et al., 2009b).