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Research Article

PROTECTIVE EFFECT OF *PARACALYX SCARIOSA* (ROXB.) ALI AGAINST ETHANOL INDUCED HEPATIC TOXICITY IN BRL 3A CELL LINES

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ABSTRACT
Paracalyx scariosa (Roxb.) Ali. is a woody twiners belongs to the family Fabaceae. The use of plant has been mentioned in folk medicine to treat liver disorders. The review has been reported the presence of flavonoids like quercetin, rutin and kaempferol etc. There are scientific proofs available regarding the hepatoprotective effect of these flavonoids. However, no scientific record is available for the hepatoprotective activity of *Paracalyx scariosa* (Roxb.) Ali. Hence the present study was designed to evaluate the protective activity of *Paracalyx scariosa* (Roxb.) Ali against ethanol induced toxicity. Protective effect of extracts (methanol, benzene and acetone) and fractions (ethyl acetate, aqueous) of methanol was tested against ethanol induced toxicity in BRL 3A cell lines. The degree of hepatoprotection was determined by measuring cell viability, cytotoxicity marker LDH and hepatic marker enzymes AST, ALT and ALP. Silymarin was used as the standard drug for comparison. The extracts and fractions significantly reduced the ethanol induced cytotoxicity and elevated the reduced levels of LDH, AST, ALT, and ALP after treatment. The results were analyzed by one way ANOVA followed by Dunnett's test. The present study suggests that the extracts (methanol, benzene and acetone) and fractions (ethyl acetate, aqueous) of *Paracalyx scariosa* (Roxb.) Ali. possess significant hepatoprotective activity which was found to be dose dependent in BRL 3A cell lines.
Keywords: *Paracalyx scariosa* (Roxb.) Ali; Hepatoprotective; Ethanol; BRL 3A; *In vitro*

INTRODUCTION

The liver occupies a vital role in the main functions of the organism. It is particularly susceptible to chemically induced injury due to its extensive metabolic capacity and cellular heterogeneity. Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) formation and scavenging by antioxidants. Excess generation of ROS can cause oxidative damage to bio molecules resulting in lipid peroxidation, mutagenesis and carcinogenesis[1]. *In vitro* liver systems represent a better experimental approach to screen potential hepatotoxic compounds and to investigate the mechanism by which chemicals induce liver lesions[2]. Liver cell lines are characterized by unlimited sub cultivation and cell availability in large number[3]. The BRL 3A cell line is an epithelial cell lines from buffalo rat liver which is able to divide in the absence of serum. Alcohol abuse, alcohol intolerance, alcohol dependence and other alcohol related disabilities are some of the most challenging public health problems. The biochemical, pharmacogenetic and pathological disturbances observed in humans after acute and chronic intake of alcohol are exceedingly complex and varied. Chronic alcohol abuse provokes successive hepatic changes consisting of hepatic steatosis, fibrosis, alcoholic hepatitis and cirrhosis. However the exact mechanism by which alcohol causes liver damage remains obscure and controversial and also factors involved are not yet fully understood[4]. One of the long term goals of developing a natural hepatoprotective agent is to use it for prevention and treatment of alcohol liver diseases. Hepatoprotectives are a class of therapeutic agents that includes synthetic as well as natural product which offer protection to liver from damage or help in regeneration of hepatic cells. Medicinal herbs are significant source of hepatoprotective drugs. It has been reported that about 170 phytoconstituents isolated from 110 plants belonging to 55 families do possess hepatoprotective activity[5]. Only a small portion of hepatoprotective plants as well as formulations used in traditional medicine are pharmacologically evaluated for its efficacy[6]. *Paracalyx scariosa* (Roxb.) Ali. also called as *Paracalyx scariosus* and *Cylista scariosa* is a woody twiners belongs to the family Fabaceae, is one such medicinal plant reported to cure liver disorders is not evaluated pharmacologically. In this contest an attempt is made to screen the hepatoprotective activity of aerial parts of *Paracalyx scariosa* (Roxb.) Ali.

MATERIALS AND METHODS

Plant collection, preparation of extracts and fractions

The fresh areal parts including leaves, flowers and stem were collected from Shimoga district of Gajanuru of Karnataka state, India. The plant was authenticated in Botanical Survey of India, Coimbatore, India. (BSI/SRC/5/23/2010-Tech. 1616). The air dried plant material was coarsely powdered and extracted with methanol in soxhlet extractor with 100 g for 48 h and the solution was evaporated to dryness under reduced pressure and controlled temperature by using rotary flash evaporator. The dried methanol extract was dissolved in 30 ml of distilled water and partitioned with double the volume of ethyl acetate successively for 3 times (60 ml x 3) in a separating funnel. The ethyl acetate soluble fraction and aqueous soluble fractions were collected and concentrated in vacuum using rotary flash evaporator. The solvent was removed completely over the water bath and finally desiccator dried. Similarly, the dried areal parts were extracted with two different solvents, acetone and benzene with 100 g each for 48 h. The benzene extract was macerated with 150 ml of methanol and filtered and concentrated and the solvent was removed completely. The extracts and fractions so obtained were used for the study[7].

Hepatoprotective study of *Paracalyx scariosa* (Roxb.) Ali in BRL3A cell line

Chemicals

3-(4, 5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. Tris base and antibiotics from Hi-Media Laboratories Ltd., Mumbai, India. Dimethyl Sulfoxide (DMSO), ethanol and Propanol from E.Merck Ltd., Mumbai, India.

Cell lines and Culture medium

BRL3A (Rat, Liver cell line) cell culture was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of BRL3A were cultured in DMEM supplemented with 10 % inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5 % CO_2 at 37 C until confluent. The cells were dissociated with Trypsin Phosphate Versene Glucose (TPVG) solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2 % inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of Cell viability by MTT Assay

The MTT assay was performed according to the method of Francis *et al*[8]. The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM medium containing 10 % FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37° C for 3 days in 5 % CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37° C in 5 % CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a micro plate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50 % (CTC_{50}) values is generated from the dose-response curves for each cell line.

% Growth Inhibition = 100 – {(Mean OD of individual test group ÷ Mean OD of control group) × 100}

Determination Hepatoprotective activity

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM medium containing 10 % FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium. 50 µl of DMEM with 100 mM ethanol and 50 µl of different non-toxic test concentrations of test drugs were added. The plates were then incubated at 37° C for 24 h in 5 % CO_2 atmosphere. After 24 h, the cell supernatants were collected separately, centrifuged and stored at -20 ^{0}C for biochemical estimation. 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5 % CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage cell viability was determined, based on which the percentage protection offered by test and standard drugs was calculated over the ethanol control[9].

% Protection over toxicant control = {(Mean OD of test group × 100 ÷ Mean OD of ethanol) - 100}

The experimental groups were carried out in quadruplicate as

- Group 1- Cells + media as a vehicle control
- Group 2- Cells + 150 mM ethanol for induction of toxicity as a negative control
- Group 3 – Cells + 150 mM ethanol + 62.5 µg/ml and 125 µg/ml of test drug samples individually (Benzene, Acetone, Ethyl acetate fraction)
- Group 4 – Cells + 150 mM ethanol + 250 µg/ml and 500 µg/ml of test drug samples individually (Methanol and Aqueous fraction)
- Group 5- Cells + 150 mM ethanol + 250 µg/ml of Silymarin as positive control

Biochemical parameters

The cell supernatants collected separately were centrifuged and stored at -20°c for the estimation of biochemical parameters. Liver function parameters aspartate amino transaminase (AST) and alanine amino transaminase (AST), according to Reitman *et al*[10], alkaline phosphatase (ALP) activity according to Bessaey *et al*[11] and lactate dehydrogenase (LDH) assay according to the procedures recommended by commercial kit (ABANOVA) were studied.

Statistical Analysis

Linear regression analysis was used to calculate the IC_{50} values and biochemical parameters were analyzed by one way ANOVA fallowed by Dunnett's test

***P < 0.001, **P < 0.01, *P < 0.05

RESULTS

MTT Assay for cell viability determination

The IC_{50} values of Benzene, Acetone, methanol extracts, ethyl acetate fraction and aqueous fraction were found to be 170 µg/ml, 155 µg/ml, 650 µg/ml, 145 µg/ml and 800 µg/ml respectively. Taking these values into consideration two working concentrations 62.5, 125 µg/ml was chosen for benzene extract, acetone extract and ethyl acetate fraction. For methanol extract and aqueous fraction 500 and 250 µg/ml were chosen as the working concentrations. A standard working concentration 250 µg/ml was chosen for positive control drug Silymarin and 150 mM concentration of ethanol was chosen for induction of hepatotoxicity. Figure 1 shows the % growth inhibition of extracts and fractions

Hepatoprotective activity of *Paracalyx scariosa* (Roxb.) Ali. in BRL 3A cell lines

Ethyl acetate fraction was found to have more percentage of hepatoprotection (97.7), followed by aqueous fraction (96.1), methanol (95.3), acetone (72.1) and benzene (55.4) at their respective higher concentrations. Whereas the standard drug Silymarin has shown 71.3 % hepatoprotection over toxic control. In lower concentrations aqueous fraction (84.9), has shown the highest percentage hepatoprotection followed by ethyl acetate (83.3), methanol (82.9), acetone (58.9) and benzene (37.6) respectively. Overall the test samples have shown percentage hepatoprotection dose dependently [Figure 2]

Estimation of hepatoprotective parameters (AST, ALT, ALP)

Ethanol treated group significantly increased the AST, ALT, ALP levels ($P < 0.001$) compared to the cell control. Silymarin in 250 µg/ml, Acetone, Benzene Extracts in 125 µg/ml methanol in 500 µg/ml, ethyl acetate in 125 µg/ml and aqueous fraction in 500 µg/ml significantly reduced the AST, ALT, ALP levels ($P < 0.001$) [Figure 3,4,5] compared to the ethanol induced group. Overall the test samples have significantly reduced the elevated AST, ALT, ALP levels in a dose dependent manner.

Estimation of Lactose Dehydrogenase (LDH)

Ethanol treated group has significantly ($P < 0.001$) increased the LDH levels. Ethyl acetate fraction at 125 µg/ml, Silymarin, aqueous fraction at 500 µg/ml, methanol at 500 µg/ml, acetone at 125 µg/ml and benzene at 125 µg/ml has significantly reduced the LDH levels ($P < 0.001$) [Figure 6] compared to the ethanol treated group. Overall the test samples have significantly reduced the LDH levels in a dose dependent manner.

DISCUSSION

In the present study the ethanol treated group has shown a significant decrease in the cell viability comparing with the cell control indicating the toxic effect. Cell viability depends directly on the structure of the membrane and the damage to the cellular membrane can be detected by enzyme leakage. The increased ROS produced due to ethanol toxicity might have formed adducts with nucleic acids leading to cell membrane damage, thereby increasing the leakage of enzymes. The presence of enzymes outside the cell represents damage to the hepatic cell. Ethanol treatment has significantly reduced the cell viability as well as increased the leakage of LDH and the liver function enzymes such as ALT, AST, and ALP. Treatment with Benzene, Acetone extracts, methanolic extract and its fractions of *Paracalyx scariosa* (Roxb) Ali. caused a dose dependent increase in the percentage of cytoprotection and dose dependent decrease in the LDH levels. At the same time they have significantly reduced the elevated hepatic marker enzyme (AST, ALT, ALP) levels in the medium indicating the hepatoprotective action which may be a consequence of membrane stabilization as well as free radical scavenging effect of flavonoids present in the plant extracts. The flavonoids may have interrupted the reaction of ROS with cellular proteins and nucleic acids and preventing the formation of adducts by acting as scavengers there by stabilizing the cell membrane resulting in decreased leakage of hepatic biomarkers. Results have shown that the test samples have shown a dose dependent hepatoprotection. The obtained results are in harmony with those reported by Kamel *et al*[12], Santhosh *et al*[13], Bhagyashree *et al*[14].

CONCLUSION

It is evident from the present study that *Paracalyx scariosa* (Roxb) Ali could be a potential source of natural hepatoprotective that could have great importance as therapeutic agent in preventing or slowing the hepatic disorders. Of all the 3 extracts and 2 fractions studied, methanol extract and its ethyl acetate, aqueous fractions are found to have more prominent hepatoprotective effect. Further work regarding isolation of bioactive compounds responsible for this potent activity and in vivo studies will provide more insight about the role of plant.

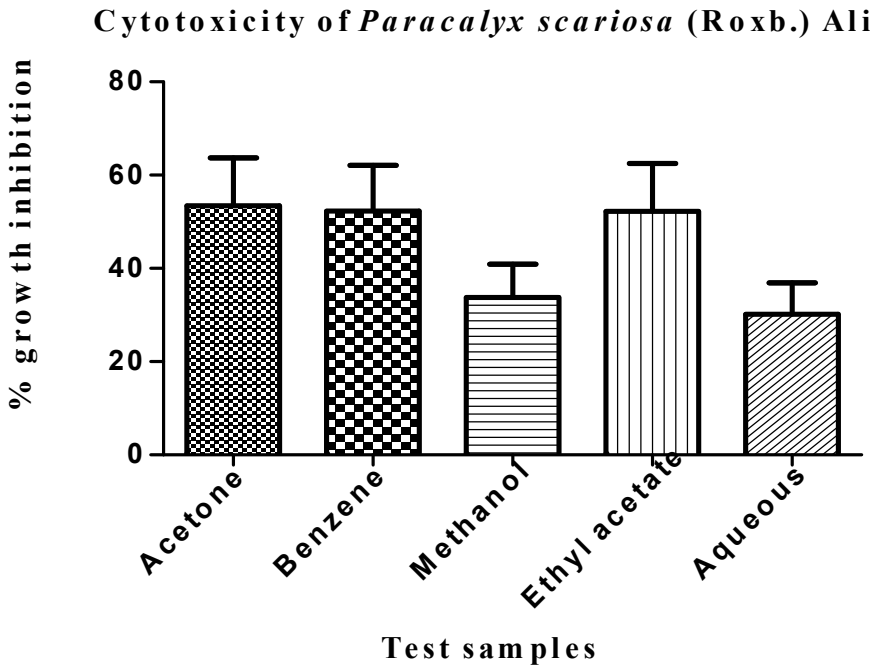

Figure 1: Cytotoxicity of *Paracalyx scariosa* (Roxb.) Ali

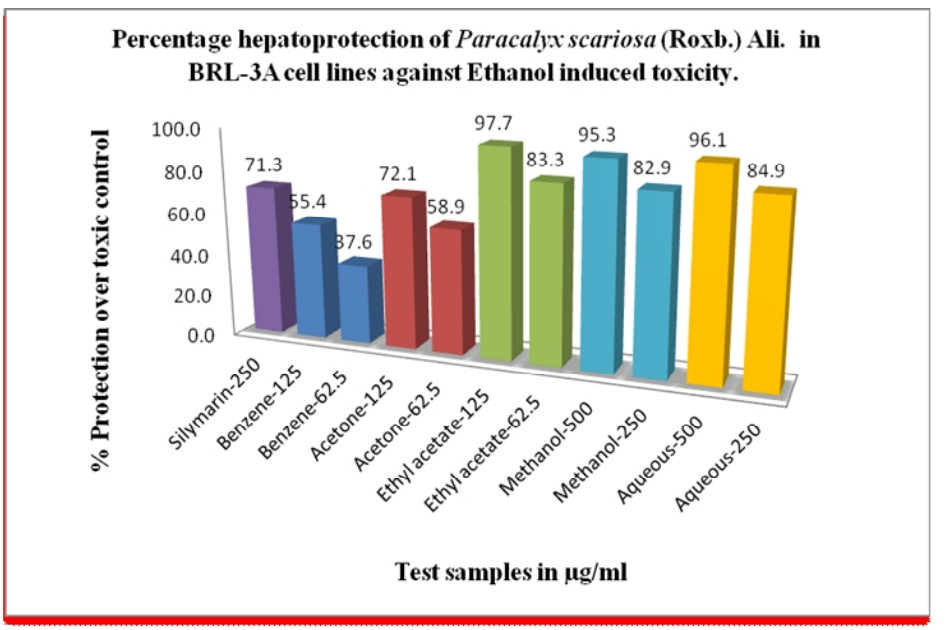

Figure 2: Percentage hepatoprotection offered by *Paracalyx scariosa* (Roxb.) Ali over toxic control

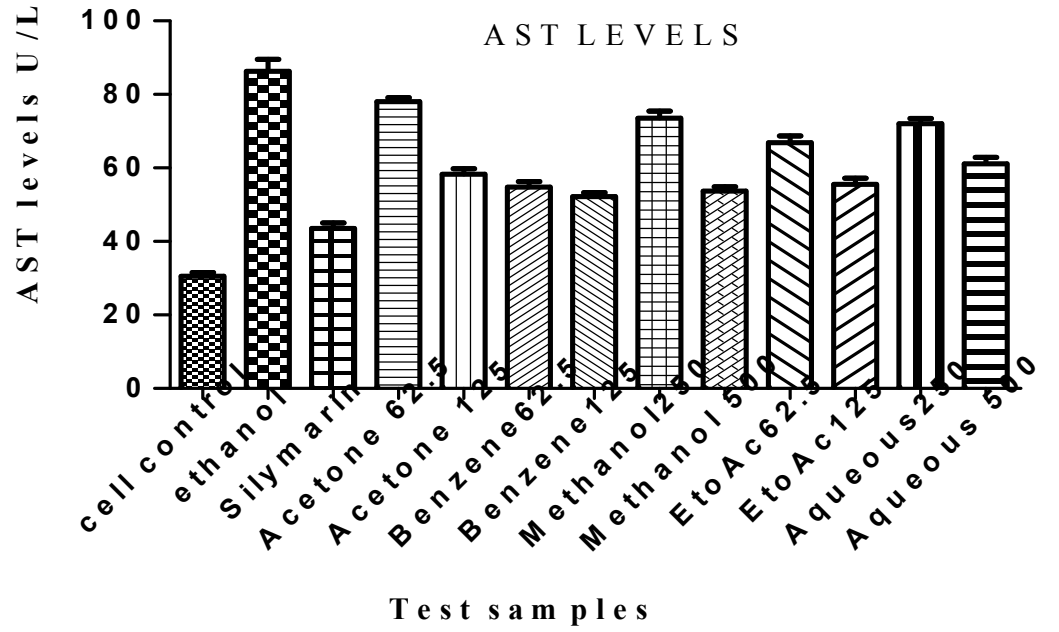

Figure 3: Effect of *Paracalyx scariosa* (Roxb.) Ali on AST levels

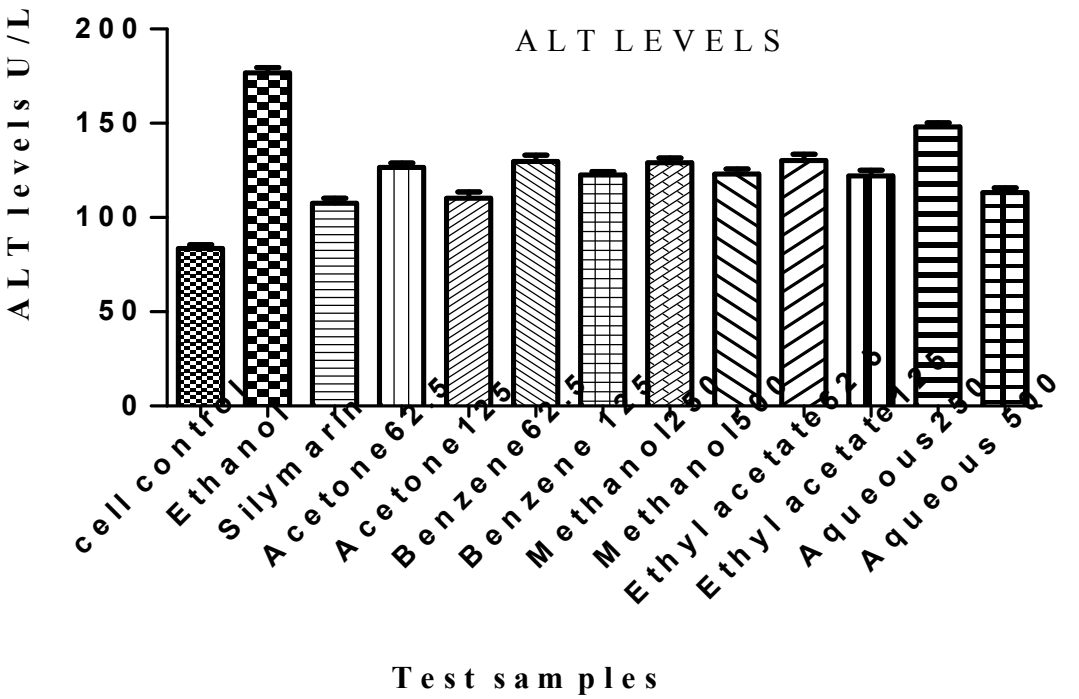

Figure 4: Effect of *Paracalyx scariosa* (Roxb.) Ali on ALT levels

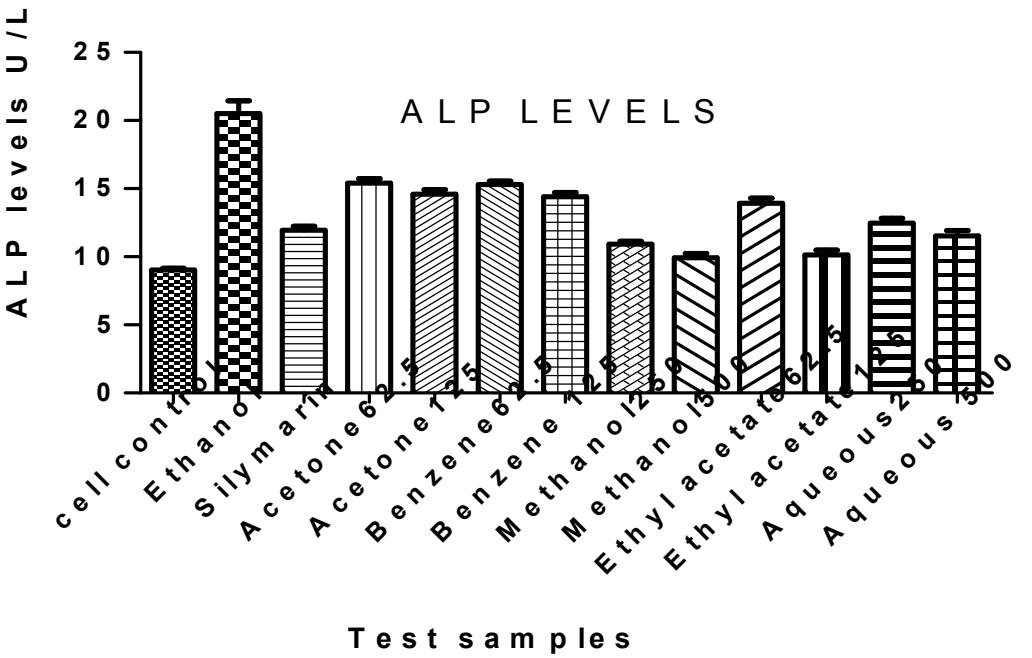

Figure 5: Effect of *Paracalyx scariosa* (Roxb.) Ali on ALP levels

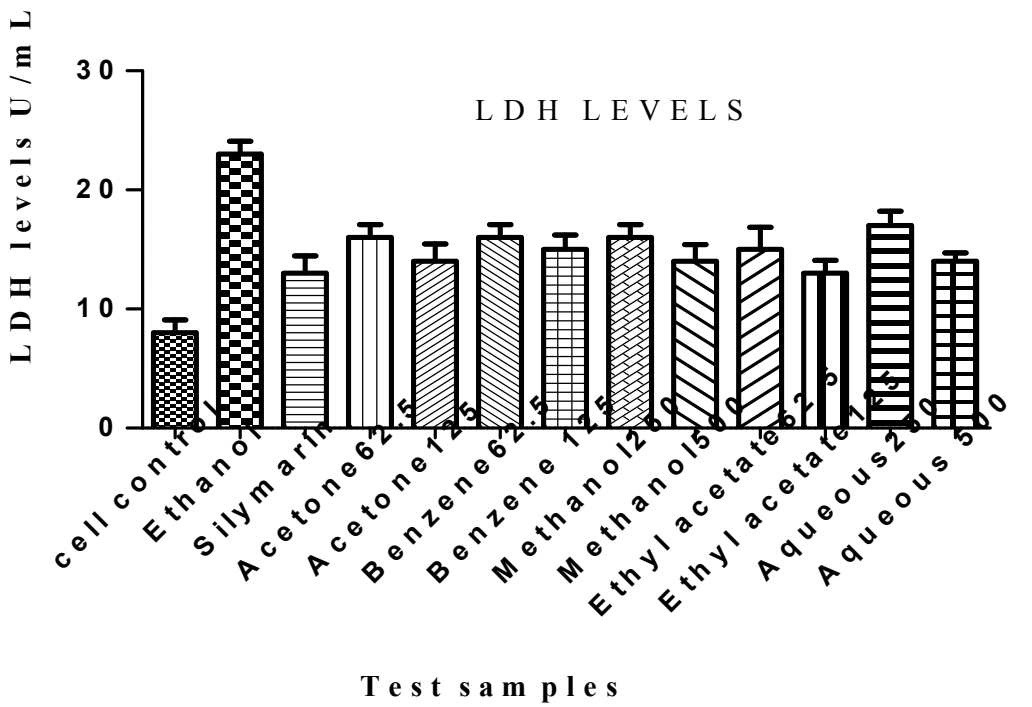

Figure 6: Effect of *Paracalyx scariosa* (Roxb.) Ali on LDH levels

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