Bulletin of Pure and Applied Sciences
Zoology (Animal Science), Vol.43B, No.1s,
January-June 2024: P.711-717

Print version ISSN 0970 0765
Online version ISSN 2320 3188

Original Research Article *Content Available online at: https://www.bpasjournals.com/*

Identification of Napthoquinone from *Rhinacanthus nasutus* against Alcohol Liver Disease

[*1]Nissar Ahmad Reshi., [1] Manasi Vinay Damale., [1] Razia Ahmad Kutty

[1]Department of Biological Sciences, School of Science, Sandip University Nashik, India

***Corresponding Author**
Dr. Nissar Ahmad Reshi
Associate Professor, Department of Biological Sciences
School of Science, Sandip University Nashik
[*]Email: nissarreshi@gmail.com

ABSTRACT:
In the current study, leaf and leaf derived callus extracts of *R. nasutus* were evaluated *in vitro* for their hepatoprotective potential against alcohol induced liver necrosis using HepG2 cells. Callus from leaf explants was induced on Murashige and Skoog (MS) medium fortified with various plant growth hormones. Dry leaf callus and coarse leaf powder were sequentially extracted in various organic solvents using Soxhlet apparatus. Crude callus and leaf extracts were subjected to toxic dose study prior to their determination of hepatoprotective property. MTT assay was used to determine the *in vitro* hepatoprotective potential of extracts, which was measured by the percentage of cell viability using 96 well plate method. Prior to alcohol intoxication, HepG2 cells were pretreated for 72 hrs with different individual extracts. Result confirmed that cells pretreated with ethanolic leaf extract (200 ug / ml) exhibited 90% viability and the cells pretreated with ethanolic callus extract showed 84% viability after intoxication with alcohol. The standard drug used in the study was silymarin, which showed the 81% cell viability after alcohol intoxication. The phytochemical analysis of leaf ethanolic extract was carried out by HPLC, LCMS, IR and NMR. The spectral data analysed confirmed the presence of predominant naphthoquinone; rhinacanthin - C.

Keywords:
Alcohol Live Necrosis, Hepatoprotection, HepG2 cell line, MTT assay, rhinacanthin - C.

How to cite this article: Nissar Ahmad Reshi., Manasi Vinay Damale., Razia Ahmad Kutty (2024). Identification of Napthoquinone from Rhinacanthus nasutus against Alcohol Liver Disease. *Bulletin of Pure and Applied Sciences-Zoology*, 43B (1s), 711-717.

Introduction

As per the WHO 2014 report, alcohol consumption claims approximately 3.3 million lives annually (18). The consumption of alcohol beyond a border line is considered a grave healthcare global problem with deleterious economic, clinical and social consequences. Alcohol consumption over a long period nearly takes a toll on almost every internal body organ.

However, the earliest and highest degree of damage occurs in liver tissues, because it being the primary alcohol metabolism site (6). The excessive ROS production due to alcohol consumption deteriorates the hepatic cells, which eventually leads to liver dysfunction. Diseases associated with alcohol consumption like cirrhosis, alcoholic hepatitis and steatosis are attributed to the continuous surge in the prolonged alcohol consumption (1). Number of adverse metabolic changes are induced in liver by alcohol. Earlier studies on HepG2 cells have revealed that alcohol is apoptotic and cytotoxic in nature predominantly in liver (19). The increasing mortality rate associated with Alcohol consumption has forced pharmaceutical industries to dig deep to find the novel and effective drugs to combat the alcohol liver necrosis and associated ailments.

Plant based medicine has always been first choice of a man to alienate the human ailments. India is blessed with almost 8000 medicinal plant species comprising nearly 50% of higher flowering plants and has a rich history of using plants as medicine. Since last few decades, there has been a surge in the interest of identification and characterisation of biologically active secondary metabolites of medicinal plants. Secondary metabolites are considered as medicinally potent molecules with complex structures and have been employed to combat many diseases. As per the previous reports, about 170 phytoconstituents from various plant families have been reported to have hepatoprotective potential (10).

It has been reported that about 170 phytoconstituents isolated from 110 plants belonging to 55 families possess hepatoprotective activity (10). In the present investigation, we attempted to investigate the potential of *Rhinacanthus nasutus*, an ethnomedicinal herb for its hepatoprotective property using HepG2 cells and its phytochemical analysis to identify the biologically active compounds. *R. nasutus* is a bushy herb, belongs to the family Acanthaceae. It has a history of being used in traditional and folk medicine for various human ailments. Traditionally, the plant has been used to treat hepatitis, diabetes, herpes and several other fungal skin diseases (8).

Materials and Methods

R. nasutus was collected from Karnataka Western Ghats India and maintained in Departmental medicinal plant garden. Young leaf explants were collected and surface sterilised as described by Reshi et al 2017 (12) and inoculated on solid MS medium supplemented with various growth regulators. The cultures were left for incubation for callus induction and proliferation. The proliferated callus was harvested after 4 - 6 weeks, oven dried at 60 ^{0}C and stored for further use. Dry leaf and callus were coarse powdered and sequentially extracted in various organic solvents (Petroleum ether, chloroform, Ethyl Acetate, Methanol, Ethanol) using Soxhlet apparatus (16). Aqueous extraction of leaf and callus was separately carried out as described in earlier studies (16). Anti-liver necrosis evaluation of leaf and callus extracts was carried out using HEpG2 cells. All the leaf and callus extracts and silymarin (standard drug) were analysed for toxic dose concentration by MTT method as described by (2). IC_{50} value of alcohol was determined as described earlier (10).

Anti-liver necrosis potential of extracts was determined by a method described earlier by (11). Briefly, cells were transferred (after subculture and trypsinisation) to 96 well (1 x 10^5 cells in each well). To allow the formation for monolayer of cells in each individual well, plates were incubated for 72 hrs. Each well containing HepG2 cell monolayer was treated with DMEM (200 µl), which contained leaf and callus extracts. Control plates were maintained with silymarin as standard drug. Followed by the treatment of extracts, (after 72 hrs) the extract treated wells containing HepG2 cells were intoxicated with alcohol (150 mM). After 72 hrs of alcohol treatment, the cell viability was determined by MMT assay. The plates were treated with DMEM medium containing 5 % MTT reagent and allowed to incubate for 3 hrs.

followed by incubation, the pates were treated with solubilisation solution of 1 % DMSO. The plates were lightly shaken on shaker till the solubilisation of formazan (2). The absorbance of plates was measured by microplate reader to determine the grow inhibition percentage using a formula:

$$\left\{\frac{\text{Mean OD of the individual test group}}{\text{Mean OD of control group}}\right\} \text{ x } 100$$

Percentage growth inhibition =

Ethanolic leaf extract treated wells exhibited the maximum cell viability percentage. The said extract was investigated for the phytochemical composition to identify the biologically active compound. The crude ethanolic was purified by column fractionation, followed by spectral studies (HPLC, LCMS & NMR) for the characterisation of bioactive compound (5,15).

Results

Callus formation was induced in the 3rd week post inoculation. Varied callus induction percentage was reported with respect to the growth hormones used, with highest callus inducing percentage in MS medium supplemented with 2,4 - D**.** Toxic dose studies of callus extracts, leaf extracts and silymarin by MTT assay revealed that callus and leaf extracts, if used above the concentration of 175 µg/ml reduce the cell viability 35%**.** Silymarin was found toxic to cells if used above the concentration of 75 µg/ml**.** IC_{50} value of alcohol was found to be 150 mM.

The anti-liver necrosis potential of extracts (leaf and leaf callus) and silymarin was evaluated by MTT assay. HepG2 cells were treated with alcohol (ethanol intoxication, 150 mM) for 72 hrs. Prior to alcohol intoxication, cells were treated for 72 hrs with different leaf and callus extracts (150 µg/ml and 175 µg/ml). After the alcohol intoxication, the cell viability percentage of wells (containing extract pre – treated HepG2 cells) was determined by MTT assay. The result showed that cell viability is dose dependent and extract pretreated cells sustained the alcohol intoxication by showing the viability from 58% to 90%. Among the leaf extracts, ethanolic extract exhibited potential anti-necrosis potential. The ethanolic extract (leaf) pretreated cells (175 µg/ml) showed 90% cell viability after alcohol intoxication.

The anti-liver necrosis potential was also found in callus extracts. From callus extract pre-treatment cells, highest cell viability percentage was found in HepG2 cells which were treated with callus ethanolic extract (84%). Silymarin, a standard drug pretreated cells showed the viability of 81%. The cell viability percentage of cells (HepG2) pretreated with various extracts is given in Table 1 & 2.

Out of various extracts (leaf and leaf callus), ethanolic leaf extract treated cells (HepG2) exhibited maximum viability percentage of 90%. The potential bioactive ethanolic extract was investigated for its phytochemical profiling. Preliminary investigations revelated the presence of alkaloids, naphthaquinones, flavonoids, terpenoids and sterols. The column fractionation of ethanolic extract revealed four elutes and each elute upon evaluation of hepatoprotection exhibited the anti – liver necrosis. The elute with highest cell viability retention potential against alcohol intoxication was further subjected to spectral analysis to identify the potential hepatoprotective biomolecule. Ethanolic leaf extract, which exhibited the maximum cell viability percentage post alcoholic intoxication, was found to contain four elutes upon column fractionation. Individual column fractions were evaluated for their anti-liver necrosis potential. The potential bioactive fraction was further analyses for identification of biomolecule. Two major peaks were seen in HPLC, the dominating peak, with absorbance at 20 nm exhibited elution at 3.32 min. The LCMS (**Fig. 1)** confirms the mass of the compound to be 410.5026.

H-NMR (**Fig 2)** studies confirms the presence of 30 H^+ and C-NMR **(Fig. 3)** spectra dictates the presence of 25 carbons. The elementary spectral analysis suggests the presence of H (7.37%), C (73.15%) and O (19.49%), which further puts forth molecular formula as C25H30O5. The metabolomics reveals that the compound is napthoquinone, rhinacanthin – C.

Discussion

The current study has revealed the varying effect of auxins on the callus induction and proliferation. 2,4-D has been predominantly the only growth hormone (auxin) which has positively contributed to callus induction and proliferation. In the previous studies, many authors have also confirmed in their respective studies that out of the various auxins, 2,4-D helps to induce and proliferate the callus more in leaf explants **(13,07**).

Plant based drugs, crude or purified are subjected to cytotoxic studies to identify the level of toxic dose concentration, which forms the basis of drug development (4). In corroborations with the previous studies, toxic dose study of leaf and leaf callus extracts was carried out. In the present investigation, alcohol was used to intoxicate the HepG2 cells, which has deleterious effects on hepatic cells. Alcohol causes hepatic injury, necrosis, lesions accompanied by alcoholic cirrhosis, alcoholic hepatisis and fibrosis (9). In the current investigation, the HepG2 cells treated with leaf and leaf callus extracts for 48 – 72 hrs followed by alcohol intoxication sustained the necrosis by showing the cell viability percentage (MTT assay) of 90%. The MTT assay is based on the action of an enzyme, succinate dehydrogenase, present in the inner mitochondrial membrane of living cells. The enzyme cleaves the tetrazolium dye, therefore gives the confirmation of a viability of a cell.

The increase in the cell viability percentage upon their treatment with the extracts may be due the membrane stabilisation boosted by phytochemicals and strengthening the tissue antioxidant mechanism system to counter alcoholic oxidative stress **(4**). The dose dependent cell viability percentage has been previously confirmed in *Cassia roxburghii* (1), *Andrographis paniculata* (17) and *Rumex vesicarius* **(2**). *It* has been suggested from earlier studies that cell viability depends on membrane structure and any damage to it leads to the leakage of the enzyme which eventually leads to cell death (10). It is believed that increase in cell viability is attributed to the action of biologically active phytochemicals, which give a boost to structural organisation of a cell membrane and interrupt the reactions of ROS with cellular proteins, thereby preventing the oxidative damage caused by alcohol and stabilise the membrane. Similar inferences have been drawn by authors in earlier studies (14; 3; 11).

Conclusion

Production of ROS due to alcohol consumption leads to hepatic damage. The current study corroborates with earlier findings that plant secondary metabolites boots the cellular defence mechanism to combat the radical stress. The metabolomics revealed that napthoquinone has a strong potential to combat the alcoholic oxidative stress and therefore could be employed as tool to further strengthen the hepatic drugs. Further, callus cell lines may be identified and explored for the mass production of bioactive molecule, which therefore may arrest the threat posted to natural population of *R. nasutus.*

Acknowledgement

Authors are grateful to Department of Life Science and Microbiology, School of Science Sandip University Nashik, India for providing all the necessary facilities to carry out this research.

Conflict of Interest The authors do not have any conflict of interest.

References

1. Arulkumaran K, Rajasekaran A, Ramasamy R, Jegadeesan M, Kavimani S. Cassia roxburghii seeds protect liver against toxic effects of ethanol and carbon tetrachloride in rats. *International Journal of Pharma Tech Research.* 2009, 1, 273 - 246.
2. Asha T, Londonkar R, Nayaka H. Cytotoxicity and hepatoprotective attributes of methanolic extract of *Rumex vesicarius* L. *Biological Research*. 2015, 48, 1 - 9
3. Bhagyashree R, Sanjeevani H, Ujwala R. *In vitro* protection of hepatocytes by *Alocasia macrorrhiza* leaf juice against CCl4 and tylenol mediated hepatic injury. International Journal of Pharmaceutical Applications. 2011, 2, 122 - 128
4. Ibrahim M, Khaja N, Aara A, Khan A, Habeeb A, Devi P, et al. Hepatoprotective activity of *Sapindus mukorossi* and *Rheum emodi* extracts. *World Journal of Gastroenterology*. 2008, 14, 2566 - 2571.
5. Kalsi, P.S. Spectroscopy of organic compounds – 5th Edition, New Age and Allied (P) Ltd, Kolkata, India, pp. 2002, 719-724.
6. Lieber, C.S. Alcoholic liver disease: New insights in pathogenesis lead to new treatments. *Journal of Hepatology*. 2000, 32, 22, 113 - 128
7. Manickam V, Elangomadhavan R, Antonisamy R. Regeneration of Indian ginseng plantlets from stem callus. *Plant Cell Tissue and Organ Culture.* 2000, 14, 55 - 58
8. Muthukumaraswamy S., Mohan R.V., Kumaresan S., Chelladurai V. Herbal remedies of Palliyar tribe of Grizzled Giant Squirrel wildlife sanctuary, Western Ghats, Rivilliputhur, Tamil Nadu for poisonous bites. *Journal of Economic and Taxonomic Botany*. 2003, 27, 761-764
9. OL K. Alcoholic liver disease: Pathobiological aspects. *Journal of Hepatology*. 1995, 23, 7 - 15
10. Ramoji A., Kuppast I., Mankani K.L. Protective effect of *Paracalyx scariosa* against ethanol induced hepatic toxicity in BRL 3A cell lines. *International Research Journal of Pharmacy*. 2014, 5, 495 - 501.
11. Reshi N. A., Sudarshana M.S., Girish V.H. Evaluation of hepatoprotective potential of leaf and leaf callus extracts of *Anisochilus carnosus* (L) wall. *International Journal of Phytomedicine*. 2018, 10, 156 - 161
12. Reshi N.A, Sudarshana M.S, Girish H.V. *In Vitro* Micropropagation of *Anisochilus carnosus* (L) Wall. *J App Pharm Sci*. 2017, 7, 98-102.
13. Rout G.R., Saxena C, Samantaray C, Das P. Rapid plant regeneration from callus cultures of *Plumbago zeylanica*. *Plant Cell Tissue and Organ Culture*. 1999, 56, 47 - 51.
14. Santhosh S, Sini K, Anandan R, Mathew P. Hepatoprotective activity of chitosan against isonaizid and rifampicin induced toxicity in experimental rats. *European Journal of Pharmacology*. 2007, 57, 69 - 73
15. Sharma, M., Rahim, M. S., Kumar, P., Mishra, A., Sharma, H., & Roy, J. (2020). Large-scale identification and characterization of phenolic compounds and their marker–trait association in wheat. *Euphytica.* 2020, 216, 1 - 18.
16. Sheikh, H.M., Reshi, N.A. Characterisation of secondary metabolites from Rhinacanthus nasutus L (Kurz) for the identification of novel antibacterial leads. *Tropical Biomedicine.* 2020, 37, 812 - 821
17. Sivaraj A, Vinothkumar P, Sathiyaraj K, Sundaresan S, Devi K, Senthilkumar B. Hepatoprotective potential of *Andrographis paniculata* aqueous leaf extract on ethanol induced liver

toxicity in albino rats. *Journal of Applied Pharmaceutical Science*. 2011; 6, 24 - 28

18. World Health Organization (WHO). *Global Status Report on Alcohol and Health*. Geneva, WHO, 2014, Available at http://www.who.int/substance_abuse/publications/
19. Wu D, Caderbaum A. l. Ethanol induced apoptosis to stable HepG2 cell line expressing human cytochromeP-4502E1. Alcoholism: *Clinical and Experimental Research*. 1993, 23, 67 - 76.

Table 1: HepG2 cell viability post their treatment with extracts of leaf

Leaf Extracts	Concentration (µg/ml)	% Cell viability
Aqueous	150	77±0.32
	175	79±0.26
Ethanol	150	83±0.51
	175	90±0.56
Methanol	150	72±0.57
	175	76±0.38
Ethyl acetate	150	72±0.20
	175	74±0.35
Petroleum ether	150	66±0.31
	175	68±0.32
Chloroform	150	60±0.38
	175	60±0.42
Silymarin	75	81±0.53
Control	Only cells	100

*Assays carried in triplicates; value shows Mean±S.D

Table 2: HepG2 cell viability post their treatment with extracts of leaf callus

Callus Extracts	Concentration (µg/ml)	% Cell viability
Aqueous	150	70±0.67
	175	74±0.31
Ethanol	150	80±0.20
	175	80±0.43
Methanol	150	74±0.43
	175	77±0.21
Ethyl acetate	150	60±0.42
	175	62±0.32
Petroleum ether	150	62±0.45
	175	65±0.40
Chloroform	150	56±0.23
	175	58±0.45
Silymarin	75	81±0.53
Control	Only cells	100

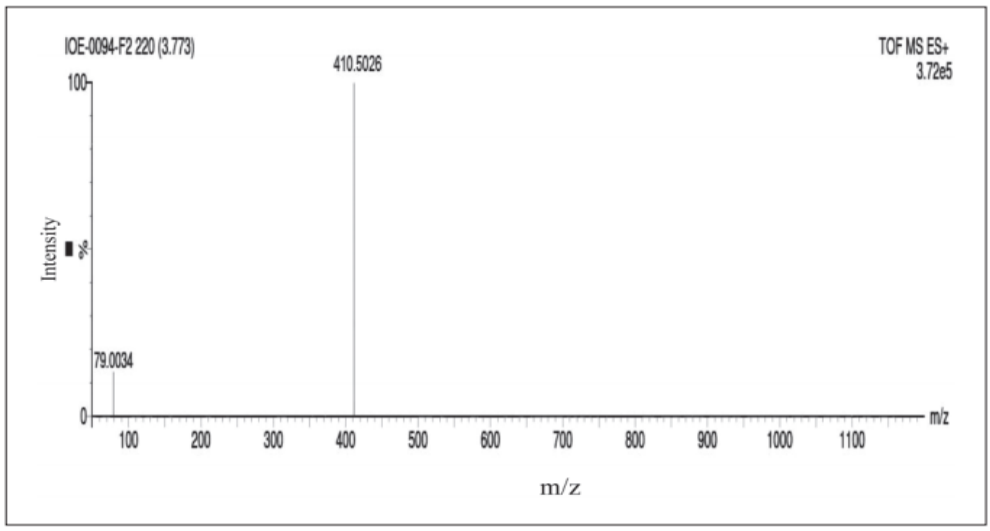

Fig. No. 1: LCMS spectra of Leaf extract (Ethanolic) of *R. nasutus*

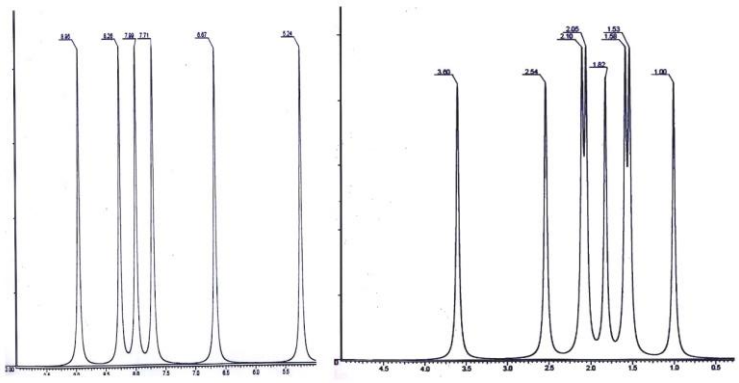

Fig. No.2: NMR (H) spectra of Leaf extract (Ethanolic) of *R. nasutus*

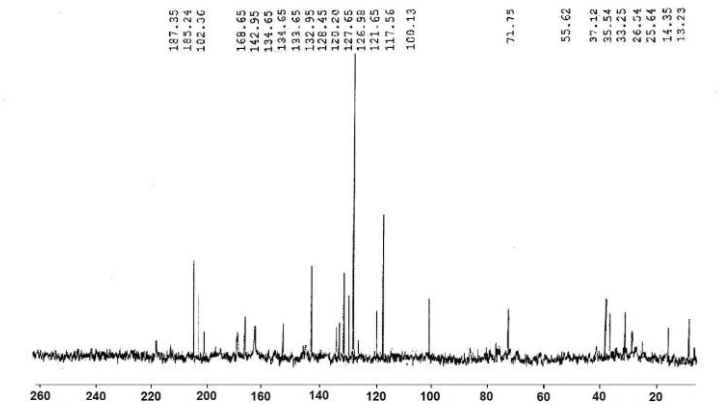

Fig. No. 3: NMR (C) spectra of Leaf extract (Ethanolic) of *R. nasutus*