Evaluation of Rasna panchaka (indigenous drug) as oxidative stress down-regulator using serum-free explant culture system

Manjula Talluri, Srinivasa Reddy Yathapu, Dinesh Kumar Bharatraj

Abstract:
CONTEXT: The importance of phytochemicals/natural products as potential therapeutic agents in the present context is gaining a lot of importance. India with a rich heritage of such preparations needs evaluation as potent drugs. Explant culture system is a method, which is sensitive, reliable, reproducible and is capable of mimicking the in situ conditions maintaining the tissue in sufficiently high level of integration.
AIM: The current study aimed to test the antioxidant activity of test compounds, namely, traditional aqueous (4212) and aqueous-methanolic (4308) extracts of Rasna panchaka using liver explant cultures.
MATERIALS AND METHODS: Dose-response optima of extracts (0.2–10 µg/mL) were determined using mouse liver explant culture system up to 48 h. The antioxidant property of extracts was assessed by primary oxidative defense parameters, namely, superoxide-dismutase (SOD), catalase, reduced glutathione (GSH), and malondialdehyde (MDA).
RESULTS: The results indicated that the cellular architecture of the cultured tissue was well conserved in the first 6 h with a gradual display of specific changes in the next 24 h. There was a significant increase in MDA levels in experimental groups indicating the oxidative stress induction in explants. A dose of 2.0 µg/mL extracts have shown statistically significant (P<0.05) protection against oxidative stress. MDA levels, a measure of lipid peroxidation, were significantly (P<0.01) reduced by 50% in extract treated explants compared to control. This effect was accompanied by the increase in the first defense enzymes SOD (50%) and catalase (18%) with no change in reduced GSH levels.
CONCLUSION: The study enforces the importance of “explant culture system,” as it not only reduces the use of nonclinical/animal model but also is rapid and sensitive. Further, results of the current study also suggest that aqueous-methanolic extract of Rasna panchaka is having superior antioxidant activity compared to traditional water extract.

Keywords: Liver explant culture, oxidative stress, Rasna panchaka

Introduction

The use of in vitro models in many areas of research has become essentiality owing to the research community’s desire to reduce traditional animal testing models.

Among the most recent advances in this field are those in vitro methods using human/animal tissues and cells, since these exhibit the greatest promise for modeling/comparing the interactions of chemicals with human systems.[1-3] These in vitro systems also enhance our understanding of the mechanisms of drug-induced and/or chemical-induced
efficacy or toxicity. In contrast, in vivo models are too complex to unveil the mechanistic details due to their structural and functional heterogeneity.

Among the various organ systems; the liver is the most elaborately evaluated organ since it is the major site of biotransformation of drugs. In addition, many studies indicate common use of hepatocyte-based systems as in vitro models for studying the hepatic function and other biochemical changes like oxidative stress. The monolayer and occasionally suspension cell cultures have been widely used to study many different aspects of liver function and to predict hepatotoxicity. More recently, there is a revival for explant model or tissue slice model which maintains intact three-dimensional tissue architecture (unlike monolayer cell culture systems) and which have recently proven to maintain differentiated hepatic function for several days in culture. The fundamental reason for their efficacy is the heterogeneity of the cell population maintained in their natural assemblage and juxtaposition.

We have optimized and validated traditional preparations used for the management of several chronic ailments like arthritis, asthma for their antioxidant activity and were successful in establishing the same in a battery of in vitro tests along with a pilot clinical study. The current study was aimed to ascertain the antioxidant activity of an Ayurvedic formulation “Rasna panchaka” using a serum-free explant culture system. The antioxidant properties of the extract (s) were evaluated using a battery of oxidative stress parameters such as reduced glutathione (GSH), malondialdehyde (MDA), superoxide-dismutase (SOD), and catalase.

Materials and Methods

The plants used for the study, namely, Pluchea lanceolata, Ricinus communis, Cedrus deodara, Tinospora cordifolia, and Zingiber officinale were procured from Government Ayurvedic College, Hyderabad, India. Plants were authenticated by authorized taxonomist (Heritage Bio-Natural Systems Pvt. Ltd., Hyderabad, India). Unless otherwise specified, all the fine chemicals, Medium 199 with HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) (serum-free), sterile disposable culture-ware were procured from Sigma-Aldrich Co., (Bengaluru, India). Other chemicals such as methanol, ethanol, formaldehyde, and xylene (AR grade) were purchased from standard deviation fine chemicals, India.

Plant extracts

Aqueous and aqueous-methanolic extracts of selected plants were prepared and coded as described previously by Parekh et al.. Briefly, various parts of the plants (P. lanceolata, R. communis, C. deodara, T. cordifolia, Z. officinale) were air-dried and pulverized into powder. Powders of all the plants were taken in equal ratios and separately imbibed overnight at room temperature either in aqueous or aqueous-methanol (50:50) mixture and were extracted subsequently. The aqueous extract was lyophilized to dryness and coded as 4212. The aqueous-methanol extract was initially subjected to remove methanol by evaporation using rotary evaporator (Buchi Rotavapor R-205), subsequently lyophilized and coded as 4308. The residues were stored in desiccated tight screw cap containers at 4°C. These residues were appropriately diluted with water on the day of the experiment.

Animals

Normal adult Swiss Albino male mice weighing 20–25 g were obtained from National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition, Hyderabad, India. Each animal was placed in a separate polypropylene cage fitted with a stainless wire bottom and top with a provision for pellet diet and water as ad libitum. Mice were maintained at 22°C ± 2°C, with 75 air changes per hour and a relative humidity of 54%–57% with a 12 h light/dark cycle. The animals were fed on standard sterile pellet diet supplied by NCLAS, Hyderabad containing the standard composition of all macro and micronutrients and access to purified water collected through activated charcoal filter and exposed to Ultraviolet rays. The study and procedures were approved by the “Institutional Animal Ethics Committee (IAEC)” of the National Institute of Nutrition, Hyderabad (IAEC/Proj-03/2004/4065).

Liver tissue collection and preparation of explant cultures

The process of liver tissue collection and preparation of explant cultures was followed as described previously by Sharma et al.. Briefly, mice were sacrificed by cervical dislocation followed by thorough washing with mild soap, cleaned with sterile water. Then, fur was shaven and wiped with 70% ethyl alcohol. The liver was excised aseptically in the laminar flow, placed in chilled “Medium 199” and explanted into 1–2 mm³ cubes (1–2 mg each). They were cultured in serum-free “Medium 199 with HEPES” (pH 7.4) at 37°C in humidified chamber containing 5% CO² (Thermo Electronic Corp., Model No. 3111). The pH of the medium was maintained at 7.4 by the buffering activity of HEPES, despite changes in carbon dioxide concentration. A fixed number of explants were placed into each petri-dish (60 mm) containing 5 ml of medium. Then, aqueous-methanolic plant extract–4308 (1–10 µg/mL) and traditional “water extract–4212” (2.0 µg/mL) were added to the explants to determine the effective dose by percentage normal cells with time up to 48 h. The explants were maintained in the
medium in the absence and presence of extract (4308) for a period of 2 h, 4 h, 8 h, 12 h, 24 h, and 48 h, respectively, to determine the “histopathological changes in the cells.” Each experiment was repeated thrice under identical conditions.

Histopathological procedures
The histopathological changes in the cultured liver explants were examined as described previously by Luna\textsuperscript{[16]} with slight modifications. Briefly, the cultured liver explants collected at various time points of incubation and at various concentrations of extract were fixed in 10% formaldehyde. They were dehydrated with increasing concentrations of ethanol followed by treatment with a mixture of absolute alcohol plus xylene and finally cleared with two changes of xylene alone. The tissues were then embedded in paraffin wax and 5 µm sections were cut on microtome (Leica RM 2125) followed by staining with hematoxylin and eosin for evaluating cytoarchitectural features using Nikon light microscope (Model No. Eclipse E800) and photographed with a 35-mm camera. To determine the degree of change, a minimum of 500 cells were counted and classified as normal, pyknotic, faint and enlarged based on their structure, nuclear membrane integrity, and optimal staining.

Quantification of oxidative/antioxidant status
Postmitochondrial fractions (PMF) were prepared from liver explants to determine the oxidative/antioxidant activity as described previously by Lodish et al.\textsuperscript{[16]} Liver explants were incubated in the medium in the presence (2.0 µ/mL) and absence of the extracts (4308 and 4212) for the specified time period of 2 h and 4 h. Subsequently, they were sonicated in chilled 1.15% KCl to prepare 10% homogenate solution and centrifuged at × 1000g for 20 min at 4°C. The supernatant obtained was further centrifuged at × 12000g for 20 min at 4°C to separate the PMF. PMF was then evaluated for oxidative/antioxidant status by correlating it to the measured oxidative/anti-oxidant status, namely, GSH, MDA, SOD, Catalase as described previously.

Statistical analysis
The mean and SD/standard error of the mean were calculated for all the variables. Between groups, statistical analysis was performed using one-way analysis of variance and pairwise significances by least significant difference test. Where ever heterogeneity in the data of a particular group was observed, nonparametric, Kruskal-Wallis was performed to identify the difference at 95% confidence interval (P < 0.05). The results were considered statistically significant at P < 0.05. The data were analyzed using SPSS version 15.0 for Windows (Chicago, IL, USA).

Results
Effect of varying doses of extracts
An effect of aqueous-methanolic extract (4308) with a dose range of 0.2–10.0 µg/mL and traditional water extract 4212 (2.0 µg/mL) on liver explants cells with time are given in Figure 1. The percentage of normal cells was decreasing with time up to 48 h. A concentration of 2.0 µg/mL of both the extracts have shown normal cells as compared to controls, whereas other concentrations have shown detrimental effect on explants. In addition, the highest concentration (10.0 µg/dL) of extract-4308 seems to be toxic to the explants cells [Figure 1]. In view of this further studies were carried out with 2.0 µg/mL of the either of the extracts.

Cellular architecture
Histologically, the cultured tissue harvested at various time points clearly indicated that the cellular architecture of the tissue was well conserved during the first 6 h with a gradual display of specific changes subsequently in the next 24 h [Figure 2]. Liver explant at 0 h of incubation in the medium indicated the presence of >85% of normal nuclei. The nuclear profile and cellular morphology were intact up to 4 h among the experimental group (the treated group) compared to control [Figure 2b, c, f and g]. The cytosol was also stained uniformly displaying no visible sign of any distress. At 8 h, the extract treated group revealed nuclear loosening without hampering of the cytosolic composition [Figure 2h]. Conversely, the control group did not display a visible sign of any ill effect [Figure 2d]. At the end of 24 h, the drug-treated group demonstrated lesser nuclear integrity as compared to the corresponding control [Figure 2e and i].

Oxidative/anti-oxidant status
The PMF MDA levels, a measure of lipid peroxidation, were significantly (P < 0.01) increased in explants alone (untreated) compared to the fresh liver [Figure 3].
The explants incubated with extracts shown less degree of lipid peroxidation as evidenced from statistically significant \( (P < 0.01) \) reduction in MDA levels, i.e., by 50%. Damage reversal was more significant at 4 h time point bringing down the MDA levels to fresh liver levels [Figure 3].

SOD levels were increased in explants alone (untreated) compared to fresh liver SOD levels [Figure 4]. Further, the levels of SOD was significantly \( (P < 0.01) \) improved by 50% in the extract treated (both 4212 and 4308) explants at 2 h compared to control [Figure 4]. Whereas, the SOD levels at 4 h was reduced to normal in the explants treated with aqueous extracts compared to control. Nonetheless, no much change in SOD levels of aqueous-methanolic extract treated group was noted as compared to 2 h time point [Figure 4].

The catalase levels were also increased by 25% and 20%, respectively, for aqueous-methanolic and aqueous extracts at 2 h period as compared to respective controls (untreated) [Figure 5]. Whereas, at 4 h time point the levels of catalase have come down to normal levels of control explants. Although a significant difference was observed at all-time points (0, 2, and 4 h) between treatments of both extracts, a similar pattern of raise and fall of catalase was maintained throughout the experimental phase. Furthermore, it was found that there was no change in the reduced GSH levels for both the extracts at both time points [Figure 6].

**Discussion**

There is an increase in usage of *in vitro* systems to overcome the constraints in animal testing. Explant culture model that maintains intact three-dimensional tissue architecture (unlike monolayer cell culture systems) for biochemical functions is of recent interest in evaluating the drugs. Antioxidant properties of various traditional medicines have been well reported and characterized in *in vitro* systems and *in vivo* systems.\(^{17,18}\) The *in vitro* tests/assay data alone may not be comparable to biological responses and at the same time *in vivo* tests are time-consuming. We selected to evaluate an indigenous drug (*Rasna panchaka*) to unveil its oxidative/anti-oxidant effect using mouse liver explants, which biochemical functions are very much similar to *in vivo* conditions.\(^{14,19}\)

In the present communication, we have adapted and modified the model developed by Jat *et al.*\(^{20}\) where conditions are created by slicing tissue to undergo
physical stress, thereby creating stress-induced oxidative damage. This condition is evident from the significant increase of MDA levels. This system successfully maintains the integrity and cytoarchitecture of the liver explant close to normal. These explants maintained a normal and stable condition at the normal growth temperature of 37°C as evidenced by histopathological analysis. One interesting feature of the entire series of histological observations has been the optimal maintenance of the normal tissue in the medium which is a noteworthy fact establishing the validity of the current assay system. It further establishes the importance of such systems where the effects can be studied with no additional problems.

At longer time points, the explants are not immortal and the percentage of normal nuclei decreased significantly. The characteristics of the explants were dramatically influenced by the concentration of the extract added. High concentrations resulted in apparently transformed cells with no nuclei or shrunken nuclei. The present system has been standardized that at a concentration of 2 µg/ml of extract and at a time interval of 2–4 h liver explants are similar to in situ condition.

To evaluate oxidative biochemical changes in the PMF obtained from these explants after incubating with the extract was assessed for antioxidant enzymes that play a role in the first stage of the defense mechanism, namely, SOD, catalase, and GSH peroxidase. SOD reacts with superoxide to form hydrogen peroxide and then water before the free radical can cause damage to cellular structures. The reactive oxygen-hydrogen peroxide is then counteracted by the enzyme catalase to dissociate into the water. Both SOD and catalase enzymes show increased levels in reaction to injury and this response is clearly evident from our study.

It is also evident that explants incubated with extracts had better levels of the defense enzymes and also exhibited reduced lipid peroxide levels. The extracts are enhancing/triggering the release of defense enzymes. It was found that the levels of reduced GSH were not changed by the addition of extracts. In contrast, the extracts have shown a significant increase in SOD and CAT levels suggesting the action of extracts in down regulating the oxidative stress.

Andrés and Cascales have shown that the presence of Vitamin E, a strong antioxidant, in culture media reduces oxidative stress through the inhibition of lipid peroxidation and an increase of catalase, SOD, and GPx activity. There were also other studies showing antioxidant properties of various plants. We also observed similar changes in the first-line defense mechanism, strengthening the hypothesis of antioxidant activity of test compounds.

### Conclusion

The present investigation concludes that the aqueous and aqueous-methanolic extracts of *Rasna panchaka* possess
potential antioxidant activity as tested by explant culture system. An important application of this novel technique is the preliminary screening of xenobiotics for potential pharmacological effects. The current study also enforces that this novel approach gives scope for rapid screening of drugs of therapeutic activity. This system is a sensitive and reliable method and can, therefore, help in reducing the in vivo animal/human experiments.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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