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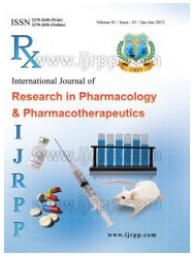

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Estimation of *Tecoma stans* as oxidative stress down-regulator via serum-free explants culture

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ABSTRACT

In the current sense, phytochemicals/natural products are gaining a lot of popularity as potential therapeutic agents. India, which has a long history of such preparations, needs to be assessed as potent products. Explant culture system is a process that is adaptive, repeatable, and capable of simulating in situ conditions while maintaining a sufficiently high degree of tissue integration. Using liver explants cultures, researchers tested the antioxidant activity of test compounds, namely standard aqueous (4212) and aqueous-methanolic (4308) extracts of Tecoma stans. The dose-response maxima of extracts (0.2–10 g/mL) were calculated over a 48-hour span using a mouse liver explants culture system. Key oxidative protection parameters, such as superoxide-dismutase (SOD), catalase, reduced glutathione (GSH), and malondialdehyde, were used to evaluate the antioxidant properties of extracts (MDA). The results showed that the cultured tissue's cellular architecture was well conserved in the first 6 hours, accompanied by a steady display of specific changes over the next 24 hours. MDA levels in experimental groups increased significantly, suggesting oxidative stress induction in explants. A dosage of 2.0 g/mL extracts provided statistically significant (P 0.05) oxidative stress safety. When extract treated explants were compared to control, MDA levels, a measure of lipid peroxidation, were significantly (P 0.01) reduced by 50%. With no improvement in GSH levels, this effect was followed by a 50% rise in the first defensive enzymes SOD (50%) and catalase (18%). The study illustrates the benefit of an "explants culture method," which not only eliminates the use of nonclinical/animal models but is also simple and responsive. The current study's findings also indicate that the aqueous-methanolic extract of *Tecoma stans* has higher antioxidant activity than the typical water extract.

Keywords: Oxidative stress, *Tecoma stans,* Liver explants culture,catalase,oxidative stress.

INTRODUCTION

Because the research community wants to reduce the use of traditional animal testing models, in vitro models have become essential in many areas of research. In vitro methods using human/animal tissues and cells are among the most recent developments in this area, as they show the most potential for modeling and comparing chemical interactions with human systems. [1-3] These in vitro systems also help us learn more about the mechanisms of drug and chemical-induced efficacy and toxicity. In vivo models, on the other hand, are too complicated to reveal mechanistic details due to structural and functional heterogeneity.

The liver is the most thoroughly studied of the various organ systems because it is the primary site of drug biotransformation. Furthermore, many studies show that hepatocyte-based systems are commonly used as in vitro models for studying hepatic function and other biochemical changes such as oxidative stress. [4, 5] Monolayer and suspension cell cultures have been widely used to investigate many aspects of liver function and to predict hepatotoxicity. [6] Explant models or tissue slices, which maintain intact three-dimensional tissue architecture (unlike monolayer cell culture systems) and have recently proven to maintain differentiated hepatic function for several days in culture, have recently seen resurgence. [9] The heterogeneity of the cell population maintained in their natural assemblage and juxtaposition is the fundamental reason for their efficacy. We successfully optimized and validated traditional preparations used for the management of several chronic ailments such as arthritis and asthma for their antioxidant activity in a battery of in vitro tests as well as a pilot clinical study. [8] The current study used a serum-free explants culture system to determine the antioxidant activity of an Ayurvedic formulation called Tecoma stans. A battery of oxidative stress parameters, including reduced glutathione (GSH), [9] malondialdehyde (MDA), [10] superoxide-dismutase (SOD), [11]and catalase, were used to assess the antioxidant properties of the extract (s). [12]

MATERIALS AND METHODS

Tecoma stans, the plant used in the research, was obtained from Government Ayurvedic College in Hyderabad, India. An authorized taxonomist confirmed the plant's authenticity (Heritage Bio-Natural Systems Pvt. Ltd., Hyderabad, India). All fine chemicals, Medium 199 with HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) (serum-free), and sterile disposable culture-ware were obtained from Sigma-Aldrich Co., unless otherwise stated (Bangalore, India). Other chemicals were purchased from standard deviation fine chemicals in India, including methanol, ethanol, formaldehyde, and xylene (AR grade).

Plant extracts

Tecoma stans aqueous and aqueous-methanolic extracts were prepared and coded. The plant's different parts were air-dried and ground into powder. The plant powder was taken and soaked overnight at room temperature in either aqueous or aqueous-methanol (50:50) mixtures before being removed. 4212 was assigned to the aqueous extract after it was lyophilized to dryness. Initially, the aqueous-methanol extract was evaporated to isolate the methanol using a rotary evaporator (Buchi Rotavapor R-205), then lyophilized and coded as 4308. At 4°C, the residues were held in desiccated tight-screw-cap containers. On the day of the experiment, these residues were correctly diluted with water.

Animals

The National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition, Hyderabad, India, given regular adult Swiss Albino male mice weighing 20–25 g. Each animal was housed in its own polypropylene cage with a stainless steel bottom and top, as well as access to a pellet diet and unlimited water. Mice were held at a constant temperature of 22°C 2°C, with 75 air changes per hour and a relative humidity of 54–57 percent, with a 12 hour light/dark period. The animals were fed a standard sterile pellet diet provided by NCLAS, Hyderabad, which contained the standard composition of both macro and micronutrients, as well as filtered water collected via an activated charcoal filter and exposed to UV rays.

Liver tissue collection and preparation of explants cultures

Sharma et al. had previously identified the procedure for collecting liver tissue and preparing explants cultures. [13 and 14]Mice were sacrificed by cervical dislocation and thorough washing with mild soap before being washed with sterile water. After that, the fur was shaved and wiped with 70% ethyl alcohol. The liver was explanted into 1–2 mm3 cubes (1–2 mg each) aseptically in the laminar flow, and then put in chilled "Medium 199." They were cultured at 37°C in a humidified chamber containing 5% CO2 in serum-free “Medium 199 with HEPES” (pH 7.4). (Thermo Electronic Corp., Model No. 3111). Despite shifts in carbon dioxide concentration, the buffering activity of HEPES kept the pH of the medium at 7.4. Every petri-dish (60 mm) containing 5 ml of medium had a fixed number of explants [12-15]. The explants were then treated with aqueous-methanolic plant extract-4308 (1–10 g/mL) and standard “water extract –4212” (2.0 g/mL) to assess the effective dose by percentage normal cells over a 48-hour period. To assess the “histopathological

changes in the cells," the explants were held in the medium for 2 hours, 4 hours, 8 hours, 12 hours, 24 hours, and 48 hours, respectively, in the absence and presence of extract (4308). Each experiment was carried out three times under the same conditions.

Histopathological procedures

The histopathological changes in the cultured liver explants were investigated as previously mentioned by Luna[15], with minor changes. Briefly, cultured liver explants were fixed in 10% formaldehyde at different time points during incubation and at various concentrations of extract. They were dehydrated with the concentrations of ethanol, then treated with a mixture of absolute alcohol and xylene, and eventually cleared with two xylene-only adjustments. After embedding the tissues in paraffin wax, 5 m parts were cut on a microtome (Leica RM 2125), stained with hematoxylin and eosin, and photographed with a 35-mm camera to test cytoarchitectural features using a Nikon light microscope (Model No. Eclipse E800). A minimum of 500 cells were counted and graded as regular, pyknotic, faint, or expanded based on their structure, nuclear membrane integrity, and optimal staining to determine the degree of change.

Quantification of oxidative/antioxidant status

As mentioned previously by Lodish et al., [16]postmitochondrial fractions (PMF) were prepared from liver explants to evaluate oxidative/antioxidant activity. Liver explants were incubated in the medium for 2 hours and 4 hours in the presence (2.0/mL) and absence (4308 and 4212) of the extracts. They were then sonicated in chilled 1.15 percent KCl to make a 10% homogenate solution, which was centrifuged at 1000g for 20 minutes at 4°C. To isolate the PMF, the supernatant was centrifuged at 12000g for 20 minutes at 4°C. The tissue's responsiveness to exogenous extract was then assessed by correlating PMF to the measured oxidative/anti-oxidant status, namely GSH, MDA, SOD, and Catalase, as previously mentioned.

STATISTICAL ANALYSIS

For each variable, the mean and SD/standard error of the mean were determined. One-way analysis of variance was used between groups, and pair wise significances were determined using the least significant difference test. Wherever there was heterogeneity in a group's results, a nonparametric Kruskal-Wallis test was used to find the difference at a 95 percent confidence interval (P 0.05). At P 0.05, the findings were found statistically important. SPSS version 15.0 for Windows was used to analyse the results (Chicago, IL, USA)

RESULTS

Effect of varying doses of extracts

[Figure 1] shows the effect of aqueous-methanolic extract (4308) at doses ranging from 0.2 to 10.0 g/mL and conventional water extract 4212 (2.0 g/mL) on liver explants cells over time. Up to 48 hours, the percentage of normal cells was decreasing. Both extract at a concentration of 2.0 g/mL showed normal cells as compared to controls, whereas other concentrations had a negative effect on explants. Furthermore, the highest concentration of extract-4308 (10.0 g/dL) appears to be toxic to explants cells [Figure 1]. As a result, additional experiments were performed using 2.0 g/mL of each of the extracts.

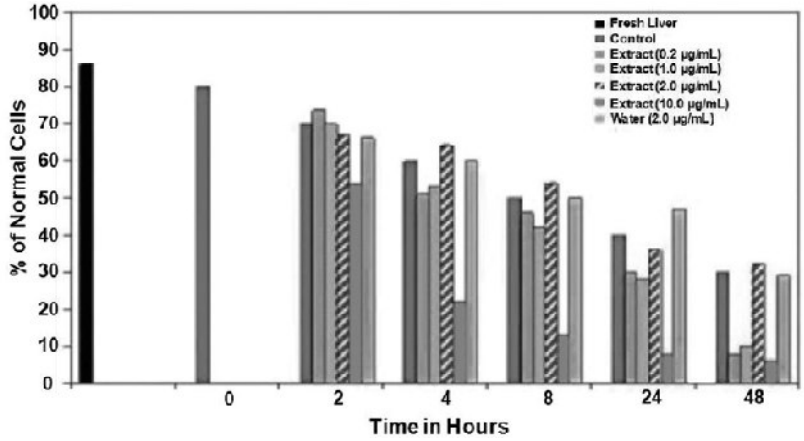

Figure 1: Percentage of normal cells with time and dose. The bars represent mean of percentage of normal cells in triplicate samples with various concentrations of extract of 4308 (0.2–10.0 µg/mL) and traditional water extract –4212 (2.0 µg/dL) up to 48 h. The percentage of normal cells was decreasing with time.

Cellular architecture

The cultured tissue collected at different time points histologically revealed that the tissue's cellular architecture was well conserved within the first 6 hours, with a gradual showing of specific changes in the next 24 hours [Figure 2]. At 0 hours of incubation in the medium, a liver explants revealed the presence of >85% of normal nuclei. When compared to control [Figure 2] b, [Figure 2] c, [Figure 2] f, and [Figure 2] g, the experimental group (the treated group) retained their nuclear profile and cellular morphology for up to 4 hours. The cytosol was also evenly stained, with no apparent signs of distress. The extract-treated group showed nuclear loosening without a change in cytosolic composition after 8 hours [Figure 2]h. The control group, on the other hand, showed no clear signs of any detrimental effects [Figure 2]d. As compared to the corresponding control [Figure 2]e and [Figure 2]i, the drug-treated community had less nuclear integrity after 24 hours.

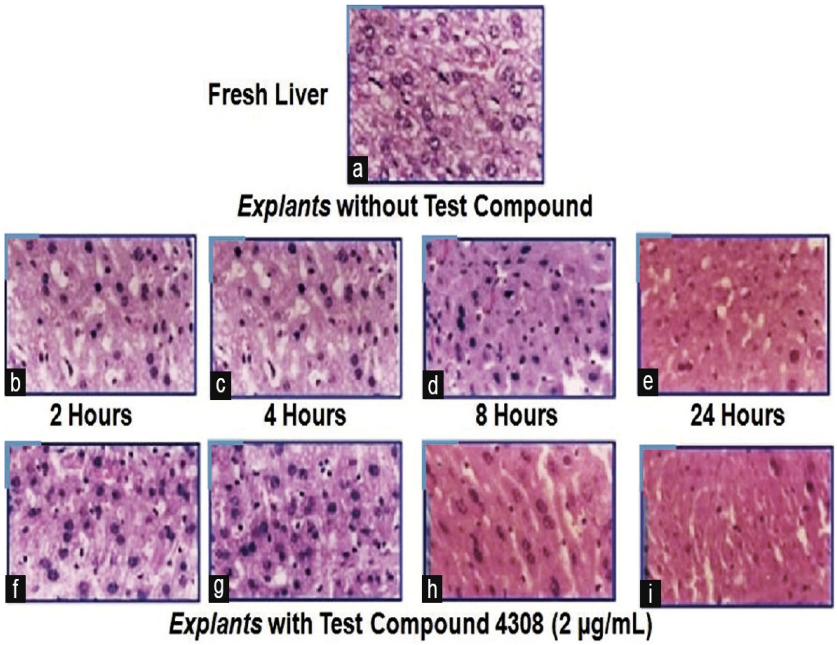

Figure 2: Cellular architecture of tissues with time and dose of test compound 4308. The photographs. (a) fresh liver. (b-e) explants incubated without extracts of test compound for 2, 4, 8, and 24 h, (f-i) explants incubated with test compound 4308 (2 µg/ml) for 2, 4, 8, and 24 h. Cellular architecture of tissue was well conserved during the first 4 h (b, c, f and g) with gradual changes subsequently in the next 24 h.

Oxidative/anti-oxidant status

In explants alone (untreated), PMF MDA levels, a measure of lipid peroxidation, were significantly (P 0.01) higher than in fresh liver [Figure 3]. Explants incubated with extracts displayed less lipid peroxidation, as demonstrated by a 50% reduction in MDA levels, which was statistically significant (P 0.01). Damage reversal was more pronounced at the 4 h time point, with MDA levels returning to fresh liver levels [Figure 3].

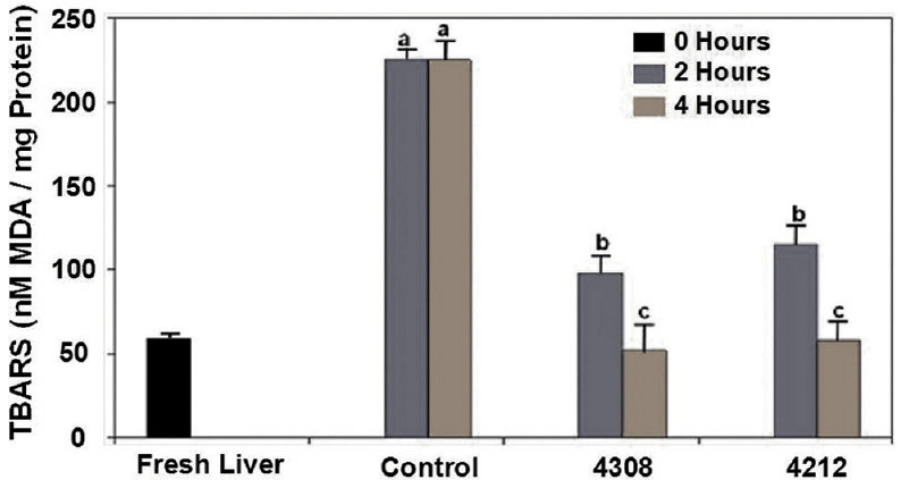

Figure 3: Levels of thiobarbituric acid reactive species in explants with and without test compounds. Bars represent the mean ± standard deviation of thiobarbituric acid reactive species in triplicates. The levels were significantly (P < 0.01) reduced in the presence of both the test compound extracts (4308 and 4212) with a concentration of 2.0 µg/mL. Different superscripts are significantly different.

Explants alone (untreated) had higher SOD levels than fresh liver SOD levels [Figure 4]. Furthermore, at 2 h, the levels of SOD in the extract handled (both 4212 and 4308) explants were significantly (P 0.01) higher than in the control [Figure 4]. When compared to control, SOD levels in explants treated with aqueous extracts were reduced to normal after 4 hours. Nonetheless, as compared to the 2 h time point, there was no substantial improvement in SOD levels in the aqueous-methanolic extract treated group [Figure 4].

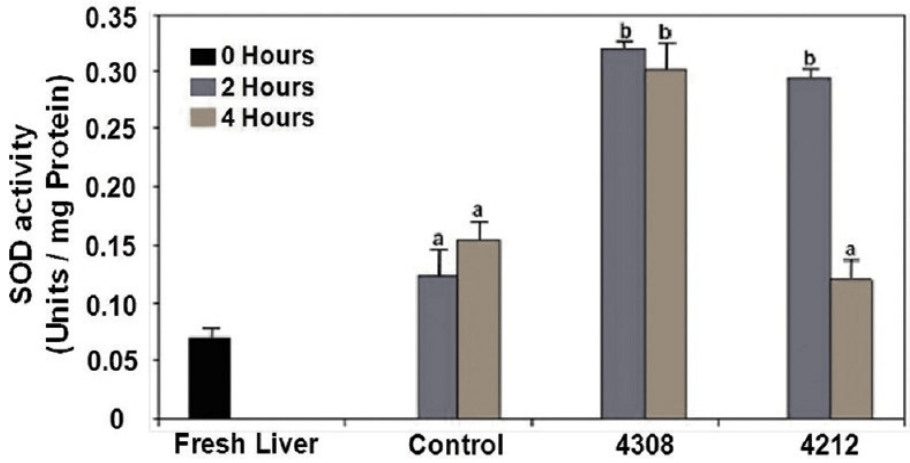

Figure 4: Levels of superoxide dismutase in explants with and without test compounds. Bars represent mean ± standard deviation superoxide dismutase levels of triplicate samples. The levels were significantly (*P* < 0.01) increased in the presence of both the test compound extracts (4308 and 4212) with a concentration of 2.0 µg/mL at 2.0 h. The 4 h superoxide dismutase levels were retained in the presence of 4308, whereas reduced to normal levels at 4 h in the presence of 4212. Different superscripts are significantly different.

At 2 h, catalase levels in aqueous-methanolic and aqueous extracts were increased by 25% and 20%, respectively, as compared to respective controls (untreated) [Figure 5]. At the 4 h time point, the levels of catalase had returned to normal in the control explants. Although there was a substantial difference between treatments of both extracts at all time points (0, 2, and 4 h), a similar pattern of catalase rise and fall was preserved during the experimental period. Furthermore, at both time points, there was no difference in the reduced GSH levels for both extracts [Figure 6].

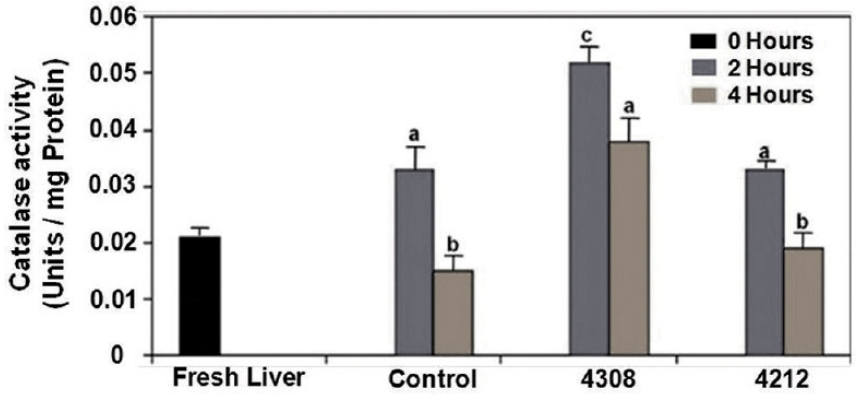

Figure 5: Levels of catalase activity in explants with and without test compounds. Bars represent the mean ± standard deviation of catalase levels of triplicate samples. The levels were significantly (P < 0.05) increased in the presence of test compound 4308 at both 2 and 4 h compared to control. No significant difference was noted with extract 4212 at 2 and 4 h as compared to control. Different superscripts are significantly different.

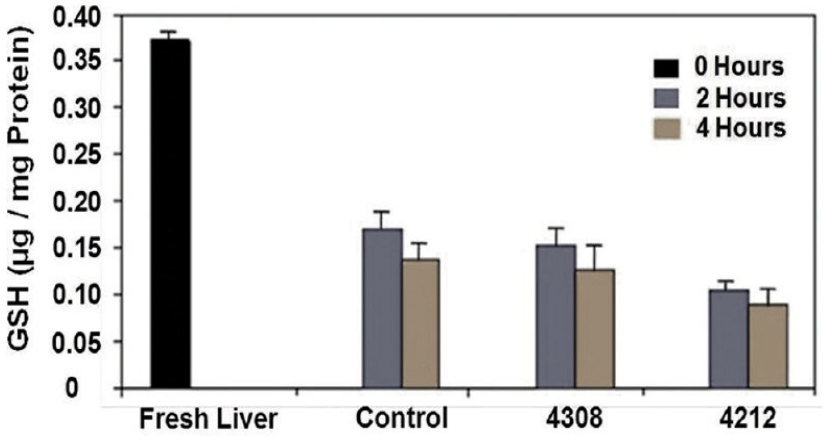

Figure 6: Levels of reduced glutathione in explants with and without test compounds. Bars represent the mean ± standard deviation of reduced glutathione levels of triplicate samples. The levels were comparable between test compound 4308 and control at 2 and 4 h. The glutathione levels were decreased in the presence of extract 4212 at 2 and 4 h as compared to control.

DISCUSSION

In vitro systems are becoming more common as a way to get around the limitations of animal testing. Explant culture model for biochemical functions that preserves intact three-dimensional tissue architecture (unlike monolayer cell culture systems) is of recent interest in drug evaluation. Antioxidant properties of various conventional medicines have been well documented in both in vitro and in vivo studies. [17, 18] The results of in vitro experiments/assays may not be equivalent to biological responses, and in vivo tests are time consuming. We chose to test Tecoma stans for its oxidative/anti-oxidant activity in mouse liver explants, which have biochemical functions that are very close to those found in vivo. [14, 19] We have adapted and modified the model developed by Jat et al.[20], in which conditions are created by slicing tissue to cause physical stress, resulting in stress-induced oxidative damage. The large rise in MDA levels reveals this state. This device effectively holds the liver explant's integrity and cytoarchitecture close to normal. Histopathological research revealed that these explants remained normal and stable at the normal growth temperature of 37°C. The optimal preservation of normal tissue in the medium, which is a noteworthy fact establishing the validity of the current assay system, has been one of the most interesting features of the entire series of histological observations. It also stresses the importance of a method that allows the results to be observed without creating any unnecessary issues.

Explants are not immortal at longer time points, and the percentage of normal nuclei decreases significantly.

The concentration of the extract added had a significant impact on the characteristics of the explants. High concentrations resulted in cells that appeared to have been transformed, with no nuclei or nuclei that had shrunk. The current system has been standardized to produce liver explants that are similar to in situ conditions at a concentration of 2 g/ml of extract and a time interval of 2–4 h. Antioxidant enzymes that play a role in the first stage of the defense mechanism, such as SOD, catalase, and GSH peroxidase, were assessed for oxidative biochemical changes in the PMF obtained from these explants after incubation with the extract. [21] a Before the free radical can cause damage to cellular structures, SOD reacts with superoxide to produce hydrogen peroxide, followed by water and oxygen. The enzyme catalase then reacts with the reactive oxygen-hydrogen peroxide, causing it to dissociate into water. In response to injury, both SOD and catalase enzyme levels rise, as evidenced by our research. It was also clear that explants incubated with extracts had higher levels of defense enzymes and lower levels of lipid peroxide. The extracts stimulate/induce the release of defense enzymes. The addition of extracts had no effect on the levels of reduced GSH, according to the findings. The extracts, on the other hand, showed a significant increase in SOD and CAT levels, indicating that they are effective in reducing oxidative stress. The presence of Vitamin E, a powerful antioxidant, in culture media reduces oxidative stress by inhibiting lipid peroxidation and increasing catalase, SOD, and GPx activity, according to Andrés and Cascales[22]. Other studies revealed that various plants have antioxidant properties. [23, 24] Similar changes in the first-line defense mechanism were also observed, bolstering the hypothesis of test compound antioxidant activity.

CONCLUSION

According to the results of this study, Tecoma stans aqueous and aqueous-methanolic extracts have potential antioxidant activity when tested using an explants culture system. The preliminary screening of xenobiotics for potential pharmacological effects is an important application of this novel technique. The current study also establishes that this novel approach allows for the rapid screening of therapeutic drugs. Because this system is sensitive and reliable, it can help to reduce the number of in vivo animal/human experiments.

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