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Protective Mechanisms of *Moringa oleifera* against CCl_4-Induced Oxidative Stress in Precision-Cut Liver Slices

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Keywords
Moringa oleifera · Free radicals · In vitro · Carbon tetrachloride · Lipid peroxidation · Oxidative stress

Summary
Objective: The present study was designed to evaluate the efficacy of *Moringa oleifera* leaves against carbon tetrachloride (CCl_4)-treated liver slices in vitro. **Material and Methods:** The study evaluated the antioxidant properties of *Moringa oleifera* leaves against CCl_4-induced oxidative damage in liver slices. **Results:** CCl_4 treatment significantly decreased the activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione S-transferase and caused decreased glutathione content and increased the thiobarbituric acid-reacting substances (TBARS). Treatment with *Moringa oleifera* extract increased the activities of antioxidant enzymes and glutathione content and reduced the levels of TBARS significantly. Observed reduction in the level of lipid peroxides showed a decreased tendency of peroxidative damage. **Discussion and Conclusion:** We conclude that, under these experimental conditions, the leaf extracts effectively suppress CCl_4-induced oxidative stress. Our findings provide evidence to demonstrate that the possible mechanism of this activity may be due to the strong antioxidant property of the leaves.

Schlüsselwörter
Moringa oleifera · Freie Radikale · In vitro · Karbontetrachlorid · Lipidperoxidation · Oxidativer Stress

Zusammenfassung
Ziel: Die vorliegende Studie hatte zum Ziel, die Wirksamkeit von *Moringa-oleifera*-Blättern auf mit Karbontetrachlorid (CCl_4) vorbehandelte Leberschnitte in vitro zu untersuchen. **Material und Methoden:** Die Studie evaluierte die antioxidativen Eigenschaften von *Moringa-oleifera*-Blättern gegen CCl_4-induzierte oxidative Schäden an Leberschnitten. **Ergebnisse:** Die Behandlung mit CCl_4 führte zu einer signifikanten Abnahme der Aktivität antioxidativer Enzyme wie Superoxiddismutase, Katalase, Glutathionperoxidase, Glutathionreduktase und Glutathion-S-Transferase, verursachte einen reduzierten Glutathiongehalt und erhöhte die Thiobarbitursäure-reaktiven Substanzen (TBARS). Die Behandlung mit *Moringa-oleifera*-Extrakt erhöhte die Aktivität antioxidativer Enzyme und den Glutathiongehalt und führte zu einer signifikanten Reduktion der TBARS-Spiegel. Die beobachtete Reduktion des Lipidperoxidspiegels zeigte eine reduzierte Tendenz zu peroxidativen Schäden. **Diskussion und Schlussfolgerung:** Wir schlussfolgern, dass unter diesen experimentellen Bedingungen die Blattextrakte CCl_4-induzierten oxidativen Stress effektiv bekämpfen. Unsere Befunde sind ein Beleg für die Demonstration, dass der mögliche Mechanismus dieser Aktivität auf die ausgeprägten antioxidativen Eigenschaften der Blätter zurückzuführen ist.

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Introduction

Liver disease is considered a serious health problem, as the liver is an important organ for the detoxification and deposition of endogenous and exogenous substances. Steroids, vaccines, and antiviral drugs, which have been used as therapeutics for liver diseases, have potential adverse effects, especially when administered long-term. Therefore, herbal products and traditional medicines with improved effectiveness and safety profiles are needed as a substitute for chemical therapeutics. Several herbal products have been shown to protect against liver injury, and many of them possess one or a combination of antioxidant, antifibrotic, immunomodulatory, or antiviral effects [1]. In recent years, there has been a substantial increase in the use of so-called complementary and alternative therapies that utilize herbal medicines in patients with liver disease [2].

Moringa oleifera Lam. (family: *Moringaceae*) is a common tree, native to India and cultivated throughout subtropical areas from West Africa to the Fiji Islands. Also known by the names of drumstick plant, kelor tree and horseradish tree, it is a source of leafy vegetable in India. *Moringa oleifera* is called 'miracle vegetable' because it is not just a food, but also a medicine and it may therefore be a functional food [3]. Phytochemicals such as vanillin, omega fatty acids, carotenoids, ascorbates, tocopherols, beta-sitosterol, kaempferol and quercetin have been reported from the flowers, roots, fruits and seeds [4]. The leaves are highly nutritious, being a significant source of beta-carotene, vitamin C, protein, iron and potassium [5]. Leaves of this plant have been studied extensively for various biological activities including hypolipidemic, antiatherosclerotic, immune-boosting agents, and tumor-suppressive effects [6–8]. The antioxidant activity and the total phenolic contents of *Moringa oleifera* leaves in two stages of maturity responsible for preventing the deleterious effects of oxidative stress were disclosed in a recent study [9]. Due to the wide medicinal uses of *Moringa oleifera* leaves, the present study was focused to evaluate their antioxidant potential at two stages of maturity against CCl_4-induced oxidative stress in liver slices in vitro.

Materials and Methods

Plant Material and Chemicals

The *Moringa oleifera* seeds were purchased from the Horticulture Research Institute, Periyakulam, Tamil Nadu, and Agricultural University, India. Good-quality seeds were sown in a pesticide-free area within the Institute campus. The plant specimen was authenticated by the office of the Joint Director, Ministry of Environment and Forests, Botanical Survey of India, Coimbatore, Tamil Nadu, Government of India (BSI/SC/5/25/05–06/Tech-908). The leaves were procured (from 1-year old trees) fresh for each estimation. The leaves (both mature and tender) were chopped to small pieces and dried in shade. The dried leaves were powdered and passed through sieve no. 20 and extracted (100 g) successively with 600 ml of water in a Soxhlet extractor for 18–20 h. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40–50 °C). The yield (w/w) of the extract from fresh leaves was about 10%. The extracts were prepared in duplicate and all analyses were carried out in triplicate. CCl_4 and other chemicals were procured from Sigma-Aldrich Company.

Precision-Cut Liver Slices and Experimental Design

Liver-slice technology is a relatively new addition to the battery of in vitro assays of xenobiotic metabolism and toxicity evaluation [10]. The in vitro model used in the present study as an alternative to live animals was goat liver slices. The effect of exposure to the leaf extracts in the presence or absence of oxidants was followed in precision-cut liver slices, which simulated the in vivo environment. All of the experiments on animals were carried out with the approval of the institutional animal ethical committee. Fresh goat liver was obtained from a local slaughterhouse and transported on ice to the laboratory. The liver was washed with isotonic KCl and processed for the assays. Liver was the organ of choice because it is *the* metabolic organ and responsible for the metabolic clearance of many xenobiotics. Precision-cut liver slices were prepared (about 1 mm thick) in ice-cold Hank's balanced salt solution using the sterile scalpel. To avoid inter-experimental variations due to the circadian rhythm, isolation of the livers was always performed between 9:00 and 11:00 a.m.

Slices were prepared from the whole liver and randomly selected for incubation and sampling [11]. Slices were stored at 4 °C in the solution until the start of the experiments. Small slices of liver were cut under constant aeration (95% O_2 and 5% CO_2). Single liver slices were gently transferred with the help of a spatula into 5.5 × 1.5 cm polypropylene vials containing 2.0 ml MEM (with Earle's salt and non-essential amino acids) supplemented with 5.6 mol/l D-glucose, 26 mmol/l $NaHCO_3$, 2 mmol/l glutamine, 10% FCS and gentamicin. The tube was aerated once, sealed and incubated horizontally in a water bath shaker. The vials were maintained at 37 °C for exactly 1 h prior to exposure to the test agent. After the 1-h equilibration period the medium was replaced and the tube was again gassed. Immediately afterwards, the following groups were set up for every assay: Group I served as untreated negative control. Group II served as CCl_4 (2.0 ml/kg tissue)-treated positive control. Group III and IV were treated with CCl_4 along with the crude aqueous extract of mature and tender leaves (500 mg/g tissue), respectively. In acute toxicity, no mortality was observed up to a dose level of 600 mg/kg body weight. Group V and VI were treated only with the crude aqueous extract of mature and tender leaves (500 mg/g tissue), respectively. After the addition of the respective agents, the tissue slices were incubated at 37 °C for 2 h with mild shaking and homogenized in a teflon homogenizer with HBSS. The estimations of various parameters indicative of the antioxidant potential were carried out in the homogenate as explained below. After exposure of the slices for 2 h, they were preserved for histological examination.

Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) activity was measured spectrophotometrically by a kinetic NADH oxidation assay at 340 nm as described previously [12]. Results are expressed as LDH activity in media, relative to total activity. The percentage of LDH released was calculated as percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that in the culture medium. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

Biochemical Assessments

Lipid peroxidation was measured by the thiobarbituric acid (TBA) reaction method described by Berton et al. [13]. Glutathione (GSH) content was measured by the method of Moron et al. [14]. Catalase (CAT) activity was measured according to the method of Aebi et al. [15]. Superoxide dismutase (SOD) was assayed based on the inhibition of production of nitro-blue tetrazolium formazone according to the method of Kakkar et

al. [16]. Glutathione peroxidase (GPX) activity was measured according to the procedure of Rotruck et al. [17]. Glutathione reductase (GR) activity was assayed by David and Richard [18] with some modifications. The amount of NADPH utilised is a direct measure of enzyme activity.

Vitamin A was assayed according to the method of Bayfield and Cole [19]. The absorbance was recorded immediately at 620 nm in a spectrophotometer and the concentration in the sample expressed as µg/g tissue. Ascorbic acid, a scavenger of oxy radicals, was assayed by the method of Roe and Kuether [20]. The method described by Rosenberg [21] was followed for the estimation of tocopherol.

Histology

Tissues were fixed in buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The slides were then visualized under light microscope.

Statistical Analysis

All the parameters studied were subjected to statistical treatment using Sigma Stat statistical package (Version 3.1). The results are expressed as mean ± standard deviation (SD), and all statistical comparisons were made by 1-way ANOVA, followed by post-hoc analysis. Fischer's LSD was adapted to all the parameters under study to test the level of statistical significance. Differences showing a p level ≤0.05 were considered statistically significant.

Table 1. Effect of *Moringa oleifera* leaf extracts on LPO and GSH in CCl_4-treated liver slices (values are mean ± SD; n = 6)

Results

The metabolic integrity of liver slices was assessed by estimating the leakage of the cytosolic enzyme LDH (table 1). The liver slices exposed to CCl_4 caused an increase in the leakage of LDH which was very significant. Treatment of cells with the leaf extract decreased the leakage significantly, denoting its ability to reduce the toxicity. The plant extract by itself did not exert any toxic effects on the liver slices within the concentration range tested (2–5000 µg/ml) as ascertained by the results of LDH leakage.

Effects of Moringa oleifera *on Lipid Peroxidation and GSH Levels*

As shown in table 1, the concentration of malondialdehyde (MDA), an end product of lipid peroxidation, in the liver slices treated with CCl_4 was increased when compared with the vehicle control, and the concentration of hepatic GSH in the CCl_4 group was decreased when compared with the vehicle control group. However, treatment with *Moringa oleifera* extract resulted in a statistically significant increase in hepatic GSH concentration in comparison with that of the CCl_4 group.

Effects of Moringa oleifera *on Enzymatic Antioxidants*

As presented in table 2, the activity of CAT in the CCl_4 group was decreased when compared with the vehicle control group. However, treatment with *Moringa oleifera* leaf extract resulted in a statistically significant increase in catalase activity when compared with the CCl_4 group. The he-

Table 2. Effect of *Moringa oleifera* extract on SOD, CAT and GPX (values are mean ± SD; n = 6)

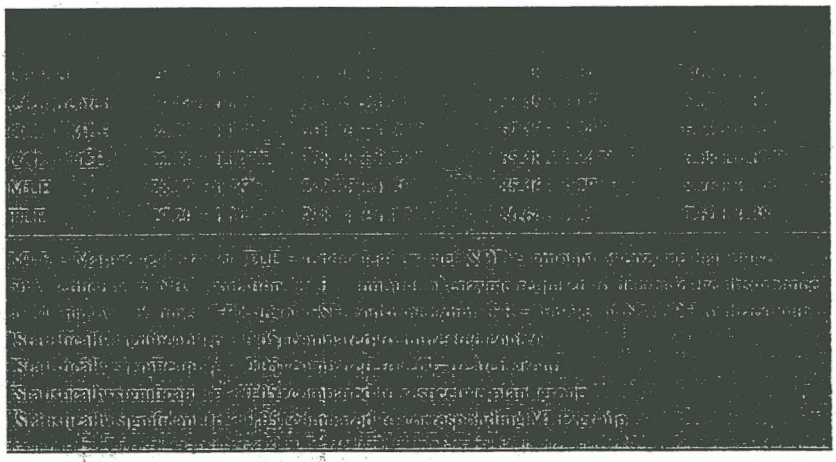

Table 3. Effect of *Moringa oleifera extract* on vitamin C, vitamin E and vitamin A (values are mean ± SD; n = 6)

Treatment	Vitamin C (mg/g)	Vitamin E (µg/g)	Vitamin A (mg/g)
Control	1.429 ± 0.01	111.36 ± 1.12	2.63 ± 0.08
CCl_4-treated	0.786 ± 0.02[a]	92.71 ± 0.16[a]	0.855 ± 0.01[a]
CCl_4 + MLE	1.216 ± 0.01[abc]	104.41 ± 0.14[abc]	1.84 ± 0.07[abc]
CCl_4 + TLE	1.06 ± 0.03[abc]	98.71 ± 0.15[abc]	1.58 ± 0.03[abc]
MLE	2.47 ± 0.08[a]	147.39 ± 0.34[a]	2.95 ± 0.03[a]
TLE	1.89 ± 0.03[a]	122.50 ± 0.19[a]	2.80 ± 0.04[a]

MLE = Mature leaf extract; TLE = tender leaf extract.
[a]Statistically significant ($p < 0.05$) compared to untreated control.
[b]Statistically significant ($p < 0.05$) compared to CCl_4-treated group.
[c]Statistically significant ($p < 0.05$) compared to respective plant group.
[d]Statistically significant ($p < 0.05$) compared to corresponding MLE group.

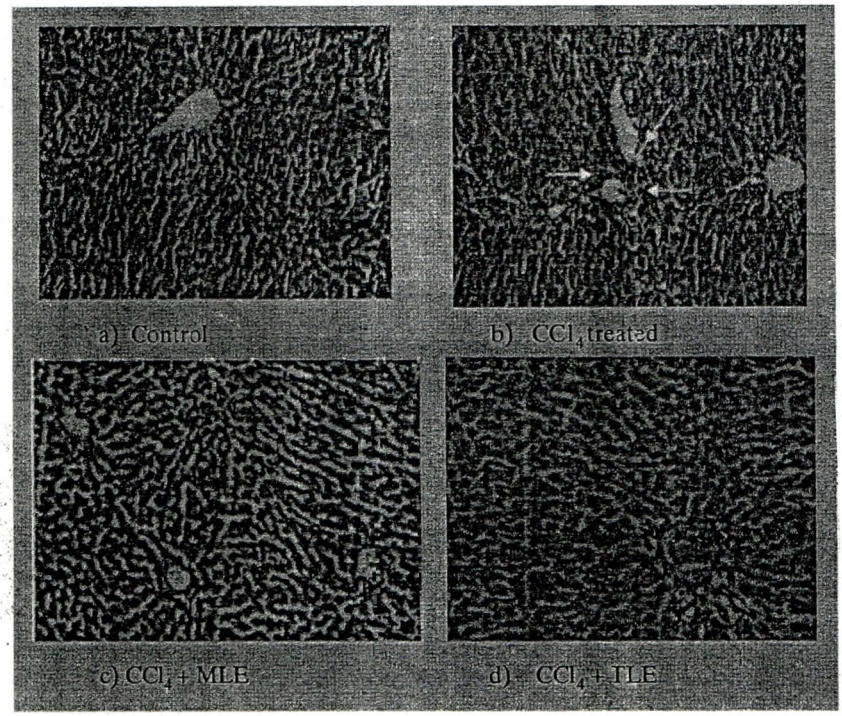

Fig. 1. Histopathologic architecture of the liver slice (H&E 100x). (**a**) Central vein surrounded by hepatic cord of cells (normal architecture). (**b**) Patches of liver cell necrosis with inflammatory collections, around central vein and focal necrosis with sinusoidal dilatation. (**c**) and (**d**) Less inflammatory cells around central vein, absence of necrosis.

patic SOD activity and GPX activity in the CCl_4 group was also reduced when compared with the vehicle control group. However, the SOD activity and GPX activity was significantly increased by *Moringa oleifera* leaf extract treatment when compared with the CCl_4 group. The activity of GR in the CCl_4 group was decreased as compared to normal control rats. However, treatment with *Moringa oleifera* leaf extract significantly protected against the depletion of GR activity induced by CCl_4.

Effect of Moringa oleifera *on Non-Enzymatic Antioxidants*

As presented in table 3, the level of vitamin A in the CCl_4 group was decreased when compared with the vehicle control group. However, treatment with *Moringa oleifera* leaf extract resulted in a statistically significant increase in the level of vitamin A when compared with the CCl_4 group. The level of vitamin C and vitamin E in the CCl_4 group was also reduced when compared with the vehicle control group. Treatment with *Moringa oleifera* leaf extract significantly increased these levels, which reiterates the antioxidant effect of the leaf extracts.

Histopathological Examination

Cellular morphology of the control slices was normal and there were hepatocellular necrosis, fatty degeneration, increased mitochondrial activity, and extensive vacuolation in CCl_4-treated liver slices (fig. 1). The leaf extract-treated group showed minimal inflammatory conditions with near-normal liver architecture possessing hepatoprotective action.

Discussion

Several studies have shown the possible benefits of antioxidants from plant sources in altering, reversing, or forestalling the negative effects of oxidative stress. *Moringa oleifera* leaves have been reported to show a number of beneficial effects against various types of degenerative diseases in humans, largely because the major ingredients of *Moringa oleifera*, the polyphenols, have potent antioxidant activity [22, 23]. Therefore, we considered that *Moringa oleifera* can be useful in the prevention of various liver injuries induced by oxidative stress. In the present study, the capability of *Moringa oleifera* to protect against CCl_4-induced hepatotoxicity and oxidative stress was investigated. In this study, we used an experimental model of CCl_4-induced acute hepatotoxicity in liver slices, because it is a potent hepatotoxin and a single exposure can rapidly lead to severe hepatic necrosis and steatosis [24, 25]. The liver, which is involved predominantly in xenobiotic metabolism, plays a major role in the assessment of hepatotoxicity. The viability of the liver slice was based on the integrity of the plasma membrane and the histology of the tissue. The response of the slices to CCl_4 explains the ability of the tissue to identify a toxic agent. This is also validated by the significant changes in the enzyme levels and histopathology of the slice also confirms the hepatic injury produced by CCl_4.

Previous studies reported that CCl_4 is activated by cytochrome P450 to form a trichloromethyl radical, $CCl_3^{\bullet}$, and with oxygen to form a trichloromethylperoxy radical, $CCl_3OO^{\bullet}$, a highly reactive species. These radicals can bind to cellular molecules (nucleic acid, protein, and lipid) impairing crucial cellular processes such as lipid metabolism, with the potential outcome of fatty degeneration. Furthermore, they can initiate the chain reaction of lipid peroxidation, which attacks and destroys polyunsaturated fatty acids, particularly those associated with phospholipids [26]. CCl_4 treatment produced high levels of oxidative damage, as evidenced by a significant elevation in LDH leakage, hepatic MDA level, and a significant decrease in GSH concentration, CAT, SOD, GPX, and GR activities, which suggest a role of oxidative stress in CCl_4 hepatotoxicity. However, treatment with *Moringa oleifera* leaf extract showed a significant protective effect against CCl_4-induced acute hepatotoxicity and oxidative stress in liver slices.

MDA is a major reactive aldehyde that appears during the peroxidation of biological membrane polyunsaturated fatty acids. Consequently, the hepatic content of MDA is used as an indicator of liver tissue damage involving a series of chain reactions [27, 28]. It has been accepted that lipid peroxidation of hepatocyte membranes is one of the principal causes of CCl_4-induced hepatotoxicity and is mediated by the production of free radical derivatives of CCl_4 [29]. In the present study, CCl_4 at 2.0 ml/kg resulted in a significant increase in the hepatic MDA concentration, indicating increased lipid peroxidation caused by administration of CCl_4. Significant decrease in the hepatic MDA concentration confirms that treatment with *Moringa oleifera* leaf extract could effectively protect against the hepatic lipid peroxidation induced by CCl_4.

Reduced levels of GSH play a key role in the detoxification of the reactive toxic metabolites of CCl_4. Liver necrosis is initiated when reserves of GSH are markedly depleted [30]. In the present study, the hepatic content of GSH was found to be decreased significantly in CCl_4-treated liver slices compared with control groups. However, treatment with *Moringa oleifera* leaf extract significantly prevented the CCl_4-induced depletion of hepatic GSH, indicating the antioxidant effect of the leaves in CCl_4-treated liver slices.

Antioxidant enzymes such as CAT, SOD, and GST are easily inactivated by lipid peroxides or reactive oxygen species, which results in decreased activities of these enzymes in CCl_4 toxicity. A major component of the antioxidant system in mammalian cells consists of three enzymes, namely, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). These enzymes work in concert to detoxify superoxide anion and H_2O_2 in cells [31]. Therefore, we theorize that the increased production of free radicals caused by administration of CCl_4 is a major cause of the significantly decreased activities of catalase, SOD, and GR in rats treated with CCl_4 compared with control rats. In liver slices treated with *Moringa oleifera* leaf extract however, the activities of these antioxidant enzymes were significantly higher, and, compared to the liver slices exposed to CCl_4 alone, they were very similar to the values noted in normal control.

Vitamin A, a fat-soluble vitamin, is essential for growth. An increase in the intake of vitamin A is reported to have beneficial effects on the nutrition systematic redox balance and immune parameters in HIV-infected persons [32]. Vitamin A administration has also been reported to prevent hepatic injury caused by CCl_4 treatment [33]. Therefore, the increase in vitamin A levels brought about by *Moringa oleifera* leaf extracts may contribute significantly to the decrease in CCl_4-induced oxidative stress. Thus, an increase in the vitamin A levels evoked by *Moringa oleifera* leaf extracts in vitro gains significance in the in vivo conditions.Vitamin E is the major chain-breaking antioxidant, protecting cell membranes against lipid peroxidation at an early stage of free-radical attack through its free-radical quenching activity [34]. Thus, it is perceivable that the increase in vitamin E levels brought about by *Moringa oleifera* leaf extracts could be reflective of the antioxidant effect. Vitamin C is an essential micronutrient required for the normal metabolic functioning in the body. It readily oxidizes to dehydroascorbic acid and interferes with the process of LPO by scavenging the superoxide anion [35]. Vitamin C has also been suggested to play an important role in diminishing the mutagenic and carcinogenic activity of the carcinogen MNNG [36]. The results are corroborative with these reports, emphasizing the antioxidant response evoked by *Moringa oleifera* leaves.

Scavenging of free radicals is one of the major antioxidation mechanisms to inhibit the chain reaction of lipid peroxidation. Recent studies on the antioxidant properties of flavonoids from

various plant extracts reveal their stimulatory action on antioxidative enzymes [37]. Chlorogenic acid, gallic acid, and quercetin that were present in appreciable quantities in *Moringa oleifera* [38] along with other phytochemicals might be responsible for the free radical-scavenging and antioxidant activity. It is further concluded that mature leaf extract with maximum inhibition of free radicals is more potent than the tender extracts. Further investigations are necessary to isolate the active principles of *Moringa oleifera* and establish their chemical nature.

Conclusion

The present study demonstrates that *Moringa oleifera* leaf extract exerts significant protective effects against CCl_4-induced oxidative stress by augmenting host antioxidant defense mechanisms. Thus, *Moringa oleifera* leaf extract is a promising agent for the prevention of chemical-induced toxicity through enhancing the antioxidant activity and lowering the extent of lipid peroxidation.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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RESEARCH ARTICLE

Anticancer Activity of *Zea mays* Leaf Extracts on Oxidative Stress-induced Hep2 Cells

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Zea mays leaves

Abstract
Cancer is one of the leading causes of death in humans. It is believed that plants can provide potential bioactive compounds for the development of "new leads" to combat cancer and other diseases. The present study focuses on the ability of the different extracts (aqueous, methanol, and chloroform) of the leaves of *Zea mays* in influencing the process of apoptosis induced by hydrogen peroxide (H_2O_2) in Hep2 (laryngeal carcinoma) cells. Various apoptosis-related parameters, such as cell viability, morphological changes, nuclear changes, and apoptotic index were characterized. sulforhodamine B and MTT assays were used to quantify the extent of cell death in the group exposed to H_2O_2, plant extracts, and their combination. Treatment with H_2O_2 caused cytotoxicity in cancer cells. The administration of leaf extract also caused an increase in the death of cancer cells. Oxidatively stressed cancer cells co-treated with all the *Z. mays* leaf extracts (except the chloroform extract) demonstrated cytotoxicity on a par with the H_2O_2-treated groups. This indicated that the aqueous and methanol leaf extracts did not influence the cytotoxic action of H_2O_2 in the cancer cells. Thus, various apoptosis-related events in Hep2 cells exposed to leaf extract throw light on the potential anticancer activity of the *Z. mays* leaves. The maximum activity was exerted by the methanolic extract followed by the aqueous and chloroform extracts.

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1. Introduction

Chemoprevention, a relatively new and promising strategy to prevent cancer, is defined as the use of natural dietary compounds and/or synthetic substances to block, inhibit, reverse, or retard the process of carcinogenesis. Various cancer chemopreventative agents can encourage apoptosis in premalignant and malignant cells *in vivo* and/or *in vitro*, which is conceivably another anticancer mechanism [1].

Thus, the search for natural products represents an area of great interest in which the plant kingdom has been documented as the most important source to provide many antioxidant and cancer chemopreventative agents with novel structures and unique mechanisms of action. With this backdrop, the present study was formulated to analyze the anticancer potential of *Zea mays* leaves. *Z. mays* is commonly known as *makkacholam* or maize. It belongs to the family Gramineae. Our preliminary studies with three different extracts (aqueous, chloroform, and methanol) revealed that it possesses strong antioxidant properties.

2. Materials and methods

2.1. Preparation of plant extracts

Z. mays seeds were procured from Tamil Nadu Agriculture University (TNAU) in Coimbatore district, Tamil Nadu. They were grown within the university campus in pots. Plant growth was observed at 5, 10, 15, 20, 25 and 30 days after sowing. The leaves on the 10^{th} day of growth were found to have maximum antioxidant activity [2]. Hence, 10-day-old plants were selected for the present study. The plantlets were uprooted and washed thoroughly with running tap water. Then the leaves were blotted dry between folds of filter paper to remove excess water.

2.2. Aqueous extract

The leaves were homogenized in water (1 g/mL) using a micropestle in a microfuge tube. They were centrifuged at low speed to clarify the extract. The supernatant corresponding to the concentration of 20 mg/20 μL was used for the assay.

2.3. Methanolic/chloroform extract

Z. mays leaves (1.0 g) were homogenized in approximately 1 mL of the solvent (methanol/chloroform). The supernatant was collected and dried at 60°C, well protected from light. The residue obtained after drying the chloroform and methanol extracts was weighed and dissolved in a known amount of dimethylsulfoxide (DMSO) to yield a concentration of 20 mg/5 μL. DMSO was maintained at a minimum level to avoid DMSO-induced events, if any. The extract preparation took around 2 hours.

2.4. Hep2 cell line culture

The cell line was procured from the National Centre for Cell Science, Pune, India. The cells were maintained in a CO_2 incubator with 5% CO_2 and 95% humidity, and supplemented with Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS). Penicillin and streptomycin (PAA) were also added to the medium to 1× final concentration from a 100 × stock.

Once the cells had attained confluent growth, the cells were trypsinized using Trypsin-EDTA (PAA) and the number of cells needed for carrying out various assays was seeded into sterile six-well and 96-well plates. In each well of the six-well plates, a clean, dry, sterile coverslip was placed before the cells were seeded. Then the plates were incubated in a CO_2 incubator with 5% CO_2 and 95% humidity.

Hydrogen peroxide (H_2O_2) at a concentration of 200μM was used as an oxidant. The concentration of plant extract used was 20 mg. The cells were treated with the oxidant, both in the presence and absence of the leaf extracts. The cells were exposed to H_2O_2 for 1 hour at 37°C. The time points were arrived at by conducting a time-related response analysis of each cell type.

After treatment, the coverslips from the six-well plates were removed and placed on a glass slide and sealed with Vaseline. These slides were used for various staining techniques; however in 96-well plates, the medium was removed and replaced with fresh medium. These were used for checking the viability status.

2.5. Parameter analysis

The apoptotic events that occurred after the oxidant treatment were analyzed using various parameters [viability assays and Giemsa, propidium iodide (PI), acridine orange/ethidium bromide (AO/EtBr), 4,6-diamino-2-phenylindol dihydro chloride (DAPI), and EtBr staining], as described below.

2.6. MTT assay

A total of $10^6/10^7$ cells per Eppendorf were seeded into 96-well plates and exposed to H_2O_2/plant extracts for 1 hour. The cytotoxicity of the drugs was assessed by the MTT assay according to the procedure used by Igarashi and Miyazawa [3]. MTT (50 μL) was added to the treated wells. The plate was incubated at 37°C for 3 hours with mild shaking. The cells were re-suspended in 200 μL of 2-propanol containing 0.04N HCl overnight in the dark. The absorbance was read at 650 nm, in a microtiter plate reader (Anthos, Germany).

2.7. Sulforhodamine B assay

Sulforhodamine B (SRB) binds to basic amino acid residues in TCA-fixed cells to provide a sensitive index of cellular protein content that is linear over a range of cell densities [4]. The cell survival was measured at 492 nm.

2.8. Giemsa staining

The incubated cells were spread on the microscopic slides with a drop of diluted Giemsa stain. The slides were mounted with cover slips and observed under a phase contrast microscope (Nikon, Japan) for morphological changes as described by Chih et al [5]. The number of cells showing apoptotic morphological changes was counted in

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each experimental group per 100 cells in 10 different fields and the experiment was repeated five times.

2.9. PI staining

PI staining was employed to discriminate apoptotic cells from normal cells, which reflects the nuclear changes during apoptosis using the protocol developed by Sarker et al [6]. After incubation with H_2O_2 and/or plant extracts, the cells were permeabilized with a mixture of acetone and methanol (1:1) at −20°C for 10 minutes. Then, 10 μL of 5 μg/mL PI was added to each slide, spread with a cover slip and incubated at 37°C for 30 minutes in the dark. The apoptotic cells were detected using the green filter of a fluorescence microscope (Nikon, Japan).

2.10. EtBr staining

The treated cells were incubated for 5 minutes with 10 μL of EtBr (50 μg/mL) and spread by placing a cover slip over it. The apoptotic cells were scored by counting the cells with condensed chromatin and fragmented nuclei under a fluorescence microscope (Nikon, Japan) using UV 2A filter at 400× magnification. The ratios of apoptotic cells to normal cells were calculated in each staining method [7].

2.11. DAPI staining

The apoptotic ratios of the oxidant-treated cells with or without the leaf extract to the untreated cells were calculated by DAPI staining [8]. The cells were then transferred to slides and were immediately fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 10 minutes at room temperature. They were then incubated with 5 μg/mL of DAPI. The apoptotic nuclei were observed under a fluorescence microscope (Motic, Germany) using a DAPI filter.

2.12. AO/EtBr staining

AO (0.1 mg/mL) and EtBr (0.1 mg/mL) were used to label nuclear DNA in Hep2 cells. Both solutions were prepared in PBS buffer (pH 7.4). PBS buffer was used to preserve normal physiological activity for unicellular cells. For cell staining, Hep2 cell samples (100 μL) were stained with AO/EtBr (5 μL) and observed under a fluorescence microscope (Nikon, Japan) with a B-2A filter [9].

2.13. Statistical analysis

Statistical significance was determined by a two-way analysis of variance ($p < 0.01$) using Agress statistical software (version 3.01).

3. Results and discussion

3.1. Cell viability assays

Cell viability was assessed by MTT assay, which is a colorimetric assay for measuring the activity of cellular enzymes

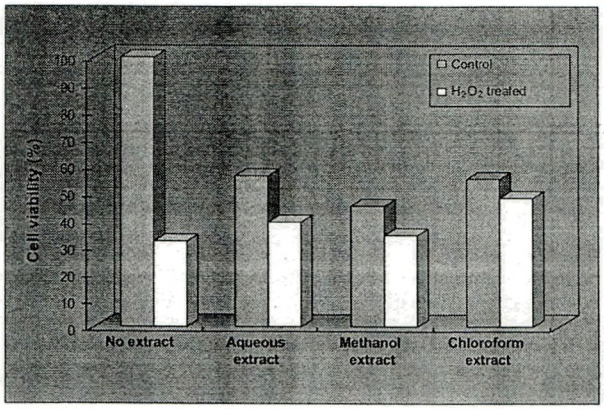

Figure 1 Effect of *Zea mays* leaf extracts on the viability of Hep2 cells subjected to oxidative stress as determined by the MTT assay. The values of the negative (untreated) control group were fixed as 100% viability and the percentage viabilities in the other groups were calculated relative to this.

that reduce the tetrazolium dye, MTT, to its insoluble formazan, giving a purple colour. Fig. 1 shows the results of evaluation of cytotoxicity of all three extracts of *Z. mays* at a concentration of 20 mg/mL and an exposure time of 1 hour. These data showed that the treatment with H_2O_2 exhibited cytotoxicity in the cancer cells. The administration of leaf extracts also caused increased death of cancer cells. Oxidatively stressed cancer cells co-treated with the *Z. mays* leaf extracts (apart from the chloroform extract) showed cytotoxicity on a par with H_2O_2-treated groups. This indicates that the aqueous and methanol leaf extracts did not influence the cytotoxic action of H_2O_2 in the cancer cells. Using the MTT assay, many researchers found time- and dose-dependent inhibition in different cells with different agents.

Inhibition of cell proliferation by sesquiterpene lactones (tomentosin and inuviscolide) from *Inula viscosa* (Compositae) leaves against human melanoma cell lines was reported by Rozenblat et al [10].

An SRB assay was also performed to assess cell survival. The results of this assay showed a similar trend to that

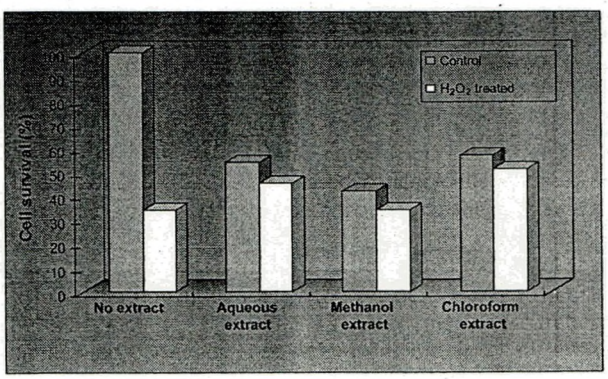

Figure 2 Effect of *Zea mays* leaf extracts on the viability of Hep2 cells subjected to oxidative stress as determined by the SRB assay. The values of the negative (untreated) control group were fixed as 100% viability and the percentage viabilities in the other groups were calculated relative to this.

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Table 1 Effect of *Zea mays* leaf extracts on the morphological changes in Hep2 cells subjected to oxidative stress as determined by Giemsa staining.

Sample	No. of apoptotic cells/100 cells		Apoptotic ratio	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	9 ± 1	52 ± 2*	0.10	1.10
Aqueous extract	45 ± 2*	60 ± 1*,†,‡	0.81	1.50
Methanol extract	63 ± 3*	68 ± 2*,†,‡	1.70	2.13
Chloroform extract	40 ± 2*	54 ± 2*,‡	0.67	1.17

The values are means ± SD of triplicates. CD value = 4.7.
* Statistically significant ($p < 0.01$) compared with untreated control group.
† Statistically significant ($p < 0.01$) compared with oxidant treated group.
‡ Statistically significant ($p < 0.01$) compared with respective plant extract treated group.

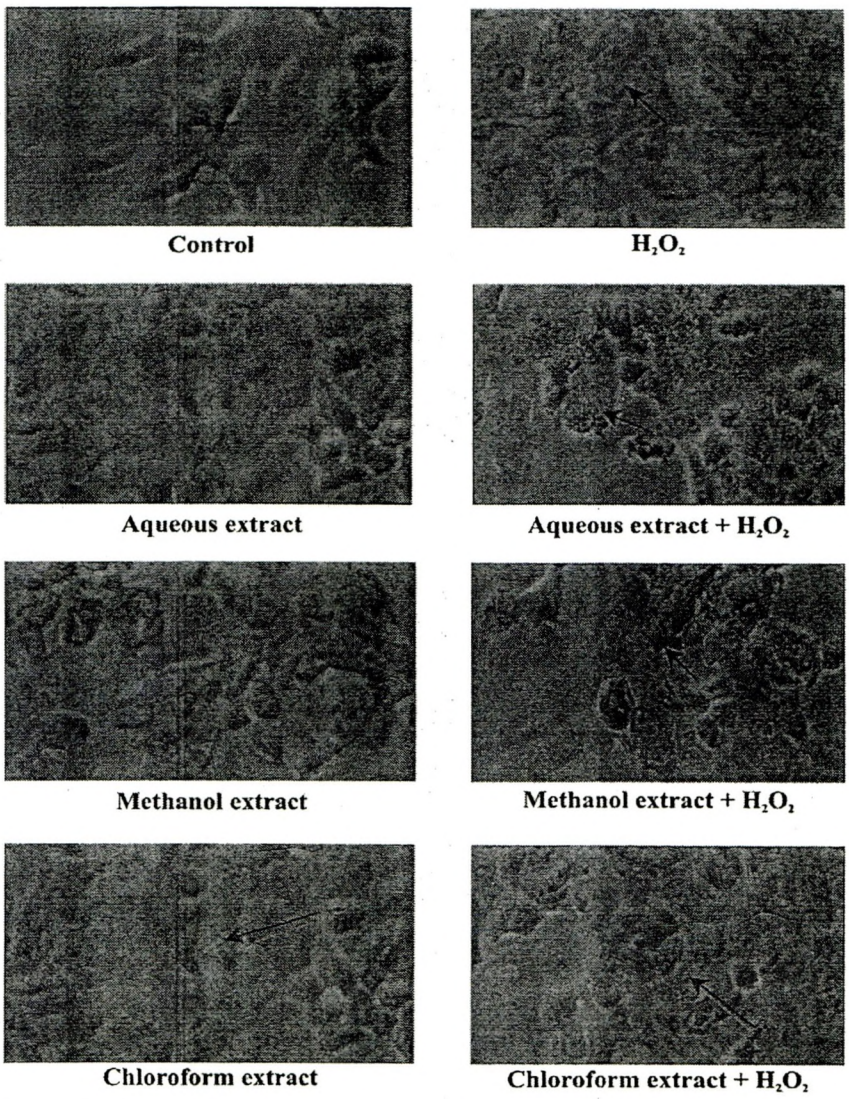

Figure 3 Apoptotic Hep2 cells showing membrane blebbing when stained with Giemsa exposed to 200 μM of H_2O_2 at 100× magnification. The number of shrunken cells was found to increase in the groups co-treated with the leaf extracts. The apoptotic cells with blebbing, cell shrinkage, nuclear fragmentation, and margination of the nucleus are indicated by arrows.

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Table 2 Effect of *Zea mays* leaf extracts on nuclear changes in Hep2 cells subjected to oxidative stress as determined by PI staining.

Sample	No. of apoptotic cells/100 cells		Apoptotic ratio	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	8 ± 3	51 ± 2*	0.09	1.04
Aqueous extract	48 ± 2*	59 ± 2*,†,‡	0.92	1.44
Methanol extract	61 ± 1*	73 ± 1*,†,‡	1.56	2.70
Chloroform extract	34 ± 2*	49 ± 1*,‡	0.52	0.96

The values are means ± SD of triplicates. CD value = 4.46.
* Statistically significant ($p < 0.01$) compared with the untreated control group.
† Statistically significant ($p < 0.01$) compared with the oxidant-treated group.
‡ Statistically significant ($p < 0.01$) compared with the respective plant extract-treated group.

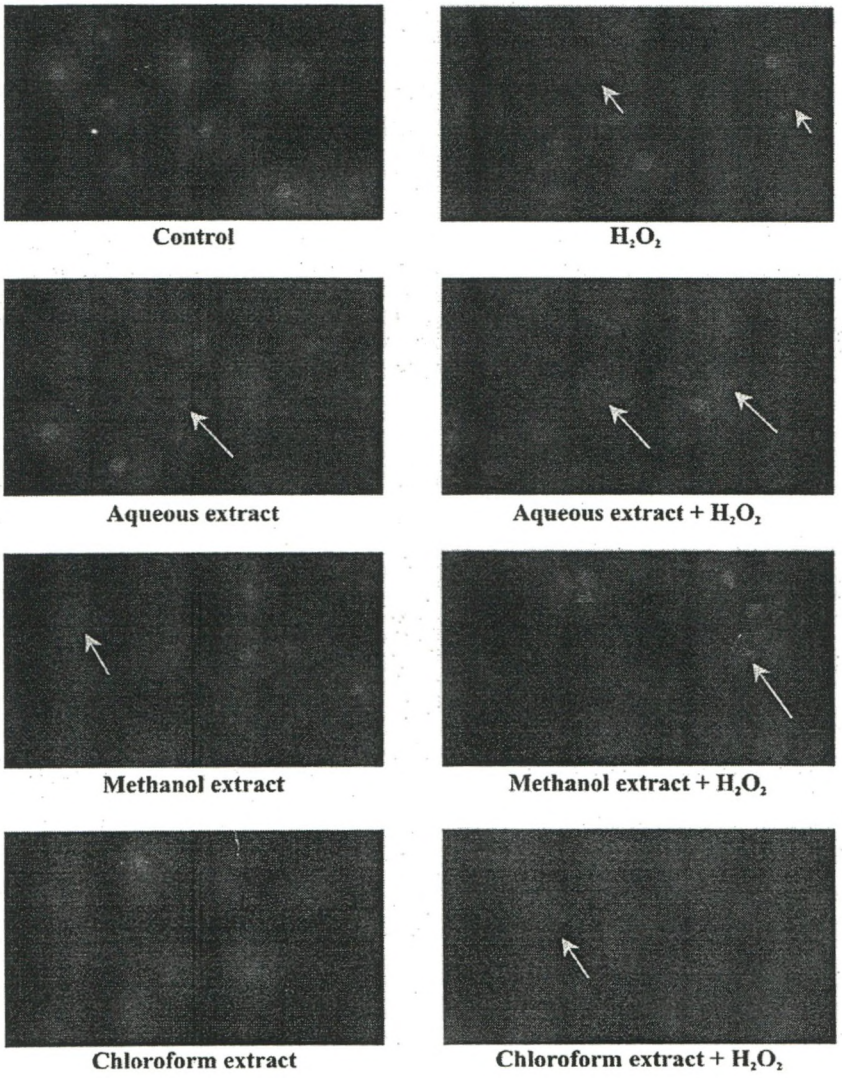

Figure 4 Photographic record of the apoptosing cells in each treatment group stained by PI. A greater number of apoptotic cells were observed in the leaf extract-treated groups exposed to oxidative stress. The apoptotic cells with blebbing, cell shrinkage, nuclear fragmentation, and margination of the nucleus are indicated by arrows.

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Table 3 Effect of *Zea mays* leaf extracts on nuclear changes in Hep2 cells subjected to oxidative stress as determined by EtBr staining.

Sample	No. of apoptotic cells/100 cells		Apoptotic ratio	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	5 ± 3	48 ± 2*	0.05	0.92
Aqueous extract	47 ± 2*	55 ± 2*†,‡	0.89	1.22
Methanol extract	61 ± 2*	70 ± 1*†,‡	1.56	2.33
Chloroform extract	35 ± 2*	50 ± 2*‡	0.54	1.00

The values are means ± SD of triplicates. CD value = 4.9.
* Statistically significant ($p < 0.01$) compared with the untreated control group.
† Statistically significant ($p < 0.01$) compared with the oxidant-treated group.
‡ Statistically significant ($p < 0.01$) compared with the respective plant extract-treated group.

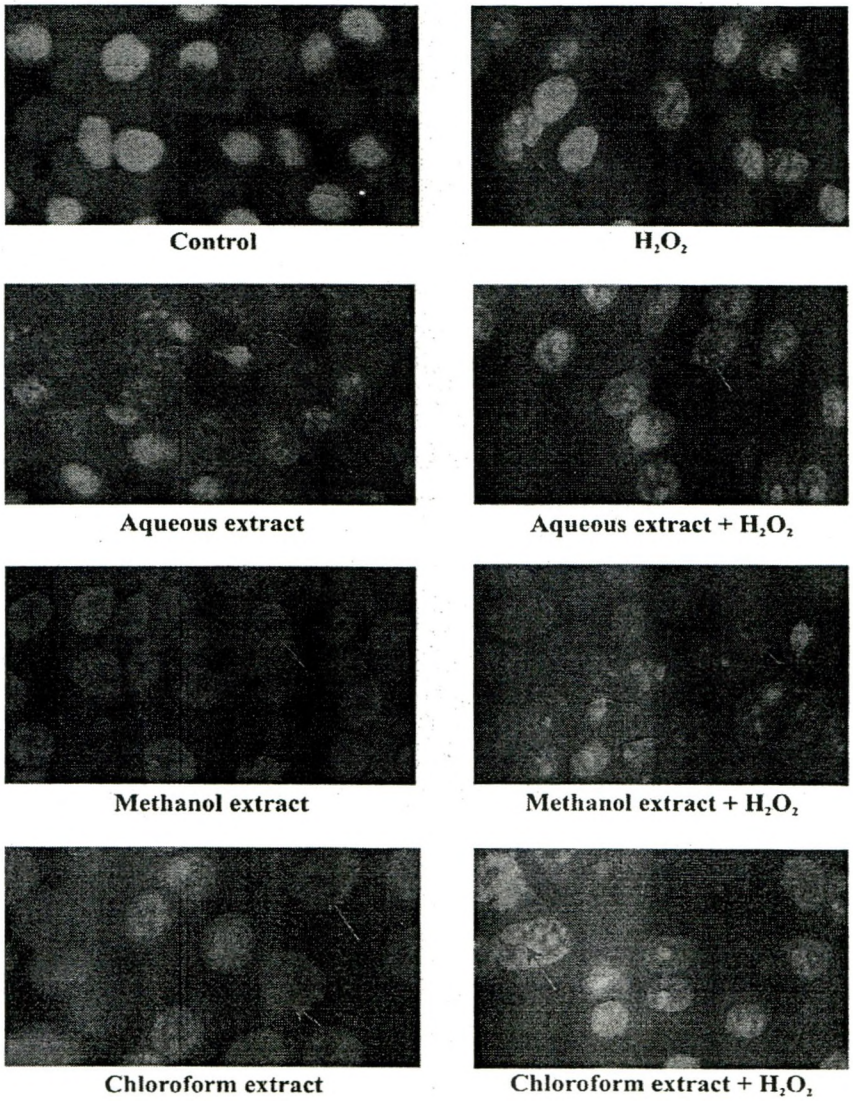

Figure 5 Apoptotic Hep2 cells showing membrane and nuclear changes when stained with ethidium bromide exposed to 200 μM of H_2O_2 at 100× magnification. These pictures indicate that the *Zea mays* leaves were highly efficient at inducing apoptosis in cancer cells, both in the presence and absence of H_2O_2. The apoptotic cells with blebbing, cell shrinkage, nuclear fragmentation, and margination of the nucleus are indicated by arrows.

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Table 4 Effect of *Zea mays* leaf extracts on nuclear changes in Hep2 cells subjected to oxidative stress as determined by DAPI staining.

Sample	No. of apoptotic cells/100 cells		Apoptotic ratio	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	6 ± 2	68 ± 2*	0.06	2.13
Aqueous extract	47 ± 2*	56 ± 1*,†,‡	0.89	1.27
Methanol extract	50 ± 3*	58 ± 3*,†,‡	1.00	1.38
Chloroform extract	43 ± 3*	53 ± 1*,†,‡	0.75	1.13

The values are means ± SD of triplicates. CD value = 5.4.
* Statistically significant ($p < 0.01$) compared with the untreated control group.
† Statistically significant ($p < 0.01$) compared with the oxidant-treated group.
‡ Statistically significant ($p < 0.01$) compared with the respective plant extract-treated group.

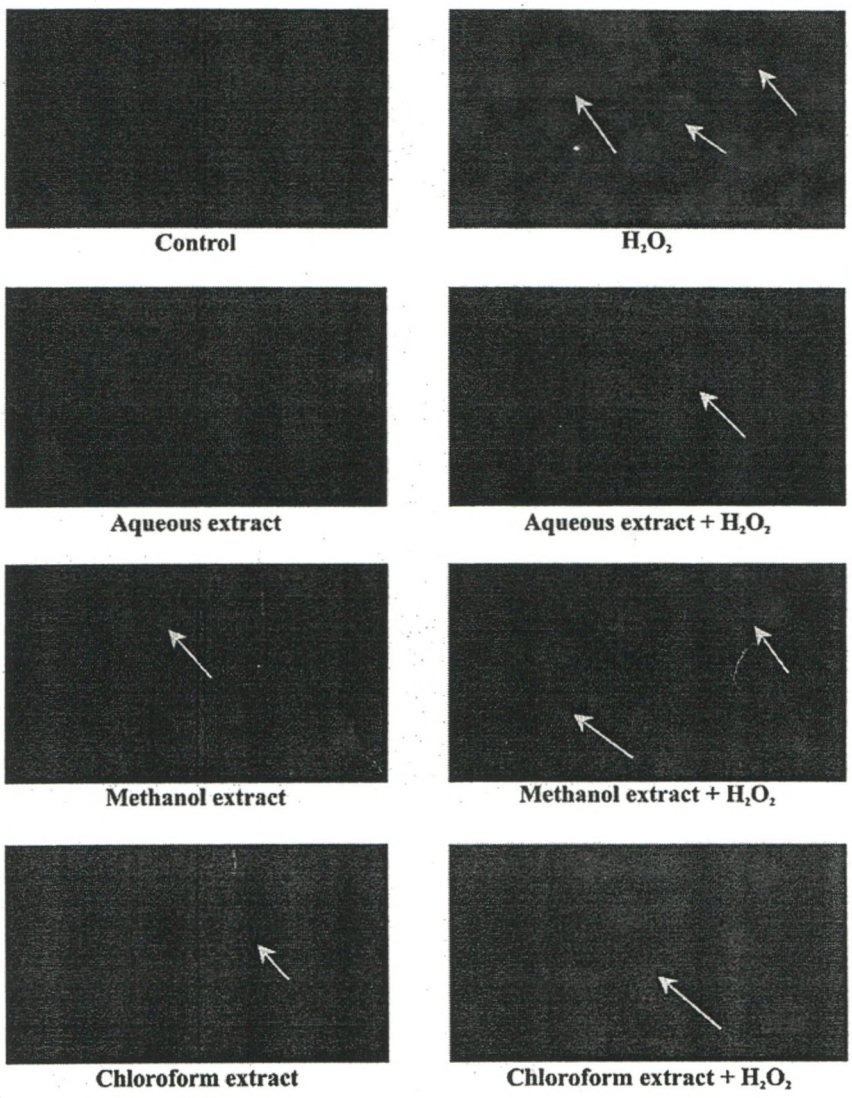

Figure 6 Apoptotic shrunken Hep2 cells with nuclear changes when stained with DAPI exposed to H_2O_2. The most apoptotic cells were found in the leaf extract-treated groups. The apoptotic cells with blebbing, cell shrinkage, nuclear fragmentation, and margination of the nucleus are indicated by arrows.

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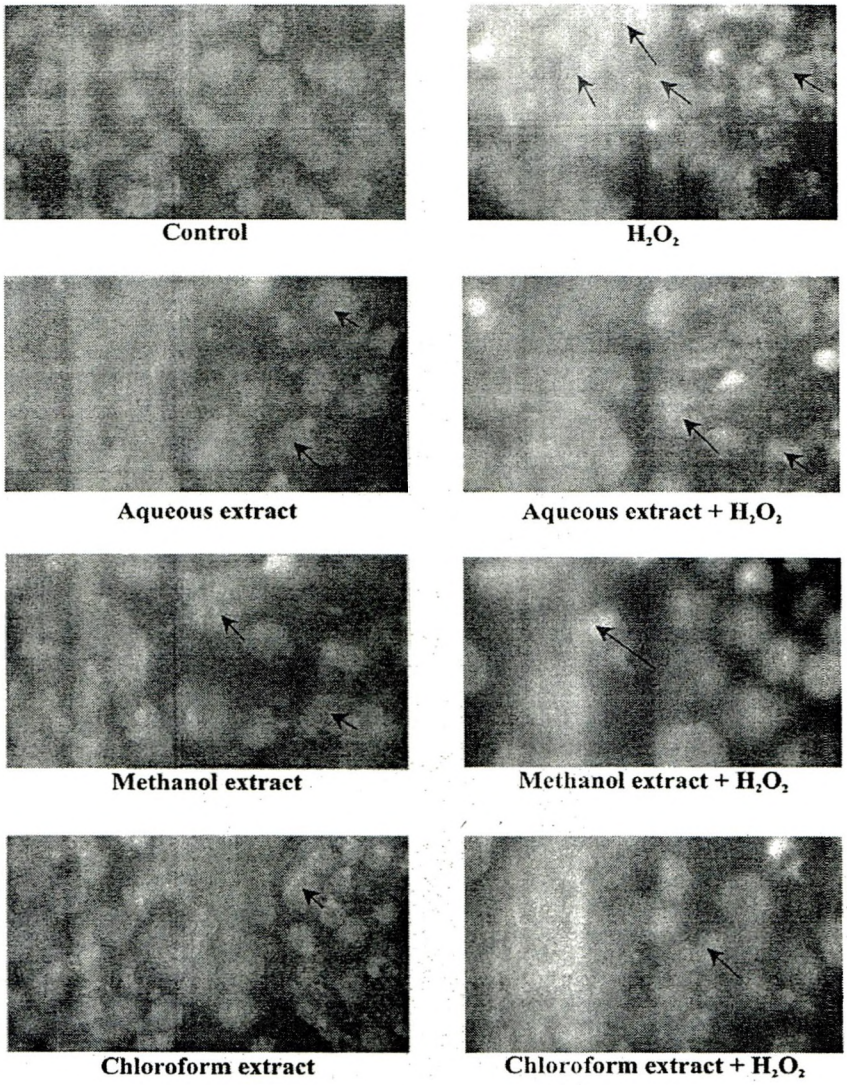

Figure 7 Hep2 cells showing red fluorescence indicated that they are undergoing apoptosis with acridine orange/ethidium bromide stain and normal cells showed green fluorescence. This staining method clearly indicates the anticancer effect of *Zea mays* leaf extracts. The apoptotic cells with blebbing, cell shrinkage, nuclear fragmentation, and margination of the nucleus are indicated by arrows.

observed in the MTT assay, and are presented in Fig. 2. An extract of the rhizomes of *Iris tectorum* Maxim exhibited a very high cytotoxicity against MCF-7 (breast cancer) and C32 (melanoma) cell lines [11].

3.2. Membrane morphological characteristics

Morphological changes, including plasma membrane blebbing, changes to the cell membrane, such as loss of membrane asymmetry and attachment and cell shrinkage, are the early stages of apoptosis that can be analyzed by Giemsa staining. The ratios of cells that undergo apoptosis to normal cells, when exposed to H_2O_2 in both the presence and absence of *Z. mays* leaf extracts, are presented in Table 1.

The results revealed that the occurrence of morphologically altered cells was greatest in the H_2O_2-treated group. Treatment with the leaf extracts also exerted an apoptosis-inducing effect in the cancer cells (Fig. 3). The number of apoptotic cells increased further upon co-exposure of the leaf extract with H_2O_2. The methanolic extract of *Z. mays* leaves was found to be very active against Hep2 cells. A crude ethanolic extract of the plant *Lycopodium clavatum* considerably inhibited the growth of HeLa cells through the induction of apoptosis via caspase-3 activation, as determined by morphological changes [12].

4. Nuclear changes

Apoptosis is a form of cell death that allows for the elimination of damaged or unwanted cells without damaging the organism. The most obvious characteristics of this form of cell death are cytoplasmic and nuclear condensation, followed by internucleosomal DNA cleavage, membrane blebbing, and finally cell fragmentation [13].

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Table 5 Effect of *Zea mays* leaf extracts on nuclear changes in Hep2 cells subjected to oxidative stress as determined by AO/EtBr staining.

Sample	No. of apoptotic cells/ 100 cells		Apoptotic ratio	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	8 ± 2	54 ± 3*	0.09	1.17
Aqueous extract	47 ± 3*	65 ± 1*†,‡	0.89	1.85
Methanol extract	64 ± 1*	72 ± 1*†,‡	1.78	2.58
Chloroform extract	39 ± 1*	54 ± 3*‡	0.64	1.17

The values are means ± SD of triplicates. CD value = 4.98.
* Statistically significant ($p < 0.01$) compared with the untreated control group.
† Statistically significant ($p < 0.01$) compared with the oxidant-treated group.
‡ Statistically significant ($p < 0.01$) compared with the respective plant extract-treated group.

Nuclear changes were observed in Hep2 cells exposed to H_2O_2 in both the presence and absence of the *Z. mays* leaf extracts using various staining techniques (PI, EtBr, DAPI, and AO/EtBr).

The data obtained with PI staining and the apoptotic indexes calculated are presented in Table 2. As shown in Fig. 4, control cells exhibited intact nuclei, but cells treated with 200 μM of H_2O_2 showed significant nuclear fragmentation. The apoptotic ratio of cells treated with oxidant was 1.04, whereas the treated cells co-exposed to leaf extracts (water, methanol, and chloroform) were 1.44, 2.70, and 0.96, which indicates that the *Z. mays* leaf extracts did not influence the cytotoxic action of H_2O_2 in the cancer cells. The effect of an ethanolic extract of *Tremella mesenterica* on the induction of apoptosis in human lung carcinoma A549 epithelial cells was also assessed by PI staining [14].

In addition to the observation of nuclear fragmentation, the anticancer potential of leaf extracts was confirmed by EtBr staining in each treatment group and the results are given in Table 3. The nuclear changes observed are shown in Fig. 5.

H_2O_2-treated cells co-treated with leaf extract exhibited morphological changes typical of apoptosis, including cell shrinkage, plasma membrane blebbing, chromatin condensation, and nuclear fragmentation, compared with the control cells with prominent rounded nuclei and defined plasma membrane contours. The leaf extracts, by themselves, also caused an induction of apoptosis in Hep2 cells. The aqueous and chloroform extracts also exhibited considerable apoptotic effects in the cancer cells.

Two phytochemicals, resveratrol and citroflavon-3-ol, and four plant extracts (grape seed polyphenols, olive oil extract, bearberry, and *Echinacea*) examined for their effect on cell viability by the EtBr assay provided evidence for strong protection against oxidative stress in H_2O_2 stress-induced U937 cells [15].

DAPI staining is used to observe the nuclear contents in shrunken cells. DAPI has been used to study pamidronate, anti-proliferative, apoptotic, and anti-migratory effects in hepatocellular carcinoma cells [16]. The extent of nuclear changes observed during H_2O_2-induced apoptosis after DAPI staining in the different treatment groups of Hep2 cells are presented in Table 4.

As is deducible from the values listed in Table 4, a trend similar to that of EtBr was noted with DAPI staining. The cells with altered nuclear changes were found to be greatest in the H_2O_2-treated group, which shows the apoptotic ratio of 0.92. The extracts of *Z. mays* leaves were also highly efficient in inducing apoptosis in cancer cells, both in the presence and in the absence of H_2O_2 (Fig. 6). Human breast cancer cells treated with the extracts of *Astrodaucus persicus* also showed potential decrease in the cell proliferation by staining with DAPI. [17] The results with Giemsa and the nuclear stains were further affirmed by AO/EtBr staining (Fig. 7), as is evident from the data obtained (Table 5).

AO stain is membrane permeable and marks the nuclei green, and EtBr, which binds to DNA, is mainly taken up by cells when membrane integrity is lost and stains the nuclei red. Since AO intercalates in the DNA but only interacts with the RNA, viable cells do not uptake EtBr and these cells exhibit green nuclei. However, EtBr is taken up by dying cells, which turn red [18].

Treatment with a combination of AO/EtBr has been used as a reliable index of cellular degeneration [19]. Sanguinarine, a benzophenanthridine alkaloid derived from the root of *Sanguinaria canadensis*, induced apoptosis in human cancer cells, which was assessed by AO/EtBr staining [20].

Phytochemical screening of the leaves identified phenolics and flavonoids as the major antioxidant components in the leaves of *Z. mays* [21]. The cancer-protective effects of flavonoids and phenolics have been attributed to a wide variety of mechanisms, including free radical scavenging, modifying enzymes that activate or detoxify carcinogens [22].

5. Conclusion

The aim of anticancer agents is to trigger the apoptosis signaling system in these cancer cells while disturbing their proliferation. Plants have many phytochemicals with various bioactivities, including antioxidant, anti-inflammatory, and anticancer activities. Therefore, many plants have been examined to identify new and effective antioxidants and anticancer compounds, as well as to elucidate the mechanisms of cancer prevention and apoptosis. Our

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findings suggest that the *Z. mays* leaf extracts most likely have anticancer properties. The qualitative analysis revealed the presence of phenolics and flavonoids in *Z. mays* leaves, which may contribute to their medicinal properties.

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