Results

4. RESULTS

A majority of the present day diseases are reported to be due to the shift in the balance of the pro-oxidant and the antioxidant homeostatic phenomenon in the body. Pro-oxidant conditions dominate either due to the increased generation of the free radicals caused by excessive oxidative stress of the present day life, or due to the poor scavenging/quenching in the body caused by depletion of the dietary antioxidants (Muller *et al.*, 2007).

Uncontrolled production of ROS often leads to the damage of cellular macromolecules (DNA, lipids and protein) and other small antioxidant molecules. Oxidative stress induces a cellular redox imbalance, which has been found to be present in various cancer cells compared with normal cells; the redox imbalance thus may be related to oncogenic stimulation (Murphy, 2009). It is possible to reduce the risks of chronic diseases and prevent disease progression by either enhancing the body's natural antioxidant defenses or by supplementing with proven dietary antioxidants (Genestra, 2007).

The quest for natural antioxidants for dietary, cosmetic and pharmaceutical uses has become a major industrial and scientific research challenge over the last two decades. Efforts to gain extensive knowledge regarding the power of antioxidants from plants and to tap their potential are therefore on the increase.

Many plants are distributed in our country of rich biodiversity, the potential of which are not fully utilized for want of knowledge. A plant that has a high potential antioxidant property but is not being exploited fully in the society at present is the grass of maize (*Zea mays*). The present study is an attempt to analyze the various potential of the leaves at different time periods of growth and explore its uses to cure various disorders.

ANTIOXIDANT STATUS IN THE LEAVES OF *Zea mays*

In the first phase of the study, the antioxidant content was assessed in the leaves of *Zea mays* at six different time periods of growth namely 5, 10, 15, 20, 25 and 30 days after sowing, in order to determine whether any change in the antioxidant status was observed as the age of the plant increased. Both enzymic and non-enzymic components of the antioxidant defense system were quantified. The values obtained are presented below.

ACTIVITIES OF ENZYMIC ANTIOXIDANTS IN *Zea mays* LEAVES

The enzymic antioxidants in *Zea mays* leaves analyzed on the 5th, 10th, 15th, 20th, 25th and 30th days after sowing (Plate 1) were superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione S-transferase (GST) and glutathione reductase (GR). The activities obtained are presented in Table 1.

TABLE 1

ENZYMIC ANTIOXIDANT ACTIVITIES IN THE LEAVES OF *Zea mays* AT SIX DIFFERENT TIME PERIODS OF GROWTH

SAMPLE	SOD activity (Units$/mg Protein	CAT activity (Units#/mg Protein)	POD activity (Units@/mg protein)	GST activity (Units©/mg Protein)	GR activity (Units* / mg protein)
5 DAS	0.92 ± 0.01 [a]	20.2 ± 0.5 [a]	0.65 ± 0.02 [a]	0.11 ± 0.005 [a]	10.30 ± 0.10
10 DAS	0.99 ± 0.03 [a]	24.3 ± 0.7	0.99 ± 0.06	0.21 ± 0.010	12.90 ± 0.06
15 DAS	0.85 ± 0.07 [a,b]	21.7 ± 1.0 [a]	0.91 ± 0.04	0.16 ± 0.005	9.25 ± 0.05
20 DAS	0.80 ± 0.02 [b]	15.1 ± 0.1	0.77 ± 0.03 [b]	0.12 ± 0.015 [a]	6.32 ± 0.09
25 DAS	0.70 ± 0.05 [c]	8.60 ± 0.6 [b]	0.75 ± 0.10 [b,c]	0.09 ± 0.012 [a]	6.04 ± 0.04
30 DAS	0.72 ± 0.01 [c]	8.03 ± 2.0 [b]	0.70 ± 0.02 [a,c]	0.10 ± 0.005 [a]	4.00 ± 0.30
CD value	0.0778	1.795	0.0608	0.0399	0.0169

The values are Mean ± S.D. of triplicates

Values superscripted by similar alphabets within the same column indicate that they do not differ significantly at ($P < 0.001$) level.

$ 1 unit = amount of enzyme that causes 50 per cent reduction in NBT oxidation

1 Unit = Amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units/minute

@ 1 Unit = Change in absorbance at 430 nm /minute

© 1 unit = nmoles of CDNB conjugated / minute

*1 unit = mmoles NADPH oxidized

DAS – Days after sowing

The results revealed that the leaves of *Zea mays* possessed considerable activities of all the enzymic antioxidants studied, at all the different periods of growth selected. The leaves on the 10th day of growth were found to have maximum activity of the enzymic antioxidants studied, followed closely by the leaves on the 5th and 15th days of growth. There was a decline of enzyme activities thereafter. The results showed that the *Zea mays* leaves are excellent sources of antioxidants, especially at their early stages of growth.

PLATE 1

***Zea mays* LEAVES AT DIFFERENT TIME PERIODS OF GROWTH**

LEVELS OF NON-ENZYMIC ANTIOXIDANTS IN *Zea mays* LEAVES

The non-enzymic antioxidants analyzed were ascorbate, tocopherol, total carotenoids, lycopene, reduced glutathione, chlorophyll, total phenols and flavonoids. The results obtained are presented in Table 2.

From the results, it was observed that the levels of non-enzymic antioxidants (ascorbic acid, tocopherol, total carotenoids, lycopene, reduced glutathione, total phenols and flavonoids) were considerably higher in the 10^{th} day leaves. A reduction in the levels of non-enzymic antioxidants was noted in the leaves collected from the 20^{th}, 25^{th} and 30^{th} day plants. The levels of chlorophyll were higher on the 25^{th} day old plant followed by the 30^{th}, 20^{th}, 15^{th}, 10^{th} and 5^{th} day old leaves. The maximum level of reduced glutathione was observed on the 5^{th} day of growth, the values of which decreased steadily as the plants grew.

Thus, the findings of phase I revealed that the leaves on the 10^{th} day of growth was found to have maximum content of all the enzymic and non-enzymic antioxidants, followed closely by the leaves on their 5^{th} day and 15^{th} day of growth, compared to other time points tested. Therefore, the 10^{th} day leaves were selected for further studies.

PHASE II

The outcome of the first phase revealed that the leaves of *Zea mays* were found to be a rich source of both enzymic and non-enzymic antioxidants. In order to throw light on the nature of the bioactive component responsible for the antioxidant potential of the leaves of 10^{th} day plant, extracts were prepared in solvents of differing polarity namely water, methanol and chloroform and all the three extracts were taken for further analyses of the study.

In the next phase of the study, *Zea mays* leaf extracts were tested for their radical scavenging properties against DPPH, ABTS, non-radical (hydrogen peroxide), *in vitro* generation of hydroxyl radicals, superoxide and nitric oxide radicals. The effect of the extracts was also tested on DNA, lipids and cells (goat liver slices) exposed to oxidative stress *in vitro*.

TABLE 2

NON-ENZYMIC ANTIOXIDANT LEVELS IN THE LEAVES OF *Zea mays* AT SIX DIFFE

Sample	Ascorbic acid (mg / g leaf)	Tocopherol (mg / g leaf)	Total carotenoids (mg / g leaf)	Lycopene (mg / g leaf)	Reduced glutathione (nmoles/ g leaf)
5 DAS	2.03 ± 0.20[a]	1.59 ± 0.04 [a]	20.0 ±0.5	13.66 ± 0.39 [a]	233.0 ± 5.0
10 DAS	3.83 ± 0.40	2.04 ± 0.20 [b]	32.0 ± 0.8	15.80 ±0.05	204.7 ± 6.5
15 DAS	2.98 ± 0.42	2.00 ± 0.05 [b]	26.3 ± 2.5 [a]	13.46 ± 0.51 [a]	186.8 ± 6.0
20 DAS	1.70 ± 0.10 [a]	1.60 ± 0.30 [a]	26.0 ± 0.9 [a]	13.25 ± 0.02 [a,b]	113.5 ± 3.5
25 DAS	1.67 ± 0.60 [a]	1.59 ± 0.05 [a]	28.0 ± 1.0 [a]	13.14 ± 0.50 [a,b]	74.0 ± 5.0 [a]
30 DAS	1.53 ± 0.50 [a]	1.51± 0.04 [a]	16.0 ± 0.8 [a]	12.23 ± 0.62 [b]	68.0 ± 2.0 [a]
CD value	0.722	0.270	2.249	0.744	8.730

The values are Mean ± S.D. of triplicates

DAS – Days after sowing

Values superscripted by similar alphabets within the same column indicate that they do not differ s

RADICAL SCAVENGING EFFECTS OF *Zea mays* LEAF EXTRACTS

The extracts were tested for their radical scavenging effects against a battery of oxidant moieties that included the radicals DPPH, ABTS, H_2O_2 (non-radical), $OH^{\bullet}$, $SO^{\bullet}$ and NO. The results obtained are depicted below.

DPPH RADICAL SCAVENGING ACTIVITY

The per cent extent of DPPH scavenging by the *Zea mays* leaf extracts is presented in Figure 1.

It was observed that *Zea mays* leaf extracts effectively reduced the stable radical DPPH to the yellow coloured compound diphenylpicrylhydrazine. The maximum scavenging effect was observed in the methanolic extract, followed by the aqueous and chloroform extracts.

ABTS RADICAL SCAVENGING ACTIVITY

In an effort to express the antioxidant capacity of *Zea mays* leaf extracts, we also utilized another type of stable free radical species, ABTS. The values obtained are depicted in Figure 1.

All the three extracts could scavenge the ABTS free radicals, albeit to different extents. The methanolic extract was the most effective, while chloroform extract was the least effective.

HYDROGEN PEROXIDE SCAVENGING EFFECTS OF *Zea mays* LEAF EXTRACTS

The ability of *Zea mays* leaf extracts to scavenge H_2O_2 in an *in vitro* system was followed in the present study and the results are depicted in Figure 1.

All the three extracts (aqueous, methanol and chloroform) of *Zea mays* leaves exhibited strong H_2O_2-scavenging effects. The extents of scavenging were more or less similar, with the methanolic extract showing maximum effect, followed closely by aqueous and then chloroform extracts.

HYDROXYL RADICAL SCAVENGING ACTIVITY

The hydroxyl radical has high reactivity, making it a very dangerous radical with a very short half-life of approximately 10^{-9} seconds *in vivo*. 2'- deoxyribose (2'- dR) can act as a specific substrate for oxidation by hydroxyl radicals, and the extent of TBARS produced in the reaction is taken as a measure of hydroxyl radical production. The inhibition of TBARS production is, thus, considered as a measure of hydroxyl radical scavenging efficiency. The extent of damage to 2'- dR was measured in the presence and the absence of *Zea mays* leaf extracts and the values are presented in Figure 2.

The exposure to H_2O_2 caused the maximum damage to dR. This damage was very effectively counteracted by the treatment of the leaf extracts. The methanolic extract exhibited the maximum effect, followed by aqueous and then chloroform extracts.

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE *in vitro* GENERATION OF SO$^{\bullet}$ AND NO RADICALS

The per cent inhibition of SO$^{\bullet}$ and NO generation in the presence of the leaf extracts was calculated and the values are presented in Figure 3.

The three different extracts of *Zea mays* leaves were found to be very good scavengers of superoxide *in vitro* with the maximal inhibitory effect found with methanolic extract. Only a moderate reduction in NO generation was observed with all the three different extracts of *Zea mays* leaves. The extent of inhibition of nitric oxide generation *in vitro* was found to be lower than the extent of inhibition of SO$^{\bullet}$ generation. The next step was to determine the reducing and chelating activities of the leaf extracts.

REDUCING AND CHELATING ACTIVITIES OF *Zea mays* LEAF EXTRACTS

The reducing and chelating activities of *Zea mays* leaf extracts were analyzed using spectrophotometric methods. The results are presented in Figure 4.

The results indicated that the methanolic extract was very effective in both reducing and chelating metal ions. The methanolic extract exhibited the maximum activity. The reducing as well as chelating activities of methanolic extract were higher followed by aqueous and chloroform extracts.

FIGURE 1
RADICAL SCAVENGING EFFECTS OF *Zea mays* LEAF EXTRACTS

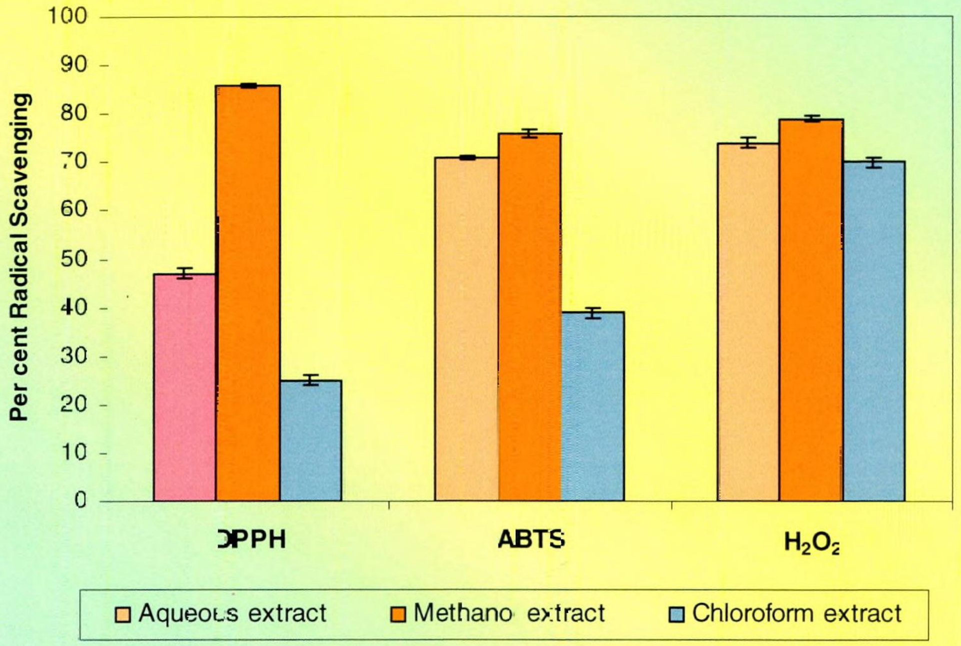
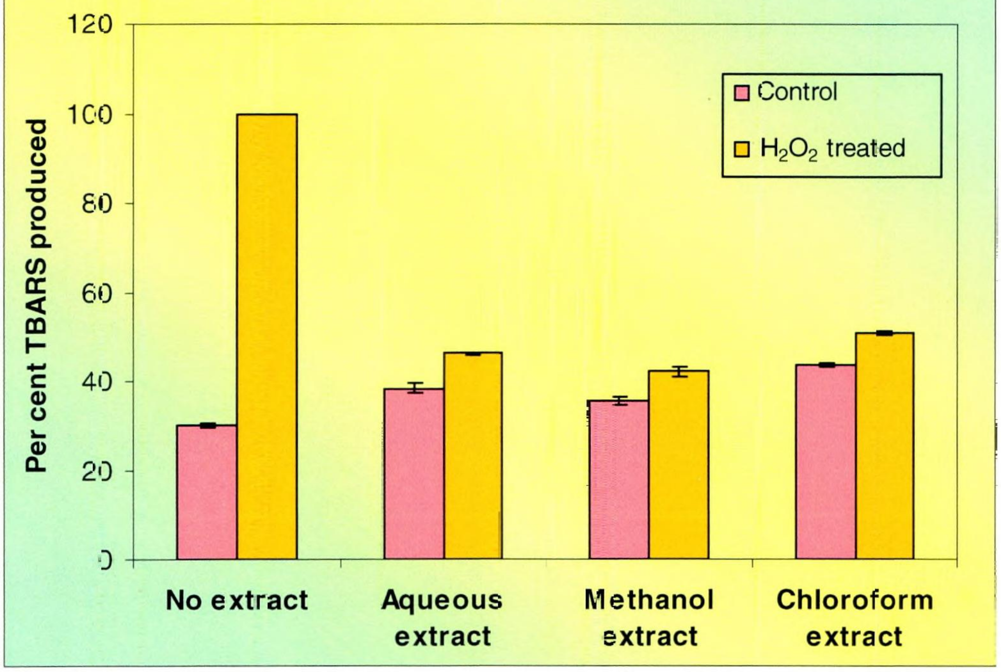
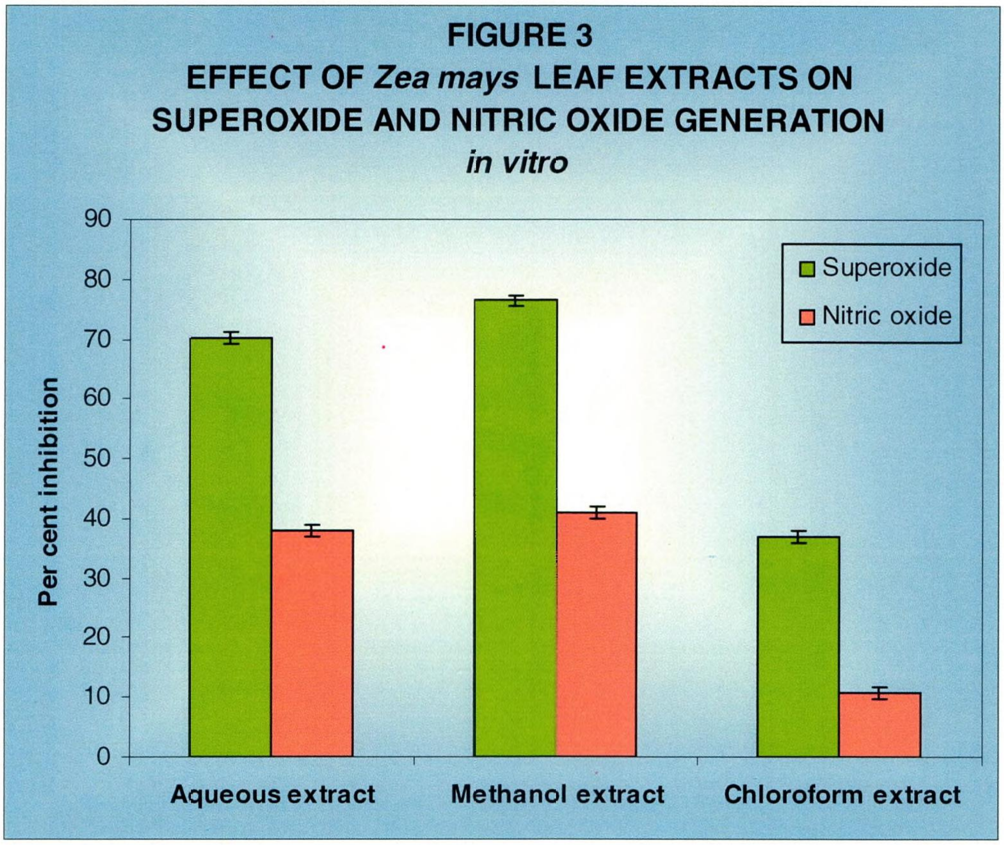
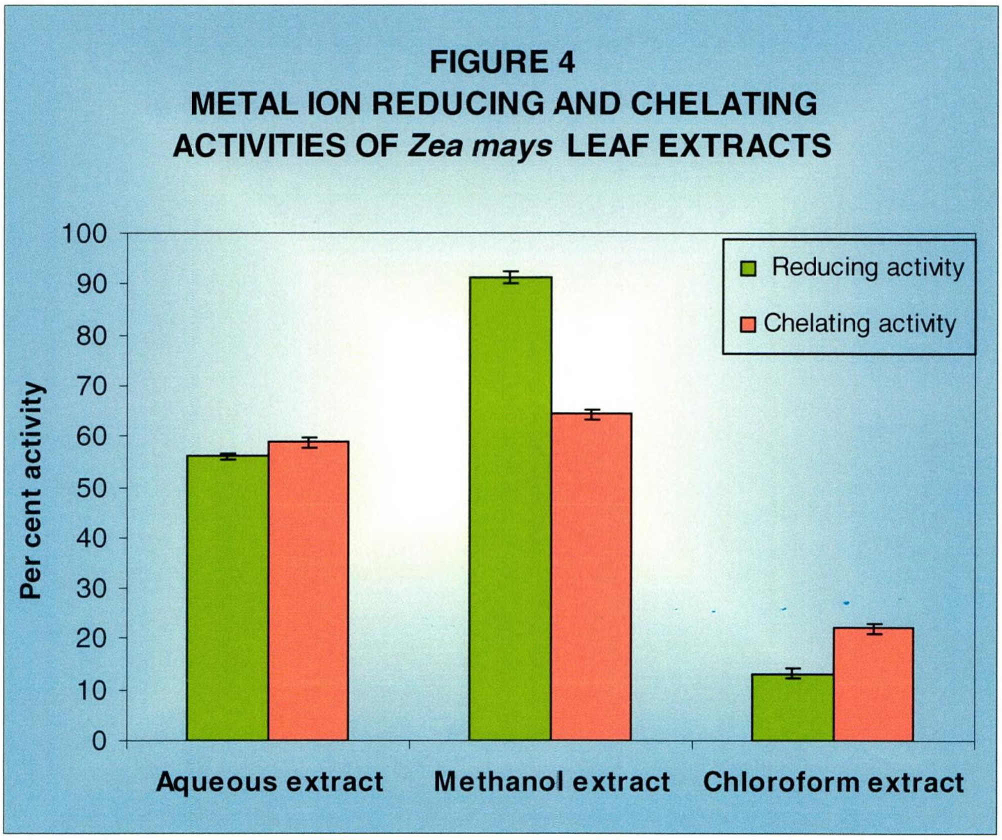


FIGURE 2
EFFECT OF *Zea mays* LEAF EXTRACTS AGAINST OXIDATIVE DAMAGE TO DEOXYRIBOSE



The values of the positive (H_2O_2 treated) group were fixed as 100% damage and the per cent damage in the other groups were calculated relative to this.





The reducing activity of BHA, a standard antioxidant was fixed as 100 per cent and the activity of the three extracts were calculated respective to this.

EFFECT OF *Zea mays* LEAVES ON OXIDATIVE DAMAGE TO BIOMOLECULES

Free radicals are prone to oxidize intracellular molecules, such as lipids, DNA and proteins, giving rise to alterations in the cell structure. It has been shown that LPO induces disturbance of fine structures, (alteration of integrity, fluidity, permeability and functional loss of biomembranes), modifies low density lipoprotein (LDL) to proatherogenic and proinflammatory forms, and generates potentially toxic products. LPO products have been shown to be mutagenic and carcinogenic (Niki, 2009).

Hence, it was felt very crucial to study the extent of oxidative damage to lipids by standard oxidants and the effect of the *Zea mays* leaves on this damage.

EXTENT OF INHIBITION OF *in vitro* LIPID PEROXIDATION

The extent of lipid peroxidation (LPO) was studied in three different systems. The first system was goat liver homogenate clarified by centrifugation to remove unbroken cells and cell debris. This system consisted predominantly of intact organelles (intracellular membrane lipids). The second system used was RBC ghosts, prepared by hypotonic lysis of RBCs. This system constituted plasma membrane lipids.

In the third system, lipid peroxidation was induced by exposure of goat liver slices *in vitro* by incubating with H_2O_2. The extent of inhibition of this LPO was studied in the presence of the leaf extracts. This system constituted the intact cell that contains both the plasma membrane and the intracellular membranes.

The per cent inhibition of *in vitro* lipid peroxidation by the leaf extracts in all the three membrane systems is presented in Figure 5.

The aqueous and methanolic extracts of *Zea mays* leaves inhibited LPO to a considerable extent in all the three systems studied. The magnitude of inhibition of LPO exhibited by the chloroform extract was considerably low. The results obtained in the present study indicated that the lipid components of both plasma membrane as well as intracellular membranes can be protected from LPO by the leaf extracts.

EFFECTS OF *Zea mays* LEAF EXTRACTS ON OXIDATIVE DAMAGE TO DNA

Several types of DNA damage are thought to be a result of ROS assault, which can attack DNA at the deoxyribose molecule or at any of the purine or pyrimidine bases. Attack at a sugar can lead to sugar fragmentation, base loss and strand breaks. Attack at a base can lead to modification of the nucleotide bases, which ultimately leads to various disorders (Blair, 2008). Hence, it was felt imperative to study the influence of the leaf extracts on DNA in different *in vitro* systems.

The protective effects of *Zea mays* leaf extracts on oxidative damage inflicted upon DNA of different hierarchical levels (λ phage DNA, haploid herring sperm DNA, diploid calf thymus DNA and intact cell DNA) were analyzed and the results are presented below.

MIGRATION PATTERN OF λ DNA TREATED WITH H_2O_2 WITH AND WITHOUT THE LEAF EXTRACTS

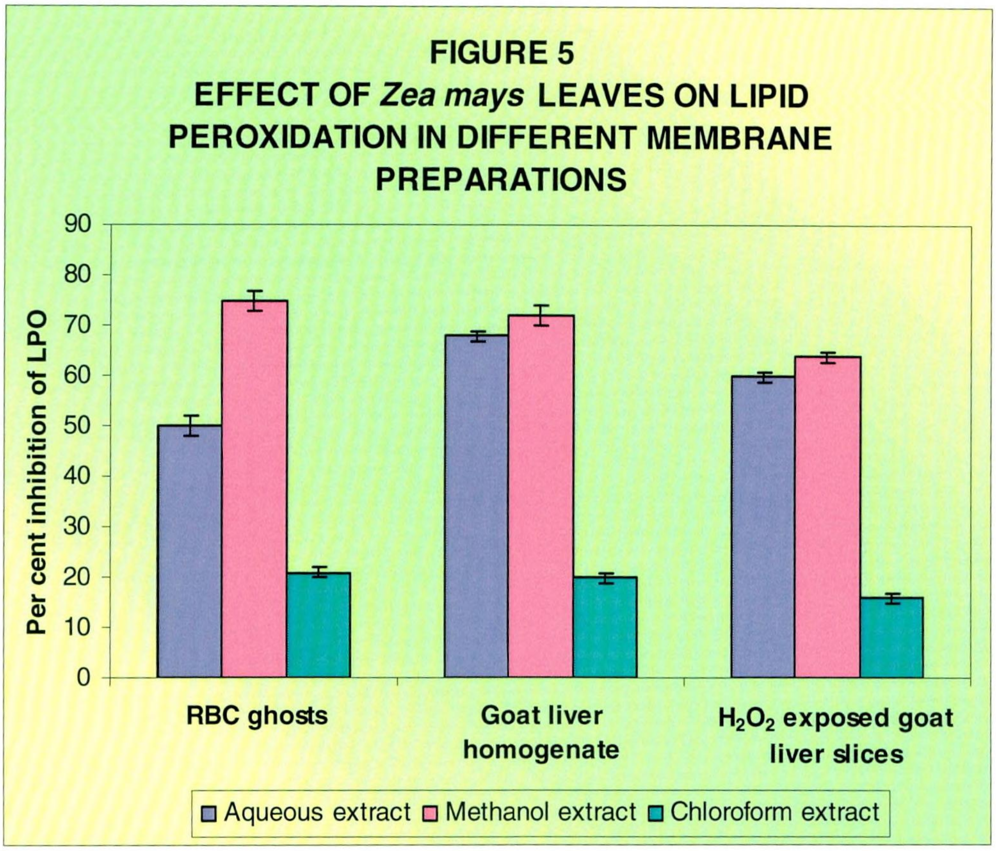
The extent of damage to λ DNA by H_2O_2 in the presence and the absence of the extracts of *Zea mays* leaves were studied by following the migration pattern of the treated DNA on agarose gels. The resultant pattern is presented in Plate 2.

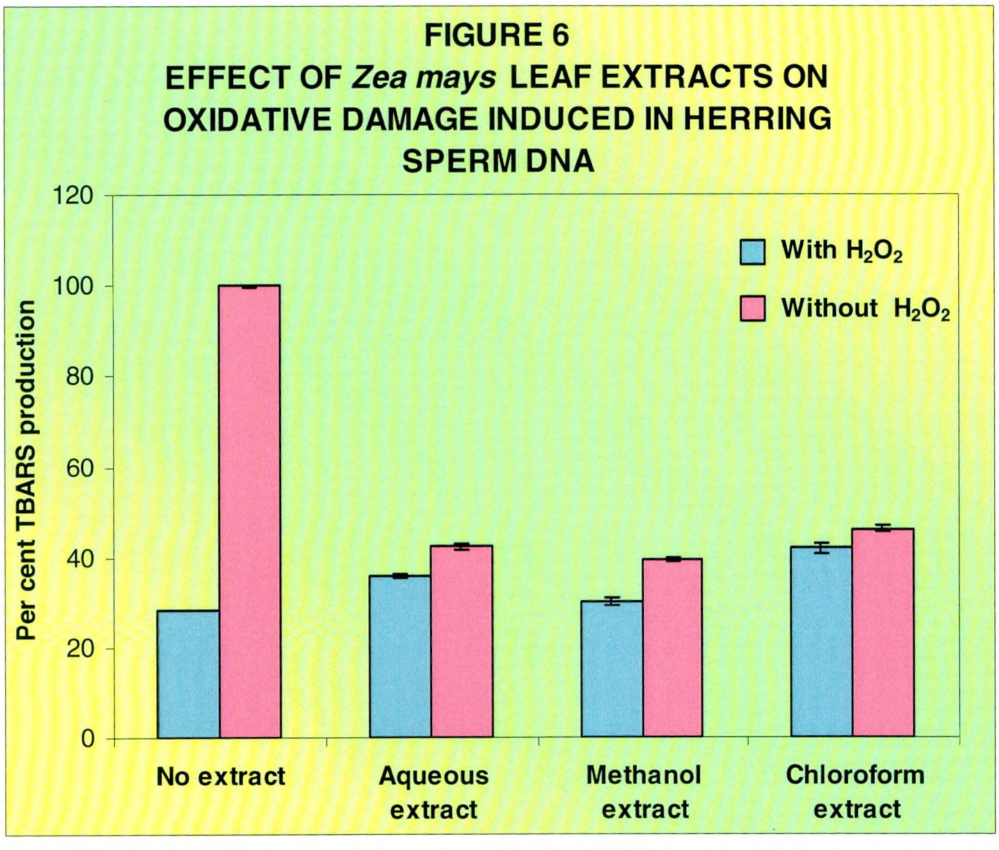
As can be seen from the picture, H_2O_2 caused extensive damage to λ DNA (lane 2). The leaf extracts, by themselves, did not cause any damage to λ DNA (lanes 3, 5 and 7). Additionally, the presence of the extracts caused a significant protection to λ DNA, signified by the presence of intact bands (lanes 4, 6 and 8).

EFFECT OF *Zea mays* LEAF EXTRACTS ON H_2O_2-INDUCED DAMAGE TO HERRING SPERM DNA

The extent of damage inflicted by H_2O_2 to herring sperm DNA was studied in the presence and the absence of *Zea mays* leaf extracts and quantified as the amount of TBARS formed. The results are represented in Figure 6.

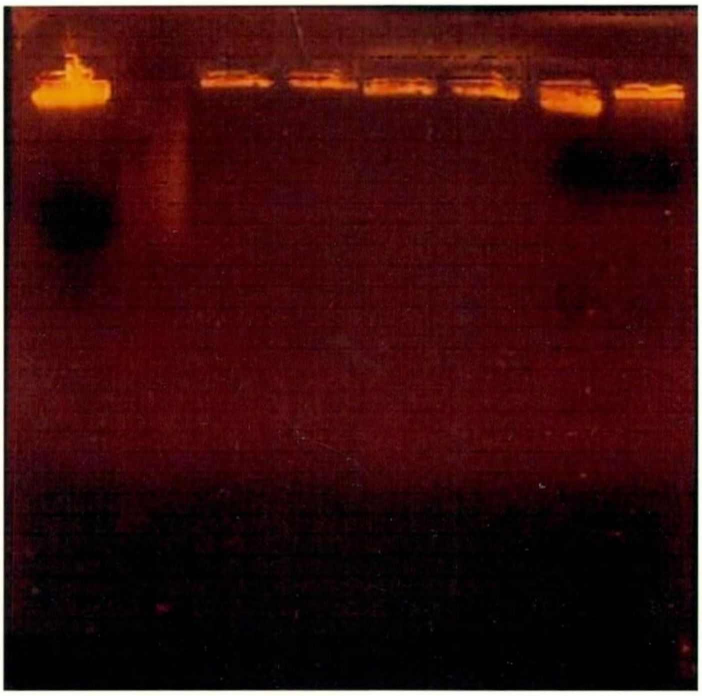
It is evident from the figure that H_2O_2 caused the maximum damage to herring sperm DNA. All the three extracts caused a highly significant decrease in the damage inflicted by H_2O_2 on herring sperm DNA. The effect of the methanolic extract of *Zea mays* leaves was more pronounced than the aqueous and chloroform extracts.





The values of the positive (H_2O_2 treated) group were fixed as 100% damage and the per cent damage in the other groups were calculated relative to this.

PLATE 2
MIGRATION PATTERN OF DNA TREATED WITH H_2O_2 WITH AND WITHOUT *Zea mays* LEAF EXTRACTS



Lane 1 – Control
Lane 2 – H_2O_2
Lane 3 – Aqueous extract
Lane 4 – H_2O_2 + Aqueous extract
Lane 5 – Methanol extract
Lane 6 – H_2O_2 + Methanol extract
Lane 7 – Chloroform extract
Lane 8 – H_2O_2 + Chloroform extract

EFFECT OF *Zea mays* LEAF EXTRACTS ON H_2O_2-INDUCED DAMAGE TO CALF THYMUS DNA

The extent of damage to calf thymus DNA was quantified spectrophotometrically and the values are presented in Figure 7.

The maximum protection was offered by the methanolic extract, followed by aqueous extract in calf thymus DNA also. The chloroform extract showed a differential effect in the two sources of DNA. The amount of TBARS formed in herring sperm DNA was higher than in the calf thymus DNA.

EFFECT OF *Zea mays* LEAF EXTRACTS ON DNA DAMAGE INDUCED BY H_2O_2 IN INTACT CELLS

The DNA damaging effect of H_2O_2 was studied by following the formation of comets in peripheral blood cells exposed to the oxidant *in vitro*. The effect of the leaf extracts on this process was also followed.

TABLE 3

EFFECT OF *Zea mays* LEAF EXTRACTS ON DNA DAMAGE INDUCED BY H_2O_2 IN PERIPHERAL BLOOD CELLS

SAMPLE	Number of cells with comets / 100 cells	
	Control	H_2O_2 treated
No extract	11 ± 1	23 ± 1^{a}
Aqueous extract	4 ± 2^{a}	$7 \pm 2^{a,b}$
Methanol extract	3 ± 2^{a}	$4 \pm 1^{a,b}$
Chloroform extract	6 ± 1^{a}	8 ± 2^{b}

The values are Means ± SD of triplicates

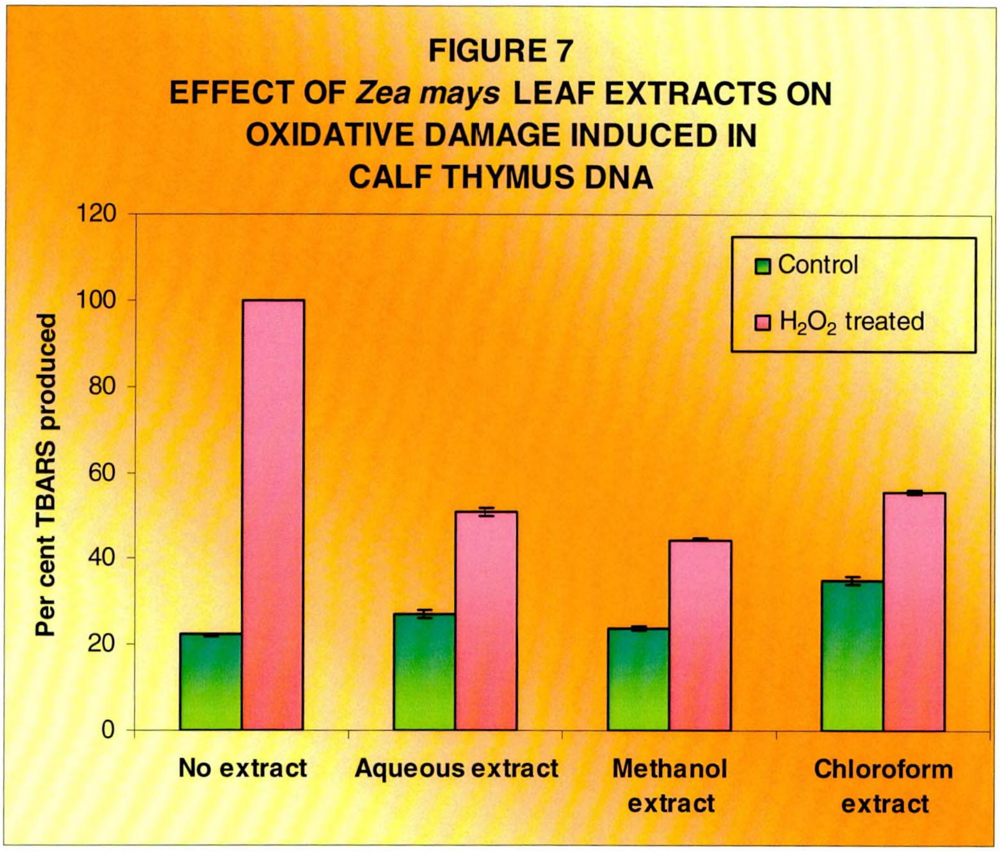
CD value = 3.77

a - Statistically significant (P<0.01) compared to untreated control

b - Statistically significant (P<0.01) compared to H_2O_2 alone treated group

c - Statistically significant (P<0.01) compared to the respective plant extract treated group

H_2O_2 exposure caused a steep increase in the number of cells with comets. In the positive control (cells treated only with H_2O_2), the DNA was severely damaged. This observation indicated that the exposure to hydrogen peroxide results in DNA damage.



The values of the positive (H_2O_2 treated) group were fixed as 100% damage and the per cent damage in the other groups were calculated relative to this.

The extracts of *Zea mays* leaves lowered the incidence of comets significantly with reference to the untreated controls. The simultaneous treatment with the leaf extracts and H_2O_2 decreased the number of cells expressing the DNA damage significantly. Surprisingly, the values remained well below the untreated group in the leaf extract exposed groups. Thus, the results indicated that the leaf extracts were able to protect the DNA in peripheral blood cells against oxidative damage. Plate 3 shows the photographic record of the comet cells in each treatment group.

EFFECT OF *Zea mays* LEAF EXTRACTS ON DNA REPAIR

The extent of DNA damage was significantly reduced by the co-administration of the cells with the leaf extracts. This reduction could be due to the prevention of DNA damage (i.e., scavenging of the DNA damaging agent by the components of leaf extracts) or to the effective repair of the damaged DNA.

In order to ascertain the exact mechanism that is operating in the system, Unscheduled DNA synthesis (UDS) was monitored. The incorporation of ^{3}HTdR was used as a measure of unscheduled DNA synthesis in live cells. Table 4 shows the radioactivity incorporated into Hep2 cell DNA in the presence or absence of H_2O_2 and /or the leaf extracts.

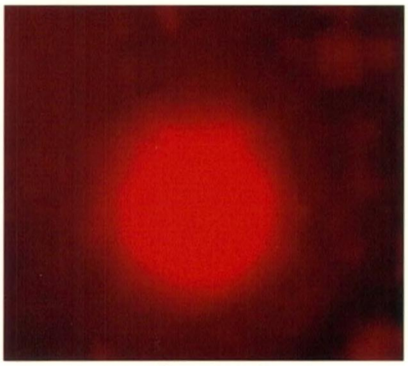
TABLE 4

EFFECT OF *Zea mays* LEAF EXTRACTS ON DNA REPAIR AFTER H_2O_2 ASSAULT *in vitro* IN Hep2 CELL LINE

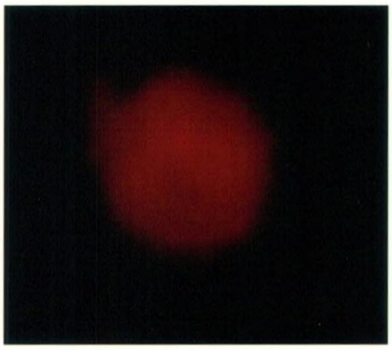
SAMPLE	Radioactivity incorporated into DNA			
	Control		H_2O_2 treated	
	dpm	Per cent incorporation	dpm	Per cent incorporation
No extract	1025	100	883	86
Aqueous extract	1002	98	1348	132
Methanol extract	1407	137	1410	138
Chloroform extract	925	90	1152	112

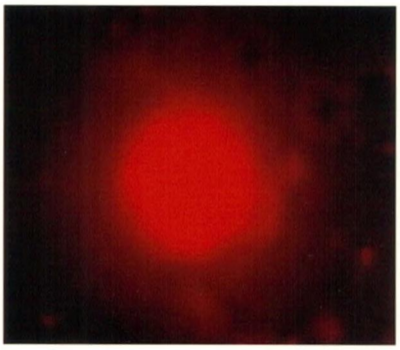
The values are Means of triplicates
The values of the negative (untreated) control group were fixed as 100% incorporation and the per cent incorporation in the other groups were calculated relative to this.

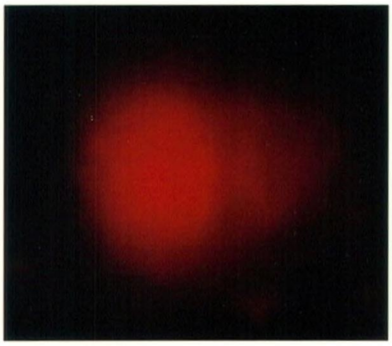
PLATE 3
COMET BEARING PERIPHERAL BLOOD LYMPHOCYTES

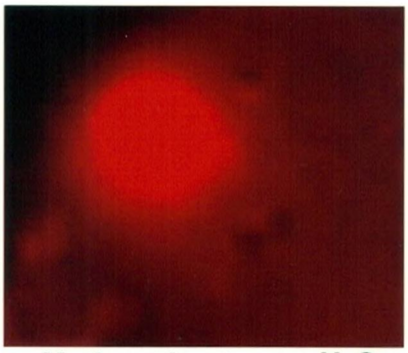

Control

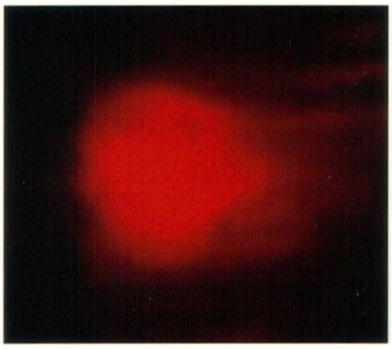

H_2O_2

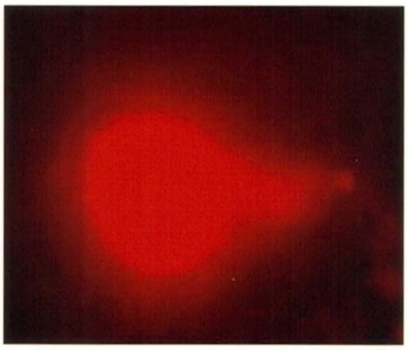

Aqueous extract


Aqueous extract + H_2O_2


Methanol extract


Methanol extract + H_2O_2


Chloroform extract


Chloroform extract + H_2O_2

The presence of H_2O_2 decreased the UDS, suggesting that DNA damage is sustained in the cells. The cells exposed to methanolic extract exhibited an increase in the incorporated radioactivity followed by the aqueous and chloroform extracts.

The results obtained thus far revealed that, among all the extracts tested, the methanolic extract of the 10 day old *Zea mays* leaves were very effective in radical scavenging, followed closely by the aqueous extract, implying that the active components contributing to the antioxidant activity are predominantly polar in nature. The *Zea mays* leaf extracts also possessed considerable levels of antioxidants, which were very effective in protecting the primary targets of oxidative assault namely lipids and DNA against oxidant-induced damage.

At the final step of the phase II study, we focused on alternate models instead of using live experimental animals. The goat liver slices were taken as the alternative model, which simulate *in vivo* conditions. The model was validated in earlier studies in our laboratory (Varier, 2002; Saraswathy, 2006; Sumathi, 2007; Vidya, 2007, Nirmala devi, 2008).

STUDIES ON THE ANTIOXIDANT STATUS IN LIVER SLICES EXPOSED TO OXIDANT AND LEAF EXTRACTS *in vitro*

Precision-cut goat liver slices were challenged with an oxidant (H_2O_2) both in the presence and in the absence of the different extracts of *Zea mays* leaves. The enzymic (SOD, CAT, POD, GST and GR) and non-enzymic (vitamin C, vitamin E, vitamin A and reduced glutathione) antioxidants were analyzed in the homogenate of the slices after one hour incubation at 37°C.

ENZYMIC ANTIOXIDANTS

The activities of the major enzymic antioxidants were assayed in the liver slice homogenate prepared after exposure to H_2O_2 and / or leaf extracts. The results are presented below.

SUPEROXIDE DISMUTASE

The activities of SOD in the slices exposed to H_2O_2 and/or *Zea mays* leaf extracts are represented in Table 5.

TABLE 5

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE SUPEROXIDE DISMUTASE ACTIVITY IN GOAT LIVER SLICES EXPOSED *in vitro* TO HYDROGEN PEROXIDE

SAMPLE	SOD ACTIVITY (Units[$]/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	13.2 ± 0.10	11.0 ± 0.50 [a]
Aqueous extract	17.0 ± 0.10 [a]	6.0 ± 0.40 [a,b,c]
Methanol extract	18.0 ± 0.05 [a]	16.9 ± 0.05 [a,b,c]
Chloroform extract	15.4 ± 0.05 [a]	13.6 ± 0.50 [b,c]

The values are Mean ± S.D. of triplicates

CD value = 0.699

$1 unit of enzyme is the amount that causes 50 per cent reduction in NBT oxidation

a - Statistically significant ($P<0.01$) compared to untreated control

b - Statistically significant ($P<0.01$) compared to H_2O_2 alone treated group

c - Statistically significant ($P<0.01$) compared to the respective plant extract treated group

The goat liver slices treated with the different extracts of *Zea mays* leaves showed a significant increase in SOD activity, compared to untreated control. Hydrogen peroxide caused a considerable decrease in the SOD activity. The co-treatment with the methanolic and aqueous extracts resulted in a significant increase compared to untreated control.

CATALASE

Table 6 depicts the effects of *Zea mays* extracts on the activities of catalase in the liver slices exposed *in vitro* to hydrogen peroxide.

TABLE 6

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE CATALASE ACTIVITY IN GOAT LIVER SLICES EXPOSED *in vitro* TO HYDROGEN PEROXIDE

SAMPLE	CAT ACTIVITY (Units[#]/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	212.0 ± 1.0	121.4 ± 1.2 [a]
Aqueous extract	377.0 ± 1.0 [a]	200.0 ± 1.0 [a,b,c]
Methanol extract	425.0 ± 1.0 [a]	308.0 ± 1.0 [a,b,c]
Chloroform extract	242.0 ± 2.0 [a]	147.8 ± 1.4 [a,b,c]

The values are Mean ± S.D. of triplicates

CD value = 2.96

#1 unit = Amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units

a - Statistically significant ($P<0.01$) compared to untreated control

b - Statistically significant ($P<0.01$) compared to H_2O_2 alone treated group

c - Statistically significant ($P<0.01$) compared to the respective plant extract treated group

The activity of catalase decreased significantly (P<0.01) upon exposure to H_2O_2. Treatment with the leaf extracts of *Zea mays* caused a significant increase (P<0.01) in the catalase activity compared to control. The liver slice exposed to the methanolic extract of *Zea mays* leaves showed the maximum increase in catalase activity compared to the untreated control as well as the groups exposed to the other extracts of the leaves.

PEROXIDASE

The activity of peroxidase was assayed in the goat liver slices exposed to H_2O_2 both in the presence and in the absence of *Zea mays* leaf extracts and the results are presented in Table 7.

TABLE 7

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE PEROXIDASE ACTIVITY IN GOAT LIVER SLICES EXPOSED *in vitro* TO HYDROGEN PEROXIDE

SAMPLE	POD (Units[@]/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	23.3 ± 1.02	21.4 ± 1.0
Aqueous extract	29.2 ± 1.06 [a]	26.0 ± 2.0[b]
Methanol extract	30.8 ± 1.40 [a]	24.8 ± 1.0[c]
Chloroform extract	25.8 ± 1.20	23.2 ± 1.0

The values are Mean ± S.D. of triplicates
CD value = 4.159
@1 unit = change in absorbance at 430 nm/minute
a - Statistically significant (P<0.01) compared to untreated control
b - Statistically significant (P<0.01) compared to H_2O_2 alone treated group
c - Statistically significant (P<0.01) compared to the respective plant extract treated group

The peroxidase activity in the H_2O_2 treated liver slices decreased compared to the untreated control. The toxic effect of H_2O_2 was negated by the concordant treatment with *Zea mays* leaf extracts. *Zea mays* leaf extracts, by themselves, significantly (P<0.01) increased the peroxidase activity compared to control, except chloroform extract.

GLUTATHIONE S-TRANSFERASE

GSTs are active in the detoxification of numerous products, including those that cause reactive oxidant damage to DNA and lipids, such as organic epoxides, lipid hydroperoxides, and unsaturated aldehydes (Hayes *et al.*, 2005). The GST activities in the hydrogen peroxide treated liver slices in the presence and the absence of different extracts of *Zea mays* leaves are expressed in Table 8.

TABLE 8

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE GLUTATHIONE S-TRANSFERASE ACTIVITY IN GOAT LIVER SLICES EXPOSED *in vitro* TO HYDROGEN PEROXIDE

SAMPLE	GST (Units[@]/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	0.025 ± 0.003	0.020 ± 0.001
Aqueous extract	0.028 ± 0.004	0.024 ± 0.002
Methanolic extract	0.030 ± 0.002	0.024 ± 0.003 [c]
Chloroform extract	0.024 ± 0.001	0.023 ± 0.002

The values are Mean ± S.D. of triplicates
CD value = 0.0058
@1 unit = nmoles of CDNB conjugated / minute
a - Statistically significant ($P<0.01$) compared to untreated control
b - Statistically significant ($P<0.01$) compared to H_2O_2 alone treated group
c - Statistically significant ($P<0.01$) compared to the respective plant extract treated group

The activities of GST decreased upon exposure to H_2O_2. Treatment with aqueous or methanolic extracts of *Zea mays* leaves caused an increase in GST activity over the control. They also caused the reversal of the decrease caused by H_2O_2 in GST activity, to a considerable extent. The chloroform extract of the leaves did not increase GST activity by itself. However, it effectively counteracted the effect of H_2O_2, raising the activity to near control values.

GLUTATHIONE REDUCTASE

The effect of *Zea mays* leaf extracts on the GR activities of H_2O_2 exposed liver slice homogenates was analysed and the results are presented in Table 9.

The GR activity was decreased slightly by H_2O_2, but this decrease was not statistically significant. When the liver slices were exposed to the different extracts of *Zea mays* leaves, an increase in GR activity was observed, with methanolic extract eliciting the maximum increase.

TABLE 9

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE GLUTATHIONE REDUCTASE ACTIVITY IN GOAT LIVER SLICES EXPOSED *in vitro* TO HYDROGEN PEROXIDE

SAMPLE	GR (mmoles NADPH oxidized/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	2.00 ± 0.2	1.60 ± 0.3
Aqueous extract	3.38 ± 1.3	2.86 ± 1.4
Methanol extract	4.18 ± 2.1	3.12 ± 1.1
Chloroform extract	3.08 ± 1.0	2.24 ± 1.0

The values are Mean ± S.D. of triplicates
CD value = 2.84
a - Statistically significant ($P<0.01$) compared to untreated control
b - Statistically significant ($P<0.01$) compared to H_2O_2 alone treated group
c - Statistically significant ($P<0.01$) compared to the respective plant extract treated group

H_2O_2-induced damage to the liver slices was reflected by the decreased antioxidant enzyme activities when compared to controls. Co-treatment of the liver slices with *Zea mays* leaf extracts prevented these changes from occurring on exposure to H_2O_2. Among the three extracts tested, the methanolic extract was capable of improving the levels of antioxidants studied to a higher extent followed by the aqueous extract. The chloroform extract exhibited minimal protective activities.

NON-ENZYMIC ANTIOXIDANTS

The first and second lines of defense of antioxidant system includes enzymic and non-enzymic antioxidants. In the present study, as representatives of non-enzymic antioxidants, the levels of vitamins C, E, A and reduced glutathione were estimated in the liver slices exposed to H_2O_2 in the presence or the absence of *Zea mays* leaf extracts.

VITAMIN C

The influence of *Zea mays* leaf extracts on the vitamin C levels in the hydrogen peroxide exposed liver slices is presented in Table 10.

H_2O_2 treatment caused a statistically significant ($p<0.01$) decrease in vitamin C levels. The levels of vitamin C in the liver slices exposed to the leaf extracts alone showed a significant ($p<0.01$) increase compared to untreated control. The co-exposure of liver slices to oxidant and the leaf extracts caused a rise in the levels of ascorbate. The maximum increase was observed with the methanolic extract of *Zea mays* leaves.

TABLE 10

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE ASCORBIC ACID LEVELS IN THE GOAT LIVER SLICES EXPOSED *in vitro* TO HYDROGEN PEROXIDE

SAMPLE	Ascorbic acid (mg/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	2.08 ± 0.002	0.196 ± 0.002 [a]
Aqueous extract	0.233 ± 0.002 [a]	0.304 ± 0.002 [a,b,c]
Methanol extract	0.233 ± 0.003 [a]	0.249 ± 0.004 [a,b,c]
Chloroform extract	0.250 ± 0.002 [a]	0.303 ± 0.001 [a,b,c]

The values are Mean ± S.D. of triplicates
CD value = 0.00572
a - Statistically significant ($P<0.01$) compared to untreated control
b - Statistically significant ($P<0.01$) compared to H_2O_2 alone treated group
c - Statistically significant ($P<0.01$) compared to the respective plant extract treated group

VITAMIN E

The effect of *Zea mays* leaf extracts on the tocopherol levels, in the presence and the absence of hydrogen peroxide in goat liver slices, is presented in Table 11.

TABLE 11

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE VITAMIN E LEVELS IN GOAT LIVER SLICES EXPOSED *in vitro* TO HYDROGEN PEROXIDE

SAMPLE	Vitamin E (μg/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	18.37 ± 1.8	16.58 ± 1.2
Aqueous extract	35.73 ± 2.0 [a]	30.15 ± 1.0 [a,b,c]
Methanol extract	43.15 ± 3.4 [a]	34.45 ± 2.4 [a,b,c]
Chloroform extract	28.09 ± 2.1 [a]	20.59 ± 1.0 [c]

The values are Mean ± S.D. of triplicates
CD value = 4.70
a - Statistically significant ($P<0.01$) compared to untreated control
b - Statistically significant ($P<0.01$) compared to H_2O_2 alone treated group
c - Statistically significant ($P<0.01$) compared to the respective plant extract treated group

The goat liver homogenate when treated with hydrogen peroxide showed a slight decrease in the levels of vitamin E, which, however, escaped statistical significance. This depleting effect was counteracted by the co-treatment with the leaf extracts. The vitamin E levels showed a significant ($p<0.01$) increase in the methanolic and aqueous extract treated groups. The levels were found to be higher in the methanolic extract treated group. The chloroform extract reverted the depleted vitamin E levels to control level in the oxidant treated group. It is, thus, conceivable from the results, that the treatment with the leaf extracts of *Zea mays* can improve the vitamin C and E status in the liver, thereby preventing H_2O_2-induced damages.

VITAMIN A

The levels of vitamin A observed in the different treatment groups are tabulated in Table 12.

H_2O_2 treatment caused a significant depletion in the levels of vitamin A compared to untreated controls. This depleting effect was reversed by the co-treatment with the leaf extracts. The extent of increase was maximum with the methanolic extract followed by the aqueous and chloroform extracts. The liver slice homogenates prepared after exposure to the leaf extracts alone also showed a marked increase in vitamin A levels with all the three extracts.

TABLE 12

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE VITAMIN A LEVELS IN GOAT LIVER SLICES EXPOSED *in vitro* TO HYDROGEN PEROXIDE

SAMPLE	Vitamin A (µg/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	121.0 ± 2.0	96.70 ± 2.2 ^{a}
Aqueous extract	166.5 ± 2.2 ^{a}	130.0 ± 1.0 ^{a,b,c}
Methanol extract	195.2 ± 2.1 ^{a}	159.3 ± 2.1 ^{a,b,c}
Chloroform extract	137.4 ± 2.1 ^{a}	118.8 ± 1.4 ^{b,c}

The values are Mean ± S.D of triplicates
CD value = 4.53

a - Statistically significant ($P<0.01$) compared to untreated control
b - Statistically significant ($P<0.01$) compared to H_2O_2 alone treated group
c - Statistically significant ($P<0.01$) compared to the respective plant extract treated group

GLUTATHIONE

Table 13 presents the levels of GSH in the oxidant treated goat liver slices in the presence and the absence of *Zea mays* leaf extracts.

TABLE 13

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE GLUTATHIONE LEVELS IN GOAT LIVER SLICES EXPOSED *in vitro* TO HYDROGEN PEROXIDE

SAMPLE	Glutathione (nmoles/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	116.8 ± 2.6	88.0 ± 2.0 ^{a}
Aqueous extract	159.2 ± 3.0 ^{a}	144.0 ± 2.0 ^{a,b,c}
Methanolic extract	200.0 ± 2.0 ^{a}	173.6 ± 1.8 ^{a,b,c}
Chloroform extract	131.2 ± 1.4 ^{a}	88.0 ± 3.0 ^{a,c}

The values are Mean ± S.D of triplicates

CD value = 5.46

a - Statistically significant (P<0.01) compared to untreated control

b - Statistically significant (P<0.01) compared to H_2O_2 alone treated group

c - Statistically significant (P<0.01) compared to the respective plant extract treated group

The oxidant (H_2O_2) treated liver slices showed a significant (P<0.01) decrease in the levels of reduced glutathione, compared to the untreated control. The methanolic and aqueous extracts of *Zea mays* leaves were effective in mitigating the toxicity caused by H_2O_2. The chloroform extract did not exert any protective effect, albeit increasing the GSH values in the absence of H_2O_2. The GSH levels in the liver slice homogenates prepared after exposure to the leaf extracts showed a significant (p<0.01) increase with all the three extracts.

Thus, the results show that the leaf extracts of *Zea mays* can improve the antioxidant status in the goat liver slices exposed *in vitro* to oxidative stress. Since the model was carefully planned to simulate *in vivo* conditions, it is perceivable that the effects also occur in the intact system.

PHASE III

The results obtained thus far proved that the *Zea mays* leaf extracts possessed strong antioxidant efficacy in all the systems studied. It is known that excessive oxidative damage, when unchecked, can result in cell death (Kannan and Jain, 2000). Therefore, in the present study, the extent of cell death induced by H_2O_2-induced oxidative stress was followed initially in untransformed cells. The cells used under this group were *Saccharomyces cerevisiae* cells and primary cultured chick embryo fibroblasts.

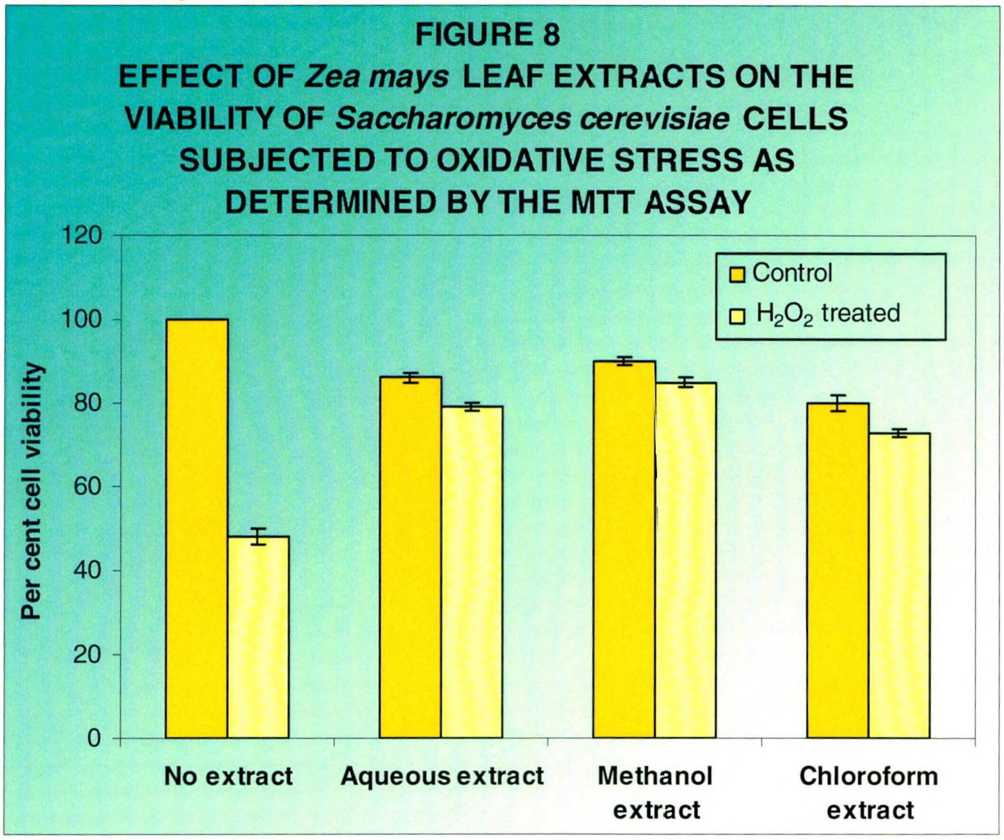
Yeast cells were chosen as they are considered to be ideal model organisms for eukaryotic cells and the entire genomic and proteomic characterization of these cells are being pursued as a prototype of all the higher eukaryotic systems (Coughlan and Brodsky, 2005). The other model system used was chick embryo fibroblasts, which constitute a primary cell culture system and is considered to be very close to human system. The study was planned in tune with one of the primary objectives of our research group, which is to standardize the use of alternative experimental systems for studying the protective effects of plant extracts and products, thereby minimizing the use of live animals in research.

EFFECT OF *Zea mays* ON H_2O_2-INDUCED DEATH IN *Saccharomyces cerevisiae* CELLS AND PRIMARY CHICK EMBRYO FIBROBLASTS

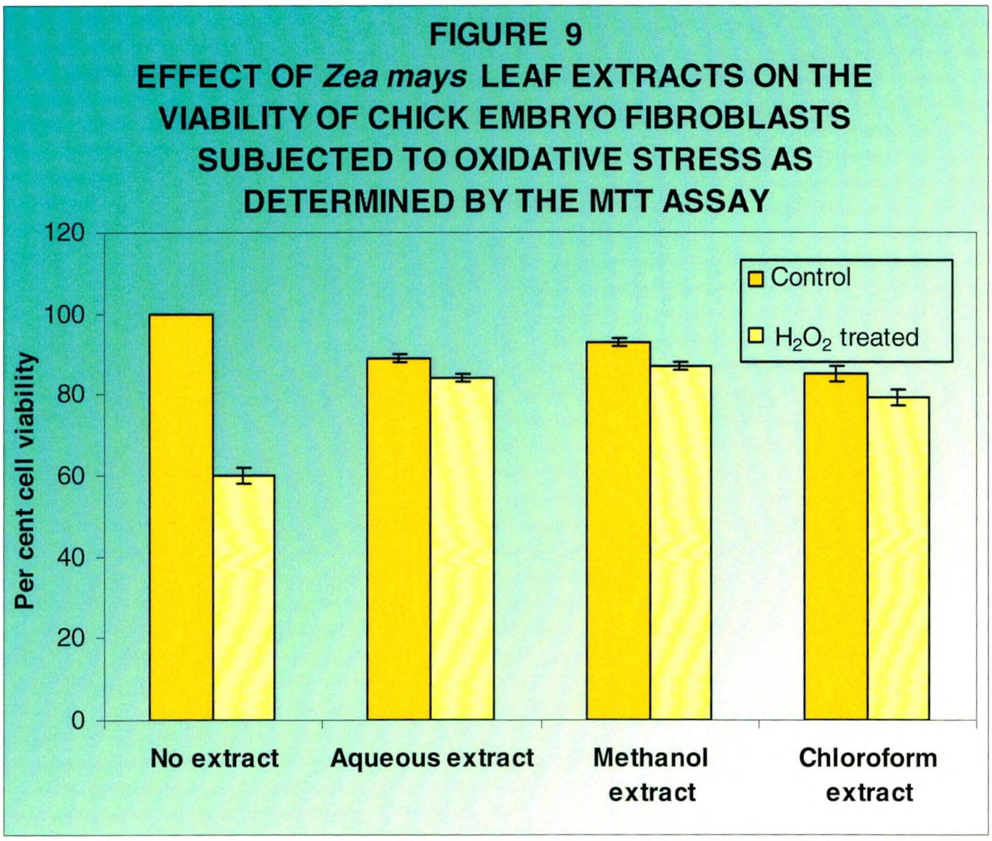
In the present investigation, the antioxidant effects of *Zea mays* leaf extracts on cell death induced in *Saccharomyces cerevisiae* cells and primary chick embryo fibroblasts by hydrogen peroxide (H_2O_2) was studied. The various parameters analysed were cell viability (MTT and SRB) and cellular and nuclear morphology (giemsa, PI, EtBr, DAPI and AO/EtBr). The results obtained for the various parameters analyzed are presented below.

CELL VIABILITY ASSAYS

MTT is used to determine the cell viability in assays of cell proliferation and cytotoxicity (Hazra *et al.*, 2007). The per cent cell viability was quantified using MTT in the different treatment groups. The extents of viabilities in the different treatment groups are shown in Figures 8 and 9.



The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.



The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.

The values presented in Figures 8 and 9 reveal that H_2O_2 exposure drastically brings down the viability of *Saccharomyces cerevisiae* cells and chick embryo fibroblasts. *Zea mays* leaf extracts increased the viability of cells subjected to oxidative stress, with the maximum cytoprotection rendered by the methanolic extract followed by the aqueous and the chloroform extracts. The leaf extracts by themselves also caused cell death to a certain extent in both the *Saccharomyces cerevisiae* cells and chick embryo fibroblasts compared to the untreated control groups.

The SRB assay provides a sensitive method for measuring the cytotoxicity of drugs. The increased absorbance of SRB is reported to be indicative of the proliferation efficiency of the cells (Vistica *et al.*, 1990).

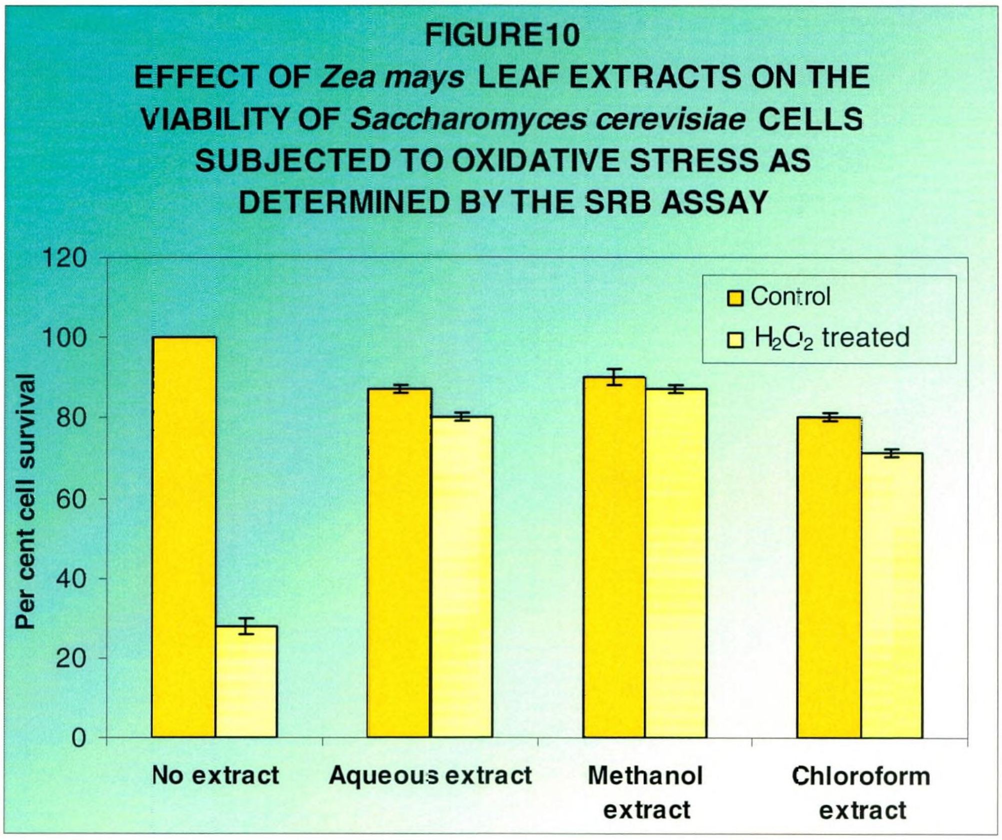
In the present investigation, SRB assay was used as an additional parameter to assess the viability and proliferative efficiency of yeast cells and primary chick embryo fibroblasts subjected to oxidative stress in the presence and the absence of *Zea mays* leaf extracts. Figures 10 and 11 summarize the data obtained.

The results obtained after the calculations for cytotoxicity as determined by the SRB assay showed that the viability reduced drastically in the H_2O_2 treated group of cells. The cell viability significantly increased in the *Zea mays* extracts treated cells even in the presence of apoptosis-inducing stress.

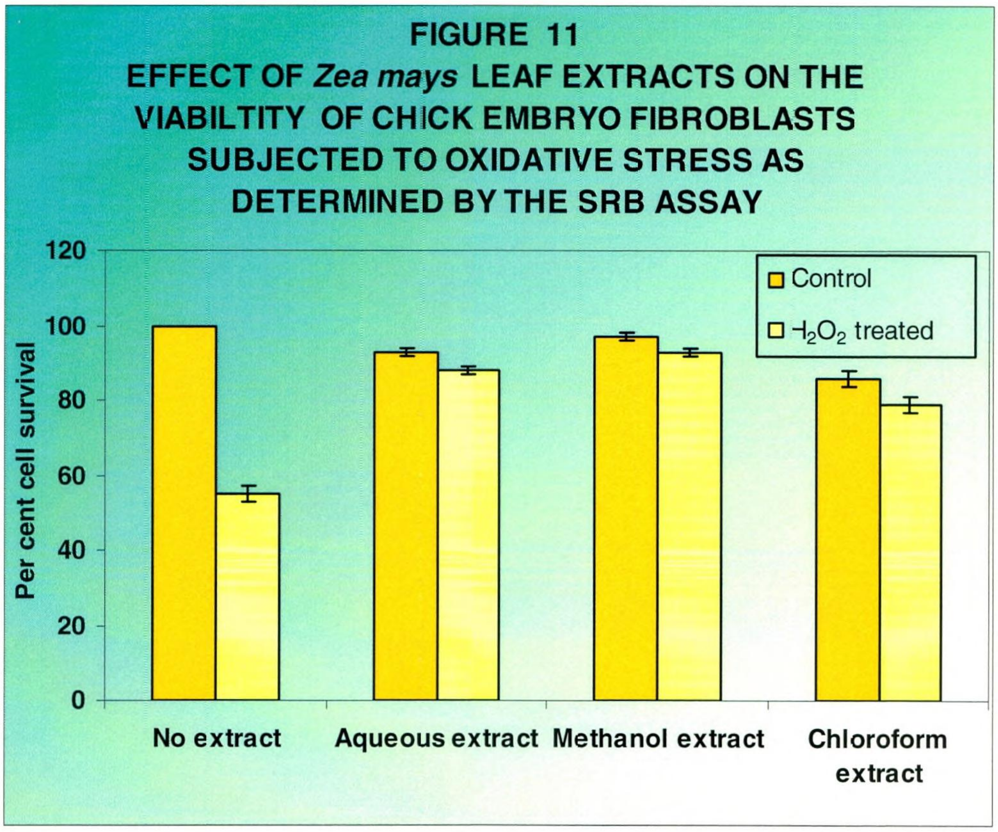
The results obtained with the MTT and SRB assays, thus, confirmed the protective effects of *Zea mays* leaves against oxidative stress-induced cell death in yeast cells and chick embryo fibroblasts.

MORPHOLOGICAL CHANGES OBSERVED IN THE YEAST CELLS

Giemsa is used to differentiate nuclear and/or cytoplasmic morphology of a variety of cells. The stain class was originally designed to incorporate cytoplasmic (pink) staining with nuclear (blue) staining (Garcia, 1999).



The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.



The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.

Apoptotic cells can be recognized by stereotypical morphological changes, which include shrinkage of cells, blebbing of plasma membrane and compaction and margination of nuclear chromatin (Mishra and Kumar, 2005). These changes were observed and quantified in the yeast cells and primary chick embryo fibroblasts subjected to oxidative stress in the presence and the absence of *Zea mays* leaf extracts, using phase contrast microscopy. The number of cells showing apoptotic morphological changes was counted in each experimental group per 100 cells in three different fields and the results are presented in Tables 14 and 15.

TABLE 14

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE MORPHOLOGICAL CHANGES IN *Saccharomyces cerevisiae* 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	4 ± 2	82 ± 2 [a]	0.04	4.5
Aqueous extract	16 ± 1 [a]	20 ± 2 [a,b]	0.19	0.25
Methanol extract	11 ± 3 [a]	15 ± 2 [a,b]	0.12	0.18
Chloroform extract	20 ± 0 [a]	25 ± 1 [a,b,c]	0.25	0.30

The values are means ± S.D of triplicates
CD value = 4.38
a - Statistically significant (P< 0.01) compared to untreated control group
b - Statistically significant (P< 0.01) compared to oxidant treated group
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

TABLE 15

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE MORPHOLOGICAL CHANGES IN CHICK EMBRYO FIBROBLASTS 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	11 ± 1	79 ± 1 [a]	0.12	3.76
Aqueous extract	20 ± 2 [a]	23 ± 3 [a,b]	0.25	0.30
Methanol extract	15 ± 2	18 ± 2 [a,b]	0.18	0.22
Chloroform extract	24 ± 0 [a]	28 ± 3 [a,b]	0.31	0.38

The values are means ± S.D of triplicates
CD value = 4.76
a - Statistically significant (P< 0.01) compared to untreated control group
b - Statistically significant (P< 0.01) compared to oxidant treated group
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

The number of apoptosing cells to normal appearing cells was calculated for each group as proposed by Cantarella *et al.* (2003). The data obtained are also represented in Tables 14 and 15.

The number of apoptotic cells increased in the oxidant treated groups (Plates 4 and 5). The plant extracts, by themselves, also exerted a cytotoxic effect to a minimal level. When administered along with H_2O_2, the leaf extracts resulted in a significantly decreased number of apoptotic cells. The maximum inhibition of H_2O_2-induced apoptosis was exhibited by the methanolic extract followed by the aqueous and chloroform extracts. These results indicate that the *Zea mays* leaves can render protection to *Saccharomyces cerevisiae* cells and chick embryo fibroblasts against H_2O_2-induced cell death.

NUCLEAR CHANGES

The nuclear morphologies that characterize apoptosis are chromatin condensation, nuclear fragmentation and cornering of the nuclear contents. These changes were observed and quantified in the present study in the yeast cells and chick embryo fibroblasts subjected to oxidative stress in the presence and the absence of the leaf extracts. The number of cells showing nuclear apoptotic morphology was counted by PI staining in each experimental group of the yeast cells and chick embryo fibroblasts and the results are presented in Tables 16 and 17.

TABLE 16

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN *Saccharomyces cerevisiae* 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	5 ± 2	85 ± 3 [a]	0.05	5.7
Aqueous extract	14 ± 1 [a]	18 ± 2 [a,b]	0.16	0.22
Methanol extract	12 ± 2 [a]	15 ± 3 [a,b]	0.14	0.18
Chloroform extract	18 ± 2 [a]	25 ± 2 [a,b,c]	0.21	0.33

The values are means ± S.D of triplicates

CD value = 5.27

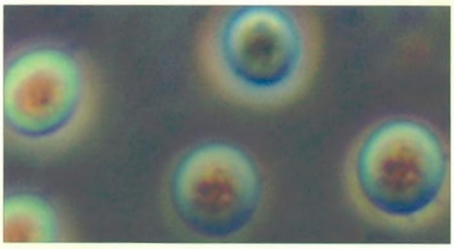
a - Statistically significant (P< 0.01) compared to untreated control group

b - Statistically significant (P< 0.01) compared to oxidant treated group

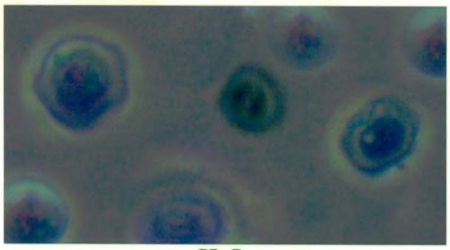
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

PLATE 4

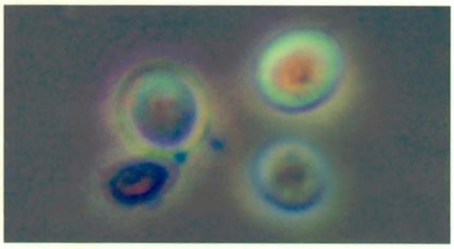
Saccharomyces cerevisiae CELLS STAINED WITH GIEMSA (OXIDANT-H_2O_2)



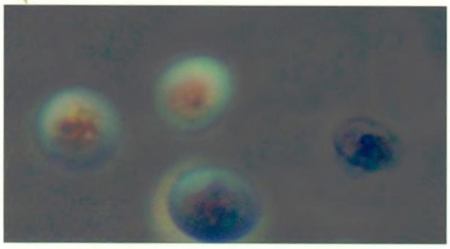
Control



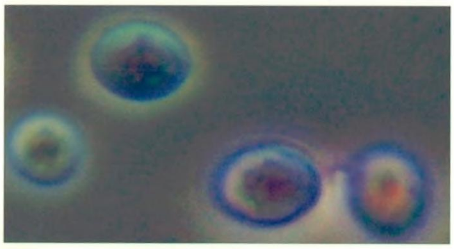
H_2O_2



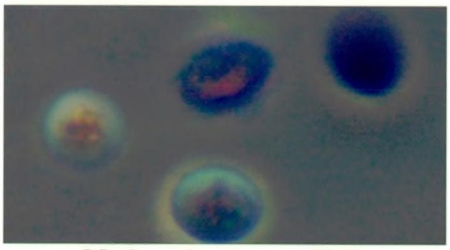
Aqueous extract



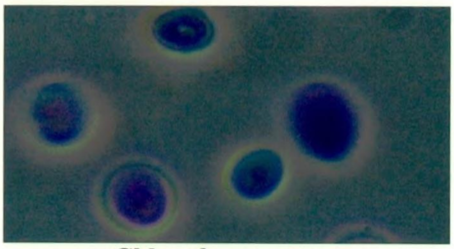
Aqueous extract + H_2O_2



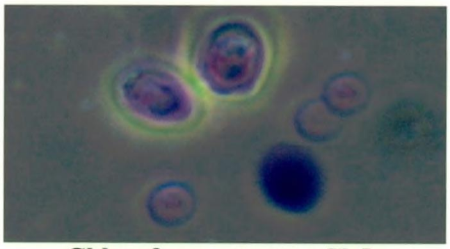
Methanol extract



Methanol extract + H_2O_2

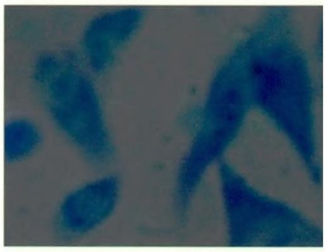


Chloroform extract



Chloroform extract + H_2O_2

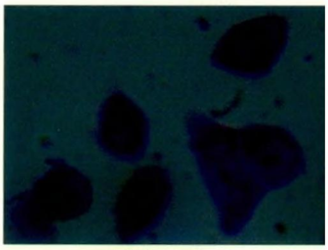
PRIMARY CHICK
G



Control

Aqueous extract

Methanol extract



Chloroform extra

TABLE 17

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN CHICK EMBRYO FIBROBLASTS 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	13 ± 2	77 ± 2 [a]	0.15	2.33
Aqueous extract	20 ± 1 [a]	24 ± 2 [a,b]	0.25	0.32
Methanol extract	13 ± 1	16 ± 2 [b]	0.15	0.19
Chloroform extract	24 ± 2 [a]	28 ± 2 [a,b]	0.32	0.39

The values are means ± S.D of triplicates
CD value = 4.29
a - Statistically significant ($P< 0.01$) compared to untreated control group
b - Statistically significant ($P< 0.01$) compared to oxidant treated group
c - Statistically significant ($P< 0.01$) compared to respective plant extract treated group

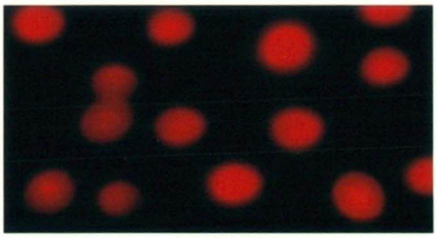
PI is a nucleic acid stain used especially in the apoptotic studies. The fragmented nuclei in apoptotic cells can be viewed clearly using this stain. Oxidative stress in *Saccharomyces cerevisiae* cells and primary chick embryo fibroblasts induced by H_2O_2 brought about a steep increase in the number of apoptotic cells (Plates 6 and 7). All the three extracts of *Zea mays* leaves significantly reduced the extent of apoptosis revealed by the nuclear changes.

The induction of apoptosis in *Saccharomyces cerevisiae* cells and primary chick embryo fibroblasts by H_2O_2 and its modulation by the *Zea mays* leaf extracts was then quantified using EtBr permeability into the cells. EtBr specifically stains DNA and has been used extensively for the detection of nuclear shrinkage, including chromatin condensation, nuclear fragmentation and the appearance of apoptotic bodies, all of which are indicative of apoptosis.

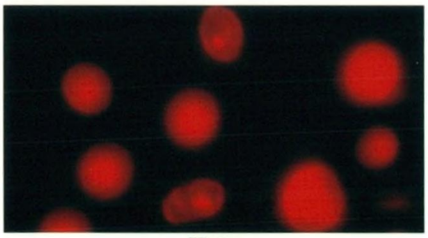
The apoptotic ratio was calculated from the number of normal and dying cells in each treatment group after EtBr staining and the values obtained are tabulated in Tables 18 and 19.

PLATE 6

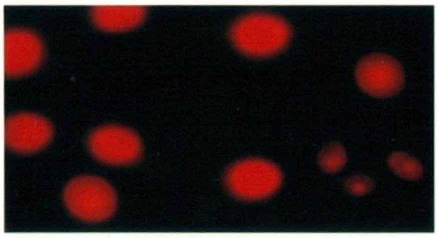
Saccharomyces cerevisiae CELLS STAINED WITH PI (OXIDANT-H_2O_2)



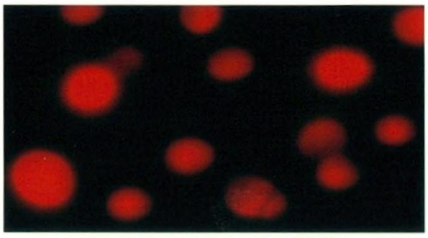
Control



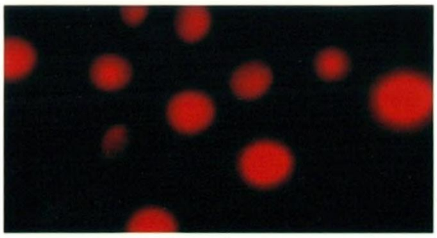
H_2O_2



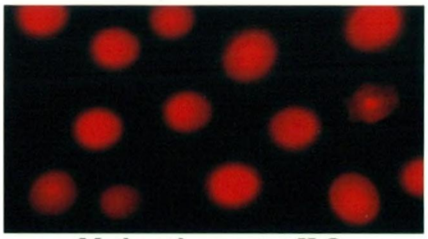
Aqueous extract



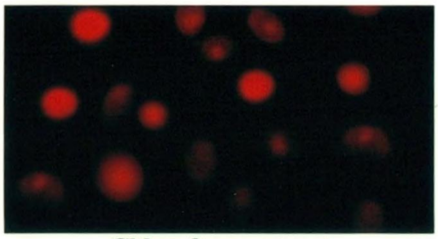
Aqueous extract + H_2O_2



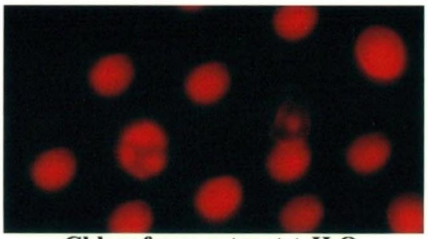
Methanol extract



Methanol extract + H_2O_2

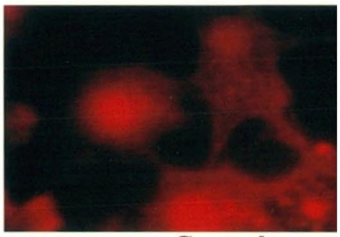


Chloroform extract

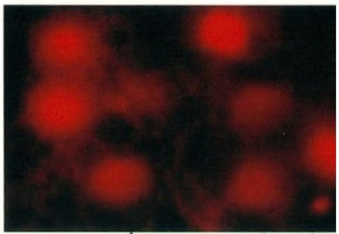


Chloroform extract + H_2O_2

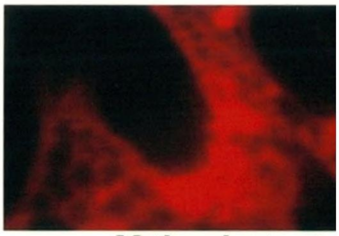
PRIMARY CHICK EM
PI



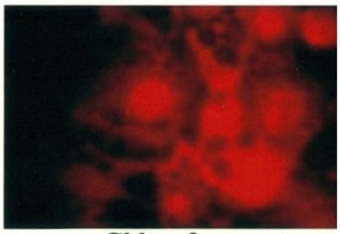
Control



Aqueous extract



Methanol extract



Chloroform extract

TABLE 18

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN *Saccharomyces cerevisiae* 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	7 ± 3	86 ± 2 [a]	0.08	6.1
Aqueous extract	15 ± 2 [a]	17 ± 3 [a,b]	0.18	0.2
Methanol extract	6 ± 2	10 ± 2 [b]	0.06	0.11
Chloroform extract	21 ± 4 [a]	28 ± 3 [a,b,c]	0.27	0.39

The values are means ± S.D of triplicates
CD value = 6.48
a - Statistically significant ($P< 0.01$) compared to untreated control group
b - Statistically significant ($P< 0.01$) compared to oxidant treated group
c - Statistically significant ($P< 0.01$) compared to respective plant extract treated group

TABLE 19

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN CHICK EMBRYO FIBROBLASTS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY EtBr STAINING

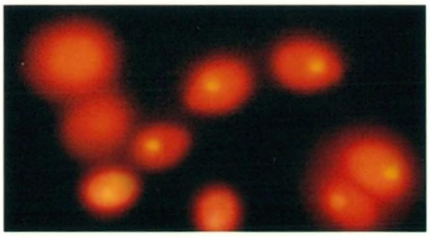
SAMPLE	No. of Apoptotic cells/ 100 cells		Apoptotic ratio	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	17 ± 2	76 ± 2 [a]	0.20	3.16
Aqueous extract	27 ± 3 [a]	25 ± 4 [a,b]	0.37	0.33
Methanol extract	14 ± 2	19 ± 1 [b]	0.16	0.23
Chloroform extract	29 ± 1 [a]	32 ± 2 [a,b]	0.40	0.47

The values are means ± S.D of triplicates
CD value = 5.53
a - Statistically significant ($P< 0.01$) compared to untreated control group
b - Statistically significant ($P< 0.01$) compared to oxidant treated group
c - Statistically significant ($P< 0.01$) compared to respective plant extract treated group

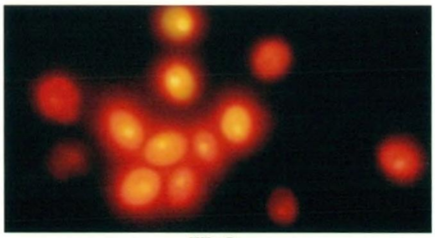
The negative control, which contained no extract or H_2O_2, harbored intact nuclei and the cells treated with H_2O_2 evidenced a significant increase in the level of nuclear fragmentation, which is indicative of apoptosis (Plates 8 and 9). The cells treated with the leaf extracts showed reduced number of apoptotic cells in the presence and absence of oxidative stress.

PLATE 8

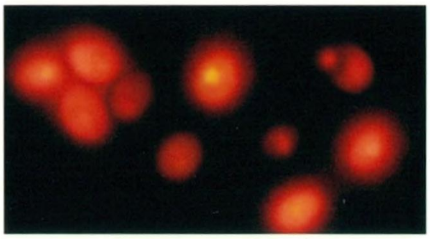
Saccharomyces cerevisiae CELLS STAINED WITH EtBr (OXIDANT-H_2O_2)



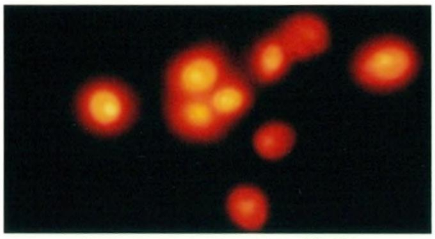
Control



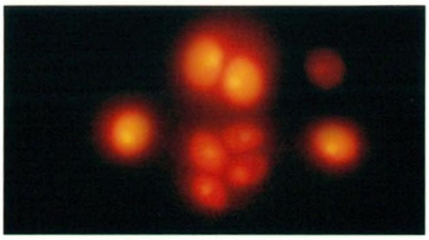
H_2O_2



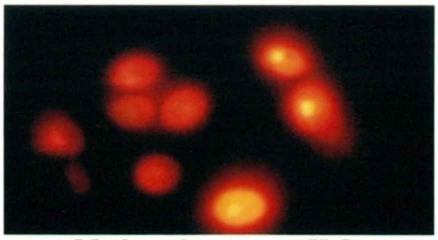
Aqueous extract



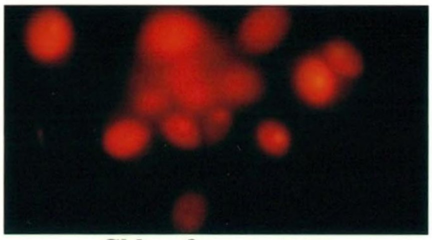
Aqueous extract + H_2O_2



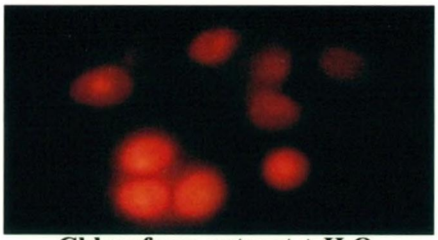
Methanol extract



Methanol extract + H_2O_2

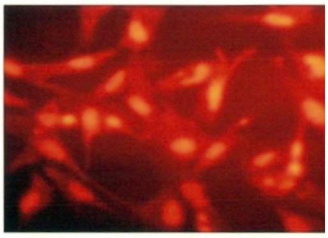


Chloroform extract

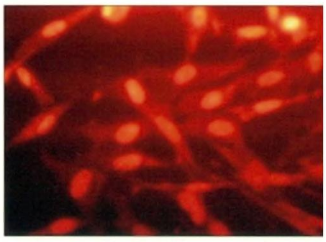


Chloroform extract + H_2O_2

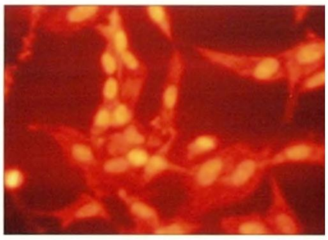
PRIMARY CHICK E
E



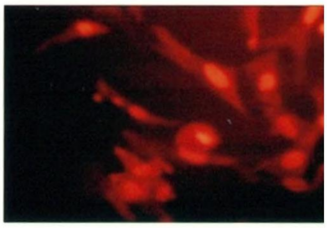
Control



Aqueous extract



Methanol extract



Chloroform extrac

DAPI is a dye that belongs to the group of indole dyes. It is a fluorescent stain that is used to highlight the nuclear changes during apoptosis (Konduri *et al.,* 2007). The number of cells that exhibited DAPI-stained apoptotic nuclear changes in the *Saccharomyces cerevisiae* cells and primary chick embryo fibroblasts exposed to H_2O_2 and/or *Zea mays* leaf extracts are presented in Tables 20 and 21.

TABLE 20

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN *Saccharomyces cerevisiae* 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	83 ± 2 [a]	0.06	4.9
Aqueous extract	16 ± 1 [a]	20 ± 2 [a,b]	0.19	0.25
Methanol extract	14 ± 2 [a]	17 ± 2 [a,b]	0.16	0.20
Chloroform extract	22 ± 2 [a]	27 ± 3 [a,b,c]	0.28	0.37

The values are means ± S.D of triplicates
CD value = 4.92
a - Statistically significant (P< 0.01) compared to untreated control group
b - Statistically significant (P< 0.01) compared to oxidant treated group
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

TABLE 21

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN CHICK EMBRYO FIBROBLASTS 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	14 ± 3	81 ± 2 [a]	0.16	4.2
Aqueous extract	21 ± 2 [a]	24 ± 1 [a,b]	0.27	0.31
Methanol extract	13 ± 2	16 ± 1 [b]	0.15	0.19
Chloroform extract	25 ± 1 [a]	28 ± 2 [a,b]	0.33	0.39

The values are means ± S.D of triplicates
CD value = 4.46
a - Statistically significant (P< 0.01) compared to untreated control group
b - Statistically significant (P< 0.01) compared to oxidant treated group
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

Our results clearly indicated that H_2O_2 exposure caused a significant number of *Saccharomyces cerevisiae* cells and primary chick embryo fibroblasts to commit to apoptosis. The presence of the *Zea mays* leaf extracts brought the proportion of apoptosing cells down, showing its protective action against the oxidative stress induced by H_2O_2. The methanolic extract showed a greater inhibition of apoptosis when compared to a moderate inhibition exhibited by the aqueous and chloroform extracts. *Zea mays* leaf extracts, by themselves, exhibited slight cytotoxic effect towards yeast cells. Plates 10 and 11 show the photographic record of the apoptosing cells in each treatment group.

Acridine orange/ethidium bromide (AO/EtBr) staining is used to visualize apoptotic and normal cells clearly with distinct coloured fluorescence. The treated cells were stained with AO/EtBr fluorescence stain and the number of apoptotic cells was counted under a fluorescent microscope. The apoptotic ratios of the AO/EtBr stained cells of *Saccharomyces cerevisiae* and primary chick embryo fibroblasts were calculated. The data are presented in Tables 22 and 23.

TABLE 22

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN *Saccharomyces cerevisiae* 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	83 ± 2 [a]	0.09	4.9
Aqueous extract	18 ± 1 [a]	20 ± 2 [a,b]	0.21	0.25
Methanol extract	9 ± 2	13 ± 3 [b]	0.09	0.15
Chloroform extract	21 ± 2 [a]	24 ± 3 [a,b]	0.26	0.32

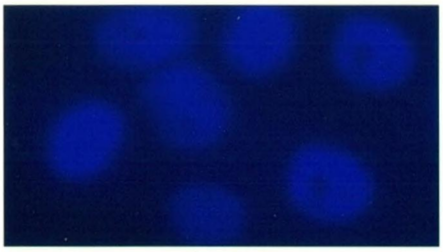
The values are means ± S.D of triplicates
CD value = 5.23
a - Statistically significant (P< 0.01) compared to untreated control group
b - Statistically significant (P< 0.01) compared to oxidant treated group
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

PLATE 10

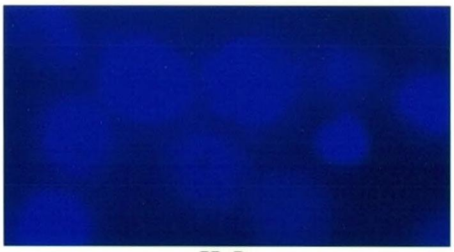
***Saccharomyces cerevisiae* CELLS STAINED WITH DAPI**

(OXIDANT-H_2O_2)

PRIMARY CHICK E

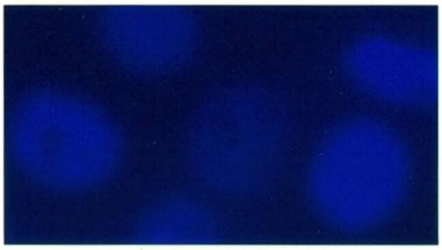


Control

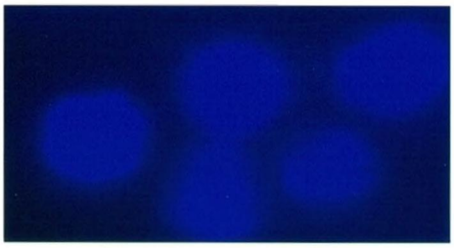


H_2O_2

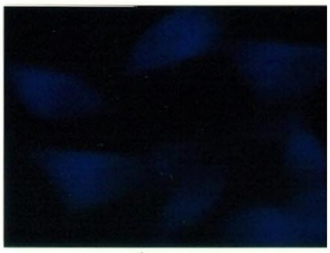
Control



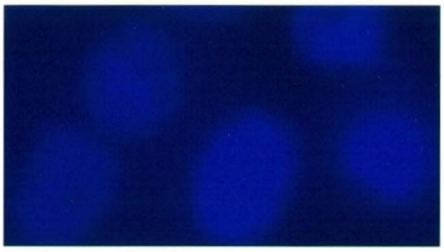
Aqueous extract



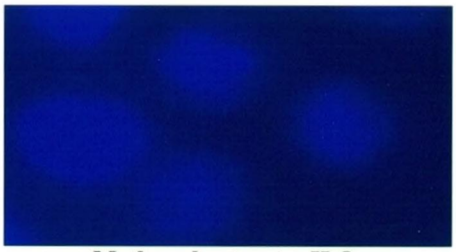
Aqueous extract + H_2O_2



Aqueous extrac

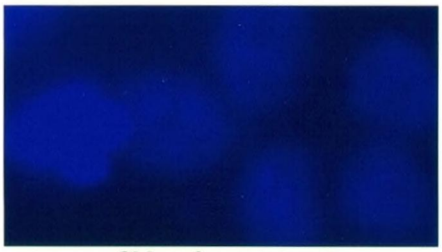


Methanol extract

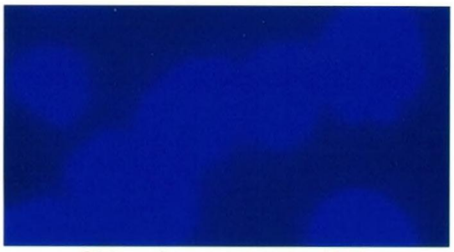


Methanol extract + H_2O_2

Methanol extrac



Chloroform extract



Chloroform extract + H_2O_2

Chloroform extra

TABLE 23

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN CHICK EMBRYO FIBROBLASTS 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	13 ± 1	80 ± 3 [a]	0.15	4.0
Aqueous extract	16 ± 2	21 ± 2 [a,b,c]	0.19	0.27
Methanol extract	11 ± 1	15 ± 1 [b]	0.12	0.18
Chloroform extract	22 ± 2 [a]	27 ± 1 [a,b,c]	0.25	0.37

The values are means ± S.D of triplicates
CD value = 4.2
a - Statistically significant ($P < 0.01$) compared to untreated control group
b - Statistically significant ($P < 0.01$) compared to oxidant treated group
c - Statistically significant ($P < 0.01$) compared to respective plant extract treated group

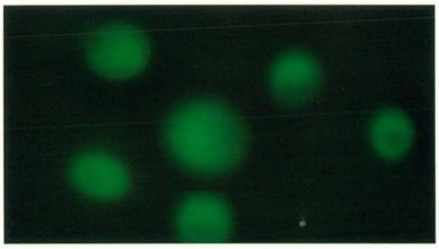
The induction of oxidative stress in *Saccharomyces cerevisiae* cells and primary chick embryo fibroblasts by H_2O_2 brought about an increase in the number of apoptotic cells (Plate 12 and Plate 13). This effect was strongly counteracted by the *Zea mays* leaf extracts. The normal cells displayed uniform green colour with organized structure, whereas the apoptotic cells showed intense orange red fluorescence with membrane morphological and nuclear changes. The aqueous and chloroform extracts, by themselves, exerted their toxicity towards yeast cells and chick embryo fibroblasts to certain extent.

It is evident from the results obtained that the leaves of *Zea mays* render strong protection to *Saccharomyces cerevisiae* cells and primary chick embryo fibroblasts against oxidative stress-induced apoptotic cell death.

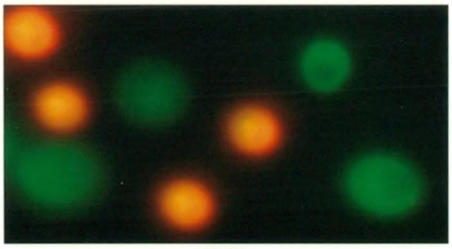
These studies demonstrated the fact that the *Zea mays* leaf extracts could influence the process of cell death in two different types of cells. Agents that influence cell death, especially those that can induce apoptosis in cancer cells, have always been sought upon as a source of anticancer drugs. Thus, having ascertained the influence of the leaf extracts on the process of apoptosis, we also studied the effect of the leaf extracts on oxidative stress-induced apoptosis in a cancer cell line (Hep2 laryngeal carcinoma).

PLATE 12

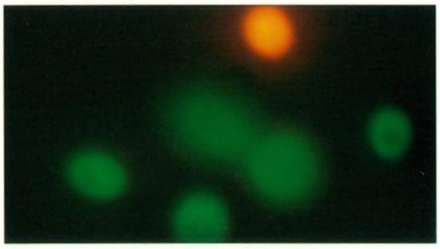
Saccharomyces cerevisiae CELLS STAINED WITH AO/EtBr (OXIDANT-H_2O_2)



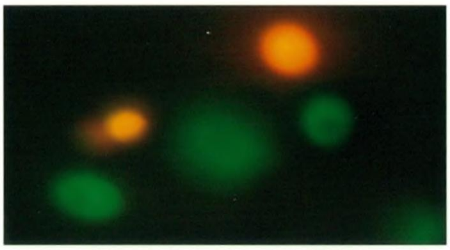
Control



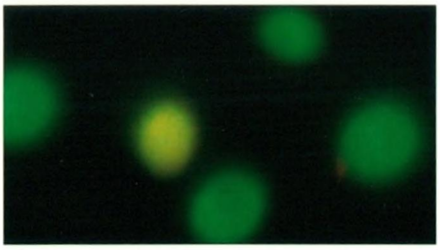
H_2O_2



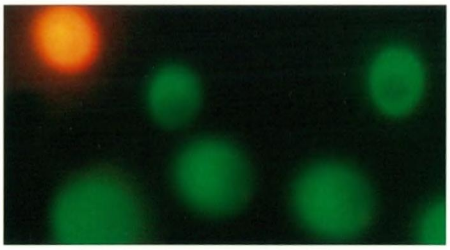
Aqueous extract



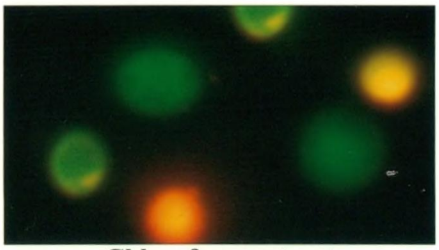
Aqueous extract + H_2O_2



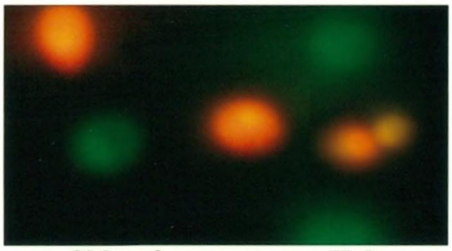
Methanol extract



Methanol extract + H_2O_2

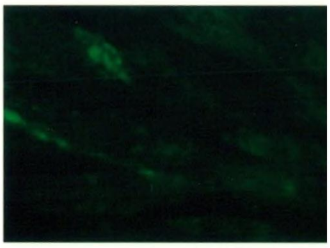


Chloroform extract

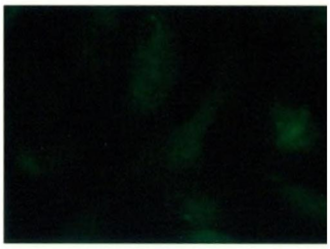


Chloroform extract + H_2O_2

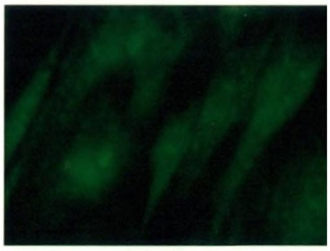
PRIMARY CHICK E
A



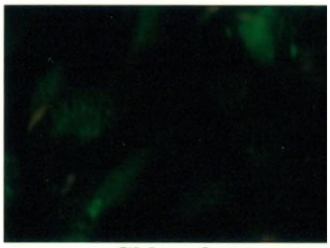
Control



Aqueous extract



Methanol extrac



Chloroform extra

EFFECT OF *Zea mays* LEAF EXTRACTS ON H_2O_2 -INDUCED APOPTOSIS IN Hep2 CELLS

The influence of *Zea mays* leaf extracts on the viability of the cancer cells (Plate 14) was studied using the cytotoxicity assays (MTT and SRB).

EFFECT OF *Zea mays* LEAF EXTRACTS ON H_2O_2 -INDUCED APOPTOSIS IN Hep2 CELLS

The influence of *Zea mays* leaf extracts on the viability of the cancer cells (Plate 14) was studied using the cytotoxicity assays (MTT and SRB).

CELL VIABILITY ASSAYS

The cytotoxity assays were performed in the Hep2 cells exposed to oxidative stress in the presence and in the absence of *Zea mays* leaf extracts.

The treatment with hydrogen peroxide exhibited cytotoxity in the cancer cells. The administration of the leaf extracts also caused increased death of cancer cells. Oxidatively stressed cancer cells co-treated with the *Zea mays* leaf extracts showed cytotoxicity on par with the H_2O_2 treated groups except chloroform extract. This indicates that the aqueous and methanol leaf extracts did not influence the cytotoxic action of hydrogen peroxide in the cancer cells.

Sulphorhodamine B assay was also performed to assess the cell survival. The results of this assay showed a similar trend as that observed in the MTT assay. The results are presented in Figure 12 (for MTT assay) and Figure 13 (for SRB assay).

The Hep2 cells subjected to H_2O_2-induced oxidative stress both in the presence and in the absence of *Zea mays* leaf extracts were studied using membrane morphological staining technique The ratio of apoptosing to normal cells are presented in Table 24.

TABLE 24

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^{a}	0.10	1.10
Aqueous extract	45 ± 2^{a}	$60 \pm 1^{a,b,c}$	0.81	1.50
Methanol extract	63 ± 3^{a}	$68 \pm 2^{a,b,c}$	1.70	2.13
Chloroform extract	40 ± 2^{a}	$54 \pm 2^{a,c}$	0.67	1.17

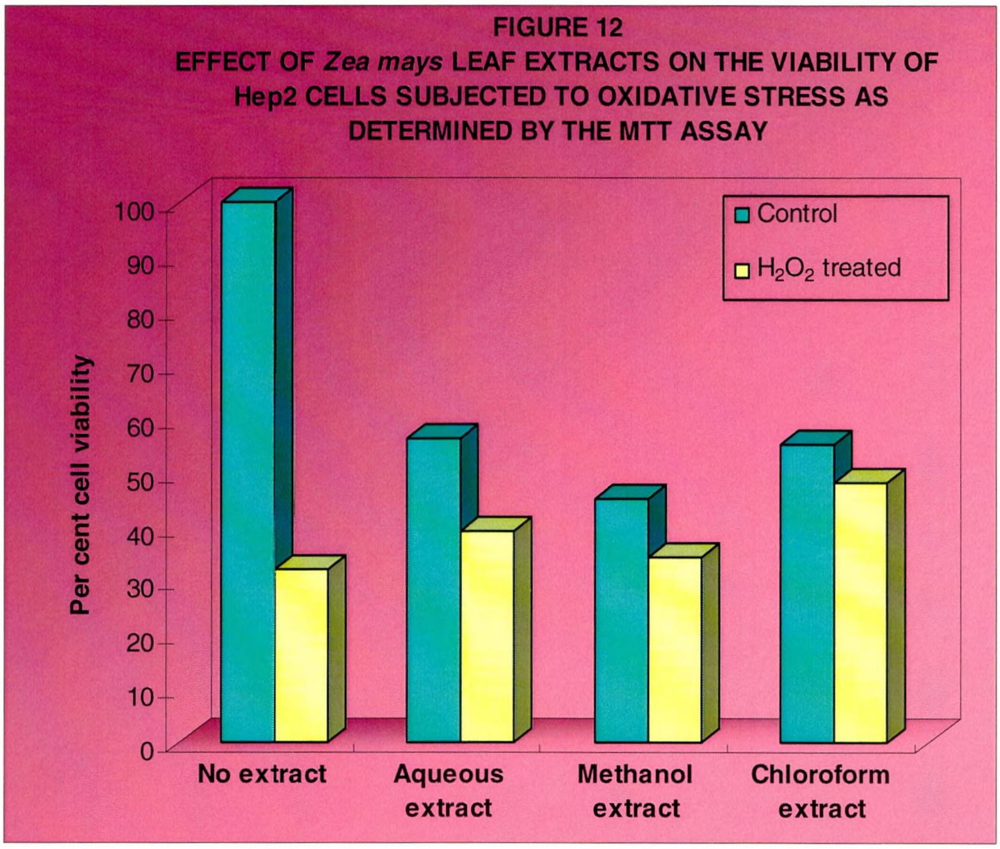
The values are means ± S.D of triplicates

CD value = 4.7

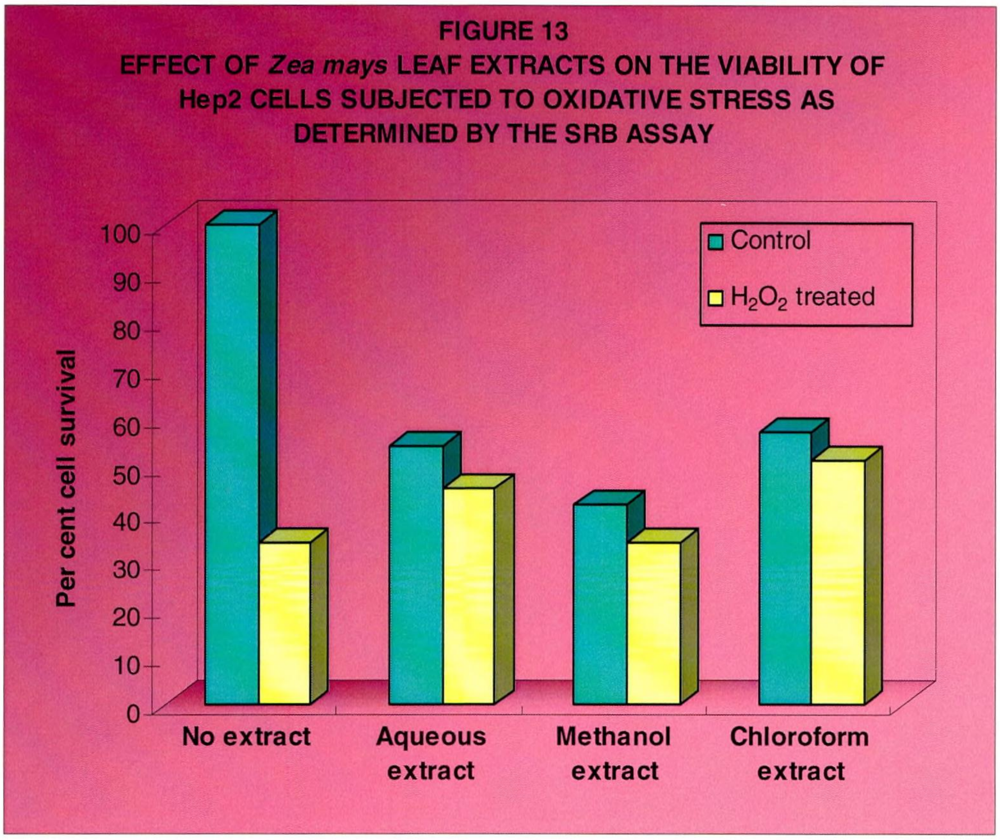
a - Statistically significant (P< 0.01) compared to untreated control group

b - Statistically significant (P< 0.01) compared to oxidant treated group

c - Statistically significant (P< 0.01) compared to respective plant extract treated group



The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.



The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.

The occurrence of morphologically altered cells was found to be maximum in the H_2O_2 treated group. The treatment with the leaf extracts also exerted an apoptosis-inducing effect in the cancer cells (Plate 15). The number of apoptotic cells increased further upon co-exposure of the leaf extract along with H_2O_2.

NUCLEAR CHANGES

The nuclear changes were observed in the Hep2 cells exposed to H_2O_2 in both the presence and absence of the *Zea mays* leaf extracts using various staining techniques (PI, EtBr, DAPI and AO/EtBr). The data obtained with PI staining and the apoptotic index calculated are presented in Table 25.

TABLE 25

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN Hep2 CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY PI STAINING

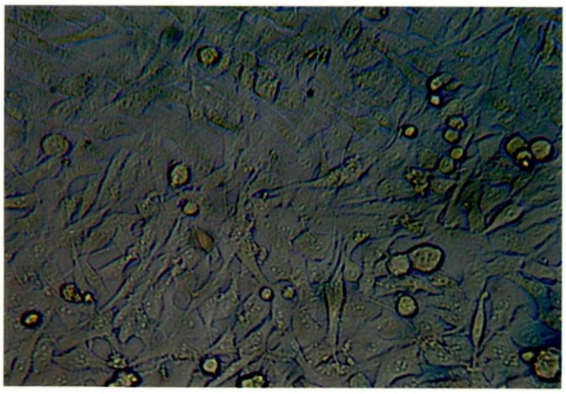
SAMPLE	No. of Apoptotic cells / 100 cells		Apoptotic ratio	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No Extract	8 ± 3	51 ± 2 [a]	0.09	1.04
Aqueous extract	48 ± 2 [a]	59 ± 2 [a,b,c]	0.92	1.44
Methanol extract	61 ± 1 [a]	73 ± 1 [a,b,c]	1.56	2.70
Chloroform extract	34 ± 2 [a]	49 ± 1 [a,c]	0.52	0.96

The values are means ± S.D of triplicates
CD value = 4.46
a - Statistically significant (P< 0.01) compared to untreated control group
b - Statistically significant (P< 0.01) compared to oxidant treated group
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

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 as compared to control cells with prominent rounded nuclei and defined plasma membrane contours (Plate 16). The leaf extracts, by themselves, also caused an induction of apoptosis in Hep2 cells.

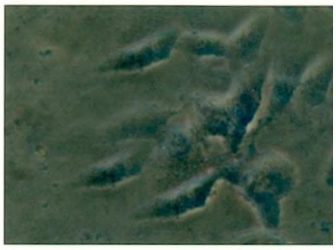
The number of cells showing nuclear apoptotic morphology was counted by ethidium bromide staining in each treatment group and the results are given in Table 26. The nuclear changes observed are shown in Plate 17.

PLATE 14

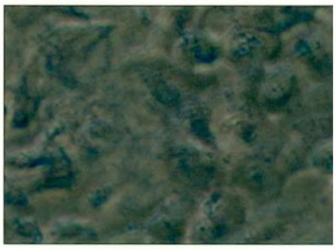


Hep2 cells

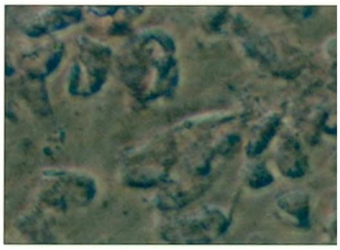
Hep2 CELI



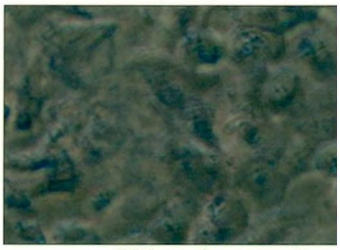
Control



Aqueous extract



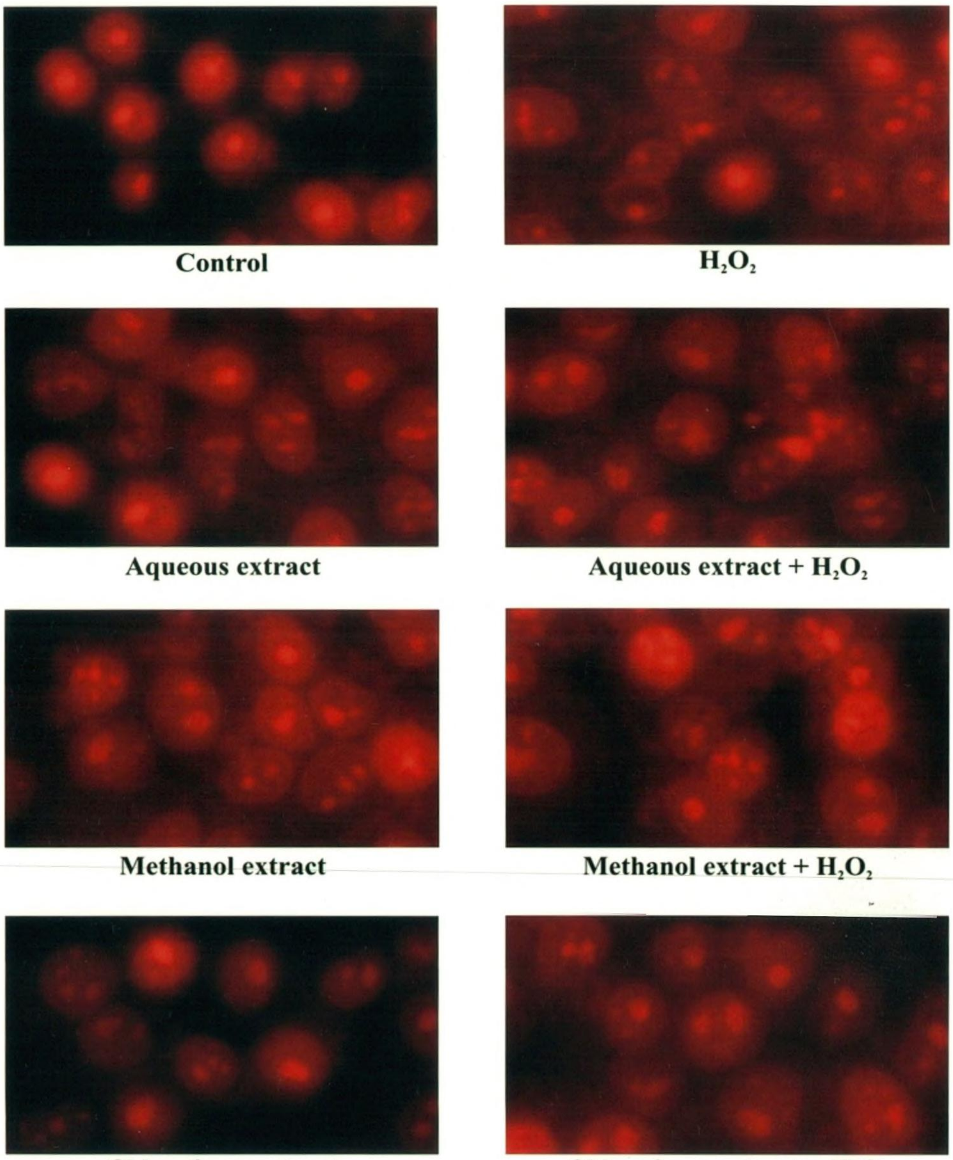
Methanol extract



Chloroform extrac

PLATE 16

Hep2 CELLS STAINED WITH PI (OXIDANT-H_2O_2)



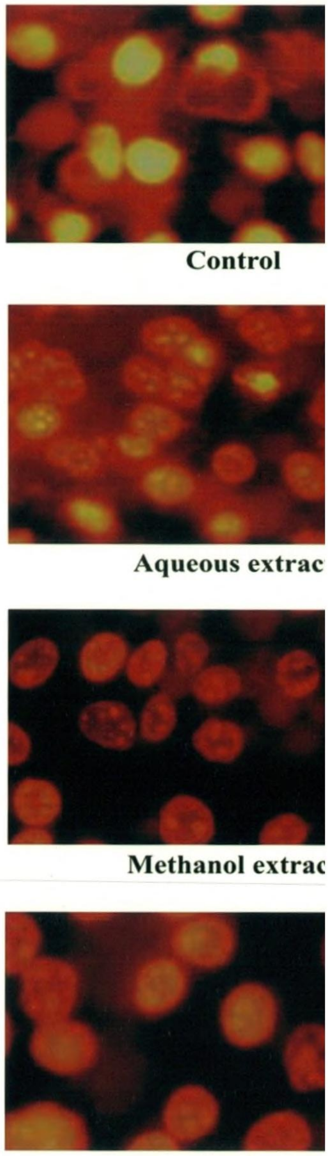
Control | H_2O_2

Aqueous extract | Aqueous extract + H_2O_2

Methanol extract | Methanol extract + H_2O_2

Chloroform extract | Chloroform extract + H_2O_2

Hep2 CELLS ST



Control

Aqueous extrac

Methanol extrac

Chloroform extra

TABLE 26

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 ^{a}	0.05	0.92
Aqueous extract	47 ± 2 ^{a}	55 ± 2 ^{a,b,c}	0.89	1.22
Methanol extract	61 ± 2 ^{a}	70 ± 1 ^{a,b,c}	1.56	2.33
Chloroform extract	35 ± 2 ^{a}	50 ± 2 ^{a,c}	0.54	1.00

The values are means ± S.D of triplicates
CD value = 4.9
a - Statistically significant (P< 0.01) compared to untreated control group
b - Statistically significant (P< 0.01) compared to oxidant treated group
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

Oxidative stress-induced (H_2O_2-exposed) Hep2 cells committed to apoptosis to an increased extent, which further increased significantly in the presence of *Zea mays* leaf extracts. The methanolic extract induced the maximum extent of apoptosis. The aqueous and chloroform extracts also exhibited considerable apoptotic effect in the cancer cells.

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 27.

TABLE 27

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 ^{a}	0.06	2.13
Aqueous extract	47 ± 2 ^{a}	56 ± 1 ^{a,b,c}	0.89	1.27
Methanol extract	50 ± 3 ^{a}	58 ± 3 ^{a,b,c}	1.00	1.38
Chloroform extract	43 ± 3 ^{a}	53 ± 1 ^{a,b,c}	0.75	1.13

The values are means ± S.D of triplicates
CD value = 5.4
a - Statistically significant (P< 0.01) compared to untreated control group
b - Statistically significant (P< 0.01) compared to oxidant treated group
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

As deducible from the values listed in Table 27, a trend similar to that of EtBr was noted with DAPI staining. The cells with altered nuclear changes were found to be maximum in the H_2O_2 treated group. The extracts of *Zea mays* leaves were also highly efficient in inducing apoptosis in cancer cells, both in the presence and in the absence of H_2O_2 (Plate 18).

The results with giemsa and the nuclear stains were further affirmed by AO/EtBr staining (Plate 19), as is evident from the data obtained (Table 28).

TABLE 28

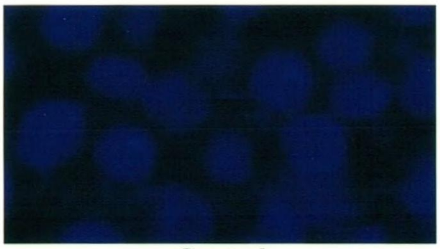
EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN Hep2 CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY AO/ EtBr STAINING

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 [a]	0.09	1.17
Aqueous extract	47 ± 3 [a]	65 ± 1 [a,b,c]	0.89	1.85
Methanol extract	64 ± 1 [a]	72 ± 1 [a,b,c]	1.78	2.58
Chloroform extract	39 ± 1 [a]	54 ± 3 [a,c]	0.64	1.17

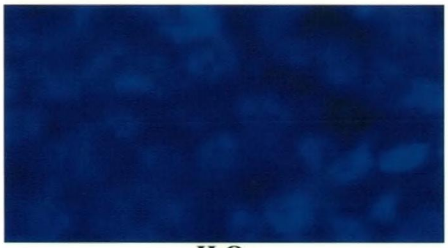
The values are means ± S.D of triplicates
CD value = 4.98
a- Statistically significant (P< 0.01) compared to untreated control group
b - Statistically significant (P< 0.01) compared to oxidant treated group
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

As can be observed from our results, the effects of H_2O_2 were similar in the non-cancerous (yeast cells and chick embryo fibroblasts) and cancerous (Hep2) cells, while the effects of *Zea mays* leaf extracts were differential. The leaf extracts, by themselves, were non-toxic to non-cancerous cells, but caused increased cytotoxicity to cancer cells. It was therefore thought that it would be interesting and worthwhile to probe the influence of the leaf extracts on the effect of a standard chemotherapeutic drug on both non-cancerous (chick embryo fibroblasts) and cancerous (Hep2) cells. The chemotherapeutic agent chosen for the present study was etoposide, which is known to induce apoptosis in cells via oxidative stress (Siitonen *et al.*, 1999).

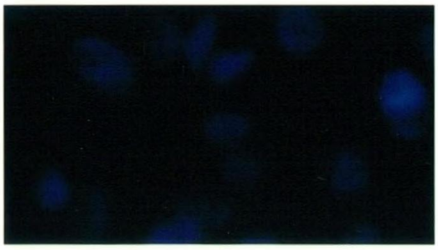
PLATE 18
Hep2 CELLS STAINED WITH DAPI (OXIDANT-H_2O_2)



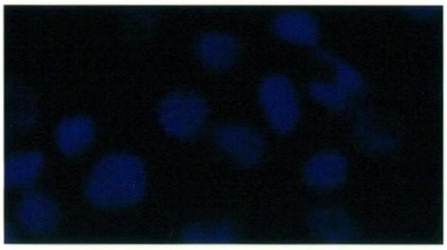
Control



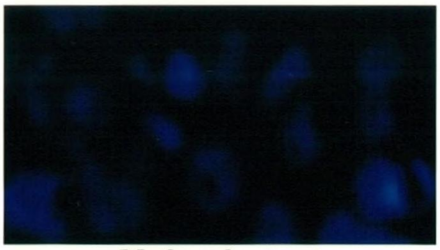
H_2O_2



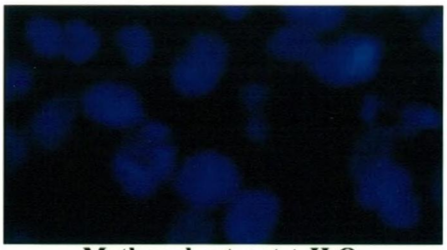
Aqueous extract



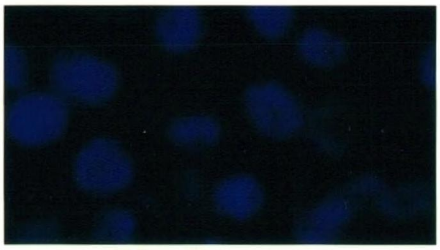
Aqueous extract + H_2O_2



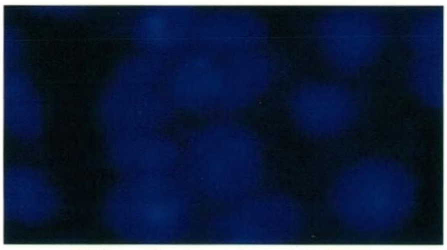
Methanol extract



Methanol extract + H_2O_2

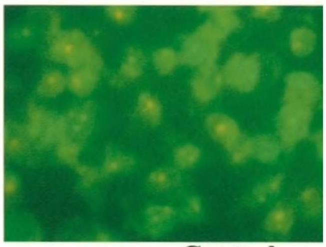


Chloroform extract

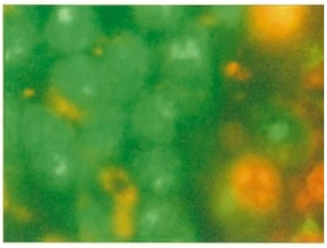


Chloroform extract + H_2O_2

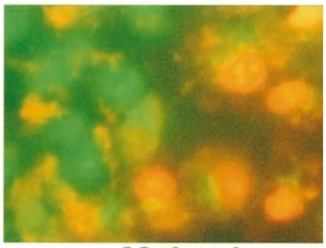
Hep2 CELLS STA



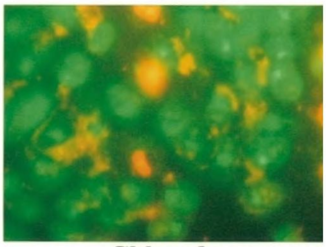
Control



Aqueous extrac



Methanol extrac



Chloroform extra

ANTI-APOPTOTIC EFFECTS OF *Zea mays* LEAF EXTRACTS ON CHICK EMBRYO FIBROBLASTS

The influence of etoposide in the presence and the absence of the *Zea mays* leaf extracts in the non-cancerous cells (primary chick embryo fibroblasts) were evaluated by employing cytotoxicity assays and various (membrane and nuclear) staining techniques.

CYTOTOXICITY ASSAYS

The cell viability was assayed by MTT after treatment of primary chick embryo fibroblasts with leaf extract and/or etoposide. The results are presented in Figure 14.

Cytotoxicity of the primary cultured cells as determined by the MTT assay showed a drastic reduction in the cell viability after treatment with etoposide. The leaf extracts increased the viability of cells subjected to oxidative stress. This indicates that *Zea mays* leaf extracts render significant protection to primary chick embryo fibroblasts against etoposide toxicity. The results of MTT assay were confirmed by the SRB cell survival assay as shown in Figure 15.

The characteristic morphological changes in apoptotic cells were analysed by giemsa staining in the presence and the absence of etoposide and plant extracts. The number of apoptotic and non-apoptotic cells was counted under phase contrast microscope and the results are listed in Table 29.

TABLE 29

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE MORPHOLOGICAL CHANGES IN CHICK EMBRYO FIBROBLASTS EXPOSED TO ETOPOSIDE AS DETERMINED BY GIEMSA STAINING

SAMPLE	No. of Apoptotic cells / 100 cells		Apoptotic ratio	
	Control	Etoposide treated	Control	Etoposide treated
No Extract	2 ± 2	81 ± 1 [a]	0.20	4.26
Aqueous extract	11 ± 1 [a]	17 ± 3 [a,b,c]	0.12	0.20
Methanol extract	8 ± 3 [a]	13 ± 4 [a,b]	0.09	0.15
Chloroform extract	15 ± 2 [a]	20 ± 1 [a,b]	0.18	0.25

The values are means ± S.D of triplicates

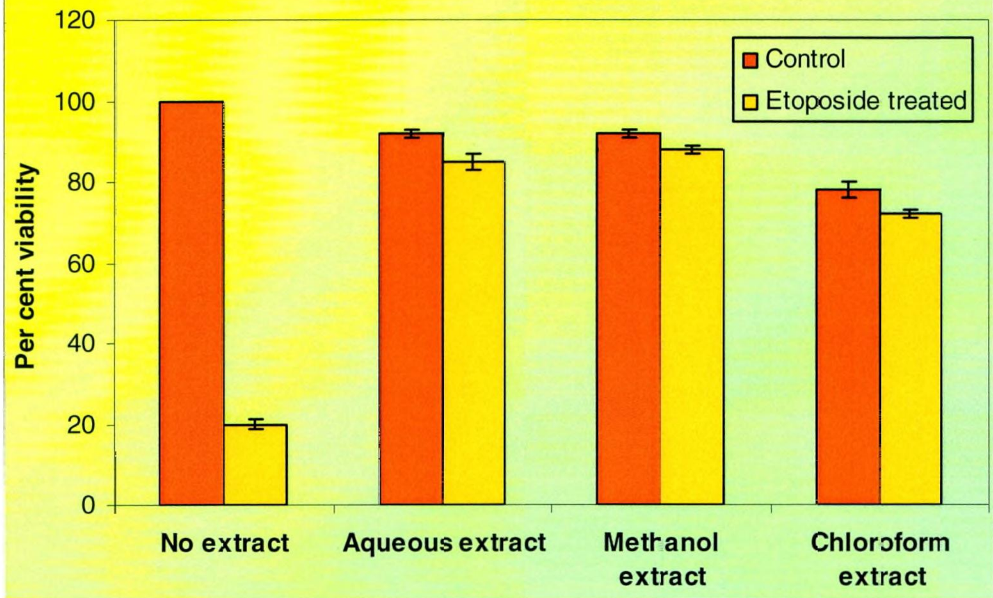
CD value = 5.7

a - Statistically significant ($P< 0.01$) compared to untreated control group

b - Statistically significant ($P< 0.01$) compared to oxidant treated group

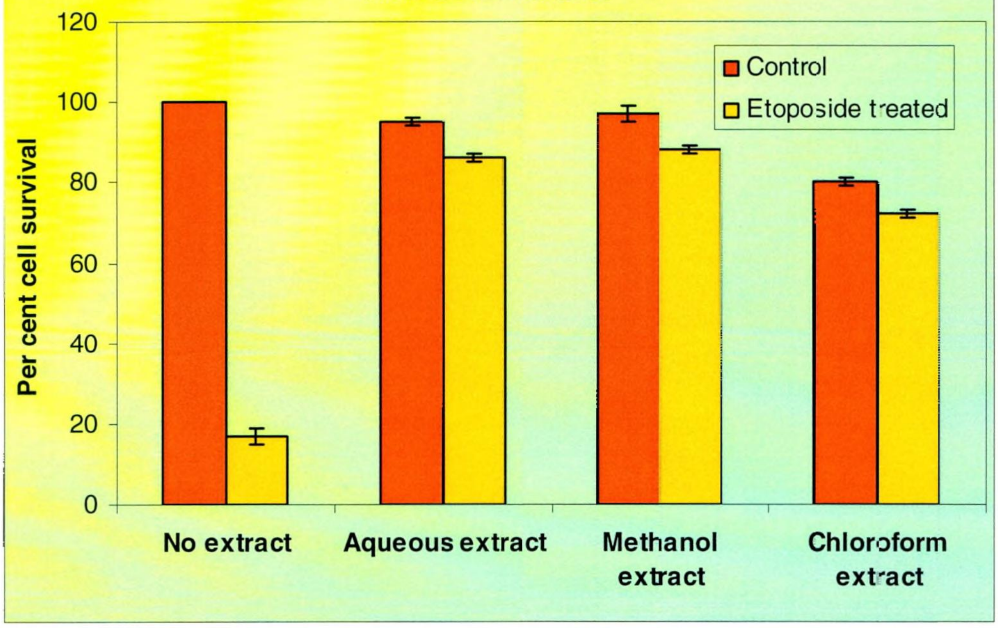
c - Statistically significant ($P< 0.01$) compared to respective plant extract treated group

FIGURE 14
EFFECT OF *Zea mays* LEAF EXTRACTS ON THE VIABILITY OF CHICK EMBRYO FIBROBLASTS EXPOSED TO ETOPOSIDE AS DETERMINED BY THE MTT ASSAY



. The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.

FIGURE 15
EFFECT OF *Zea mays* LEAF EXTRACTS ON THE VIABILITY OF CHICK EMBRYO FIBROBLASTS EXPOSED TO ETOPOSIDE AS DETERMINED BY THE SRB ASSAY



The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.

The number of cells undergoing apoptosis when subjected to oxidative stress with etoposide increased to a greater extent. When the *Zea mays* leaf extracts were co-administered, a significant decrease was observed in the number of apoptosing cells. These results indicate that *Zea mays* leaves can render protection against etoposide-induced cell death. The effect of the methanolic extract of *Zea mays* leaves was more pronounced than that of aqueous and chloroform extracts. The changes in the morphological features observed are shown in Plate 20.

NUCLEAR CHANGES

The apoptosis induction in chick embryo fibroblasts by etoposide and its modulation in the presence of *Zea mays* leaf extracts was quantified using propidium iodide staining. The values obtained are listed in Table 30.

TABLE 30

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN CHICK EMBRYO FIBROBLASTS EXPOSED TO ETOPOSIDE AS DETERMINED BY PI STAINING

SAMPLE	No. of Apoptotic cells / 100 cells		Apoptotic ratio	
	Control	Etoposide treated	Control	Etoposide treated
No Extract	4 ± 3	84 ± 1 [a]	0.04	5.25
Aqueous extract	14 ± 2 [a]	20 ± 2 [a,b,c]	0.16	0.25
Methanol extract	10 ± 1 [a]	15 ± 3 [a,b]	0.11	0.18
Chloroform extract	17 ± 3 [a]	24 ± 2 [a,b,c]	0.20	0.32

The values are means ± S.D of triplicates

CD value = 5.4

a - Statistically significant (P< 0.01) compared to untreated control group

b - Statistically significant (P< 0.01) compared to oxidant treated group

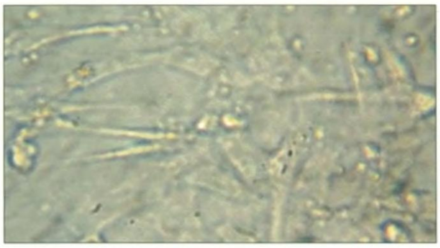
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

As deducible from the table values, upon etoposide exposure, a remarkable increase was observed in the number of dying cells. This number decreased sharply upon co-exposure to the *Zea mays* leaf extracts. Plate 21 shows the photographic records of all the groups stained with PI.

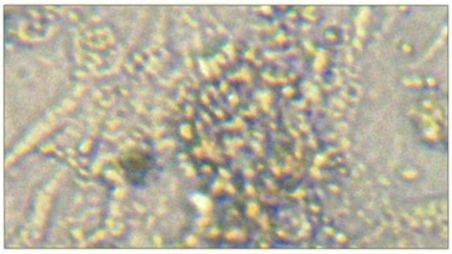
PLATE 20

PRIMARY CHICK EMBRYO FIBROBLASTS STAINED WITH GIEMSA (OXIDANT-ETOPOSIDE)

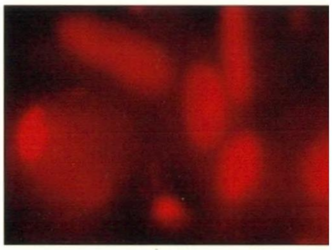
PRIMARY CHICK I WITH I



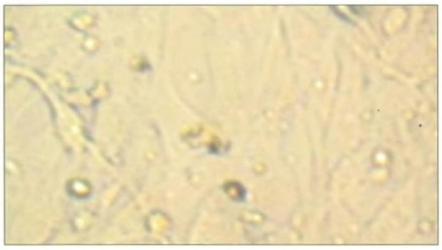
Control



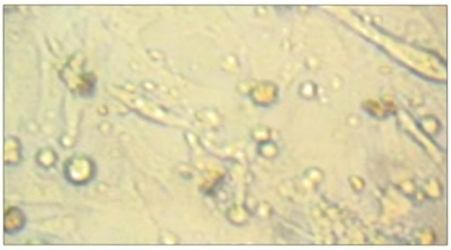
Etoposide



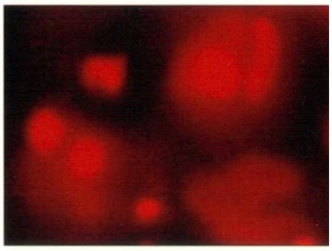
Control



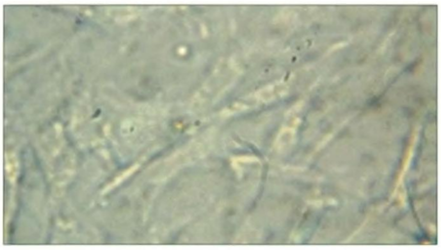
Aqueous extract



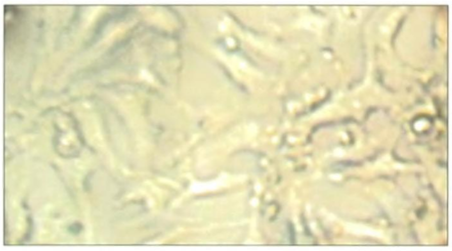
Aqueous extract + Etoposide



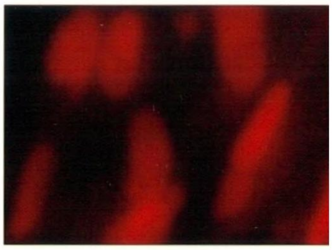
Aqueous extract



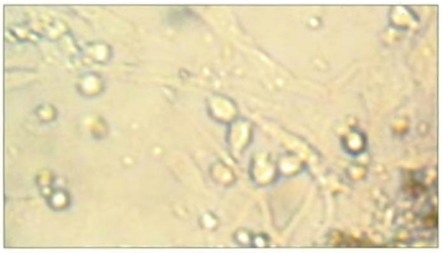
Methanol extract



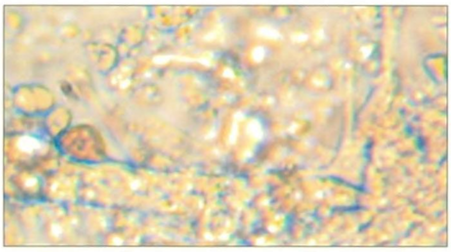
Methanol extract + Etoposide



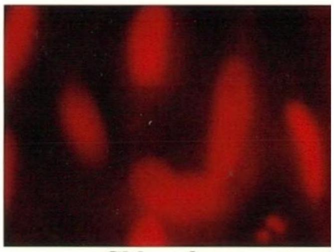
Methanol extract



Chloroform extract



Chloroform extract + Etoposide



Chloroform extract

The number of apoptotic cells and their ratios of apoptotic to normal cells were calculated in the various treatment groups exposed to EtBr or DAPI and the values are schematically represented in Table 31 (EtBr) and 32 (DAPI) respectively.

TABLE 31

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN CHICK EMBRYO FIBROBLASTS EXPOSED TO ETOPOSIDE AS DETERMINED BY EtBr STAINING

SAMPLE	No. of Apoptotic cells / 100 cells		Apoptotic ratio	
	Control	Etoposide treated	Control	Etoposide treated
No Extract	8 ± 2	85 ± 1 [a]	0.09	5.67
Aqueous extract	14 ± 3 [a]	20 ± 2 [a,b,c]	0.16	0.25
Methanol extract	12 ± 1	16 ± 1 [a,b]	0.14	0.19
Chloroform extract	18 ± 4 [a]	22 ± 2 [a,b]	0.22	0.28

The values are means ± S.D of triplicates
CD value = 5.3
a - Statistically significant (P< 0.01) compared to untreated control group
b - Statistically significant (P< 0.01) compared to oxidant treated group
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

TABLE 32

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE NUCLEAR CHANGES IN CHICK EMBRYO FIBROBLASTS EXPOSED TO ETOPOSIDE AS DETERMINED BY DAPI STAINING

SAMPLE	No. of Apoptotic cells / 100 cells		Apoptotic ratio	
	Control	Etoposide treated	Control	Etoposide treated
No Extract	6 ± 1	80 ± 1 [a]	0.06	4.00
Aqueous extract	14 ± 2 [a]	19 ± 1 [a,b]	0.16	0.23
Methanol extract	12 ± 4 [a]	15 ± 3 [a,b]	0.14	0.18
Chloroform extract	18 ± 2 [a]	23 ± 3 [a,b]	0.22	0.30

The values are means ± S.D of triplicates
CD value = 5.7
a - Statistically significant (P< 0.01) compared to untreated control group
b - Statistically significant (P< 0.01) compared to oxidant treated group
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

Etoposide treatment caused a hike in the number of cells committed to apoptosis. Treatment of the cells with the *Zea mays* leaf extracts reduced the number of cells undergoing apoptosis, despite the strong oxidative stress imposed by etoposide. These results indicate that *Zea mays* leaves can render protection against etoposide-induced cell death, with the methanolic extract faring better over the aqueous and chloroform extracts of *Zea mays* leaves. Plates 22 and 23 are the photographic records of the apoptosing cells with EtBr and DAPI respectively.

The numbers of dying cells in each treatment group after AO/EtBr staining and the corresponding apoptotic ratios are tabulated in Table 33.

TABLE 33

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN CHICK EMBRYO FIBROBLASTS EXPOSED TO ETOPOSIDE AS DETERMINED BY AO/EtBr STAINING

SAMPLE	No. of Apoptotic cells / 100 cells		Apoptotic ratio	
	Control	Etoposide treated	Control	Etoposide treated
No Extract	4 ± 2	83 ± 2 [a]	0.04	4.90
Aqueous extract	13 ± 2 [a]	16 ± 1 [a,b]	0.15	0.19
Methanol extract	8 ± 1	15 ± 2 [a,b,c]	0.09	0.18
Chloroform extract	20 ± 4 [a]	26 ± 1 [a,b,c]	0.25	0.35

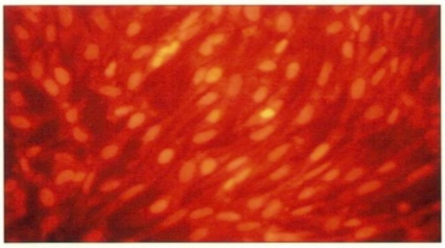
The values are means ± S.D of triplicates
CD value = 4.98
a - Statistically significant ($P< 0.01$) compared to untreated control group
b - Statistically significant ($P< 0.01$) compared to oxidant treated group
c - Statistically significant ($P< 0.01$) compared to respective plant extract treated group

Most nuclei in the control and the leaf extract treated groups displayed uniform chromatin with organized structure. Intensely stained fragmented nuclei, which indicate cells undergoing apoptosis, were frequently observed in the etoposide alone treated group. The leaf extract treatment restored the number of normal cells to near control levels. The apoptotic index increased significantly in cells exposed to etoposide, which was effectively counteracted by the leaf extracts (Plate 24).

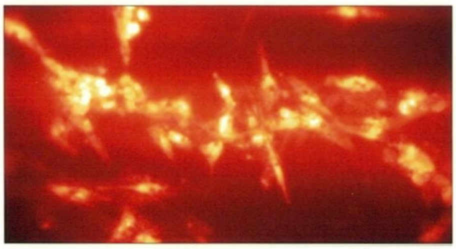
The results of this phase of the study revealed that etoposide exerted its toxicity on normal cells. *Zea mays* leaf extracts effectively counteracted the etoposide-mediated cytotoxicity, implying the protective effect of *Zea mays* leaves towards the non-cancerous cells.

PLATE 22

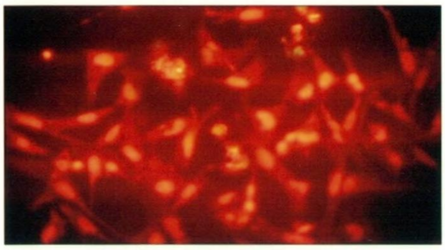
PRIMARY CHICK EMBRYO FIBROBLASTS STAINED WITH EtBr (OXIDANT-ETOPOSIDE)



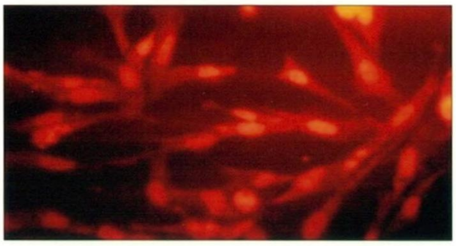
Control



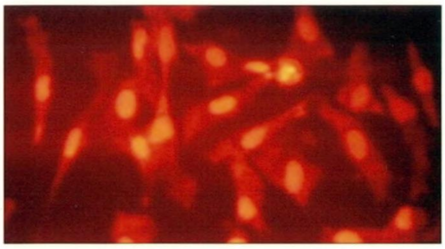
Etoposide



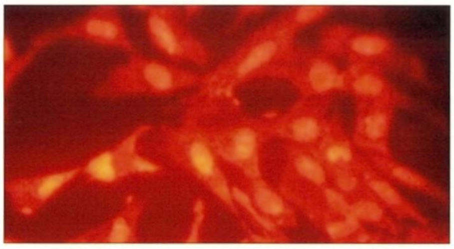
Aqueous extract



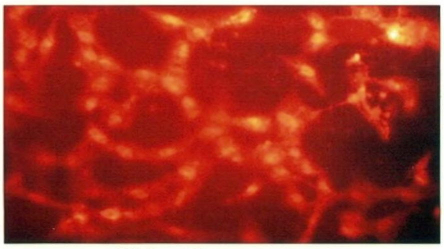
Aqueous extract + Etoposide



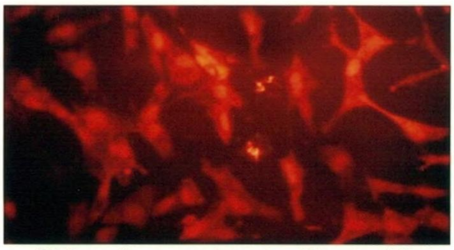
Methanol extract



Methanol extract + Etoposide

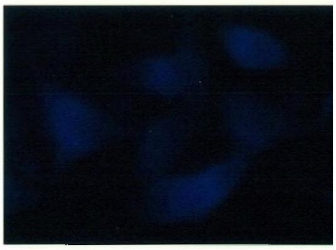


Chloroform extract

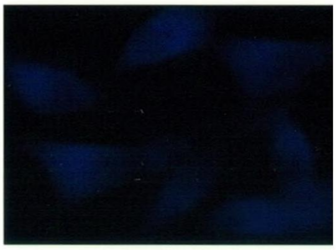


Chloroform extract + Etoposide

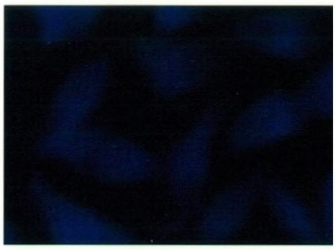
PRIMARY CHICK E
DAPI



Control



Aqueous extract



Methanol extract

Chloroform extrac

EFFECT OF *Zea mays* LEAF EXTRACTS ON ETOPOSIDE-INDUCED APOPTOSIS IN Hep2 CELLS

Apoptotic changes and cell viability were observed in Hep2 cells as for the earlier test systems and the results are presented below.

CYTOTOXICITY ASSAYS

The cytotoxicity was assayed by MTT and SRB, after treatment of Hep2 cells with leaf extract and/or etoposide. The values obtained are presented in Figure 16 and Figure 17 respectively.

The results presented in Figure 16 demonstrate the cytotoxic effect of etoposide administration to the Hep2 cells, which exerts a steep decline in the cell survival. The leaf extracts of *Zea mays* also caused a marked decrease in the viability of Hep2 cells, which decreased further in the presence of etoposide. The methanolic extract did not influence the extent of death induced by etoposide in Hep2 cells whereas the aqueous and the chloroform extracts exerted a slight increase.

The results of MTT assay was confirmed by the SRB assay. These results showed that *Zea mays* leaf extracts exhibit anticancer properties and can enhance the chemotherapeutic action of etoposide on cancer cells.

The morphological changes in the Hep2 (laryngeal carcinoma cell line) cells in the presence and the absence of the leaf extract and/or etoposide were quantified by phase contrast microscopy. The results obtained are presented in Table 34.

TABLE 34

EFFECT OF *Zea mays* LEAF EXTRACTS ON THE MORPHOLOGICAL CHANGES IN Hep2 CELLS EXPOSED TO ETOPOSIDE AS DETERMINED BY GIEMSA STAINING

SAMPLE	No. of Apoptotic cells / 100 cells		Apoptotic ratio	
	Control	Etoposide treated	Control	Etoposide treated
No Extract	18 ± 2	65 ± 2 [a]	0.22	1.85
Aqueous extract	45 ± 1 [a]	67 ± 3 [a,c]	0.82	2.03
Methanol extract	58 ± 3 [a]	72 ± 2 [a,b,c]	1.38	2.57
Chloroform extract	36 ± 4 [a]	55 ± 5 [a,b,c]	0.56	1.22

The values are means ± S.D of triplicates

CD value = 7

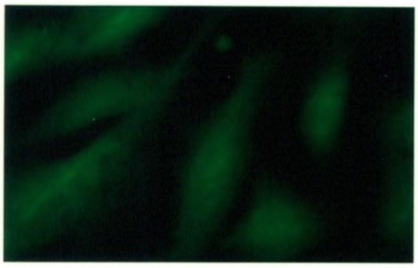
a - Statistically significant (P< 0.01) compared to untreated control group

b - Statistically significant (P< 0.01) compared to oxidant treated group

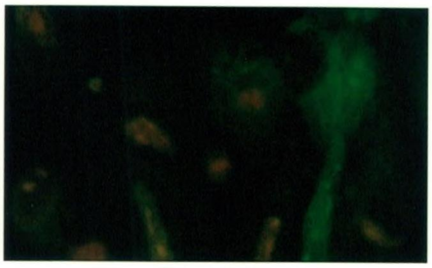
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

PLATE 24

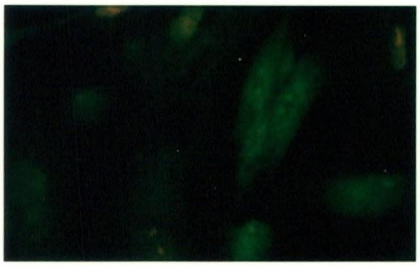
PRIMARY CHICK EMBRYO FIBROBLASTS STAINED WITH AO/EtBr (OXIDANT-ETOPOSIDE)



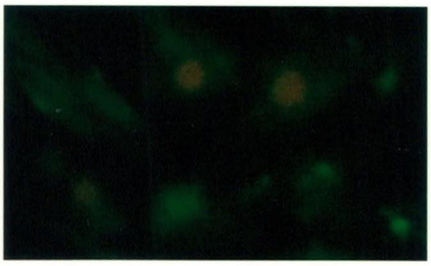
Control



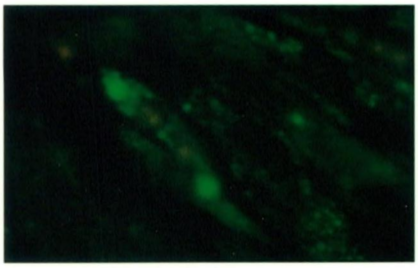
Etoposide



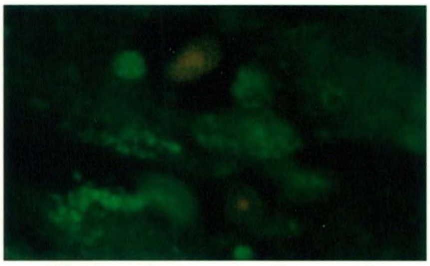
Aqueous extract



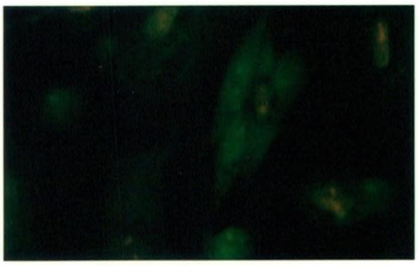
Aqueous extract + Etoposide



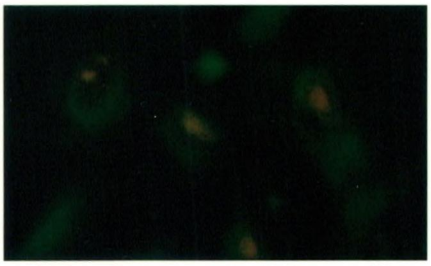
Methanol extract



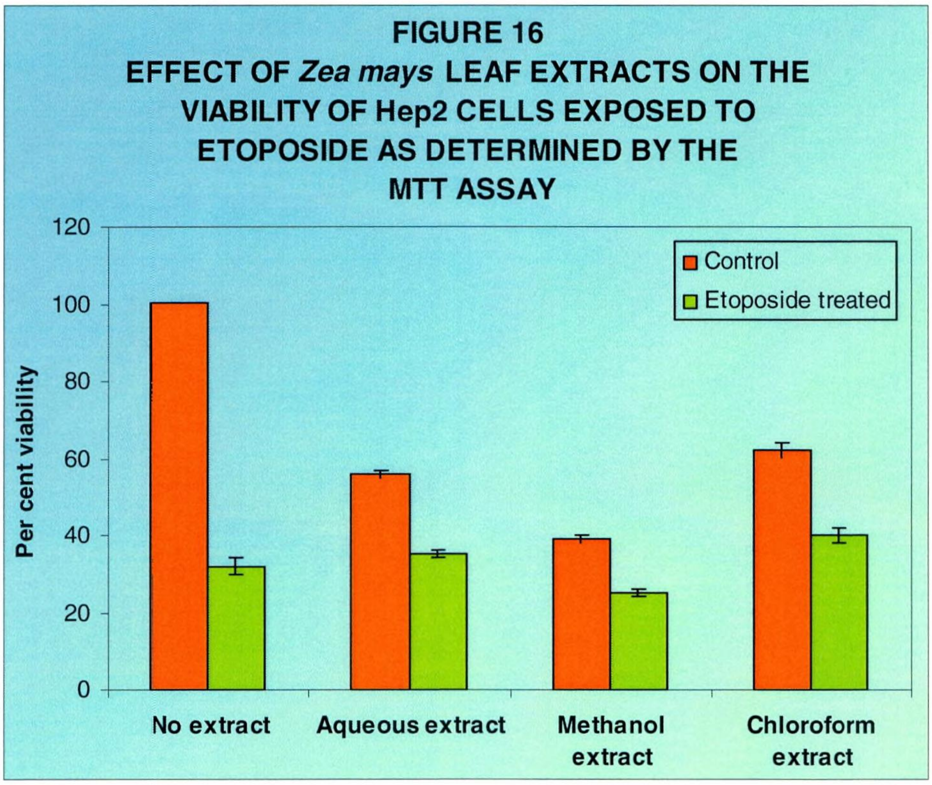
Methanol extract + Etoposide



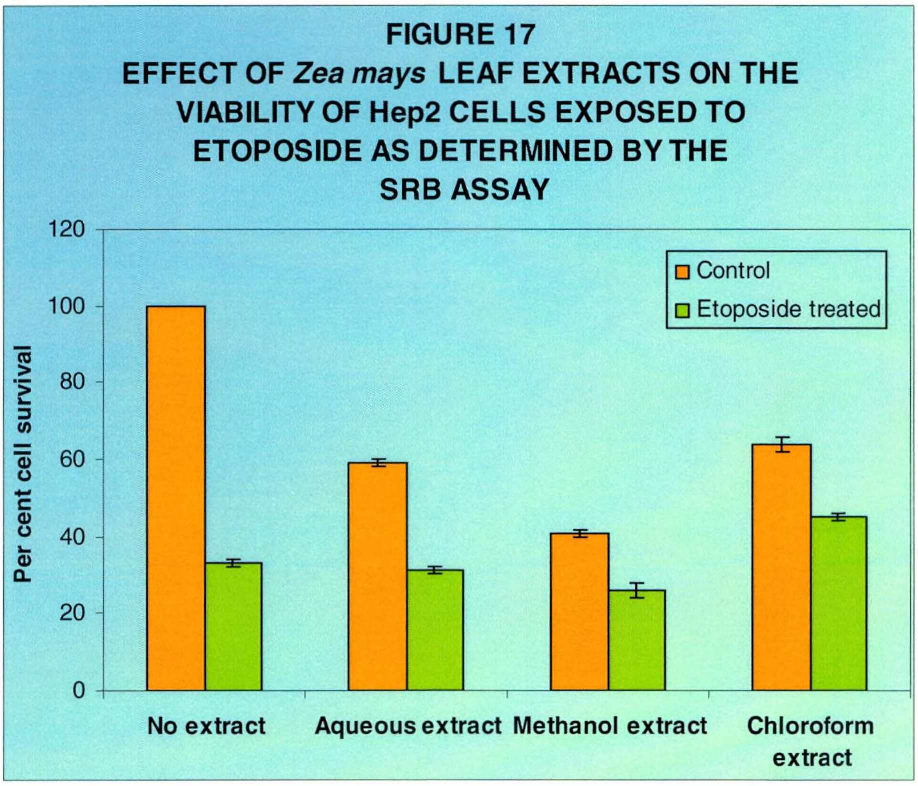
Chloroform extract



Chloroform extract + Etoposide



The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.



The values of the negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.

The administration of etoposide caused a steep increase in the number of cells showing apoptotic morphological changes (Plate 25). The administration of extracts alone also increased the number of Hep2 cells showing apoptotic morphology. When the cells were exposed to both etoposide and the leaf extracts, the extent of cell death increased further, compared to that induced by etoposide alone. This observation reveals that the leaf extracts augment the chemotherapeutic activity of etoposide in the cancer cells. This was clearly evident when the ratio of apoptosing to normal cells was calculated in each treatment group, as presented in Table 34.

NUCLEAR CHANGES

The nuclear changes in Hep2 cells by etoposide and its modulation in the presence of *Zea mays* leaf extracts were quantified using propidium iodide staining. The numbers of normal and dying cells in each treatment group after staining are tabulated in Table 35.

TABLE 35

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN Hep2 CELLS EXPOSED TO ETOPOSIDE AS DETERMINED BY PI STAINING

SAMPLE	No. of Apoptotic cells / 100 cells		Apoptotic ratio	
	Control	Etoposide treated	Control	Etoposide treated
No Extract	6 ± 1	64 ± 2 [a]	0.06	1.78
Aqueous extract	43 ± 2 [a]	69 ± 1 [a,b,c]	0.75	2.22
Methanol extract	60 ± 1 [a]	70 ± 2 [a,b,c]	1.50	2.33
Chloroform extract	39 ± 1 [a]	58 ± 4 [a,b,c]	0.64	1.38

The values are means ± S.D of triplicates

CD value = 5

a - Statistically significant (P< 0.01) compared to untreated control group

b - Statistically significant (P< 0.01) compared to oxidant treated group

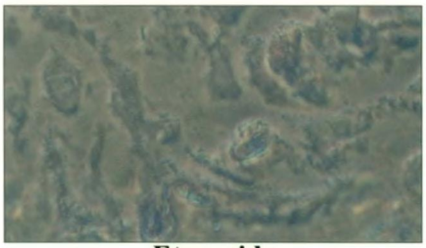
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

Our results showed that upon etoposide exposure, an increased number of cells become permeable to PI, showing their commitment to apoptosis (Plate 26).

PLATE 25

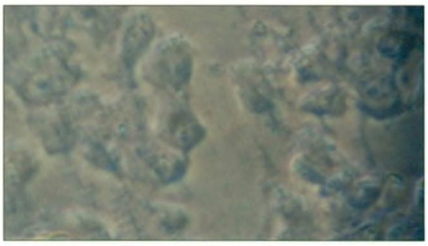
Hep2 CELLS STAINED WITH GIEMSA (OXIDANT-ETOPOSIDE)

Control



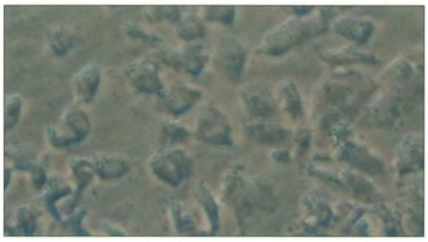
Etoposide

Aqueous extract



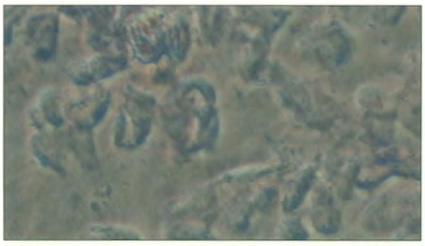
Aqueous extract + Etoposide

Methanol extract



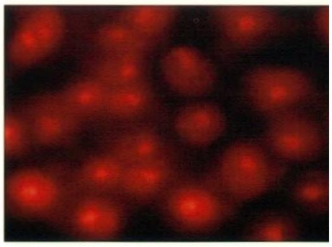
Methanol extract + Etoposide

Chloroform extract

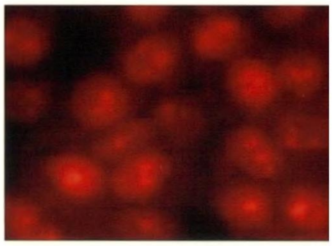


Chloroform extract + Etoposide

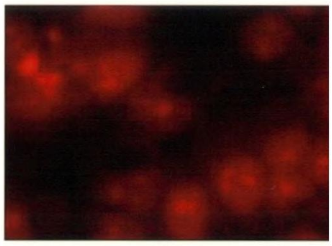
Hep2 (
(O



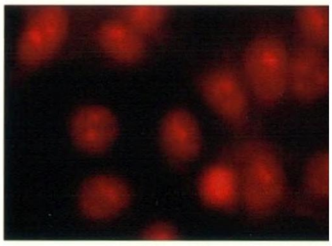
Control



Aqueous extract



Methanol extract



Chloroform extrac

All the three extracts showed considerable increase in the number of apoptosing cells. But the maximum induction of apoptosis was exhibited by the methanolic extract treated group. These results indicate that *Zea mays* leaves can render toxic effects specifically against cancer cells, with the methanolic and aqueous extracts faring better than the chloroform extract.

The apoptosis induction in Hep2 cells by etoposide and its influence in the presence of the leaf extracts of *Zea mays* was also quantified using ethidium bromide and DAPI staining. The data obtained are presented in Tables 36 and 37 respectively.

TABLE 36

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN Hep2 CELLS EXPOSED TO ETOPOSIDE AS DETERMINED BY EtBr STAINING

SAMPLE	No. of Apoptotic cells / 100 cells		Apoptotic ratio	
	Control	Etoposide treated	Control	Etoposide treated
No Extract	5 ± 1	63 ± 2 [a]	0.05	1.70
Aqueous extract	48 ± 2 [a]	68 ± 3 [a,c]	0.92	2.13
Methanol extract	63 ± 2 [a]	75 ± 4 [a,b,c]	1.70	3.00
Chloroform extract	40 ± 3 [a]	59 ± 1 [a,c]	0.67	1.44

The values are means ± S.D of triplicates
CD value = 6.69
a - Statistically significant (P< 0.01) compared to untreated control group
b - Statistically significant (P< 0.01) compared to oxidant treated group
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

TABLE 37

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN Hep2 CELLS EXPOSED TO ETOPOSIDE AS DETERMINED BY DAPI STAINING

SAMPLE	No. of Apoptotic cells / 100 cells		Apoptotic ratio	
	Control	Etoposide treated	Control	Etoposide treated
No Extract	6 ± 3	64 ± 2 [a]	0.06	1.78
Aqueous extract	41 ± 2 [a]	67 ± 3 [a,c]	0.69	2.03
Methanol extract	62 ± 4 [a]	71 ± 1 [a,b,c]	1.60	2.44
Chloroform extract	40 ± 1 [a]	56 ± 2 [a,b,c]	0.67	1.27

The values are means ± S.D of triplicates
CD value = 5.8
a - Statistically significant (P< 0.01) compared to untreated control group
b - Statistically significant (P< 0.01) compared to oxidant treated group
c - Statistically significant (P< 0.01) compared to respective plant extract treated group

Plates 27 and 28 show the photographic record of Hep2 cells exposed to etoposide and the leaf extracts stained with EtBr and DAPI respectively.

As can be seen from the values presented in the Tables 36 and 37, the extent of apoptosis followed the same trend as that revealed by PI staining. The cytotoxicity evoked by the methanolic extract of *Zea mays* leaves was on par with that evoked by etoposide alone. When administered together, etoposide and the leaf extracts caused more cells to die. In this respect, the methanolic and aqueous extracts were better than the chloroform extracts of *Zea mays* leaves.

AO/EtBr staining was also carried out to confirm the findings of the other staining methods and the results are presented in Table 38. The results of AO/EtBr staining clearly indicate that etoposide exposure caused a significant number of Hep2 cells to commit to apoptosis (Plate 29). When the cells were exposed to etoposide and the leaf extracts, the extent of cell death was comparable to that induced by etoposide alone.

TABLE 38

EFFECT OF *Zea mays* LEAF EXTRACTS ON NUCLEAR CHANGES IN Hep2 CELLS EXPOSED TO ETOPOSIDE AS DETERMINED BY AO/EtBr STAINING

SAMPLE	No. of Apoptotic cells / 100 cells		Apoptotic ratio	
	Control	Etoposide treated	Control	Etoposide treated
No Extract	10 ± 3	70 ± 2 [a]	0.15	2.33
Aqueous extract	43 ± 1 [a]	67 ± 2 [a,c]	0.89	2.03
Methanol extract	62 ± 2 [a]	73 ± 2 [a,c]	1.63	2.70
Chloroform extract	40 ± 2 [a]	55 ± 2 [a,b,c]	0.67	1.20

The values are means ± S.D of triplicates

CD value = 4.77

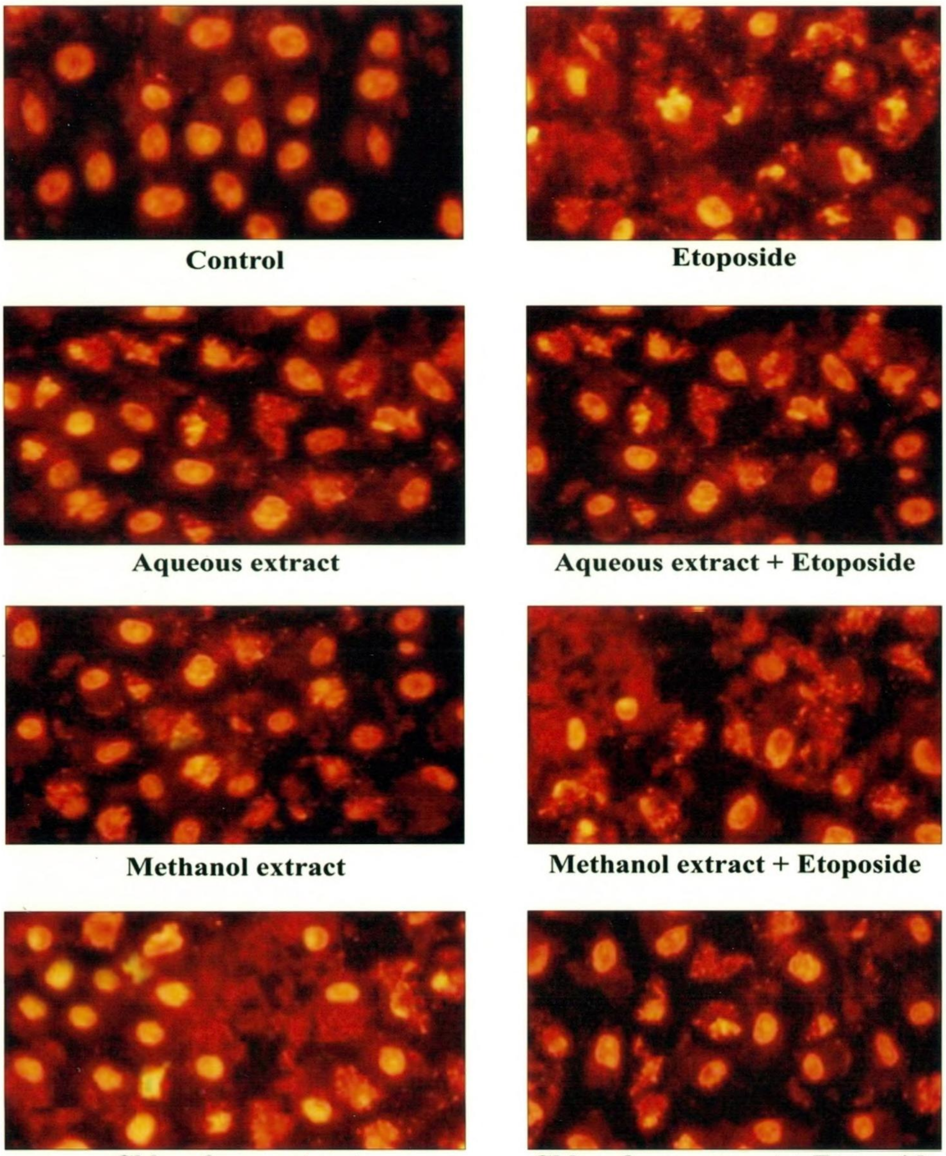
a - Statistically significant ($P< 0.01$) compared to untreated control group

b - Statistically significant ($P< 0.01$) compared to oxidant treated group

c - Statistically significant ($P< 0.01$) compared to respective plant extract treated group

PLATE 27

Hep2 CELLS STAINED WITH EtBr
(OXIDANT-ETOPOSIDE)



Control

Etoposide

Aqueous extract

Aqueous extract + Etoposide

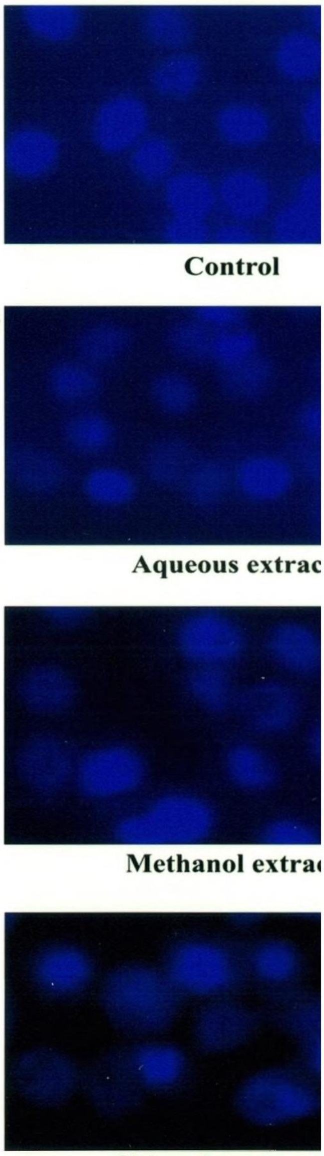
Methanol extract

Methanol extract + Etoposide

Chloroform extract

Chloroform extract + Etoposide

Hep2 C
(C



Control

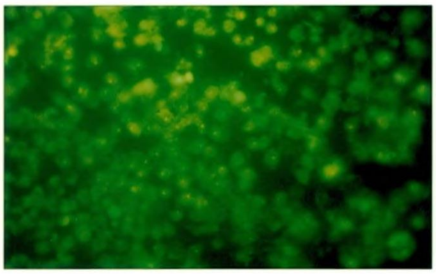
Aqueous extrac

Methanol extra

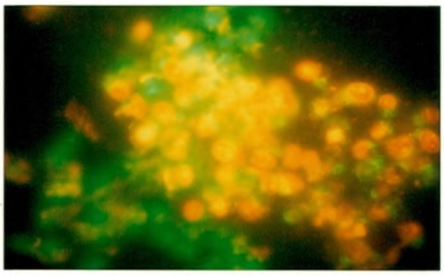
Chloroform extr

137

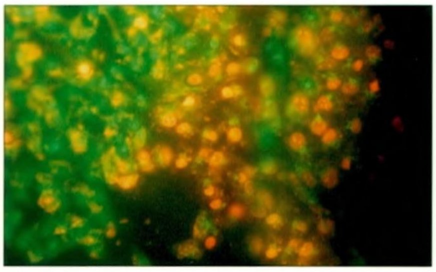
PLATE 29
Hep2 CELLS STAINED WITH AO/EtBr
(OXIDANT-ETOPOSIDE)



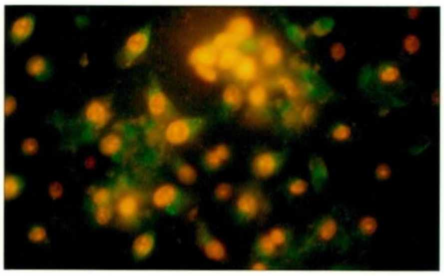
Control



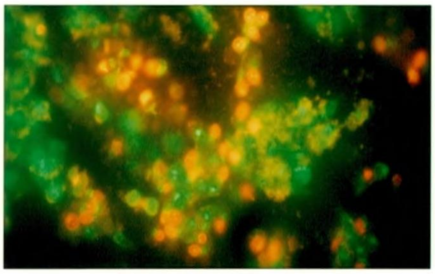
Etoposide



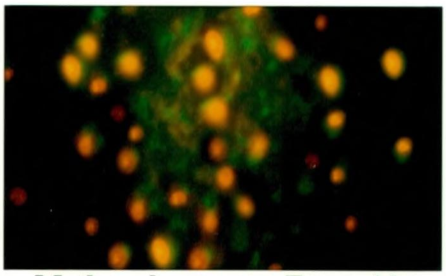
Aqueous extract



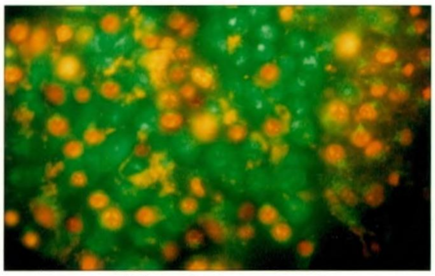
Aqueous extract + Etoposide



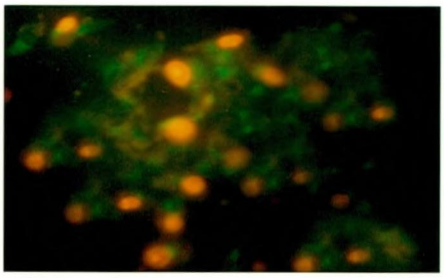
Methanol extract



Methanol extract + Etoposide



Chloroform extract



Chloroform extract + Etoposide

The results of Phase II and Phase III revealed that *Zea mays* leaf extracts can very effectively counteract oxidative stress-induced damage in purified biomolecules (lipids and DNA), tissue slices (goat liver slices), non-cancerous cells and cancerous cells. Eventhough these results strongly imply the protective action of the extracts, unequivocal proof can be provided only by conducting studies *in vivo*. It is possible that a very effective component in the plant extract, which results in good protection *in vitro*, does not render the same extent of protection under *in vivo* conditions, due to several factors like lower bioavailability, faster elimination and uneven distribution.

However, in order to overcome this lacuna, one needs to conduct the study in experimental rodents. One of the major focuses of our research activities is to identify and standardize the use of viable alternative systems that can simulate the conditions *in vivo* in the human system.

In tune with this effort, in the present study, *Drosophila melanogaster* was used as the experimental animals. The flies were chosen because it has been shown by earlier studies that they elicit a similar response as humans at the cellular level to the exposure of various substances (Arya *et al.*, 2007).

We studied the antioxidant activity of *Zea mays* using Drosophila as a model system under conditions of carbon tetrachloride (CCl_4) and hydrogen peroxide (H_2O_2) induced oxidative stress. Two different concentrations of each oxidant were used for the study. CCl_4 was used at the doses of 130mM and 195mM and H_2O_2 was used at the doses of 20mM and 30mM. These doses were selected based on the results of a pilot study, wherein varying doses of CCl_4 (50 to 200mM) and H_2O_2 (5 to 30mM) were studied. These two standard compounds were chosen to check if there existed a difference in the stress induced by a direct-acting oxidant (H_2O_2) and the one that requires metabolic activation (CCl_4). This study was conducted to analyze the differential response elicited by the two oxidants and leaf extracts under *in vivo* conditions.

ASSESSMENT OF ENZYMIC AND NON-ENZYMIC ANTIOXIDANTS DURING OXIDATIVE STRESS *in vivo*

The antioxidant status was assessed in the male and female flies exposed to oxidants (H_2O_2 and CCl_4) in the presence and the absence of the methanolic extract of *Zea mays* leaves.

TABLE 39

EFFECT OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES ON THE ACTIVI THE OXIDATIVE STRESS INDUCED *Drosophila mela*

SEX	GROUP	SUPEROXIDE DISMUTASE (Units / 30		
		WITHOUT OXIDANT	WITH H_2O_2	
			LOW DOSE	HIGH DOSE
Female	NE	36 ± 0.11	28 ± 0.08 [a]	25 ± 0.15 [a,d]
	ME	39 ± 0.5 [a]	32 ± 0.30 [a,b,c]	27 ± 0.26 [a,b,c,d]
Male	NE	32 ± 0.38 [e]	25 ± 0.2 [a, e]	23 ± 0.32 [a,d, e]
	ME	35 ± 0.10 [a, e]	29 ± 0.85 [a,b,c, e]	27 ± 0.50 [a,b,c,d]

The values are means ± SD of triplicates
CD value = 1.18
1 Unit = Amount of enzyme that causes 50% reduction in NBT oxi
NE – No extract; ME – methanolic extract
a-Statistically significant ($P < 0.01$) compared to untreated contr
b-Statistically significant ($P < 0.01$) compared to respective oxidant trea
c-Statistically significant ($P < 0.01$) compared to respective plant g
d-Statistically significant ($P < 0.01$) compared to lower dose of the o
e- Statistically significant ($P < 0.01$) compared to respective plant groups i

TABLE 40

EFFECT OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES ON THE ACTIVIT
STRESS INDUCED *Drosophila melanogaster*

SEX	GROUP	CATALASE (Units / 30 mg of flie			
		WITHOUT OXIDANT	WITH H_2O_2		
			LOW DOSE	HIGH DOSE	L
Female	NE	190.6 ± 0.10	78.7 ± 0.02 [a]	56.3 ± 1.11 [a,d]	1
	ME	170 ± 0.00 [a]	130 ± 0.05 [a,b,c]	115 ± 0.10 [a,b,c,d]	14
Male	NE	102 ± 1.15 [e]	50 ± 0.07 [a,e]	35 ± 0.26 [a,d,e]	7
	ME	104 ± 0.61 [a,e]	76 ± 0.40 [a,b,c,e]	67 ± 0.40 [a,b,c,d,e]	8

The values are means ± SD of triplicates
CD value = 1.08
1 Unit = Amount of enzyme required to decrease the absorbance at 240 nm b
NE – No extract; ME – methanolic extract
a-Statistically significant (P < 0.01) compared to untreated control
b-Statistically significant (P < 0.01) compared to respective oxidant treate
c-Statistically significant (P < 0.01) compared to respective plant grc
d-Statistically significant (P < 0.01) compared to lower dose of the oxi
e- Statistically significant (P < 0.01) compared to respective plant groups in t

TABLE 41

EFFECT OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES ON THE ACTIVITY STRESS INDUCED *Drosophila melanogaster*

SEX	GROUP	PEROXIDASE (Units / 30 mg of f		
		WITHOUT OXIDANT	WITH H_2O_2	
			LOW DOSE	HIGH DOSE
Female	NE	1.08 ± 0.05	0.68 ± 0.04 [a]	0.57 ± 0.01 [a]
	ME	1.29 ± 0.01 [a]	0.99 ± 0.03 [b,c]	0.75 ± 0.04 [a,b,c,d]
Male	NE	0.84 ± 0.06 [e]	0.57 ± 0.00 [a]	0.31 ± 0.10 [a,d, e]
	ME	0.92 ± 0.03 [e]	0.70 ± 0.01 [a,b,c, e]	0.57 ± 0.05 [a,b,c,d,e]

The values are means ± SD of triplicates

CD value = 0.12

1 Unit = Change in absorbance at 430 nm per minute

NE – No extract; ME – methanolic extract

a-Statistically significant (P < 0.01) compared to untreated contr

b-Statistically significant (P < 0.01) compared to respective oxidant treat

c-Statistically significant (P < 0.01) compared to respective plant gr

d-Statistically significant (P < 0.01) compared to lower dose of the o

e- Statistically significant (P < 0.01) compared to respective plant groups in

CHANGES IN THE ACTIVITIES OF ENZYMIC ANTIOXIDANTS

The antioxidant enzymes analyzed were SOD, CAT and POD. The results obtained are presented in the Tables 39, 40 and 41 respectively. The female flies showed significantly higher activities of all enzymes compared to the males in all the groups including the controls.

The activities of all the enzymes analysed were found to decrease upon CCl_4/H_2O_2 administration in the male and female flies. The activity was decreased to a greater extent when exposed to H_2O_2 than to CCl_4. The results also showed that the depletion was more severe as the concentration of the oxidants increased.

Co-administration of the methanolic extract of *Zea mays* leaves significantly increased the activities of these enzymic antioxidants. The reversal in the activity of SOD to near normal range was obtained in the male and female flies subjected to stress induced by the lower dose of CCl_4. A similar trend was obtained in the case of peroxidase. The catalase activity was not reverted to control range in flies exposed to even the lower dose of CCl_4.

The level of non-enzymic antioxidants, vitamins C, E and reduced glutathione were also analyzed and the results are depicted in Tables 42, 43 and 44 respectively. There was a reduction in the level of these non-enzymic antioxidants in the flies subjected to oxidative stress induced by H_2O_2/CCl_4 at both the concentrations. The extent of decrease in the levels was related to the concentration of the oxidant used. The higher the concentration, the more severe was the depletion in the levels of vitamins C, E and reduced glutathione. The higher dose of H_2O_2 caused the antioxidant level to decrease to very low levels in both the male and female flies.

The levels of vitamin C increased in both the oxidant treated groups but significantly in the H_2O_2 treated group. The co-treatment of the methanolic extract of *Zea mays* leaves increased the levels of vitamin E in the H_2O_2 and CCl_4 treated groups to significant levels. There was no significant increase in the levels of glutathione after the treatment with methanolic extract of *Zea mays* leaves in the oxidant treated groups. The non-enzymic antioxidants were increased to near normal levels in both the male and female flies exposed to the lower dose of CCl_4. The levels of non-enzymic antioxidants were also found to be higher in the female flies than in male flies. It is evident from the table that the plant extracts help in suppressing the damage caused by H_2O_2 and CCl_4 in both male and female flies.

TABLE 42

EFFECT OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES ON THE LEVEL: STRESS INDUCED *Drosophila melanogaster*

SEX	GROUP	VITAMIN C (μg / 30mg of flie		
		WITHOUT OXIDANT	WITH H_2O_2	
			LOW DOSE	HIGH DOSE
Female	NE	3.94 ±0.15	3.06 ±0.23 [a]	2.39 ±0.01 [a,d]
	ME	4.30 ±0.24 [a]	3.58 ±0.15 [a,b,c]	3.26 ±0.03 [a,b,c]
Male	NE	3.55 ±0.22 [e]	2.29 ±0.40 [a,e]	2.20 ±0.03 [a]
	ME	3.70 ±0.15 [e]	3.20 ±0.14 [b,c,e]	3.01 ±0.04 [a,b,c]

The values are means ± SD of triplicates
CD value =0.35
NE – No extract; ME – methanolic extract
a-Statistically significant (P < 0.01) compared to untreated contr
b-Statistically significant (P < 0.01) compared to respective oxidant treat
c-Statistically significant (P < 0.01) compared to respective plant g
d-Statistically significant (P < 0.01) compared to lower dose of the o
e- Statistically significant (P < 0.01) compared to respective plant groups ir

TABLE 43

EFFECT OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES ON THE LEVELS STRESS INDUCED *Drosophila melanogaster*

SEX	GROUP	VITAMIN E (µg / 30mg of flies			
		WITHOUT OXIDANT	WITH H_2O_2		
			LOW DOSE	HIGH DOSE	I
Female	NE	5.6 ±0.04	3.8 ±0.11 [a]	2.3 ±0.05 [a,d]	4.
	ME	6.6 ±0.09 [a]	4.9 ±0.23 [a,b,c]	3.7 ±0.01 [a,b,c,d]	5.
Male	NE	4.0 ±0.07 [e]	3.12 ±0.03 [a,e]	2.8 ±0.01 [a,d,e]	3.
	ME	4.6 ±0.10 [a,e]	3.46 ±0.02 [a,b,c,e]	3.2 ±0.06 [a,b,c,d,e]	3.

The values are means ± SD of triplicates

CD value = 0.19

NE – No extract; ME – methanolic extract

a-Statistically significant ($P < 0.01$) compared to untreated contr

b-Statistically significant ($P < 0.01$) compared to respective oxidant treat

c-Statistically significant ($P < 0.01$) compared to respective plant g

d-Statistically significant ($P < 0.01$) compared to lower dose of the o

e- Statistically significant ($P < 0.01$) compared to respective plant groups in

TABLE 44

EFFECT OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES ON THE LEVELS OXIDATIVE STRESS INDUCED *Drosophila melanog*

SEX	GROUP	GLUTATHIONE (μg / 30mg of f		
		WITHOUT OXIDANT	WITH H_2O_2	
			LOW DOSE	HIGH DOSE
Female	NE	4.3 ±0.01	3.6 ±0.02 [a]	1.9 ±0.00 [a,d]
	ME	4.5±0.08	3.8 ±0.10 [a,c]	2.29 ±0.002 [a,c,d]
Male	NE	3.5 ±0.20 [e]	2.9 ±0.70 [a,e]	2.5 ±0.06 [a, e]
	ME	3.80 ±0.34 [e]	3.2 ±0.12 [c, e]	3.0 ±0.36 [a,b,c, e]

The values are means ± SD of triplicates
CD value = 0.49
NE – No extract; ME – methanolic extract
a-Statistically significant ($P < 0.01$) compared to untreated contr
b-Statistically significant ($P < 0.01$) compared to respective oxidant treat
c-Statistically significant ($P < 0.01$) compared to respective plant g
d-Statistically significant ($P < 0.01$) compared to lower dose of the o
e- Statistically significant ($P < 0.01$) compared to respective plant groups ir

Thus, the study clearly demonstrates that the exposure to oxidants (H_2O_2 and CCl_4) causes a significant depletion of the major antioxidant components in *Drosophila melanogaster*. Administration of *Zea mays* leaf extract restored the depleted levels of both enzymic and non-enzymic antioxidants. These results show that the leaves of *Zea mays* exert a significant antioxidant action against oxidative stress induced by H_2O_2 and CCl_4 *in vivo*.

PHASE IV

Having ascertained the antioxidant potential of the *Zea mays* leaf extracts both *in vitro* and *in vivo*, a preliminary phytochemical screening was done in order to identify the chemical nature of the active component that is maximally responsible for rendering the leaves its antioxidant property.

The extract when tested with the Mayer's, Dragendroff's and Wagner's tests gave negative results, which indicated the absence of alkaloids. The presence of phenolics was detected by treating an aliquot of the extract with $FeCl_3$ reagent or lead acetate solution which gave a deep blue-black colour and a white precipitate respectively. These results indicated the presence of phenolics.

The presence of flavonoids was tested using aqueous sodium hydroxide, sulphuric acid and Schinodo's test. The results obtained confirmed the presence of flavonoids. Thus, the qualitative analysis of the extract revealed the presence of phenolics and flavonoids in the methanolic extract of *Zea mays* leaves.

UV ABSORPTION SPECTRUM

An absorbance scan of the methanolic extract of *Zea mays* leaves in the wavelength ranging from 190nm-350nm (Figure 18) revealed the presence of two major peaks at 195 and 250nm.

In order to confirm the results obtained after qualitative analysis, spectral analysis using HPLC, HPTLC, IR spectrum and GC-MS analyses were carried out.

HPLC ANALYSIS

The HPLC analysis of the methanolic extract of *Zea mays* leaves were carried out using the C18 column (Shimadzu equipped with UV detector). The results are depicted in Figure 19.

Three peaks (2 major and one minor) were observed in the spectrum showing the presence of three principle components in the methanolic extract of *Zea mays* leaves at retention times 5.774, 14.351 and 19.317 respectively. The retention time, height and area of the peaks are presented in Table 45.

From the data, it can be observed that the third peak with a retention time of 19.317 minutes was the biggest peak followed by the peak 2 and peak 1 with retention times of 14.351 and 5.774 respectively.

TABLE 45

RETENTION TIME, HEIGHT AND PEAK AREAS OF THE HPLC SEPARATED FRACTIONS OF THE METHANOLIC EXTRACT OF THE *Zea mays* LEAVES

Peak no	Retention Time	Height	Area
1	5.774	15306	772725
2	14.351	19754	1777379
3	19.317	65899	6730358

HPTLC

The methanolic extract of *Zea mays* leaves was tested for the presence of alkaloids, phenolics and flavonoids using HPTLC analysis as mentioned in the methodology chapter.

ALKALOIDS

Alkaloid profile was done with the reference standard nicotine. Two spots were detected but it did not stain for alkaloids using Dragendroff reagent (Plate 30). This may be due to their presence in undetectable amount. Further studies are needed to confirm the presence or the absence of alkaloids, especially as qualitative tests showed their absence.

FIGURE 18
UV SPECTRUM OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES

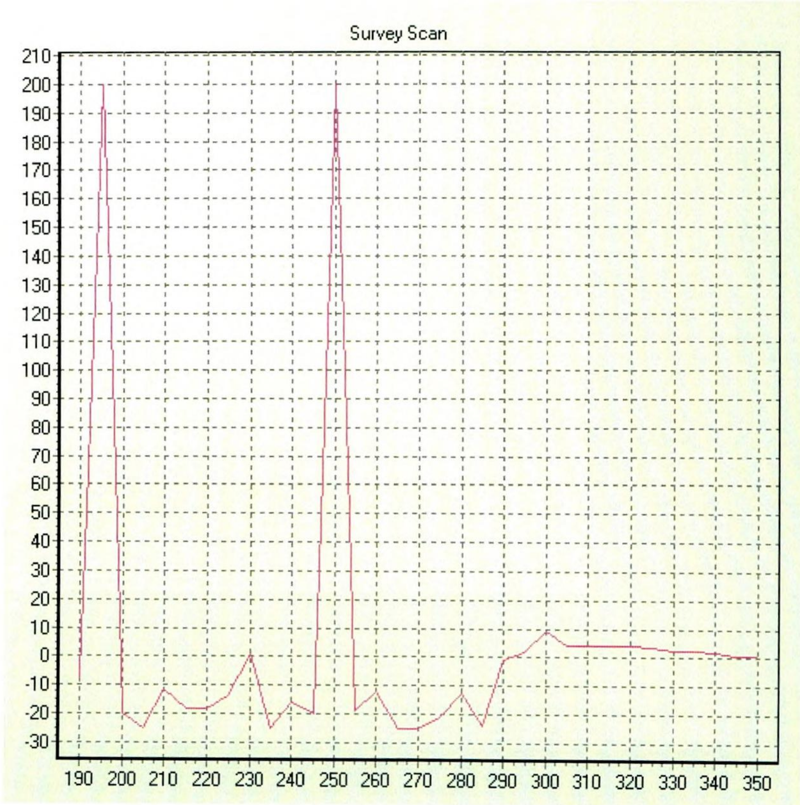
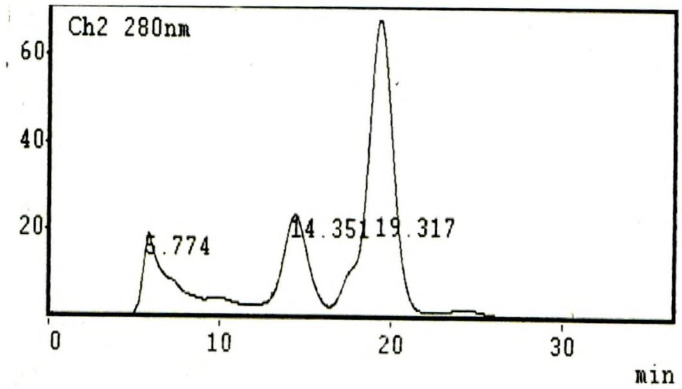


FIGURE 19
HPLC PROFILE OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES



*** Peak Report ***

PKNO	ChNO	TIME	AREA	HEIGHT	CONC
1	2	5.774	772725	15306	
2	2	14.351	1777379	19754	
3	2	19.317	6730358	65899	
			9280461	100959	

The Rf values, height and area of the peaks of the two compounds are shown in the Table 46 and the peak densitogram in Figure 20.

TABLE 46

PEAK TABLE FOR ALKALOIDS OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES BY HPTLC

Track	Peak	Rf	Height	Area	Assigned substance
ME	1	0.73	130.0	7883.0	Unknown 1
ME	2	0.80	220.9	10010.2	Unknown 2

ME – Methanolic extract of *Zea mays* leaves

PHENOLICS

The phenolics present in the methanolic extract of *Zea mays* leaves were analysed using quercetin as reference standard. The results showed the presence of blue or blue-grey coloured zone in daylight (Plate 31), which showed the presence of phenolic compounds. Eight phenolic compounds were observed with the Rf values, height and area as shown in Table 47. The peak densitogram of the same is presented in Figure 21.

TABLE 47

PEAK TABLE FOR PHENOLICS OF THE METHANOLIC EXTRACT OF THE *Zea mays* LEAVES BY HPTLC

Track	Peak	Rf	Height	Area	Assigned substance
ME	1	0.04	39.3	585.0	Phenolic compound 1
ME	2	0.08	31.9	506.3	Phenolic compound 2
ME	3	0.11	25.4	608.0	Phenolic compound 3
ME	4	0.23	65.0	1514.8	Phenolic compound 4
ME	5	30	29.7	873.9	Phenolic compound 5
ME	6	38	49.1	1618.3	Phenolc compound 6
ME	7	48	78.2	3907.0	Phenolic compound 7
ME	8	61	123.3	4773.0	Phenolic compound 8

ME – Methanolic extract of *Zea mays* leaves

FLAVONOIDS

The methanolic extract of *Zea mays* leaves was analysed for the presence of flavonoids using rutin as the standard. Yellow and yellow green fluorescence zone at UV 366nm was observed from the chromatogram (Plate 32), which confirmed the presence of flavonoids. Two positive peaks for flavonoids and one unknown peak were obtained, which are presented in Table 48. Finally, the plate was scanned at 366nm. The peak densitogram (Figure 22) was recorded.

TABLE 48

PEAK TABLE FOR FLAVONOIDS OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES BY HPTLC

Track	Peak	Rf	Height	Area	Assigned substance
ME	1	0.38	90.9	3062.4	Unknown
ME	2	0.42	46.4	2258.3	Flavonoid 1
ME	3	0.54	49.6	2035.6	Flavonoid 2

ME – Methanolic extract of *Zea mays* leaves

IR ANALYSIS

The methanolic extract of *Zea mays* leaves was analysed for the IR spectrum using Shimadzu FT-IR spectrophotometer using KBr pellet method (Figure 23). The results of IR spectrum showed major peaks at 3000-3400 cm^{-1} (broad and strong indication of ^{-}OH) indicating the presence of polyphenolics and 1670.24 cm^{-1} (indicative of carbonyl group), which suggests the presence of flavonoids.

GC-MS ANALYSIS

The GC-MS analysis of the methanolic extract of *Zea mays* leaves was carried out to identify the nature of the components present. The GC-MS output showed the presence of various components at retention times 6.843, 9.795, 10.175, 10.715, 13.376, 13.809 and 17.594 (Figure 24).

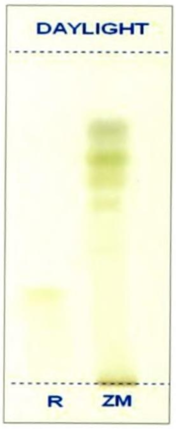
PLATE 30
CHROMATOGRAMS OF ALKALOIDS

Before derivatization

After derivatization

PLATE 31
CHROMATOGRAMS OF PHENOLICS

Before derivatization



After derivatization

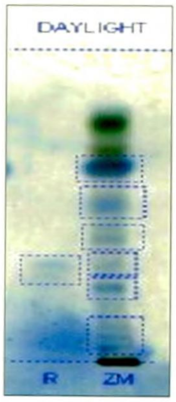


PLATE 32
CHROMATOGRAMS OF FLAVONOIDS

Before derivatization

After derivatization

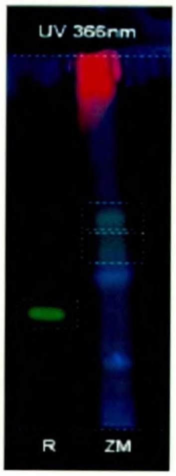


FIGURE 20
PEAK DENSITOGRAM OF ALKALOIDS OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES BY HPTLC

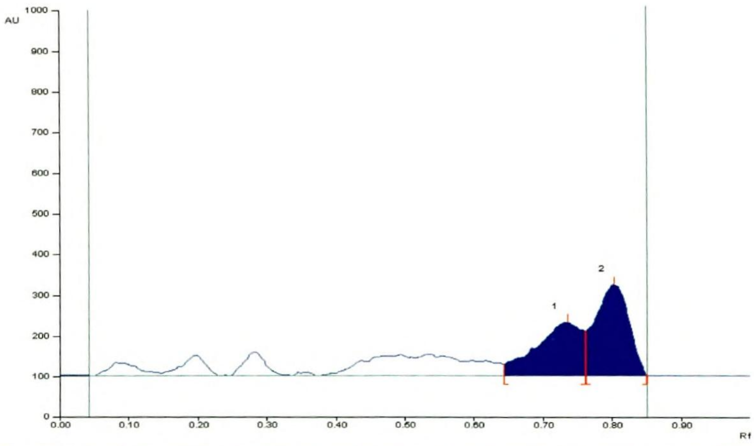


FIGURE 21
PEAK DENSITOGRAM OF PHENOLICS OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES BY HPTLC

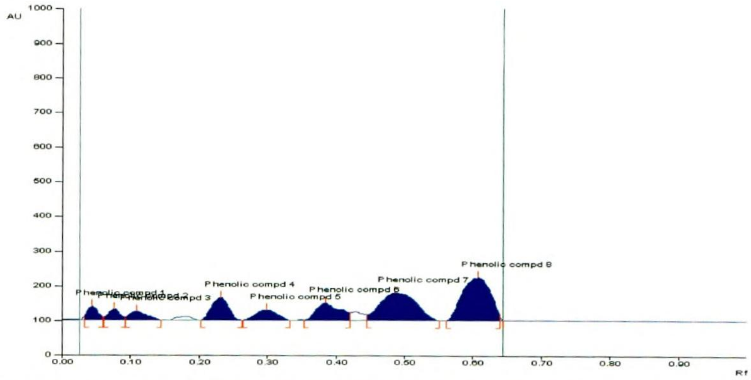
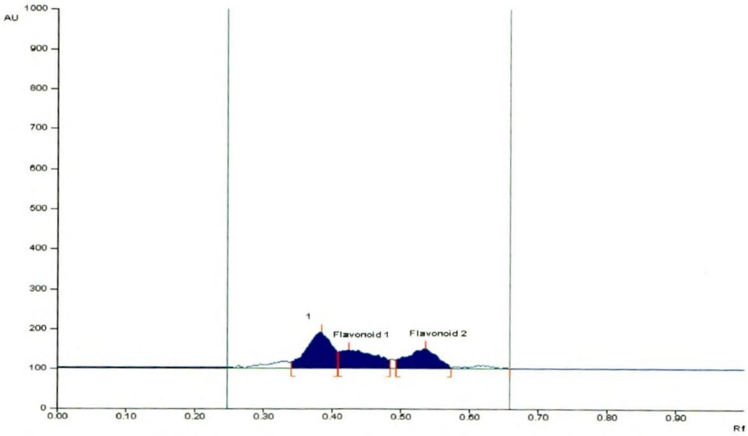


FIGURE 22
PEAK DENSITOGRAM OF FLAVONOIDS OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES BY HPTLC



The compound with retention time 6.843 showed the presence of molecular ion peak at 150.2 as presented in Figure 25. Two (M-17) peaks were observed at m/z 134.7 and m/z 117.2, which are characteristic of the presence of OH groups, suggesting that the compound may be of polyphenolic type.

The compound with retention time 9.795 gave five major (m/z) peaks. The molecular ion peak was observed at m/z 137.3 and the base peak at m/z 110.7, which is presented in Figure 26. Two (M-28) peaks were observed at m/z 151.8 and m/z 80.7 respectively, which are characteristic of (M-CO) peaks. This indicates that the fraction may contain two carbonyl groups.

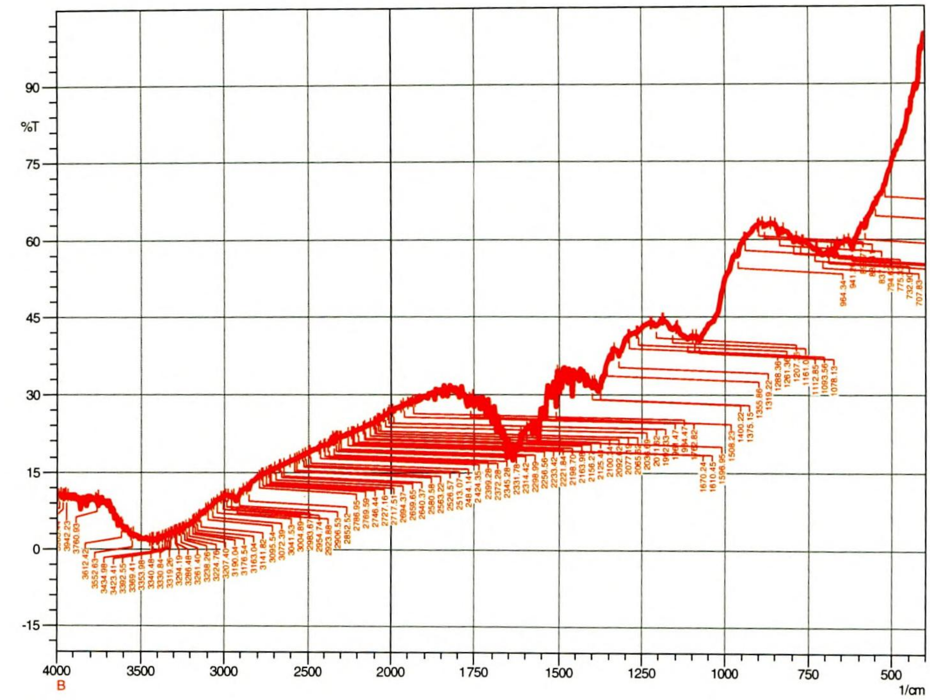
The mass spectrum with retention time 10.175 showed five major peaks (m/z), as shown in Figure 27. The molecular ion peak was obtained at m/z 124.4 and the base peak at m/z 67.9. The fragmentation pattern shows characteristic pattern of $C_nH_{2n-6}O_2$ type of compounds.

The spectrum analysis of the compound with retention time 10.715 gave four major peaks with molecular ion peak at 109.4 and the base peak at 57.9 (Figure 28). The element combination for the molecular ion peak was observed at 109.4 and the base peak at 57.9. The fragmentation shows characteristic pattern of $C_nH_{2n-1}O_2$ which is indicative of saturated aliphatic alcohols and saturated aliphatic ethers and saturated aliphatic esters. Therefore, it may be concluded that the compound with retention time 10.175 could be some hydroxylated compound.

The mass spectrum of the compound with retention time 13.376 gave five major peaks (m/z), as shown in Figure 29. The molecular ion peak was observed at m/z 123.3 and the base peak at m/z 70.9. Two (M-28) peaks were observed at m/z 165.4, 136.9 respectively, indicating the presence of two 'CO' groups.

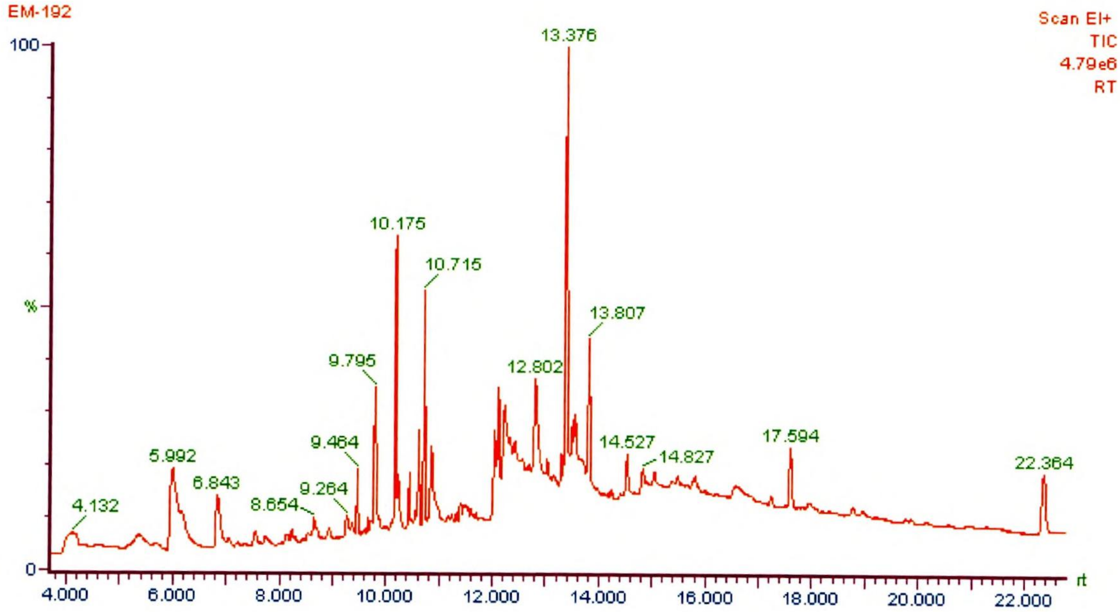
The compound with retention time 13.807 gave seven major peaks with the molecular ion peak at m/z 108.5 and base peak at m/z 78.5, as depicted in Figure 30. The (M-44) peaks were observed at m/z 193.3, indicating the presence of carboxylic acid group.

FIGURE 23



IR SPECTRUM OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES

FIGURE 24



GC-MS PROFILE OF THE METHANOLIC EXTRACT OF *Zea mays* LEAVES

FIGURE 25
PEAK FRAGMENTATION OF GC-MS SPECTRUM (6.843)

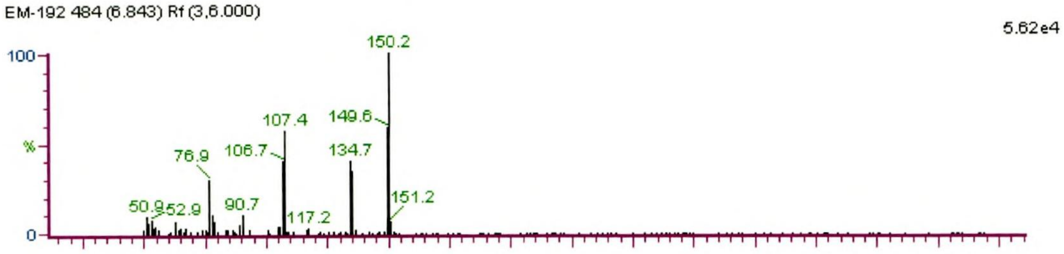


FIGURE 26
PEAK FRAGMENTATION OF GC-MS SPECTRUM (9.795)

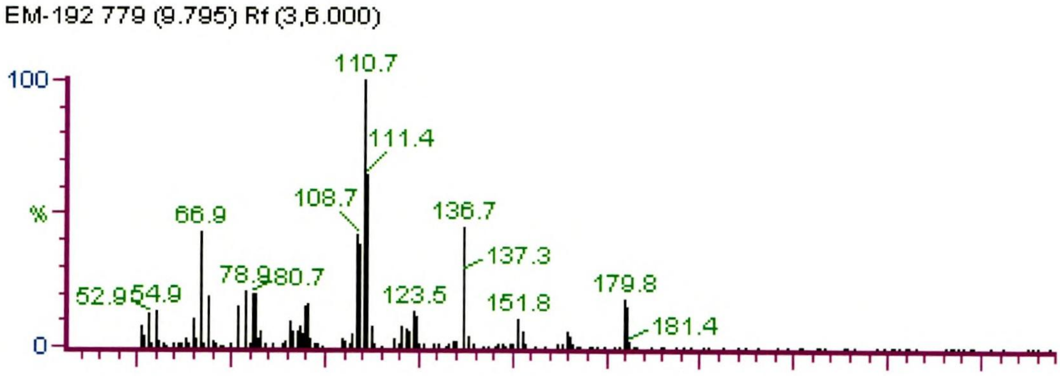


FIGURE 27
PEAK FRAGMENTATION OF GC-MS SPECTRUM (10.175)

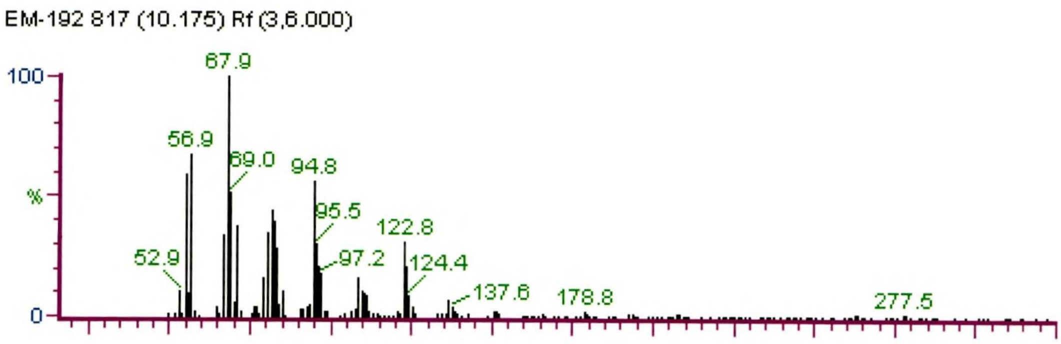


FIGURE 28
PEAK FRAGMENTATION OF GC-MS SPECTRUM (10.715)

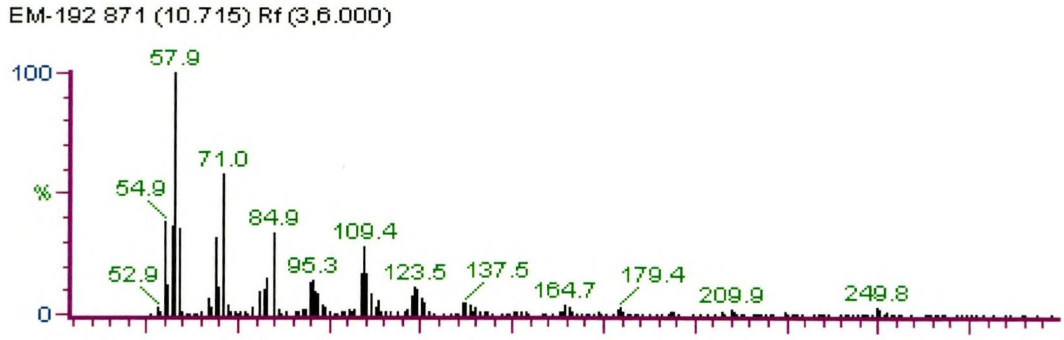


FIGURE 29
PEAK FRAGMENTATION OF GC-MS SPECTRUM (13.376)

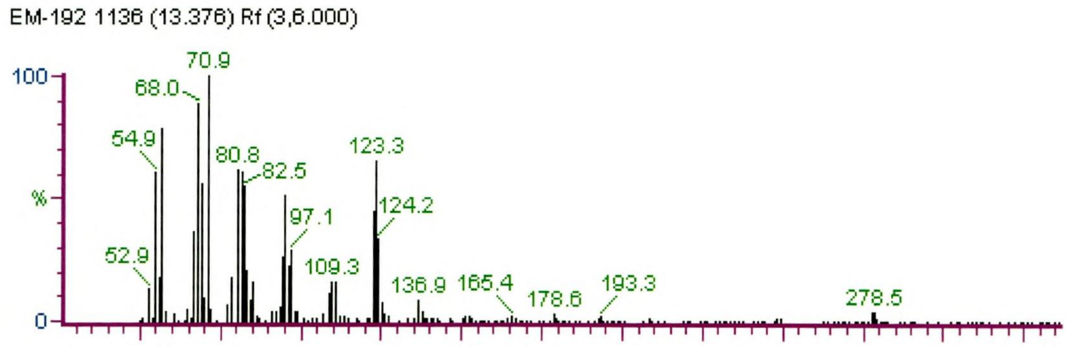


FIGURE 30
PEAK FRAGMENTATION OF GC-MS SPECTRUM (13.807)

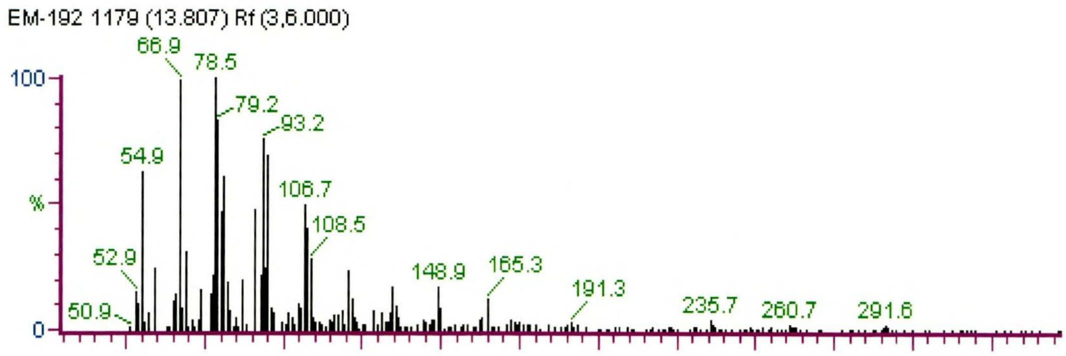
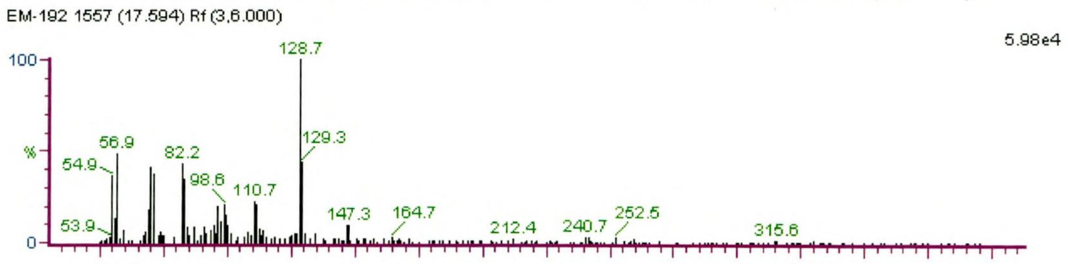


FIGURE 31
PEAK FRAGMENTATION OF GC-MS SPECTRUM (17.594)



The compound with retention time 17.594 (Figure 31) showed the presence of two (M-17) peaks at m/z 82.2 and m/z 147.3, which are characteristic of the presence of OH groups. Hence, the GC-MS profile indicates the presence of polyphenolic and flavonoid type of compounds in the methanolic extract of *Zea mays* leaves.

Thus, the phytochemical analysis of *Zea mays* leaves revealed polyphenolic and flavonoid type of compounds to be the major constituents. Further analyses are required to identify the exact structural aspects of the active principles involved.

The salient observations made in the study and the results presented in this chapter, are discussed in the light of the relevant literature in the next chapter.