Results

4. RESULTS

Free radicals are highly reactive atomic or molecular species that contain an unpaired electron, which contributes to the high reactivity. They react quickly with the nearest stable molecule to capture the electron they need to gain stability. The injured molecule loses its electron, becoming a free radical itself. Free radicals can damage vital cellular components like nucleic acids and cell membranes, resulting in subsequent cell death. Normally, there is a balance between free radical generation and scavenging. Excessive generation of free radicals and insufficient antioxidant defense mechanism leads to oxidative stress which cause tissue damage and has been implicated in a number of human diseases (Buonocore and Groenendaal, 2007).

Antioxidants are capable of neutralizing free radicals at different stages. The screening studies for antioxidant properties of medicinal and food plants have been performed increasingly in the last few decades in the hope of finding an efficient remedy for several present-day diseases and means to delay ageing symptoms (Halliwell, 2008).

Natural compounds, especially those derived from dietary sources, provide a large number of antioxidants. Apart from the dietary sources, medicinal plants can also act as sources of antioxidants. Ethnobotanical studies reveal that the plant *Artemisia vulgaris* is used as an anthelmintic, antiplasmodic and antiseptic. The plant is also used as an antimalarial agent for several decades. The present study was formulated to assess the antioxidant potential of the *Artemisia vulgaris* leaves (Plate 1).

PHASE I

In the first phase of the study, the antioxidant contents were analyzed in the leaves of *Artemisia vulgaris*. Both enzymic and non-enzymic antioxidants were quantified. The values obtained are presented below.

ENZYMIC ANTIOXIDANT ACTIVITIES IN THE LEAVES OF *Artemisia vulgaris*

The enzymic antioxidants analysed in the leaves of *Artemisia vulgaris* were superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione reductase (GR), glutathione S-transferase (GST) and polyphenol oxidases (PPO). The activities obtained are presented in Table 1.

TABLE 1

ENZYMIC ANTIOXIDANT ACTIVITIES IN *Artemisia vulgaris* LEAVES

ENZYMES	ACTIVITY
Superoxide dismutase ($U^{\#}$/g leaf)	33.97 ± 0.06
Catalase ($U^{\$}$/g leaf)	292.81±19.89
Peroxidase (U*/g leaf)	23.60 ± 0.09
Glutathione reductase (U^{+}/g leaf)	2.22 ± 0.03
Glutathione S-transferase ($U^{@}$/g leaf)	0.03 ± 0.001
PPO - Catechol oxidase (Units$^{¢}$x10^{-3}/g leaf)	0.46 ± 0.01
PPO - Laccase (Units$^{¢}$x10^{-3}/g leaf)	0.41 ± 0.003

The values are mean ± S.D of triplicates.
1 Unit = Amount of enzyme that causes 50% reduction in NBT oxidation
$ 1 Unit = Amount of enzyme required to decrease the absorbance at 240nm by 0.05 units/minute
* 1 Unit = Changes in absorbance at 430 nm/minute
+ 1 Unit = mmoles of NADPH oxidized/minute
@ 1 Unit = nmoles of CDNB conjugated/minute
¢ 1 unit of catechol oxidase/laccase = Amount of enzyme which transforms 1 unit of dihydrophenol to quinine /minute

The values presented in Table 1 show that the leaves of *Artemisia vulgaris* possess considerable activities of all the enzymes analyzed. It is evident from the values that the leaf is a good source of enzymic antioxidants.

NON-ENZYMIC ANTIOXIDANT LEVELS IN THE LEAVES OF *Artemisia vulgaris*

The levels of non-enzymic antioxidants, namely ascorbic acid, tocopherol, reduced glutathione, total carotenoids, lycopene, total phenols, total flavonoids and total chlorophyll, are presented in Table 2.

TABLE 2

NON-ENZYMIC ANTIOXIDANT LEVELS IN *Artemisia vulgaris* LEAVES

PARAMETERS	LEVELS
Ascorbic acid (mg/g leaf)	2.69 ± 0.03
Tocopherol (µg/g leaf)	6.68 ± 0.01
Reduced glutathione (nmoles/g leaf)	243.25 ± 13.42
Total carotenoids (mg/g leaf)	7.45 ± 0.34
Lycopene (mg/g leaf)	1.88 ± 0.17
Total phenols (mg/g leaf)	7.96 ± 0.33
Total flavonoids (mg/g leaf)	5.83 ± 0.07
Total chlorophyll (mg/g leaf)	2.01 ± 0.002

The values are mean ± S.D of triplicates.

The results revealed that the leaves of *Artemisia vulgaris* exhibited considerable amounts of all the non-enzymic antioxidants analysed.

In order to understand the active principle and the solvents into which the maximum amount of antioxidants got extracted, the leaves of *Artemisia*

vulgaris were serially extracted into solvents of increasing polarity (petroleum ether, benzene, chloroform, ethyl acetate and methanol) using Soxhlet apparatus. An aqueous extract was also prepared (as detailed in the previous chapter). These extracts were then tested for their radical scavenging effects against a battery of oxidant moieties that included a stable radical DPPH, a cation radical ABTS, superoxide and nitric oxide radicals.

RADICAL SCAVENGING EFFECTS OF *Artemisia vulgaris* LEAF EXTRACTS

The radical scavenging effects of different solvent extracts of *Artemisia vulgaris* were studied on DPPH, ABTS, $SO^{\bullet}$ and NO radicals. The ability of the leaf extracts to scavenge DPPH was tested in a rapid dot blot screening and quantified using a spectrophotometric assay. The picture obtained in the dot blot screening is shown in Plate 2.

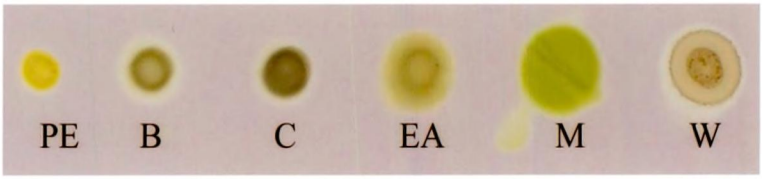
The results of rapid screening of the extracts of *Artemisia vulgaris* leaf against DPPH revealed that the methanolic extract exhibited the maximum scavenging activity, followed closely by the aqueous extract. Chloroform, ethyl acetate and benzene extracts expressed almost equal ability to scavenge DPPH. Out of all the six extracts used, the least ability to quench DPPH was observed in the petroleum ether extract.

The extent of DPPH and ABTS radical scavenging by the leaf extracts of *Artemisia vulgaris* is presented in Figure 1. The maximum extent of both DPPH and ABTS radical scavenging was elicited by the methanolic extract, followed by the aqueous extract. DPPH and ABTS scavenging effects of other solvent extracts were found to be moderate.

PLATE 1

Artemisia vulgaris **LEAVES**

PLATE 2



DPPH DOT BLOT ASSAY

PE- Petroleum ether extract
B - Benzene extract
C - Chloroform extract
EA - Ethyl acetate extract
M - Methanol extract
W - Water extract

FIGURE 1
DPPH AND ABTS RADICAL SCAVENGING EFFECTS OF *Artemisia vulgaris* LEAF EXTRACTS

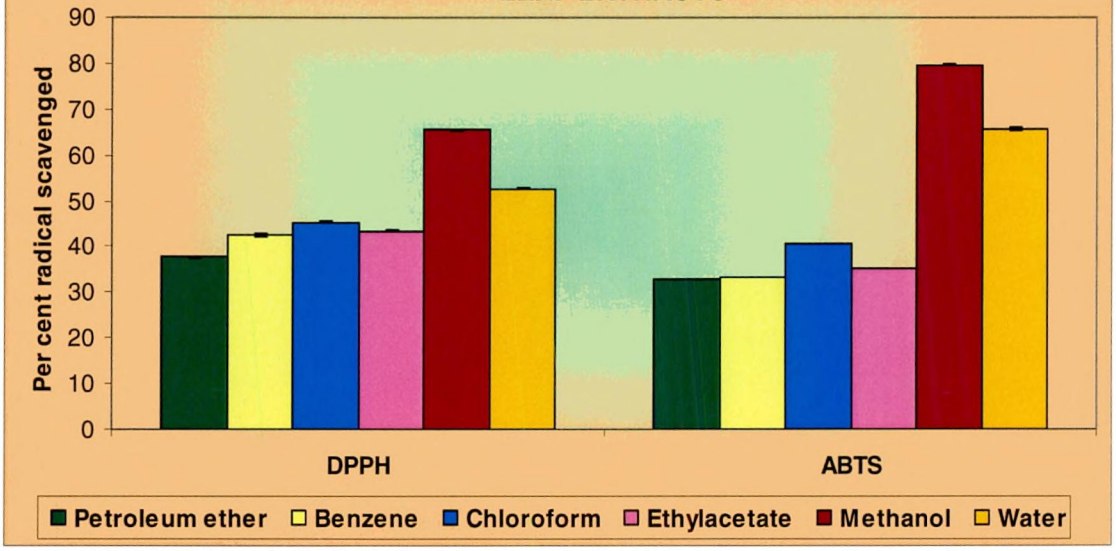
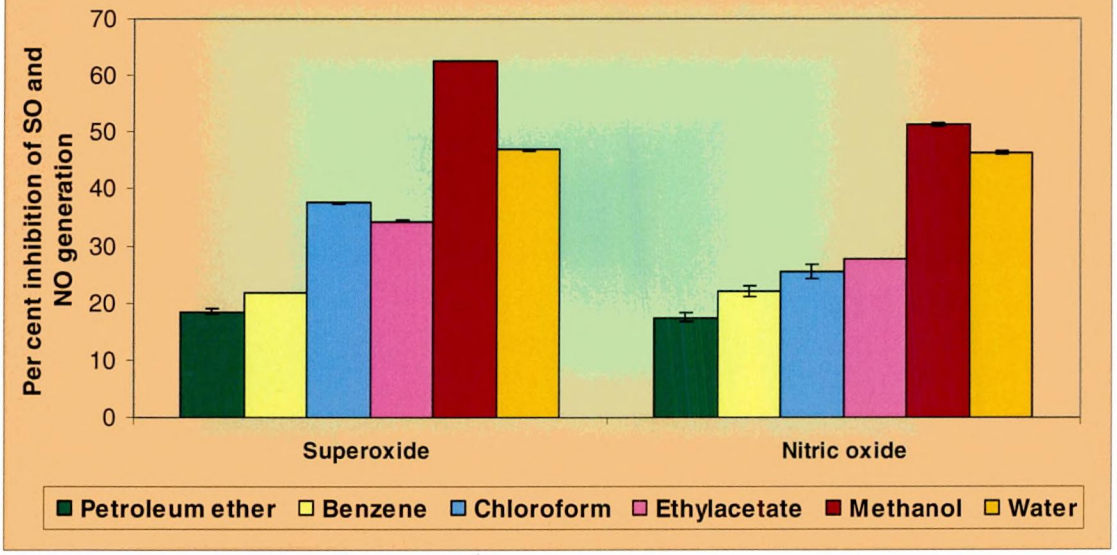


FIGURE 2
SUPEROXIDE AND NITRIC OXIDE SCAVENGING EFFECTS OF *Artemisia vulgaris* LEAF EXTRACTS



Following this, the extracts were individually tested on the *in vitro* generation of $SO^{\bullet}$ and NO. Figure 2 shows the per cent inhibition of $SO^{\bullet}$ and NO generation *in vitro* by the leaf extracts of *Artemisia vulgaris*. The results of the study showed that the maximum extent of inhibition of $SO^{\bullet}$ and NO was mediated by the methanolic extract, followed by the aqueous extract. Ethyl acetate and chloroform extracts showed moderate ability to inhibit both $SO^{\bullet}$ and NO radicals, while this was found to be low in benzene and petroleum ether extracts.

Though the methanolic extract showed the maximum scavenging activity in all the radicals tested thus far (DPPH, ABTS, $SO^{\bullet}$ and NO), there was considerable activity in the other extracts also. Therefore, further studies were carried out with aqueous (polar), methanol (partially polar and non-polar) and chloroform (non-polar) extracts of *Artemisia vulgaris* leaves.

HYDROXYL RADICAL SCAVENGING EFFECTS OF *Artemisia vulgaris* LEAF EXTRACTS

Among various oxygen-derived free radicals, the hydroxyl radical is one of the most highly reactive and harmful oxygen-derived free radicals in living organisms. Hydroxyl radicals cause 2'-deoxyribose damage and generate malondialdehyde (MDA) like product. This compound forms a pink chromogen with thiobarbituric acid (Nobushi and Uchikura, 2010). The extent of TBARS produced is a measure of hydroxyl radical formation.

The effects of *Artemisia vulgaris* leaf extracts on H_2O_2-induced damage to deoxyribose was quantified as the amount of TBARS formed, and the results are represented in Figure 3. The value of H_2O_2 treated group was fixed as 100 per cent and the other groups were calculated relative to this.

H_2O_2 exposure resulted in a steep increase in TBARS formation. The efficiency of the inhibition of TBARS formation was better in methanolic extract treated group. Aqueous and chloroform extracts also strongly inhibited the formation of TBARS.

HYDROGEN PEROXIDE SCAVENGING EFFECTS OF *Artemisia vulgaris* LEAF EXTRACTS

The efficiency of *Artemisia vulgaris* leaf extracts to scavenge H_2O_2 in an *in vitro* system was performed and the results obtained are depicted in Figure 4. The maximum scavenging of H_2O_2 was exhibited by the methanolic extract followed closely by the aqueous extract. The H_2O_2 scavenging ability was lower in the chloroform extract.

PHASE II

The second phase of the study involved the biomolcule protective effects of *Artemisia vulgaris* leaf extracts *in vitro*.

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON BIOMOLECULE PROTECTION

Reactive oxygen species (ROS) are highly reactive oxidant molecules that are reacting with cellular components causing oxidative damage to such critical biomolecules as lipids and DNA. These biomolecules were exposed to oxidant (H_2O_2) *in vitro*, and the effect of the leaf extracts against the oxidant-induced damage was studied. The results obtained are explained below.

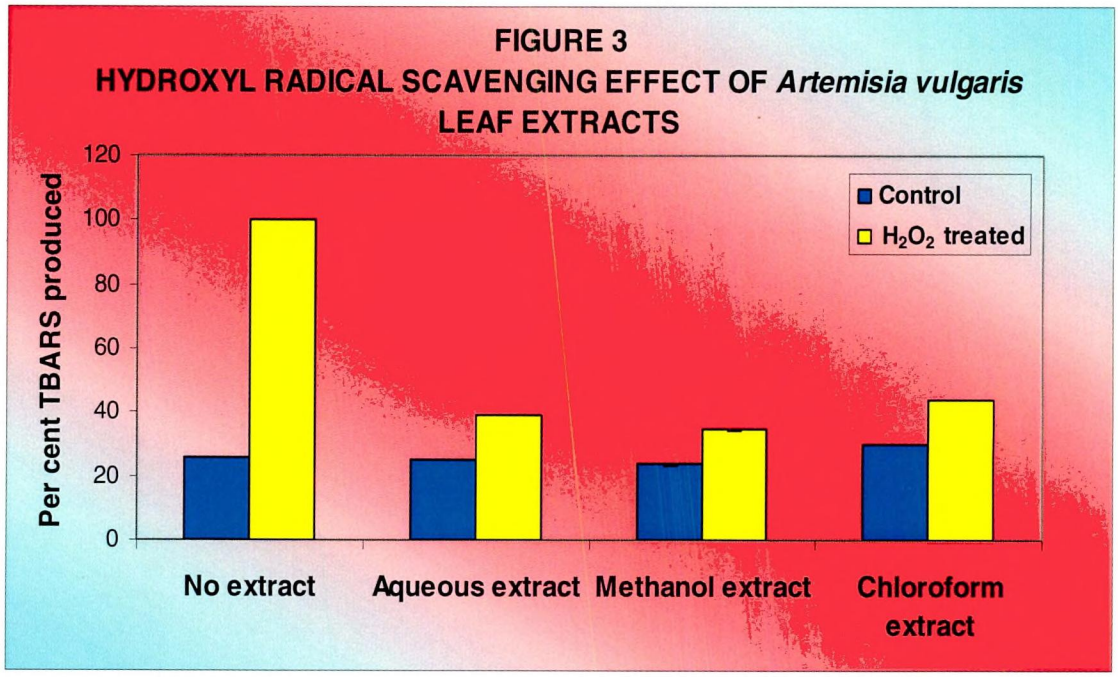


FIGURE 3
HYDROXYL RADICAL SCAVENGING EFFECT OF Artemisia vulgaris LEAF EXTRACTS
Per cent TBARS produced
120
100
80
60
40
20
0
Control
H2O2 treated
No extract
Aqueous extract
Methanol extract
Chloroform extract

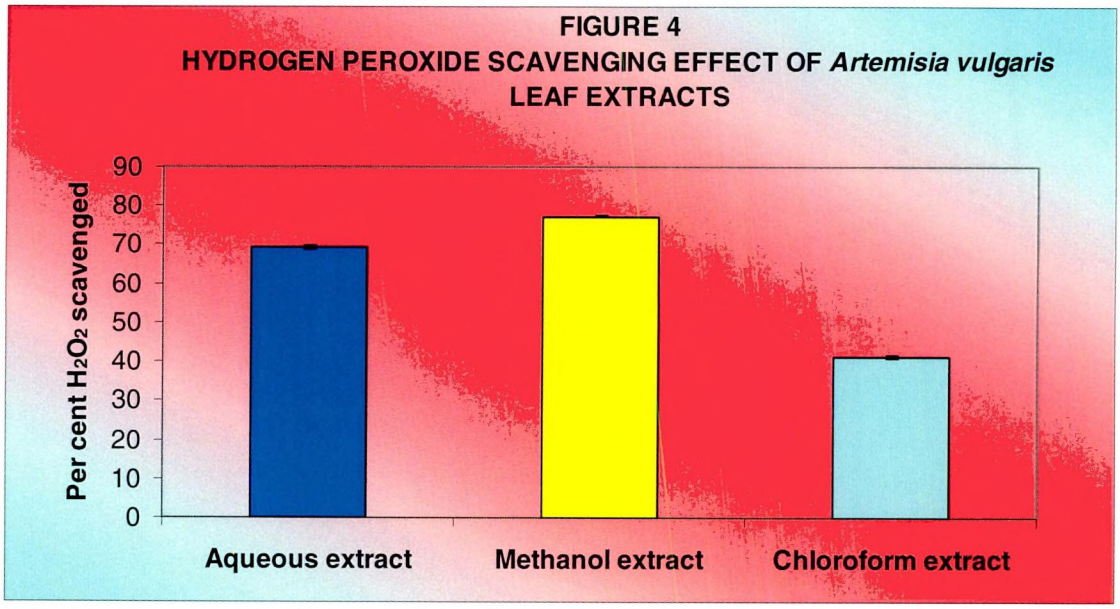


FIGURE 4
HYDROGEN PEROXIDE SCAVENGING EFFECT OF Artemisia vulgaris LEAF EXTRACTS
Per cent H2O2 scavenged
90
80
70
60
50
40
30
20
10
0
Aqueous extract
Methanol extract
Chloroform extract

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON OXIDANT-INDUCED DAMAGE TO MEMBRANE LIPIDS

The extent of LPO was followed in three different membrane preparations in order to check whether the nature of the membrane and its composition influences the extent of antioxidant action. The systems used were goat RBC ghosts (plasma membrane devoid of intracellular membranes), goat liver homogenate (mixture of plasma membrane and internal membranes) and goat liver slices (intact cells). Inhibition of *in vitro* LPO in different membrane preparations by *Artemisia vulgaris* leaf extracts are represented in Figure 5.

All the three extracts caused a substantial decline in the extent of LPO in all the three membrane preparations. The extent was more pronounced in the liver homogenate. Among the three extracts used, the methanolic extract evinced a better protection in all three lipid preparations compared to the aqueous and chloroform extracts.

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON OXIDANT-INDUCED DAMAGE TO DNA

In the present study, the DNA-protective effects of *Artemisia vulgaris* leaves were studied using DNA of different hierarchies, namely pUC18 DNA (circular, bacterial DNA), λ DNA (linear, phage DNA) and herring sperm DNA (genomic, haploid DNA of higher molecular weight). The DNA of different sources were exposed to H_2O_2 in the presence and the absence of the leaf extracts. The results obtained are given below.

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON OXIDANT-INDUCED DAMAGE TO pUC18 AND λ DNA

The migration pattern of pUC18 and λ DNA exposed to H_2O_2 *in vitro* in the presence and the absence of *Artemisia vulgaris* leaf extracts is shown in Plate 3.

It is evident from the picture that, H_2O_2 caused extensive damage to both pUC18 and λ DNA (lane 2). All the three extracts of *Artemisia vulgaris* leaves significantly reduced the damage to both pUC18 and λ DNA. While the damage caused to λ DNA by H_2O_2 was markedly reverted by all the three extracts, there was a residual damage to pUC18 DNA in the presence of aqueous and chloroform extracts. Methanolic extract, however, exhibited complete protection to pUC18 DNA as well.

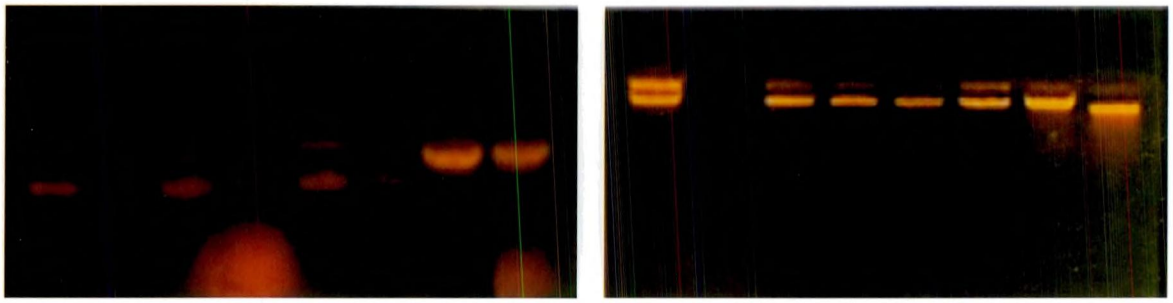
EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON OXIDANT-INDUCED DAMAGE TO HERRING SPERM DNA

The spectrophotometric results of H_2O_2-induced damage to herring sperm DNA is schematically presented in Figure 6. The value of H_2O_2-treated group was fixed as 100 per cent and the relative values in percentage were calculated for the other groups.

Exposure of H_2O_2 caused higher levels of TBARS in herring sperm DNA. The formation of TBARS was effectively brought down by the three different extracts of *Artemisia vulgaris* leaves. The H_2O_2-induced damage to herring sperm DNA was effectively counteracted by the administration of the methanolic extract, followed closely by aqueous and chloroform extracts.

PLATE 3

MIGRATION PATTERN OF pUC 18 AND λ DNA TREATED WITH H_2O_2 WITH AND WITHOUT *Artemisia vulgaris* LEAF EXTRACTS



pUC18 DNA λ DNA

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

FIGURE 5
INHIBITION OF LIPID PEROXIDATION IN DIFFERENT MEMBRANE PREPARATIONS BY *Artemisia vulgaris* LEAF EXTRACTS

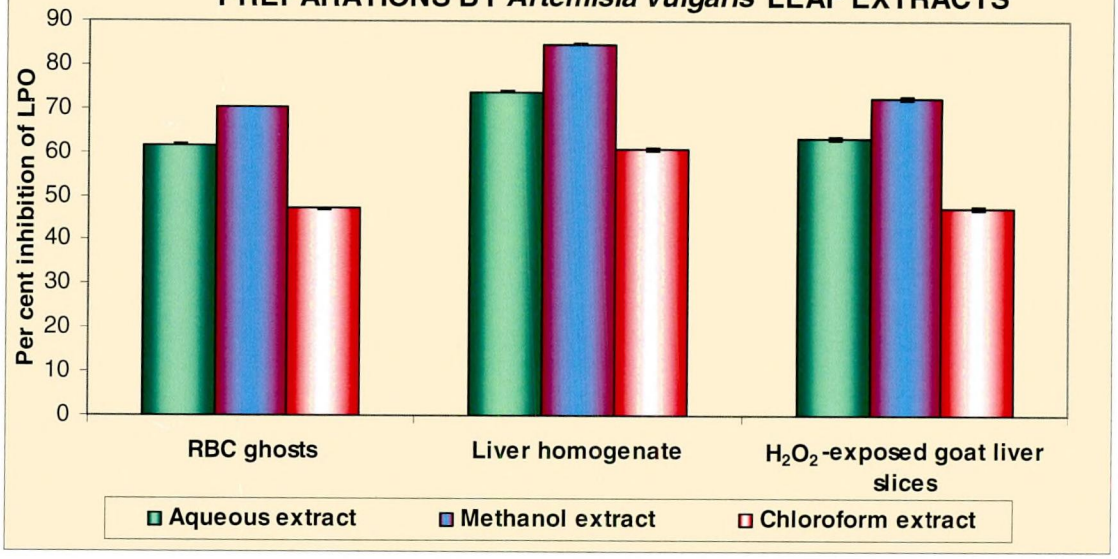
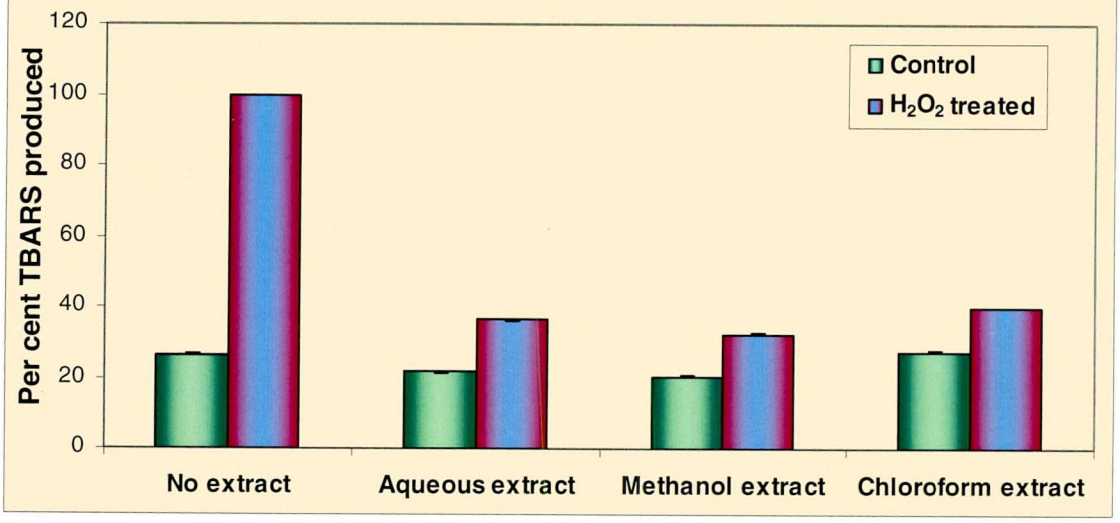


FIGURE 6
INHIBITION OF OXIDANT-INDUCED DAMAGE TO HERRING SPERM DNA BY *Artemisia vulgaris* LEAF EXTRACTS



EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON THE ANTIOXIDANT STATUS OF PRECISION-CUT LIVER SLICES EXPOSED TO H_2O_2

Precision-cut liver slices constitute an *in vitro* model representing the liver under *in vivo* conditions. Precision-cut liver slices are a frequently used *in vitro* model for toxicological studies (Staal *et al.*, 2008). Our research group gives priority to the use of *in vitro* model systems to replace animal usage. In tune with this, goat liver slices were used in the present study. The antioxidant potential of *Artemisia vulgaris* leaf extracts in goat liver slices, which were pre-exposed to H_2O_2 was analysed and the results obtained are explained below.

ENZYMIC ANTIOXIDANTS

The enzymic antioxidants analysed in the precision-cut liver slices were SOD, CAT, POD, GR and GST.

SUPEROXIDE DISMUTASE

The activities of SOD in the goat liver slices exposed to H_2O_2 and/or leaf extracts are shown in Table 3.

H_2O_2 exposure caused a significant ($P<0.05$) decrease in SOD activity compared to the control group. The co-treatment with the leaf extracts caused significant ($P<0.05$) elevation in SOD activity. The maximum activity was observed with the methanolic extract treatment. The activity in the chloroform extract treated group also significantly increased ($P<0.05$) compared to the H_2O_2-treated group. However, these values were not significantly ($P<0.05$) improved when compared to the untreated control.

TABLE 3

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON SUPEROXIDE DISMUTASE ACTIVITY IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	SOD ACTIVITY (Units#/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	35.21 ± 0.04	30.17 ± 0.06 [a]
Aqueous extract	37.79 ± 0.13 [a]	36.42 ± 0.08 [a,b,c]
Methanol extract	38.78 ± 0.12 [a]	37.58 ± 0.07 [a,b,c]
Chloroform extract	35.46 ± 0.17 [a]	32.61 ± 0.10 [a,b,c]

The values are mean ± S.D of triplicates.
#1 Unit = Amount of enzyme that caused 50% reduction in NBT oxidation
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

CATALASE

The catalase activities in H_2O_2-exposed liver slices in the presence and the absence of the leaf extracts are represented in Table 4.

TABLE 4

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON CATALASE ACTIVITY IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	CAT ACTIVITY (Units$/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	208.33 ± 7.22	136.14 ± 5.46 [a]
Aqueous extract	329.70 ± 17.85 [a]	221.94 ± 8.18 [b,c]
Methanol extract	365.18 ± 21.81 [a]	300.50 ± 14.88 [a,b,c]
Chloroform extract	276.07 ± 12.58 [a]	179.28 ± 9.45 [a,b,c]

The values are mean ± S.D of triplicates.
$1 Unit = Amount of enzyme required to decrease the absorbance at 240nm by 0.05 units.
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

A significant ($P<0.05$) decrease in catalase activity was found in H_2O_2-exposed liver slices when compared to the control group. Treatment with *Artemisia vulgaris* leaf extracts caused a significant increase ($P<0.05$) in catalase activity compared to untreated control. Co-administration of the methanolic extract and the aqueous extract with H_2O_2 caused a significant ($P<0.05$) increase in the catalase activity. The chloroform extract co-administered group showed significantly ($P<0.05$) decreased catalase activity compared to untreated control but the activity was significantly ($P<0.05$) higher than the H_2O_2-treated group. The methanolic extract elicited the maximum catalase activity compared to the other two extracts.

PEROXIDASE

The effect of *Artemisia vulgaris* leaf extracts on peroxidase activities in goat liver slices exposed to H_2O_2 *in vitro* are presented in Table 5.

TABLE 5

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON PEROXIDASE ACTIVITY IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	POD (Units[$]/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	20.07 ± 0.17	16.81 ± 0.09 [a]
Aqueous extract	25.15 ± 0.02 [a]	23.19 ± 0.03 [a,b,c]
Methanol extract	29.23 ± 0.01 [a]	26.06 ± 0.02 [a,b,c]
Chloroform extract	22.20 ± 0.02 [a]	19.01 ± 0.01 [a,b,c]

The values are mean ± S.D of triplicates.

$1 Unit = Change in absorbance at 430nm per minute.

a- Statistically significant ($P<0.05$) compared to untreated control

b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

The activity of peroxidase decreased significantly ($P<0.05$) upon exposure to H_2O_2. Treatment with the leaf extracts of *Artemisia vulgaris* caused a significant ($P<0.05$) increase in the peroxidase activity compared to the control group. The decrease in peroxidase activity by H_2O_2 was significantly ($P<0.05$) counteracted by the administration of aqueous and methanol extracts of *Artemisia vulgaris* leaves. The activity of chloroform extract revealed a trend much similar to that exhibited for catalase. The liver slices exposed to the methanolic extract of *Artemisia vulgaris* leaves showed the maximum activity.

GLUTATHIONE REDUCTASE

The glutathione reductase activities observed in the various treatment groups in the precision-cut liver slices are given in Table 6.

TABLE 6

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON GLUTATHIONE REDUCTASE ACTIVITY IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	GR ACTIVITY (Units$^+$/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	2.34 ± 0.02	1.963 ± 0.02 [a]
Aqueous extract	2.81 ± 0.02 [a]	2.660 ± 0.04 [a,b,c]
Methanol extract	2.98 ± 0.01 [a]	2.733 ± 0.02 [a,b,c]
Chloroform extract	2.73 ± 0.03 [a]	2.412 ± 0.01 [a,b,c]

The values are mean ± S.D of triplicates.
+1 Unit = mmoles NADPH oxidized per minute.
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

The glutathione reductase activities increased significantly ($P<0.05$) in the case of all the three extracts in comparison to the control group. Decreased GR activity ($P<0.05$) was found in the slices exposed to H_2O_2. This effect was reverted by the administration of all the three extracts of *Artemisia vulgaris* leaves, where the methanolic extract was found to be better in minimizing the H_2O_2-induced oxidative stress.

GLUTATHIONE S-TRANSFERASE

GST activities in the liver slices treated with H_2O_2 in the presence and the absence of the leaf extracts of *Artemisia vulgaris* are expressed in Table 7.

TABLE 7

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON GLUTATHIONE S-TRANSFERASE ACTIVITY IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	GST ACTIVITY (Units[@]/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	1.53 ± 0.02	0.997 ± 0.02 [a]
Aqueous extract	2.32 ± 0.02 [a]	1.752 ± 0.01 [a,b,c]
Methanol extract	2.64 ± 0.01 [a]	2.202 ± 0.02 [a,b,c]
Chloroform extract	1.95 ± 0.03 [a]	1.195 ± 0.04 [a,b,c]

The values are mean ± S.D of triplicates.
@1 Unit = nmoles of CBNB conjugated per minute.
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

H_2O_2 exposure caused a significant ($P<0.05$) decrease in GST activity. The depletion of GST with the exposure of H_2O_2 was counteracted by the co-administration with the leaf extracts. The methanolic extract showed significantly higher effect than the aqueous and chloroform extracts.

NON-ENZYMIC ANTIOXIDANTS

Reactive oxygen species are produced as the result of normal metabolic activities, which may lead to deleterious effects in humans. To protect the body from oxidative stress, organisms have an extensive antioxidant defense system consisting of enzymatic and non-enzymatic factors (van Stijin *et al.*, 2008).

The non-enzymic antioxidants, namely vitamins C, E, A and reduced glutathione, were estimated in the oxidant challenged liver slices with or without the leaf extracts of *Artemisia vulgaris*. The estimated levels of non-enzymic antioxidants are given below.

VITAMIN C

The levels of vitamin C in the oxidatively challenged goat liver slices in the presence and/or the absence of the leaf extracts of *Artemisia vulgaris* is presented in Table 8.

TABLE 8

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON VITAMIN C LEVELS IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	Vitamin C (mg/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	3.39 ± 0.01	2.83 ± 0.03 [a]
Aqueous extract	3.65 ± 0.04 [a]	3.54 ± 0.02 [a,b,c]
Methanol extract	3.71 ± 0.01 [a]	3.64 ± 0.05 [a,b,c]
Chloroform extract	3.63 ± 0.01 [a]	3.42 ± 0.02 [b,c]

The values are mean ± S.D of triplicates.
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

Significantly ($P<0.05$) decreased vitamin C level was found in the H_2O_2 treated group. However, the treatment of the goat liver slices with the leaf extracts of *Artemisia vulgaris* reverted the reduction. The methanolic and the aqueous extracts caused a significant ($P<0.05$) increase in the levels of vitamin C. Among the three extracts used, the methanolic extract exhibited the maximum protection, followed by the aqueous and chloroform extracts.

VITAMIN E

Vitamin E (α-tocopherol) has antioxidant benefits and is the most potent chain-breaking, fat soluable antioxidant (Mustacich *et al.*, 2010). The levels of tocopherol in the precision-cut liver slices of the different treatment groups are presented in Table 9.

TABLE 9

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON VITAMIN E LEVELS IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	Vitamin E (µg/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	11.38 ± 0.54	9.43 ± 0.29 [a]
Aqueous extract	13.66 ± 0.49 [a]	11.72 ± 0.23 [b,c]
Methanol extract	16.26 ± 0.32 [a]	13.65 ± 0.35 [a,b,c]
Chloroform extract	12.34 ± 0.74 [a]	10.78 ± 0.22 [a,b,c]

The values are mean ± S.D of triplicates.
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

It is evident from the tabulated values that a significant ($P<0.05$) decrease occurred in the vitamin E levels in oxidant-treated liver slices compared to the untreated control. Treatment with plant extracts caused a

significant increase compared to the control group. Co-administration of H_2O_2 and the methanolic extract significantly ($P<0.05$) improved the vitamin E levels. Eventhough the mean values of the activities in the H_2O_2 and the aqueous extract treated group were higher than the untreated control, the comparative analysis escaped statistical significance. The H_2O_2 action was significantly

($P<0.05$) reverted by the administration of chloroform extract; however, the vitamin E levels were significantly ($P<0.05$) lower than the untreated control in this group.

VITAMIN A

Table 10 shows the levels of vitamin A in goat liver slices exposed to oxidant in the presence and the absence of leaf extracts.

TABLE 10

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON VITAMIN A LEVELS IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	Vitamin A (µg/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	98.34 ± 0.08	60.65 ± 0.35 [a]
Aqueous extract	114.39 ± 0.14 [a]	101.48 ± 0.13 [a,b,c]
Methanol extract	138.70 ± 0.26 [a]	116.61 ± 0.31 [a,b,c]
Chloroform extract	109.54 ± 0.39 [a]	100.19 ± 0.06 [a,b,c]

The values are mean ± S.D of triplicates.
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

Hydrogen peroxide alone caused a marked decline in the levels of vitamin A, while the trend was effectively reverted by the *Artemisia vulgaris*

leaf extracts. Among all the extracts used, the liver slices treated with methanolic extract showed more increase in vitamin A level than the groups treated with the aqueous and chloroform extracts.

REDUCED GLUTATHIONE

The levels of GSH estimated in the liver slices are listed in Table 11.

TABLE 11

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON REDUCED GLUTATHIONE LEVELS IN GOAT LIVER SLICES EXPOSED TO H_2O_2 *in vitro*

SAMPLE	Glutathione (nmoles/g tissue)	
	Without H_2O_2	With H_2O_2
No extract	235.34 ± 0.60	204.40 ± 0.24 [a]
Aqueous extract	282.33 ± 0.33 [a]	263.64 ± 0.38 [a,b,c]
Methanol extract	291.37 ± 0.54 [a]	276.74 ± 0.21 [a,b,c]
Chloroform extract	250.32 ± 0.32 [a]	233.70 ± 0.20 [a,b,c]

The values are mean ± S.D of triplicates.
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

The oxidant exposure caused a significant ($P<0.05$) reduction in the levels of GSH when compared to control. The depleting effect of H_2O_2 treatment was very well counteracted by the administration of the leaf extracts, where the methanolic extract was found to be better than the other two extracts. The activity of chloroform extract revealed a trend much similar to that exhibited by vitamin E.

PHASE III

The outcome of the first two phases of the study clearly demonstrated the antioxidant potential of the *Artemisia vulgaris* leaf extracts. The results showed that the leaves possessed high levels of antioxidants, could scavenge / neutralize oxidants and free radicals and could improve the antioxidant status of tissue exposed to oxidative stress. As the next step, the effect of the leaf extracts was tested on the end-point of severe oxidative damage, namely cell death.

Apoptotic cell death is morphologically defined by chromatin condensation (pyknosis), nuclear fragmentation (karyorhensis), shrinkage of cytoplasm and formation of apoptotic bodies (Kepp *et al.*, 2009). Furthermore, apoptosis is induced *in vitro* by incubating the cells with chemicals such as antitumor agents/oxidants. In the present study, the apoptosis modulating effects of the leaf extracts were studied using oxidants namely H_2O_2 or etoposide. This effect was analysed on both untransformed (non-cancerous) and transformed (cancerous) cells.

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON H_2O_2-INDUCED STRESS IN *Saccharomyces cerevisiae* CELLS AND PRIMARY CHICK EMBRYO FIBROBLASTS

In the present study, yeast and primary chick embryo fibroblasts were the two untransformed cells used to study the effects of plant extracts on the events associated with oxidant-induced apoptosis. The oxidant used was H_2O_2. The characteristic features of apoptosis were studied and the results obtained are explained below.

CELL VIABILITY ASSAYS

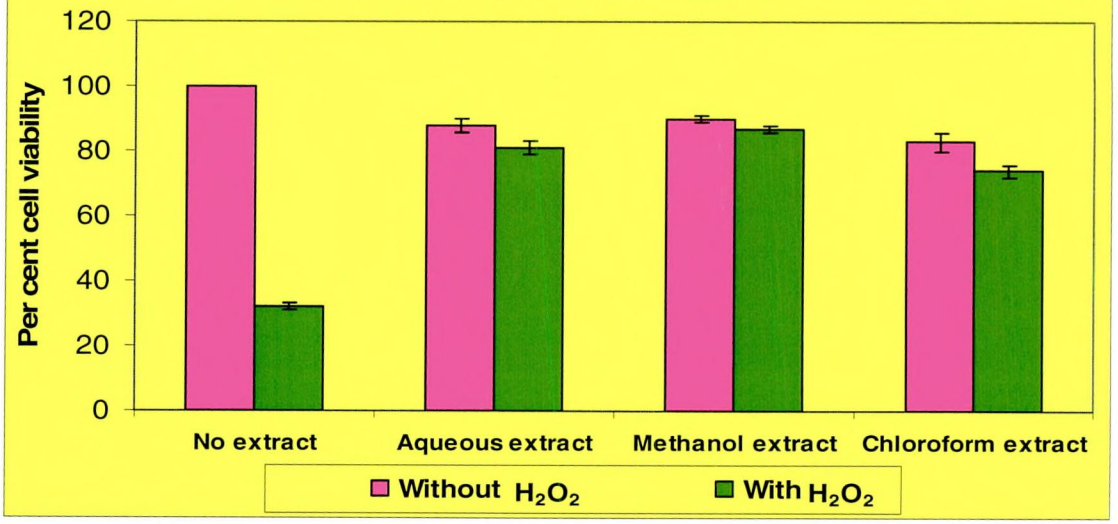
The extent of cell survival by the leaf extracts of *Artemisia vulgaris* was studied by MTT. The per cent cell viability was quantified using MTT in the different treatment groups and the values obtained are schematically represented in Figures 7 and 8.

It is evident from the values presented in Figures 7 and 8 that H_2O_2 exposure significantly decreased the viability of *Saccharomyces cerevisiae* cells and primary chick embryo fibroblasts. The *Artemisia vulgaris* leaf extracts showed improved viability of the cells subjected to oxidative stress, with the maximum protection being rendered by the methanolic extract. The plant extracts, by themselves, also caused cell death to a very slight extent in both *Saccharomyces cerevisiae* cells and chick embryo fibroblasts compared to the untreated control groups.

Sulphorhodamine B assay was used as an additional parameter to assess the viability and proliferative potential of *Saccharomyces cerevisiae* cells and primary chick embryo fibroblasts in the presence and the absence of H_2O_2 and/or the leaf extracts of *Artemisia vulgaris*. Figures 9 and 10 illustrate the results obtained.

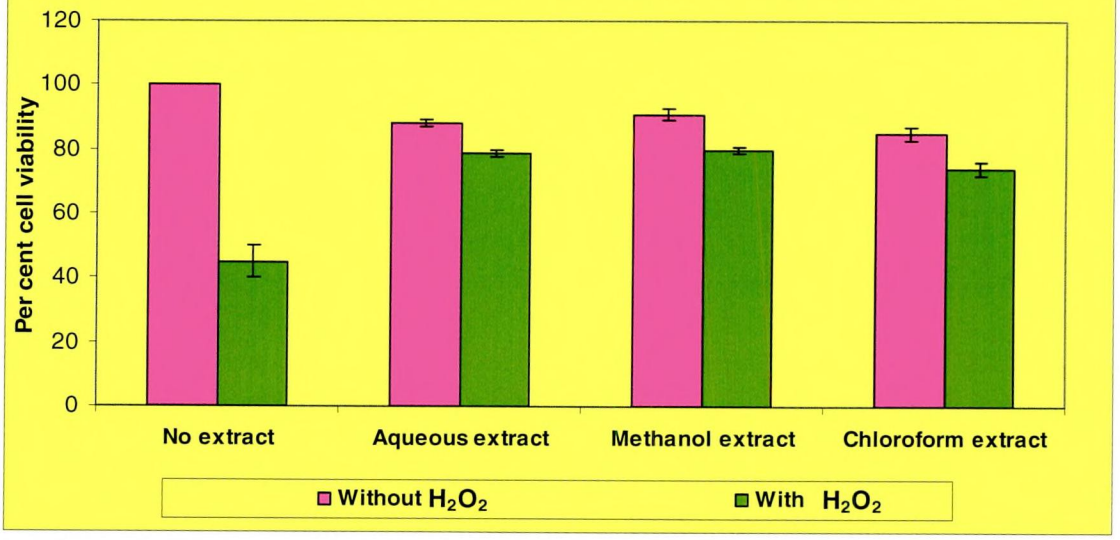
The viability of the cells was found to be decreased in the H_2O_2-treated group of both cell types. The groups treated with *Artemisia vulgaris* leaf extracts showed improved cell viability. The maximum effect was mediated by the methanolic extract, followed by aqueous and chloroform extracts.

FIGURE 7
EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON THE VIABILITY OF *Saccharomyces cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY MTT ASSAY

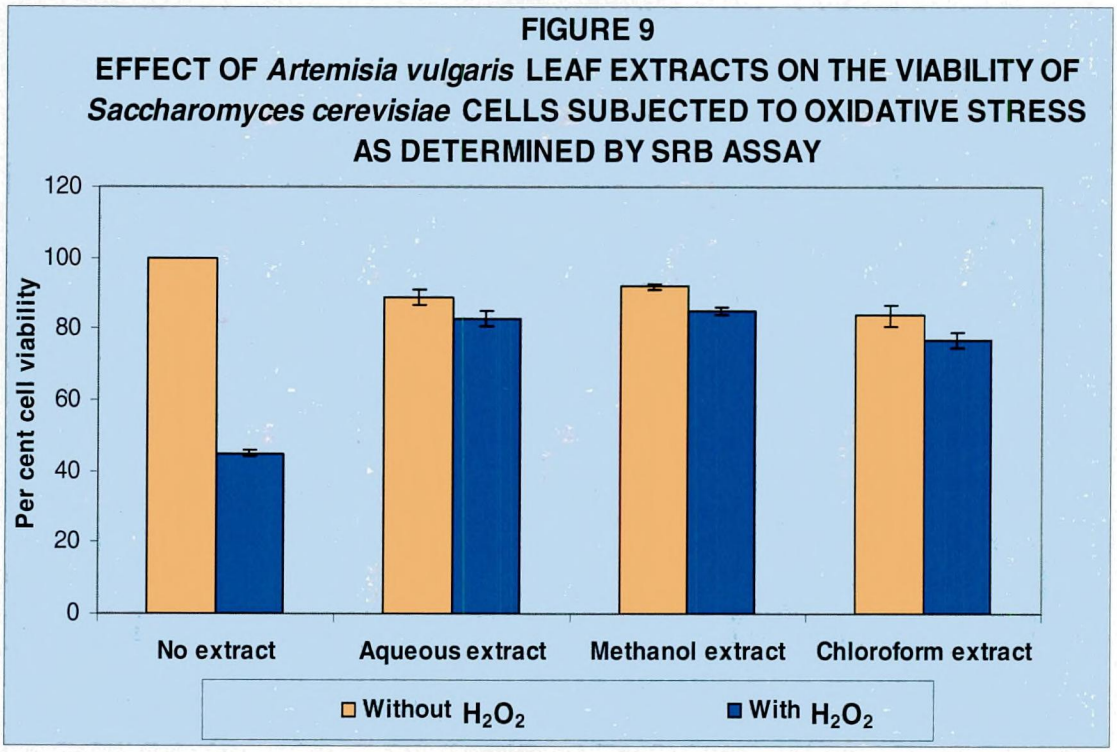


The values of the negative (untreated) control group were fixed as 100% and the per cent viabilities in the other groups were calculated relative to this.

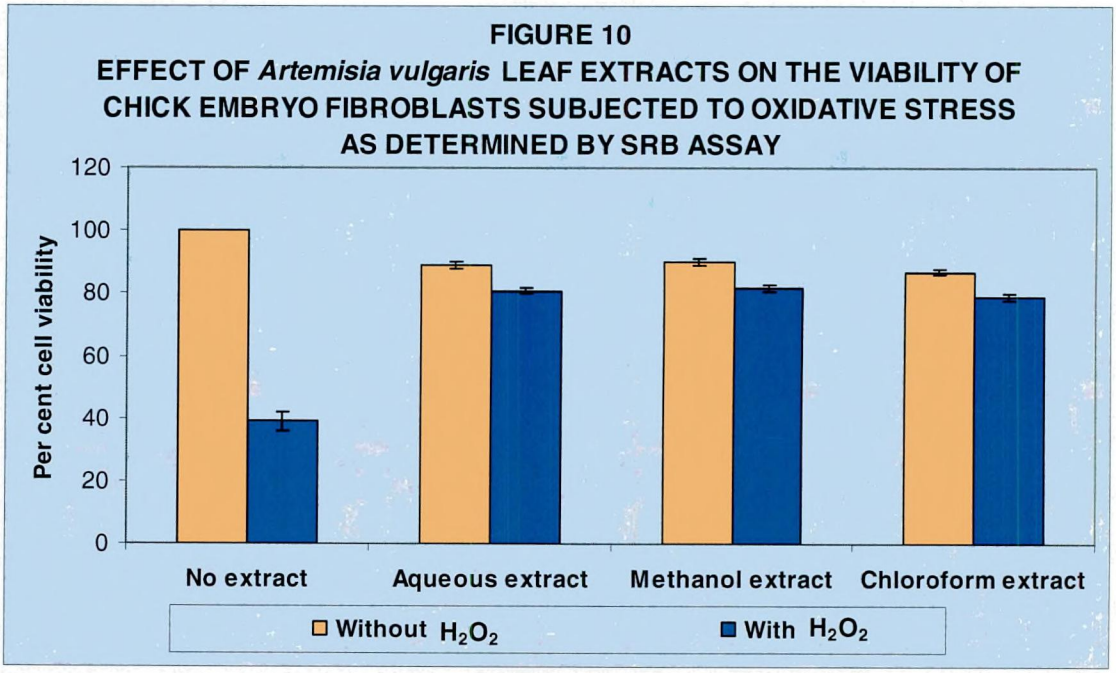
FIGURE 8
EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON THE VIABILITY OF CHICK EMBRYO FIBROBLASTS SUBJECTED TO OXIDATIVE STRESS AS DETERMINED BY MTT ASSAY



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



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



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

MORPHOLOGICAL CHANGES OF APOPTOSIS OBSERVED IN YEAST CELLS AND PRIMARY CHICK EMBRYO FIBROBLASTS

The characteristic morphological changes of apoptosis were observed and quantified in the yeast cells and primary chick embryo fibroblasts in the presence and the absence of leaf extracts and/or H_2O_2. The numbers of apoptotic cells were counted in each experimental group and the results are presented in Tables 12 and 13 respectively.

TABLE 12

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON THE MORPHOLOGICAL CHANGES IN *Saccharomyces cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS (GIEMSA STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No extract	5 ± 4	80 ± 2 [a]	0.52	4.00
Aqueous extract	15 ± 3 [a]	21 ± 2 [a,b,c]	0.18	0.27
Methanol extract	13 ± 2 [a]	20 ± 1 [a,b,c]	0.15	0.25
Chloroform extract	20 ± 2 [a]	25 ± 3 [a,b,c]	0.25	0.33

The values are mean ± S.D of triplicates.

a- Statistically significant ($P<0.05$) compared to untreated control

b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

TABLE 13

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON THE MORPHOLOGICAL CHANGES IN CHICK EMBRYO FIBROBLASTS SUBJECTED TO OXIDATIVE STRESS (GIEMSA STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No extract	8 ± 2	75 ± 3 [a]	0.09	3.00
Aqueous extract	13 ± 2 [a]	21 ± 1 [a,b,c]	0.15	0.27
Methanol extract	10 ± 1 [a]	20 ± 1 [a,b]	0.11	0.25
Chloroform extract	17 ± 3 [a]	22 ± 1 [a,b,c]	0.20	0.28

The values are mean ± S.D of triplicates.
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

Microscopic analysis revealed that, in contrast to untreated (control) cells, an increased number of H_2O_2-treated cells ($P<0.05$) were characterized by apoptotic morphology (Plates 4 and 5). The apoptosis-inducing action of H_2O_2 was significantly reverted by the administration of *Artemisia vulgaris* leaf extracts. Cells treated with the methanolic extract showed better protection against H_2O_2 compared to the aqueous and chloroform extracts. The apoptotic ratios in the treated and untreated cells were also calculated and the values obtained are also represented in Tables 12 and 13.

NUCLEAR CHANGES OF APOPTOSIS OBSERVED IN YEAST CELLS AND PRIMARY CHICK EMBRYO FIBROBLASTS

In the present study, the nuclear changes associated with apoptosis were observed and quantified in the yeast cells and chick embryo fibroblasts subjected to oxidative stress in the presence and the absence of the leaf

extracts. The apoptotic cells were counted by PI staining in each experimental group of the yeast cells and primary chick embryo fibroblasts and the results are presented in Tables 14 and 15 respectively.

After staining with PI stain, the cells were observed under the fluorescent microscope. The apoptotic nucleus stained strongly with fluorescent dyes, allowing non-apoptotic cells to be discriminated from the apoptotic ones (Plates 6 and 7). The apoptotic ratios were calculated for PI stained cells, and the values are presented in Tables 14 and 15.

Oxidative stress in yeast cells and primary chick embryo fibroblasts induced by H_2O_2 caused a significant ($P<0.05$) increase in apoptotic cells compared to the untreated group. All the three extracts significantly reduced the extent of apoptosis, where the methanolic extract was found to be better.

TABLE 14

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON NUCLEAR CHANGES IN *Saccharomyces cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS (PI STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No extract	7 ± 4	80 ± 3 [a]	0.08	4.00
Aqueous extract	16 ± 2 [a]	24 ± 2 [a,b,c]	0.19	0.32
Methanol extract	11 ± 3 [a]	19 ± 2 [a,b,c]	0.12	0.23
Chloroform extract	20 ± 2 [a]	28 ± 3 [a,b,c]	0.25	0.39

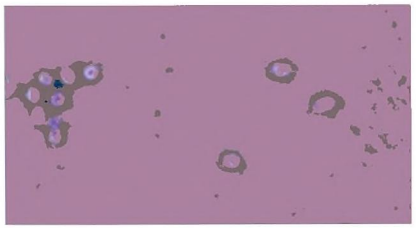
The values are mean ± S.D of triplicates.

a- Statistically significant ($P<0.05$) compared to untreated control

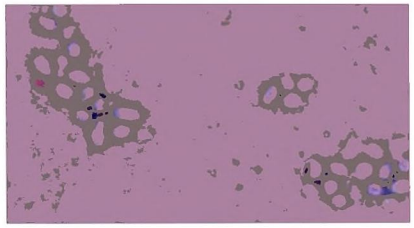
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

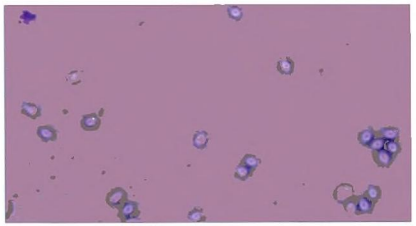
PLATE 4
Saccharomyces cerevisiae CELLS STAINED WITH GIEMSA (OXIDANT-H_2O_2)



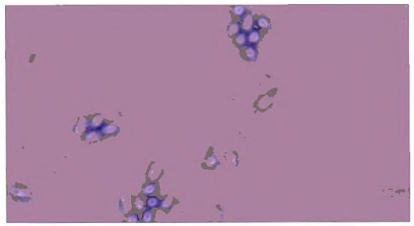
Control



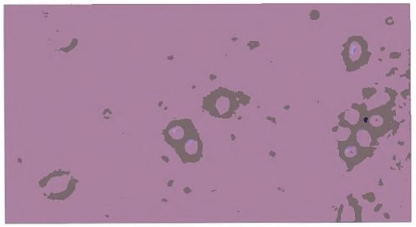
H_2O_2



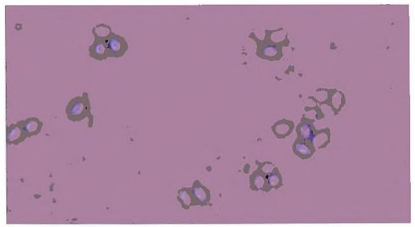
Aqueous extract



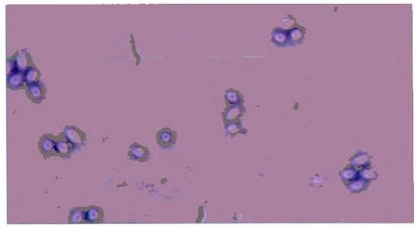
Aqueous extract + H_2O_2



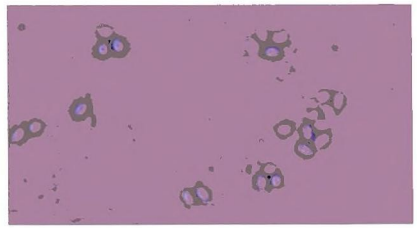
Methanol extract



Methanol extract + H_2O_2



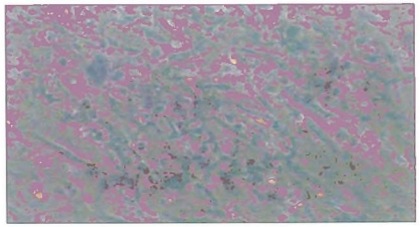
Chloroform extract



Chloroform extract + H_2O_2

PLATE 5
PRIMARY CHICK EMBRYO FIBROBLASTS STAINED WITH GIEMSA (OXIDANT-H_2O_2)

Control



H_2O_2

Aqueous extract

Aqueous extract + H_2O_2

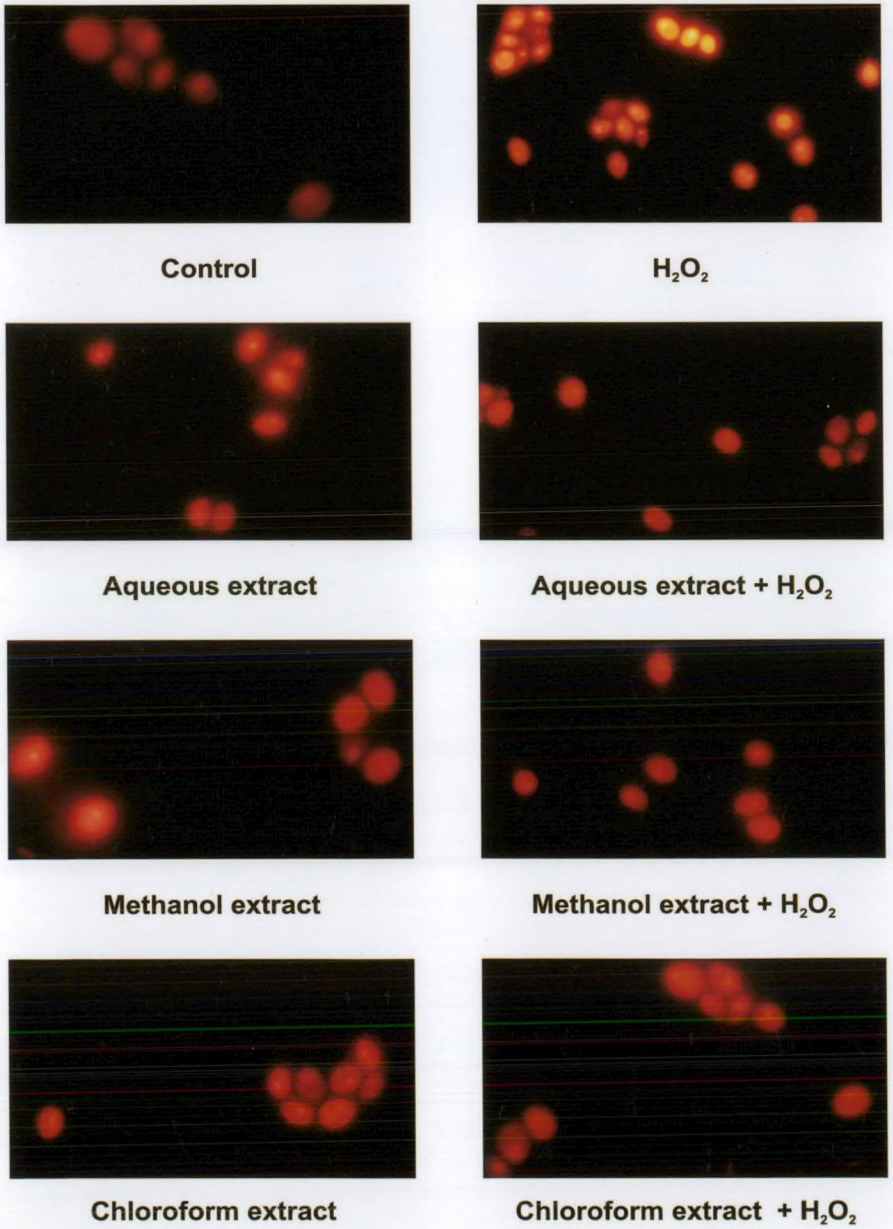
Methanol extract

Methanol extract + H_2O_2

Chloroform extract

Chloroform extract + H_2O_2

PLATE 6
Saccharomyces cerevisiae CELLS STAINED WITH PI (OXIDANT-H_2O_2)



Control

H_2O_2

Aqueous extract

Aqueous extract + H_2O_2

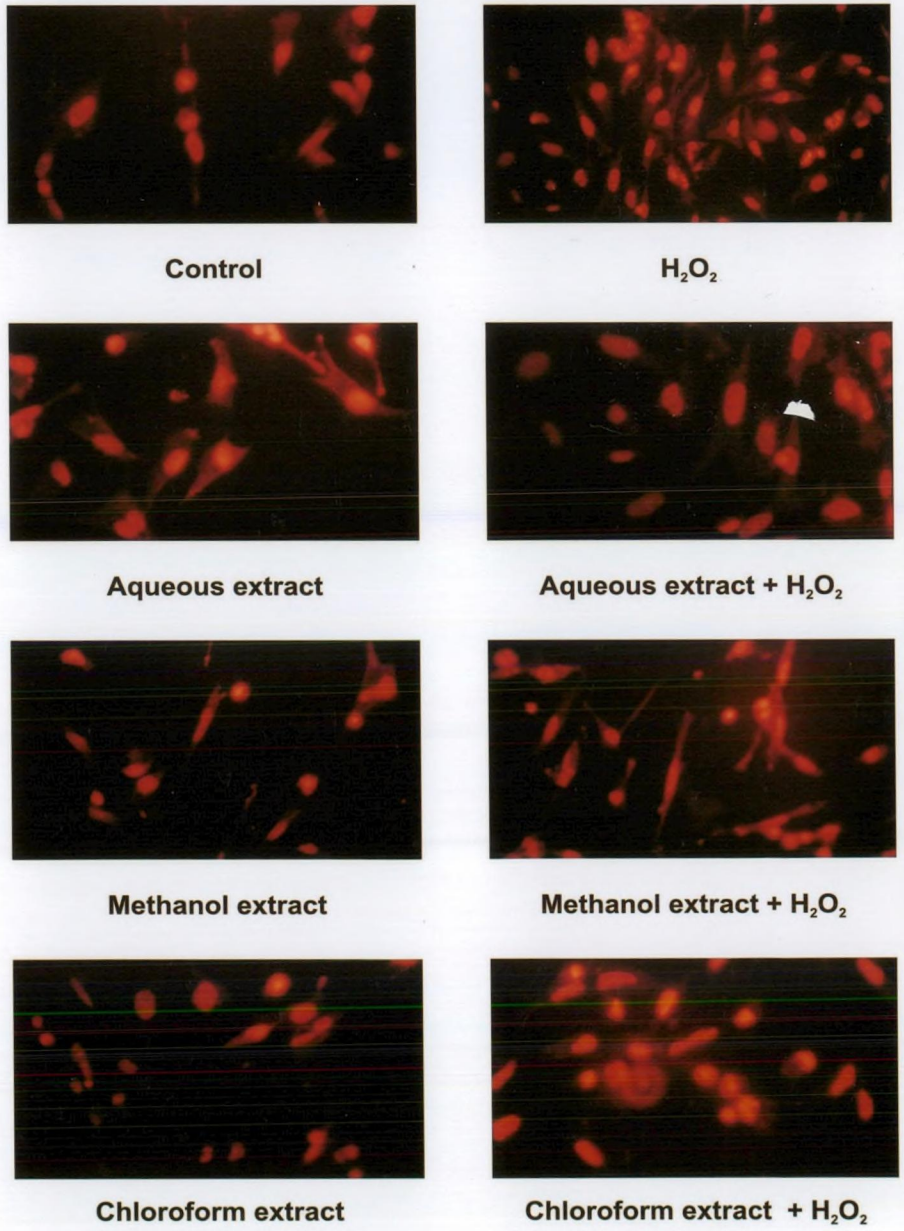
Methanol extract

Methanol extract + H_2O_2

Chloroform extract

Chloroform extract + H_2O_2

PLATE 7
PRIMARY CHICK EMBRYO FIBROBLASTS 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

TABLE 15

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON NUCLEAR CHANGES IN CHICK EMBRYO FIBROBLASTS SUBJECTED TO OXIDATIVE STRESS (PI STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No extract	9 ± 1	76 ± 3 [a]	0.10	3.17
Aqueous extract	14 ± 2 [a]	20 ± 1 [a,b,c]	0.16	0.25
Methanol extract	11 ± 1 [a]	18 ± 1 [a,b,c]	0.12	0.22
Chloroform extract	19 ± 2 [a]	22 ± 1 [a,b,c]	0.23	0.28

The values are mean ± S.D of triplicates.
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated control

The induction of apoptosis in the yeast cells and primary chick embryo fibroblasts was also analysed by EtBr staining in the presence and the absence of *Artemisia vulgaris* leaf extracts and/or H_2O_2 and the values obtained are represented in Tables 16 and 17 respectively.

As is evident from the tabulated values, oxidative stress imposed by H_2O_2 caused a steep increase ($P<0.05$) in the number of cells (both *Saccharomyces cerevisiae* cells and chick embryo fibroblasts) to commit to apoptotic death. However, this cytotoxic effect was brought down significantly ($P<0.05$) by the co-administration of all the three extracts of *Artemisia vulgaris* leaves. The protective effect rendered by the methanolic extract was superior to that of the other two extracts.

TABLE 16

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON NUCLEAR CHANGES IN *Saccharomyces cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS (EtBr STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No extract	4 ± 3	77 ± 2 [a]	0.04	3.35
Aqueous extract	14 ± 3 [a]	19 ± 1 [a,b,c]	0.16	0.23
Methanol extract	9 ± 1 [a]	17 ± 1 [a,b,c]	0.09	0.20
Chloroform extract	18 ± 2 [a]	24 ± 2 [a,b,c]	0.22	0.32

The values are mean ± S.D of triplicates.

a- Statistically significant ($P<0.05$) compared to untreated control

b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

Plates 8 and 9 depict the apoptotic nuclei that are clearly visualized after EtBr staining of yeast cells and primary chick embryo fibroblasts. From the cell numbers counted in each group, the ratios of apoptosing to normal cells were calculated and the values obtained are listed in Tables 16 and 17.

From the ratios, it is very evident that the hydrogen peroxide-imposed oxidative stress results in increased cell death. This effect is efficiently counteracted by the presence of the aqueous, methanol and chloroform extracts of *Artemisia vulgaris* leaves. Of these three extracts, the methanolic extract of the leaves exerted a very good effect that was significantly better than the other two. The effect of the chloroform extract exhibited a protective effect that was much lower in magnitude than the aqueous and methanol extract, but by itself, it caused significant protection to both the types of untransformed cells.

PLATE 8
Saccharomyces cerevisiae CELLS STAINED WITH EtBr (OXIDANT-H_2O_2)

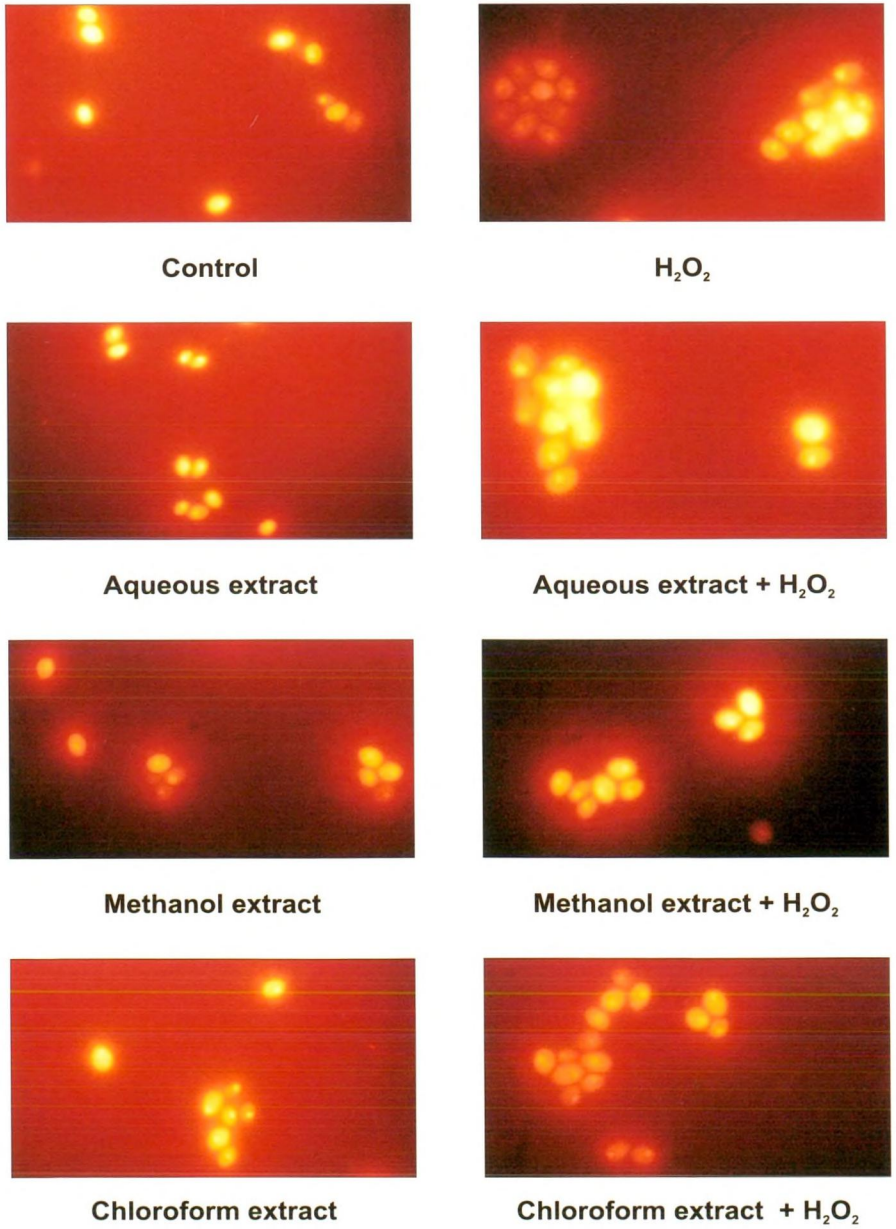


PLATE 9
PRIMARY CHICK EMBRYO FIBROBLASTS STAINED WITH EtBr (OXIDANT-H_2O_2)

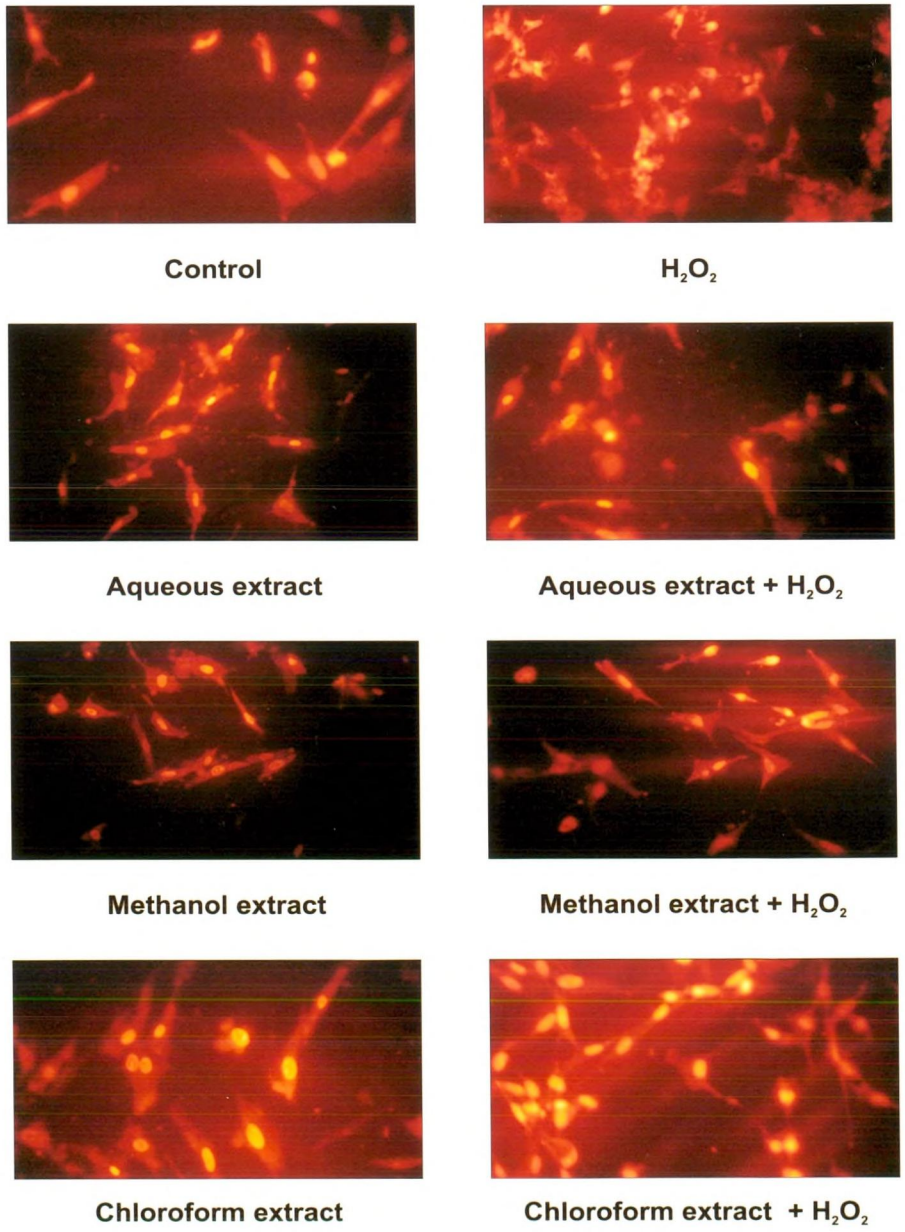


TABLE 17

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON NUCLEAR CHANGES IN CHICK EMBRYO FIBROBLASTS SUBJECTED TO OXIDATIVE STRESS (EtBr STAINING)

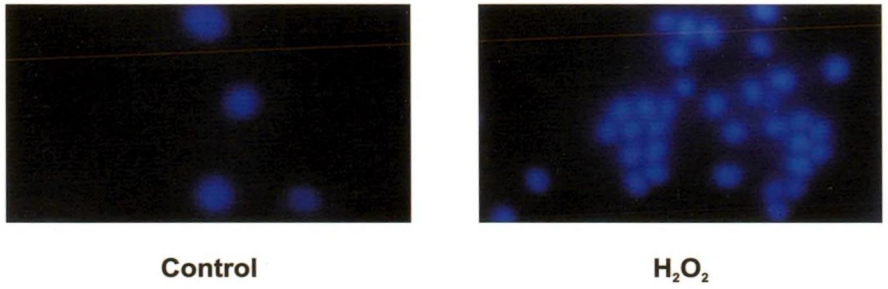
SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No extract	10 ± 2	74 ± 3 [a]	0.11	2.85
Aqueous extract	15 ± 1 [a]	24 ± 1 [a,b,c]	0.18	0.32
Methanol extract	13 ± 1 [a]	20 ± 2 [a,b,c]	0.15	0.25
Chloroform extract	18 ± 1 [a]	25 ± 1 [a,b,c]	0.22	0.33

The values are mean ± S.D of triplicates.
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

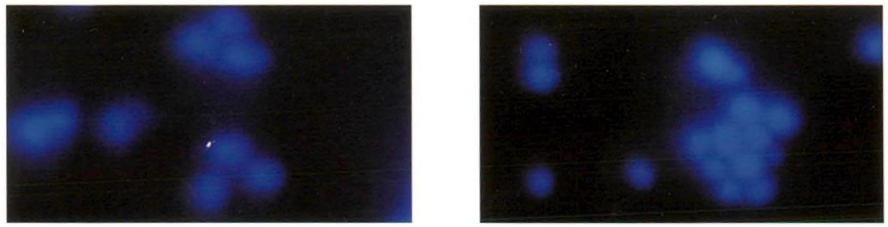
The nuclear changes of apoptosis induced by H_2O_2 in all treatment groups were examined under inverted fluorescent microscope after staining with DAPI, which specifically stains the nucleus. The apoptotic cells per 100 cells were counted for yeast cells and primary chick embryo fibroblasts and the values are tabulated in Tables 18 and 19 respectively.

The results revealed that H_2O_2-exposure caused a significant ($P<0.05$) number of yeast cells and primary chick embryo fibroblasts to commit to apoptosis, compared to control cells. The leaf extracts of *Artemisia vulgaris* brought down the apoptosis-inducing effect of H_2O_2 significantly. Among the three extracts used, the apoptosis inhibiting effect was found to be more in the methanolic extract, followed by the aqueous and chloroform extracts. The photographic record of DAPI stained apoptotic cells in each treatment group is presented in Plates 10 and 11. The apoptotic ratios are also presented in Tables 18 and 19.

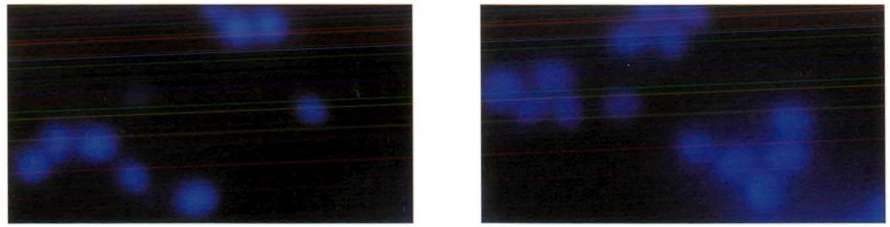
PLATE 10
Saccharomyces cerevisiae CELLS STAINED WITH DAPI (OXIDANT-H_2O_2)



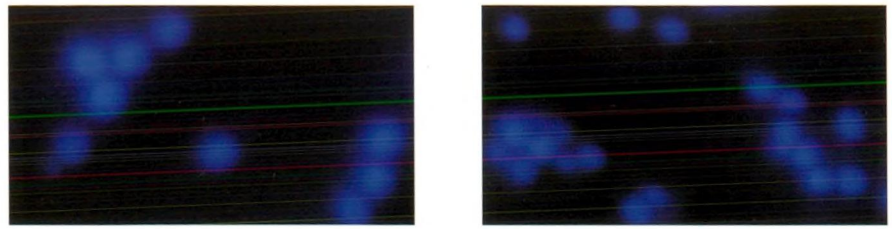
Control | H_2O_2



Aqueous extract | Aqueous extract + H_2O_2

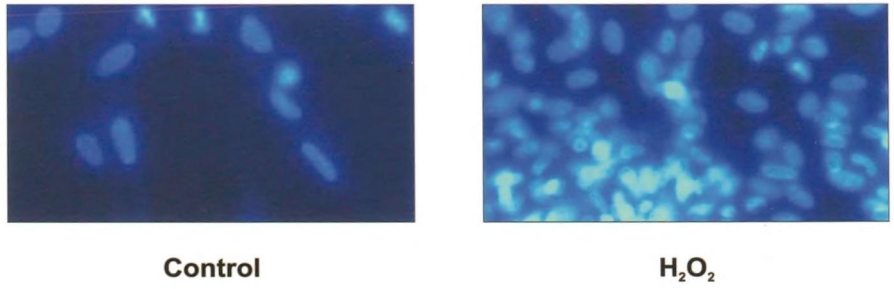


Methanol extract | Methanol extract + H_2O_2

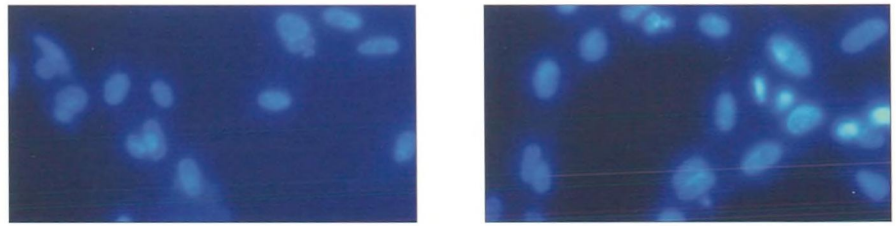


Chloroform extract | Chloroform extract + H_2O_2

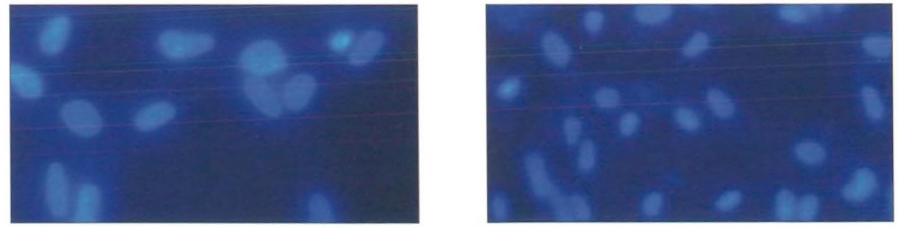
PLATE 11
PRIMARY CHICK EMBRYO FIBROBLASTS STAINED WITH DAPI (OXIDANT-H_2O_2)



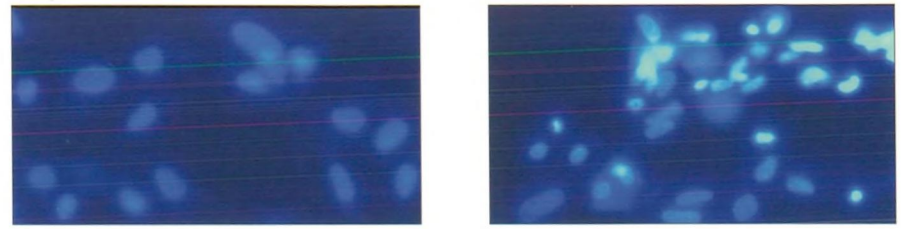
Control | H_2O_2



Aqueous extract | Aqueous extract + H_2O_2



Methanol extract | Methanol extract + H_2O_2



Chloroform extract | Chloroform extract + H_2O_2

TABLE 18

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON NUCLEAR CHANGES IN *Saccharomyces cerevisiae* CELLS SUBJECTED TO OXIDATIVE STRESS (DAPI STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No extract	6 ± 2	79 ± 3 [a]	0.06	3.76
Aqueous extract	12 ± 3 [a]	22 ± 2 [a,b,c]	0.14	0.28
Methanol extract	8 ± 2 [a]	15 ± 3 [a,b,c]	0.09	0.18
Chloroform extract	16 ± 4 [a]	24 ± 1 [a,b,c]	0.19	0.32

The values are mean ± S.D of triplicates.

a- Statistically significant ($P<0.05$) compared to untreated control

b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

TABLE 19

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON NUCLEAR CHANGES IN CHICK EMBRYO FIBROBLASTS SUBJECTED TO OXIDATIVE STRESS (DAPI STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No extract	10 ± 1	77 ± 2 [a]	0.11	3.35
Aqueous extract	15 ± 2 [a]	23 ± 1 [a,b,c]	0.18	0.30
Methanol extract	12 ± 1 [a]	21 ± 1 [a,b,c]	0.14	0.27
Chloroform extract	20 ± 2 [a]	28 ± 3 [a,b,c]	0.25	0.39

The values are mean ± S.D of triplicates.

a- Statistically significant ($P<0.05$) compared to untreated control

b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON ETOPOSIDE-INDUCED APOPTOSIS IN UNTRANSFORMED AND TRANSFORMED CELLS

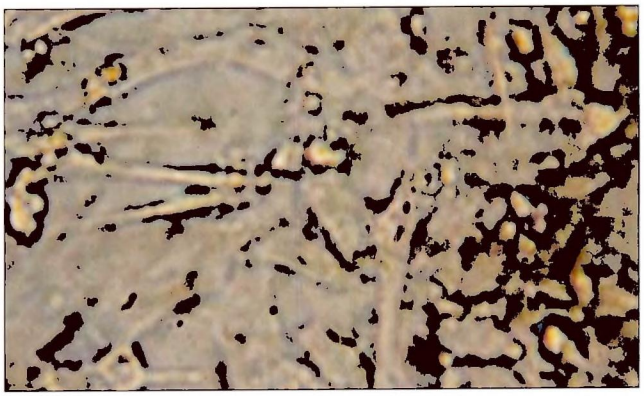
The apoptotic studies carried out in *Saccharomyces cerevisiae* cells and primary chick embryo fibroblasts gave a clear idea that the *Artemisia vulgaris* leaf extracts render protection to these untransformed cells against H_2O_2-induced oxidative stress. As the next step towards understanding the effect of leaf extracts in oxidant-induced transformed cells, Hep2 (human laryngeal carcinoma) cells were employed. In the previous experiments, H_2O_2, a standard oxidant, was used to induce oxidative stress, whereas in the present phase, etoposide, a standard chemotherapeutic agent, which is known to cause apoptotic death via an oxidative mechanism was used in Hep2 cells.

Since etoposide is a standard chemotherapeutic drug used in cancer chemotherapy, it was felt imperative to study its effect on the untransformed cells also. For this purpose, chick embryo fibroblasts were used. H_2O_2, which was used in the previous set of experiments, was replaced by etoposide.

CELL VIABILITY ASSAYS

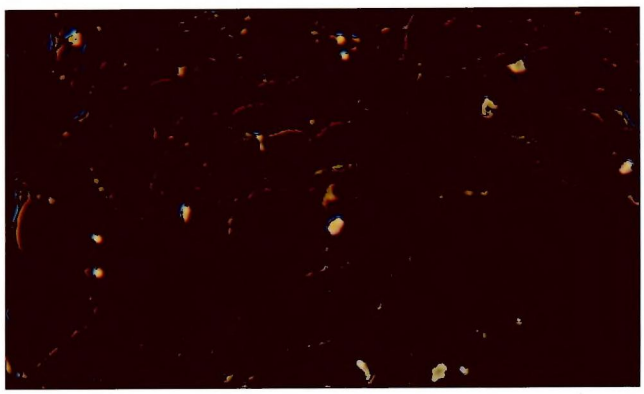
The cytotoxicity assay (MTT) was performed in chick embryo fibroblasts (Plate 12) and Hep2 cells (Plate 13) exposed to etoposide in the presence and/or the absence of *Artemisia vulgaris* leaf extracts. The results obtained are presented in Figures 11 and 12 respectively.

PLATE 12

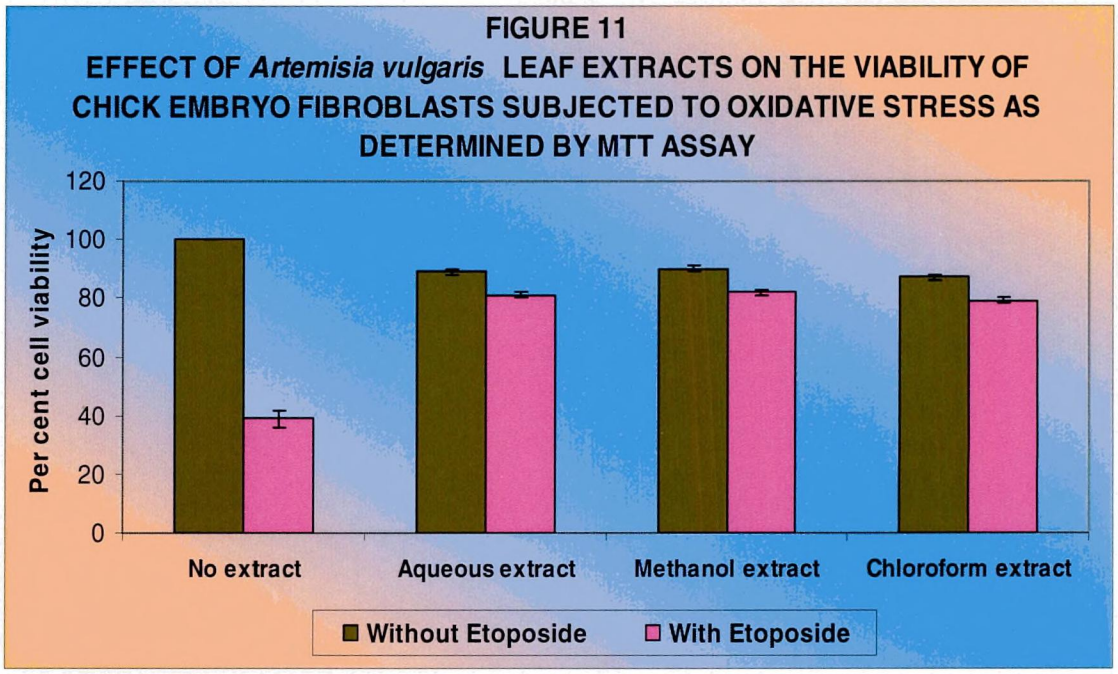


PRIMARY CHICK EMBRYO FIBROBLASTS

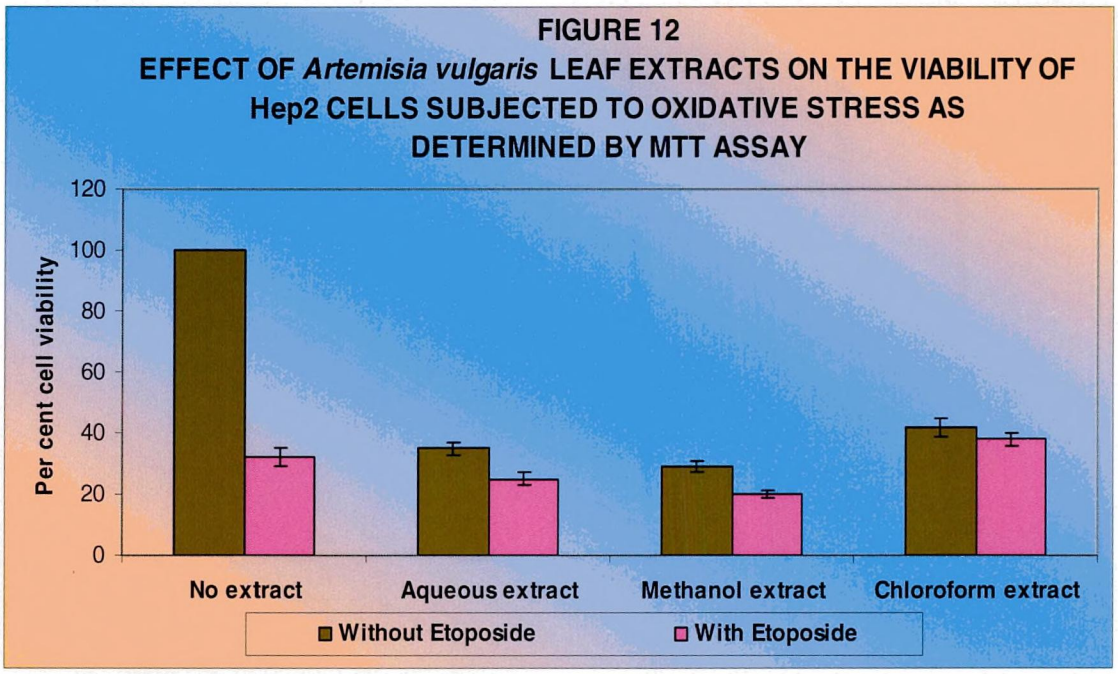
PLATE 13



Hep2 CELLS



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



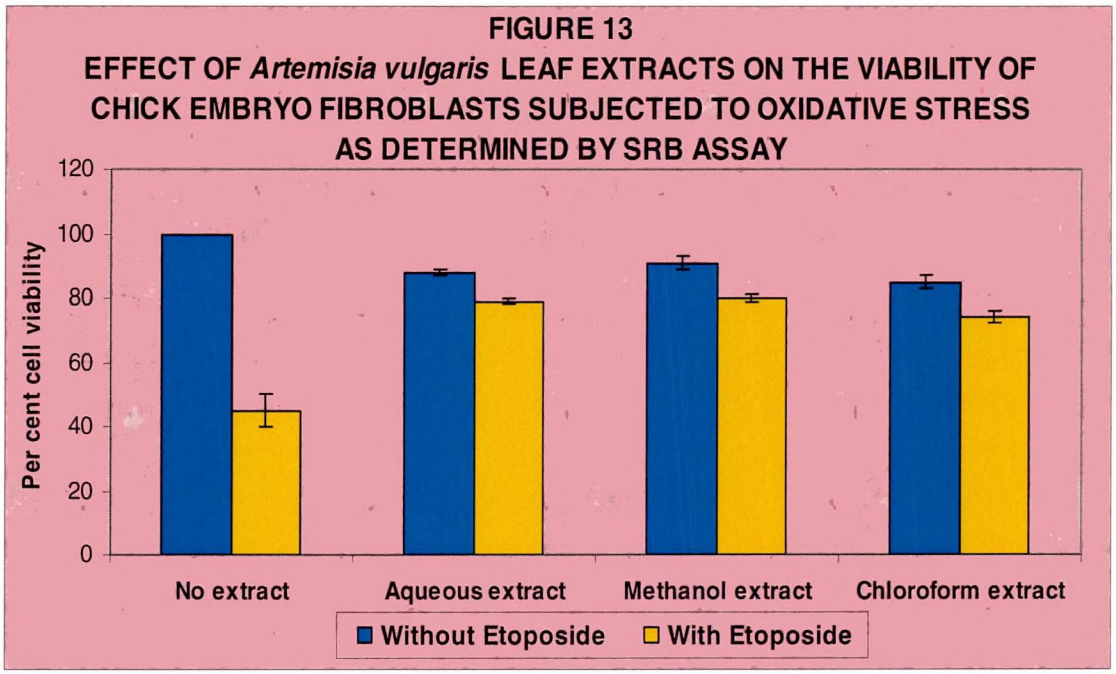
The values of negative (untreated) control group were fixed as 100% viability and the per cent viabilities in the other groups were calculated relative to this.

The results of MTT assay demonstrated that the etoposide treatment caused a significant reduction in the viability of both chick embryo fibroblasts and Hep2 cells. The cytotoxicity of etoposide to primary (untransformed) cells was effectively counteracted by the administration of *Artemisia vulgaris* leaf extracts. Surprisingly, the leaf extracts of *Artemisia vulgaris*, by themselves, caused a steep decrease in the viability of Hep2 cells, which decreased further in the presence of etoposide. These observations suggest that the plant extracts enhance the action of etoposide on Hep2 cells, while inhibiting the toxicity of etoposide to primary cells. Out of the three extracts used, the methanolic extract exhibited maximum activity in this regard.

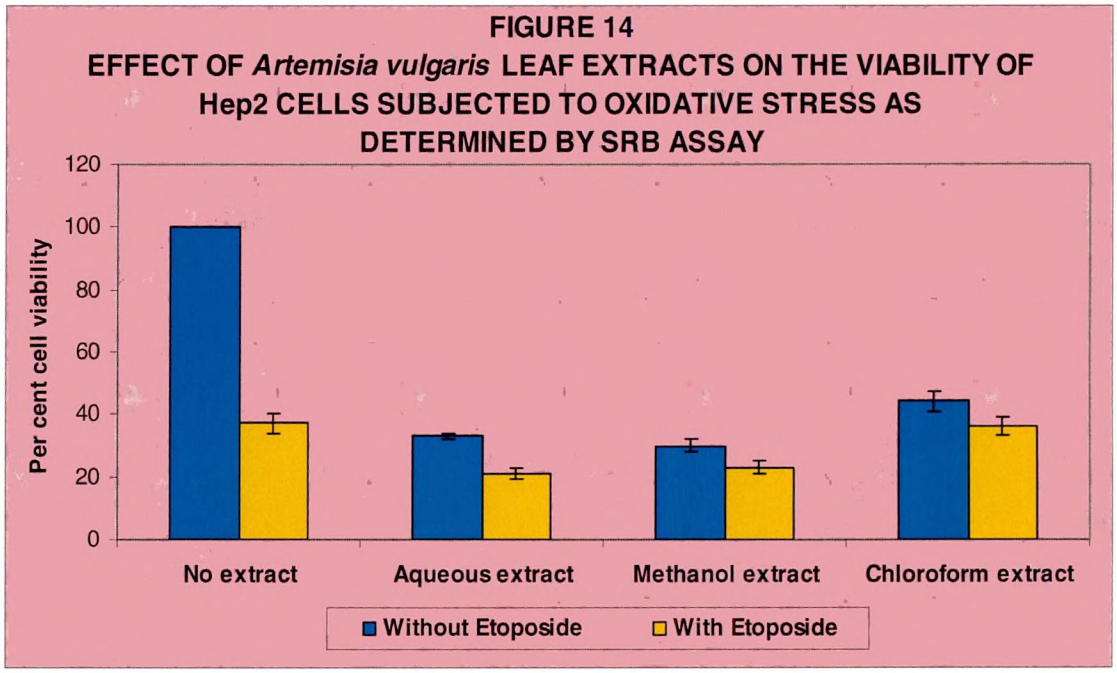
SRB assay was performed after the treatment of primary chick embryo fibroblasts and Hep2 cells with *Artemisia vulgaris* leaf extracts in the presence and/or the absence of etoposide. The results of the SRB assay of primary and Hep2 cells are depicted in Figures 13 and 14 respectively. The results of SRB assay followed the same trend as observed in MTT assay. The results of the SRB assay thus revealed that the *Artemisia vulgaris* leaf extracts showed differential effects towards etoposide-treated primary cells and Hep2 cells.

MORPHOLOGICAL CHANGES OF APOPTOSIS OBSERVED IN CHICK EMBRYO FIBROBLASTS AND Hep2 CELLS

The morphological changes in primary chick embryo fibroblasts and Hep2 cells in the presence and the absence of the leaf extracts and/or etoposide were quantified per 100 cells using phase contrast microscope. The results obtained are presented in Tables 20 and 21 respectively. The apoptotic ratios were calculated and the data are also presented in Tables 20 and 21.



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

TABLE 20

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON PRIMARY CHICK EMBRYO FIBROBLASTS SUBJECTED TO APOPTOSIS BY ETOPOSIDE (GIEMSA STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No extract	3 ± 2	75 ± 1 [a]	0.03	3.00
Aqueous extract	11 ± 2 [a]	16 ± 1 [a,b,c]	0.12	0.19
Methanol extract	7 ± 1 [a]	14 ± 1 [a,b,c]	0.08	0.16
Chloroform extract	12 ± 2 [a]	17 ± 1 [a,b,c]	0.14	0.20

The values are mean ± S.D of triplicates.

a- Statistically significant ($P<0.05$) compared to untreated control

b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

TABLE 21

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON Hep2 CELLS SUBJECTED TO APOPTOSIS BY ETOPOSIDE (GIEMSA STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No extract	18 ± 3	67 ± 1 [a]	0.22	2.03
Aqueous extract	67 ± 3 [a]	73 ± 1 [a,b,c]	2.03	2.70
Methanol extract	71 ± 1 [a]	78 ± 1 [a,b,c]	2.44	3.55
Chloroform extract	66 ± 1 [a]	71 ± 2 [a,b,c]	1.94	2.45

The values are mean ± S.D of triplicates.

a- Statistically significant ($P<0.05$) compared to untreated control

b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

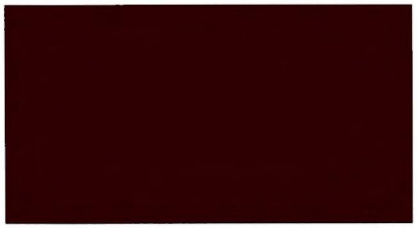
Etoposide caused a steep increase in the number of cells (both non-cancerous and cancerous) showing apoptotic morphology (Plates 14 and 15). When the different leaf extracts of *Artemisia vulgaris* were co-administered with etoposide treated primary chick embryo fibroblasts, a significant ($P<0.05$) decrease in the number of cells undergoing apoptosis was observed. Whereas, in the case of Hep2 cells, the administration of *Artemisia vulgaris* leaf extracts alone increased the number of apoptotic cells compared to untreated control, implying the anticancer activity of these extracts. Also, the co-exposure of Hep2 cells to leaf extracts along with etoposide caused further significant ($P<0.05$) increase in the number of apoptotic cells. In both the cell types, the effect of the methanolic extract was more pronounced than the aqueous and chloroform extracts.

NUCLEAR CHANGES OF APOPTOSIS OBSERVED IN PRIMARY CHICK EMBRYO FIBROBLASTS AND Hep2 CELLS

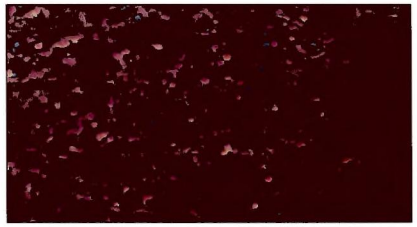
The nuclear changes in both the cancerous and non-cancerous cells by etoposide and its modulation in the presence of *Artemisia vulgaris* leaf extracts were quantified using nuclear stains namely PI, EtBr and DAPI. After staining with PI, in each treatment group, the numbers of apoptotic cells were counted using inverted fluorescent microscope and the results are listed in Tables 22 and 23.

Propidium iodide staining revealed that there was a significant ($P<0.05$) increase in the number of untransformed and transformed cells undergoing apoptosis in etoposide treated group compared to the respective control groups. When the primary chick embryo fibroblasts were exposed to both oxidant and the leaf extracts, there was a significant increase in the number of normal cells, indicating the protective effect of *Artemisia vulgaris* leaf extracts.

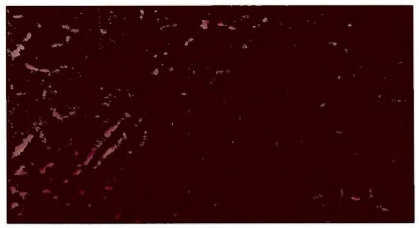
PLATE 14
PRIMARY CHICK EMBRYO FIBROBLASTS STAINED WITH GIEMSA (OXIDANT-ETOPOSIDE)



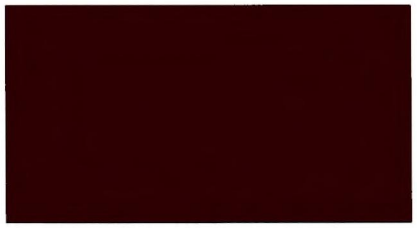
Control



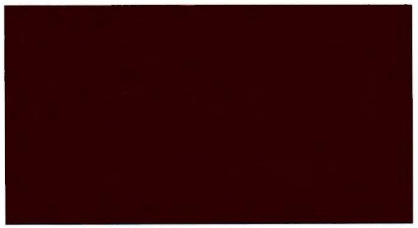
Etoposide



Aqueous extract



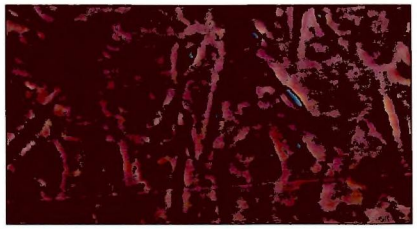
Aqueous extract + Etoposide



Methanol extract

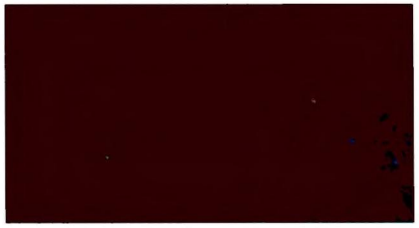
Methanol extract + Etoposide

Chloroform extract

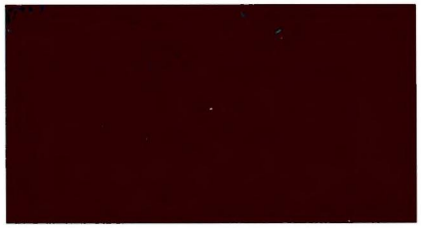


Chloroform extract + Etoposide

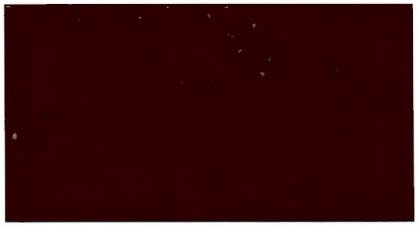
PLATE 15
Hep2 CELLS STAINED WITH GIEMSA (OXIDANT-ETOPOSIDE)



Control

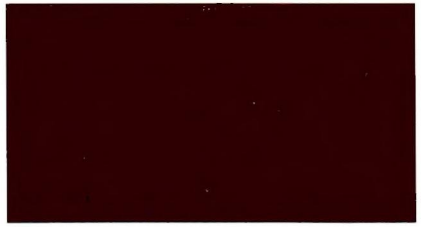


Etoposide



Aqueous extract

Aqueous extract + Etoposide



Methanol extract

Methanol extract + Etoposide

Chloroform extract

Chloroform extract + Etoposide

TABLE 22

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON CHICK EMBRYO FIBROBLASTS SUBJECTED TO APOPTOSIS BY ETOPOSIDE (PI STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No extract	5 ± 1	78 ± 2 [a]	0.05	3.54
Aqueous extract	12 ± 1 [a]	19 ± 1 [a,b,c]	0.14	0.23
Methanol extract	9 ± 1 [a]	15 ± 2 [a,b,c]	0.10	0.18
Chloroform extract	14 ± 1 [a]	20 ± 1 [a,b,c]	0.16	0.25

The values are mean ± S.D of triplicates.
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

TABLE 23

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON Hep2 CELLS SUBJECTED TO APOPTOSIS BY ETOPOSIDE (PI STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No extract	10 ± 2	66 ± 2 [a]	0.11	1.94
Aqueous extract	69 ± 1 [a]	79 ± 3 [a,b,c]	2.23	3.78
Methanol extract	70 ± [a]	81 ± 1 [a,b,c]	2.33	4.26
Chloroform extract	64 ± 2 [a]	72 ± 2 [a,b,c]	1.88	2.57

The values are mean ± S.D of triplicates.
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

In the case of Hep2 cells, all the three extracts of *Artemisia vulgaris* leaves caused significant ($P<0.05$) increase in the number of apoptosing cells.

The action of etoposide increased further in the presence of leaf extracts. Among the three extracts used, the methanolic extract exhibited the most efficient apoptosis-modulating effect. The aqueous and chloroform extracts were also efficient in their effects on apoptosis.

After staining with PI, the normal and apoptotic cells were counted using inverted fluorescent microscope and the photographs are presented in Plates 16 and 17. The apoptotic ratios of PI stained chick embryo fibroblasts and Hep2 cells are presented in Tables 22 and 23 respectively.

The extent of nuclear changes observed during etoposide-induced apoptosis after EtBr staining in the different treatment groups of the primary and Hep2 cells are presented in Tables 24 and 25 respectively.

TABLE 24

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON CHICK EMBRYO FIBROBLASTS SUBJECTED TO APOPTOSIS BY ETOPOSIDE (EtBr STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No extract	2 ± 1	76 ± 2 [a]	0.02	3.17
Aqueous extract	10 ± 1 [a]	17 ± 1 [a,b,c]	0.11	0.20
Methanol extract	9 ± 2 [a]	18 ± 1 [a,b,c]	0.10	0.22
Chloroform extract	14 ± 3 [a]	20 ± 1 [a,b,c]	0.16	0.25

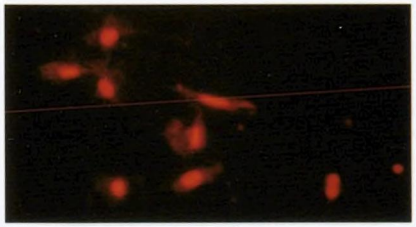
The values are mean ± S.D of triplicates.

a- Statistically significant ($P<0.05$) compared to untreated control

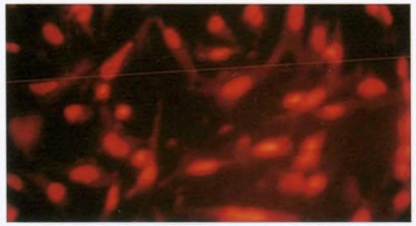
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

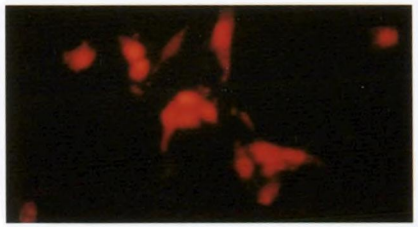
PLATE 16
PRIMARY CHICK EMBRYO FIBROBLASTS STAINED WITH PI (OXIDANT-ETOPOSIDE)



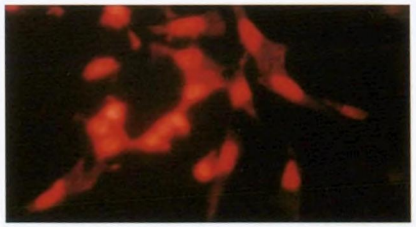
Control



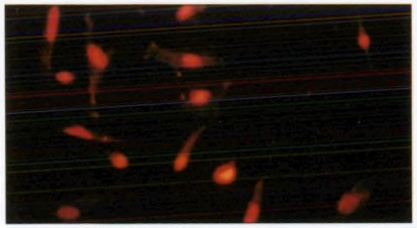
Etoposide



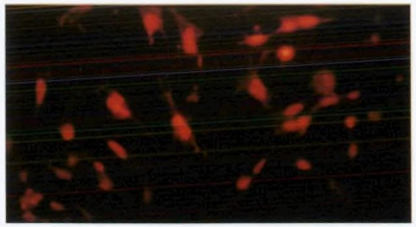
Aqueous extract



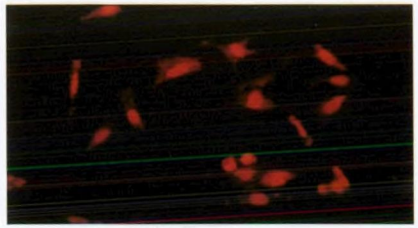
Aqueous extract + Etoposide



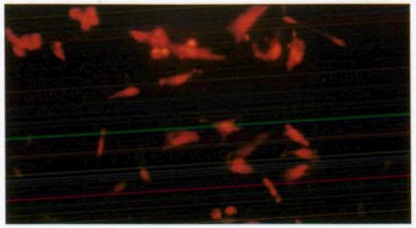
Methanol extract



Methanol extract + Etoposide

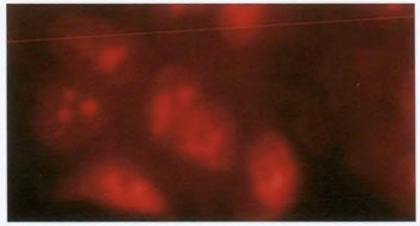


Chloroform extract

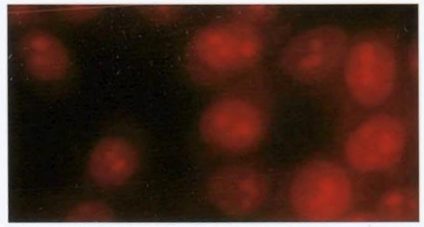


Chloroform extract + Etoposide

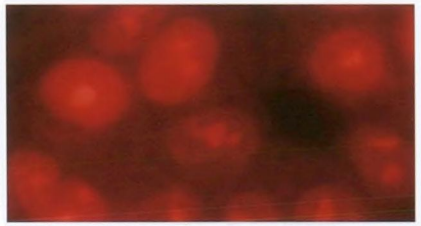
PLATE 17
Hep2 CELLS STAINED WITH PI (OXIDANT-ETOPOSIDE)



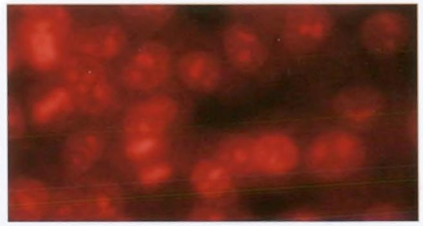
Control



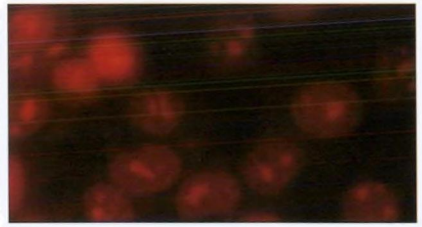
Etoposide



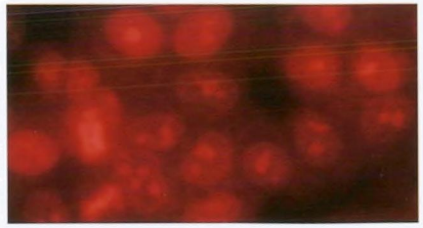
Aqueous extract



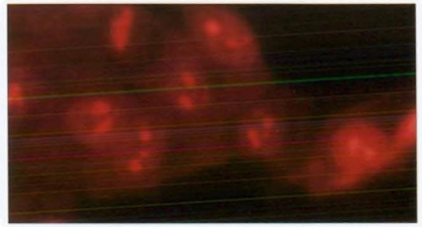
Aqueous extract + Etoposide



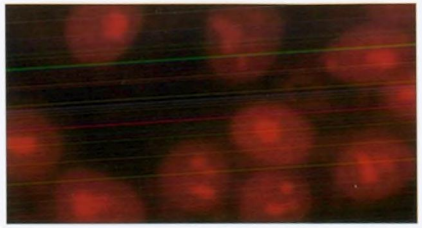
Methanol extract



Methanol extract + Etoposide



Chloroform extract



Chloroform extract + Etoposide

TABLE 25

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON Hep2 CELLS SUBJECTED TO APOPTOSIS BY ETOPOSIDE (EtBr STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No extract	15 ± 4	68 ± 2 [a]	0.18	2.13
Aqueous extract	68 ± 2 [a]	75 ± 3 [a,b,c]	2.13	3.00
Methanol extract	77 ± 3 [a]	84 ± 2 [a,b,c]	3.35	5.25
Chloroform extract	65 ± 4 [a]	70 ± 2 [a,c]	1.86	2.33

The values are mean ± S.D of triplicates.

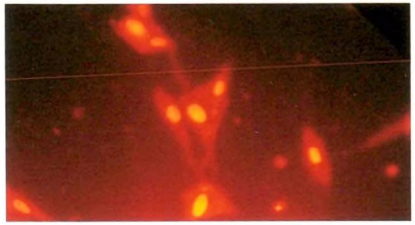
a- Statistically significant ($P<0.05$) compared to untreated control

b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group

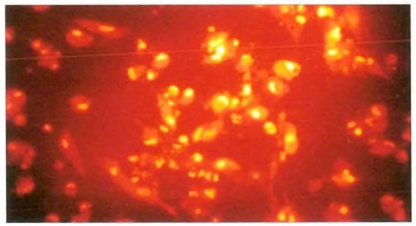
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

Etoposide treatment caused a significant proportion of cells to show nuclear apoptotic morphology (Plates 18 and 19). The action of etoposide was significantly ($P<0.05$) reverted by the co-administration of all the three extracts of *Artemisia vulgaris* leaves in chick embryo fibroblasts. The methanolic extract exhibited the maximum effect, followed closely by the aqueous and chloroform extracts. The plant extracts, by themselves, caused significant ($P<0.05$) increase in the number of dying Hep2 cells, indicating their anticancer activity. The apoptosis-inducing action of etoposide was further significantly ($P<0.05$) increased by the co-administration of *Artemisia vulgaris* leaf extracts in Hep2 cells. EtBr stained Hep2 cells showed that the maximum apoptosis-inducing action was found in the methanolic extract treated group. The aqueous and chloroform extracts also showed apoptosis-inducing effect on Hep2 cells, albeit to a lower extent.

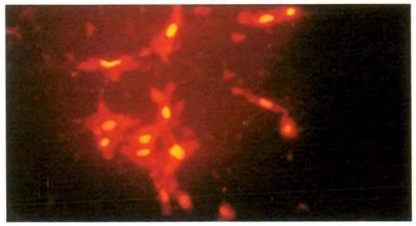
PLATE 18
PRIMARY CHICK EMBRYO FIBROBLASTS STAINED WITH EtBr (OXIDANT-ETOPOSIDE)



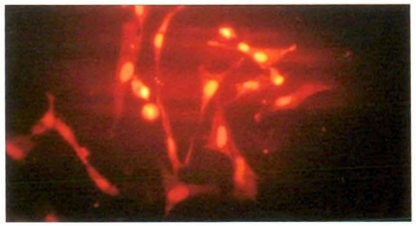
Control



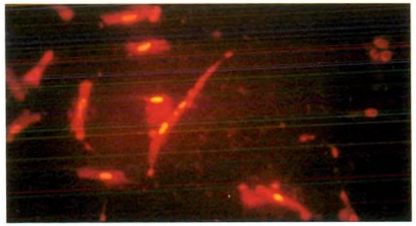
Etoposide



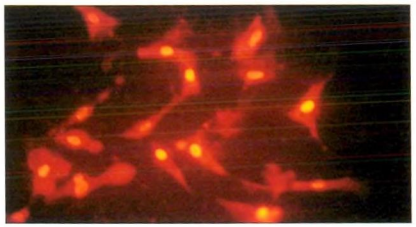
Aqueous extract



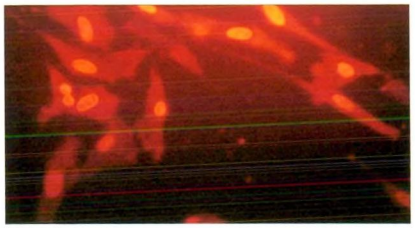
Aqueous extract + Etoposide



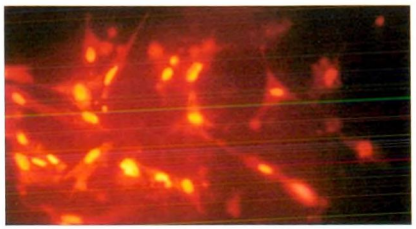
Methanol extract



Methanol extract + Etoposide

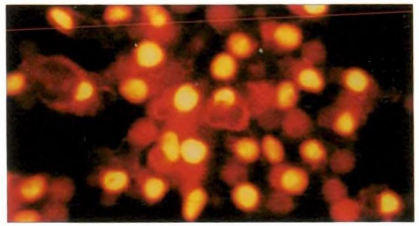


Chloroform extract

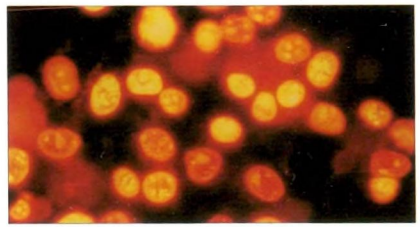


Chloroform extract + Etoposide

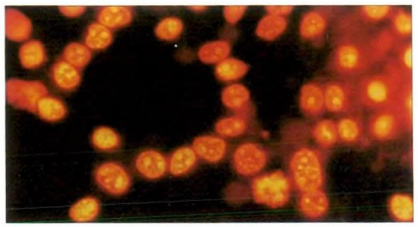
PLATE 19
Hep2 CELLS STAINED WITH EtBr (OXIDANT-ETOPOSIDE)



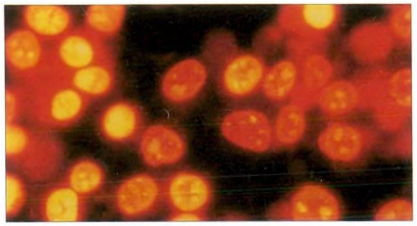
Control



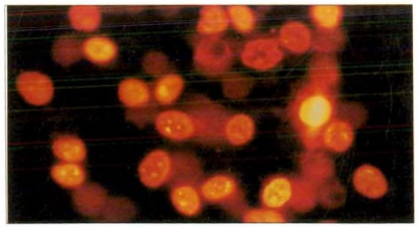
Etoposide



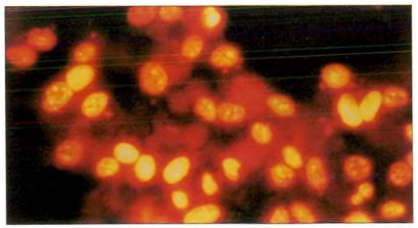
Aqueous extract



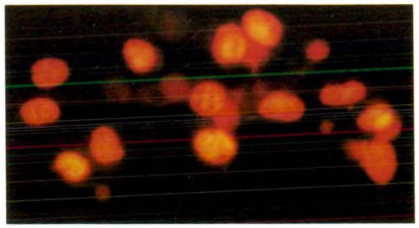
Aqueous extract + Etoposide



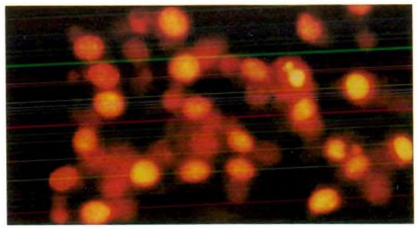
Methanol extract



Methanol extract + Etoposide



Chloroform extract



Chloroform extract + Etoposide

The apoptotic ratios for all the treatment groups were calculated and the values of the same are presented in Tables 24 and 25 respectively.

DAPI staining was also adopted to understand the nuclear changes of apoptosis in the primary and Hep2 cells subjected to oxidative stress in the presence and absence of *Artemisia vulgaris* leaf extracts. The apoptotic cells per 100 cells were counted and the values are presented in Tables 26 and 27. The DAPI stained cells were viewed under inverted fluorescent microscope and the photographic pictures are shown in Plates 20 and 21. The apoptotic ratios were also calculated and the results of each are presented in Tables 26 and 27.

TABLE 26

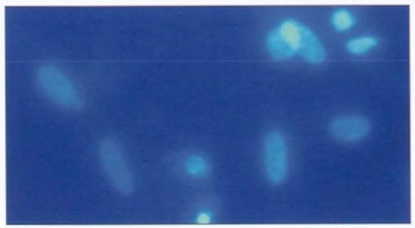
EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON CHICK EMBRYO FIBROBLASTS SUBJECTED TO APOPTOSIS BY ETOPOSIDE (DAPI STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No extract	4 ± 1	80 ± 2 [a]	0.04	4.0
Aqueous extract	9 ± 2 [a]	16 ± 1 [a,b,c]	0.09	0.19
Methanol extract	7 ± 2	13 ± 2 [a,b,c]	0.08	0.15
Chloroform extract	12 ± 1 [a]	19 ± 1 [a,b,c]	0.13	0.23

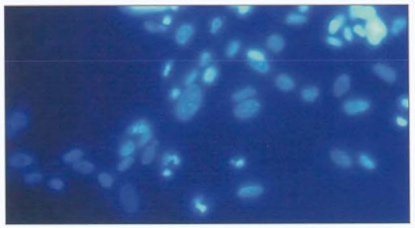
The values are mean ± S.D of triplicates.
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

The results of DAPI stained primary and Hep2 cells followed the same trend as that observed in the other nuclear staining assays.

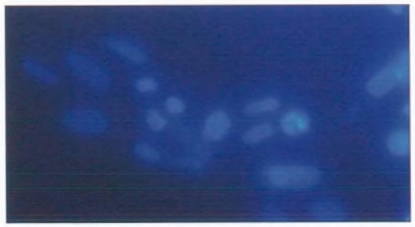
PLATE 20
PRIMARY CHICK EMBRYO FIBROBLASTS STAINED WITH DAPI (OXIDANT-ETOPOSIDE)



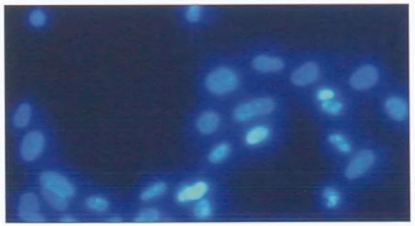
Control



Etoposide

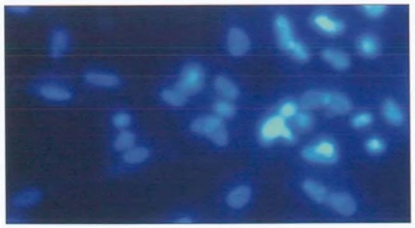


Aqueous extract

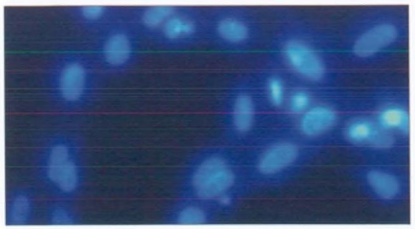


Aqueous extract + Etoposide

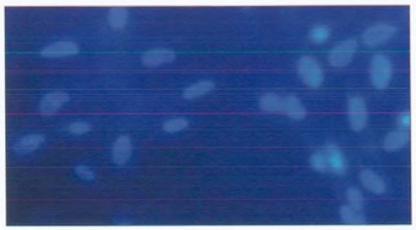
Methanol extract



Methanol extract + Etoposide

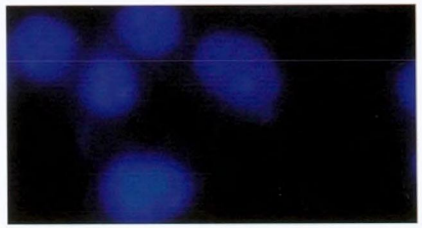


Chloroform extract

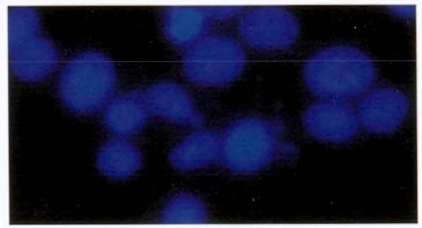


Chloroform extract + Etoposide

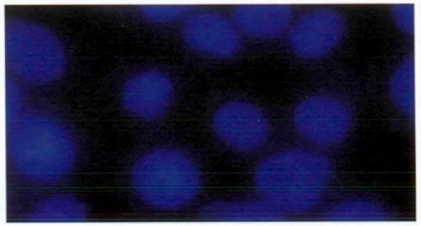
PLATE 21
Hep2 CELLS STAINED WITH DAPI (OXIDANT-ETOPOSIDE)



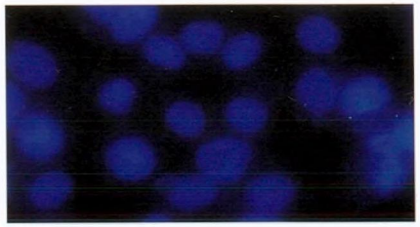
Control



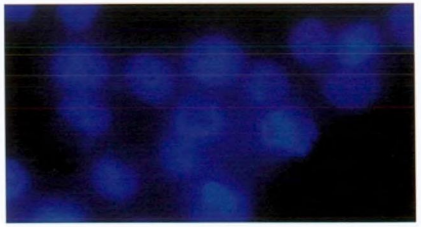
Etoposide



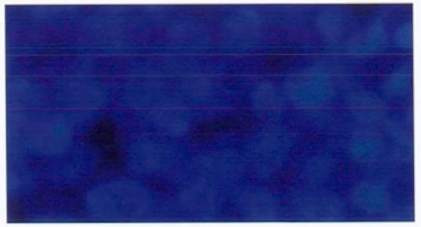
Aqueous extract



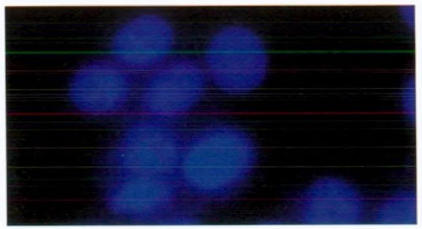
Aqueous extract + Etoposide



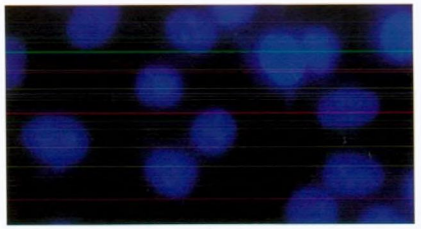
Methanol extract



Methanol extract + Etoposide



Chloroform extract



Chloroform extract + Etoposide

TABLE 27

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON Hep2 CELLS SUBJECTED TO APOPTOSIS BY ETOPOSIDE (DAPI STAINING)

SAMPLE	NO. OF APOPTOTIC CELLS/100 CELLS		APOPTOTIC RATIO	
	Without etoposide	With etoposide	Without etoposide	With etoposide
No extract	11 ± 2	64 ± 2 [a]	0.12	1.78
Aqueous extract	63 ± 2 [a]	77 ± 1 [a,b,c]	1.70	3.35
Methanol extract	73 ± 3 [a]	80 ± 1 [a,b,c]	2.70	4.00
Chloroform extract	62 ± 2 [a]	69 ± 2 [a,b,c]	1.62	2.23

The values are mean ± S.D of triplicates.
a- Statistically significant ($P<0.05$) compared to untreated control
b- Statistically significant ($P<0.05$) compared to H_2O_2 alone treated group
c- Statistically significant ($P<0.05$) compared to the respective plant extract treated group

PHASE IV

In the fourth phase of the study, preliminary phytochemical screening was conducted to identify the active principle(s) rendering the protective effects of *Artemisia vulgaris* leaves. Qualitative analysis of the methanolic extract of the plant indicated the absence of alkaloids and the presence of phenolics and flavonoids. The result of the preliminary phytochemical screening is presented in Table 28.

UV ABSORPTION SPECTRUM

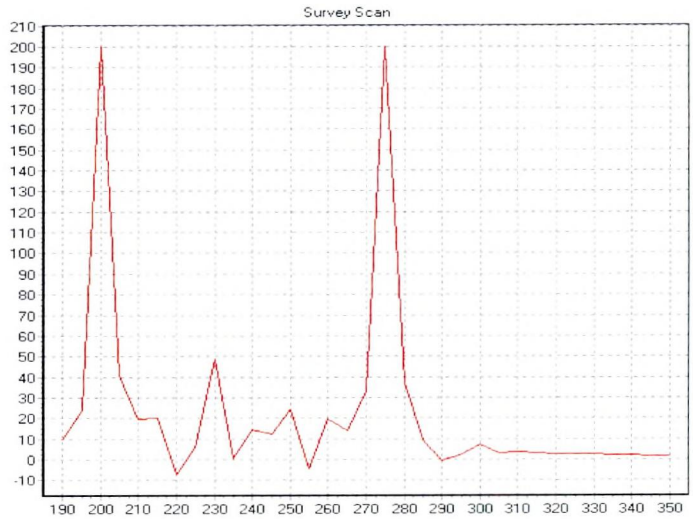
The absorbance spectrum of the methanolic extract of *Artemisia vulgaris* leaves in the wavelength ranging from 190nm to 350nm is presented in Figure 15. The methanolic extract of *Artemisia vulgaris* showed two major peaks at 200nm and 275nm and five minor peaks at 230, 240, 250, 260 and 300nm respectively.

TABLE 28

PRELIMINARY PHYTOCHEMICAL TEST

ALKALOIDS	
Mayer's test	–
Dragendroff's test	–
Wagner's test	–
PHENOLICS	
Ferric chloride test	+
Lead acetate test	+
FLAVONOIDS	
Aqueous NaOH test	+
Concentrated H_2SO_4 test	+
Aluminium chloride test	+

FIGURE 15



UV ABSORPTION SPECTRUM OF THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES

HPTLC ANALYSIS OF THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES

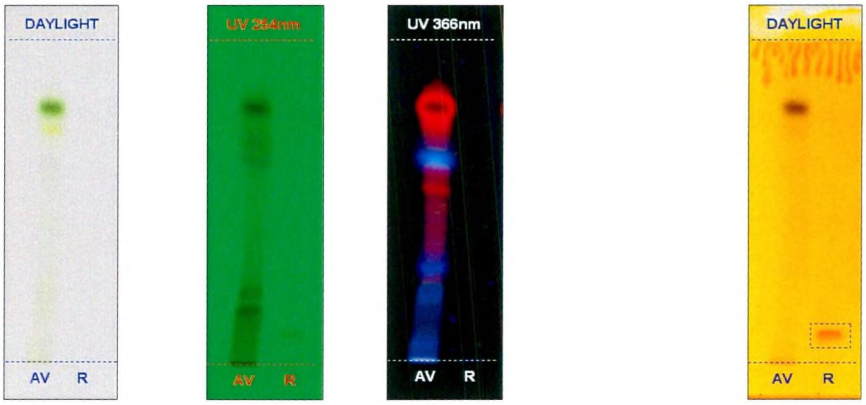
The methanolic extract of *Artemisia vulgaris* leaves was subjected to HPTLC analysis for the presence of alkaloids, phenolics, flavonoids and sesquiterpenoids. The alkaloid profile of the methanolic extract was done with the reference standard nicotine and the developed plate was sprayed with Dragendroff's reagent. The absence of bright orange colour in plate after photo-documentation at day light indicated the absence of alkaloids (Plate 22). The peak table (Table 29) and peak densitogram (Figure 16) were recorded.

The phenolics present in the methanolic extract of *Artemisia vulgaris* leaves were analysed using quercetin as reference standard. The presence of blur, blue grey colour in day light showed the presence of phenolics (Plate 23). There were totally 6 phenols identified in the methanolic extract of *Artemisia vulgaris* leaves. Two unspecific spots were also obtained. The peak table (Table 30) and peak densitogram (Figure 17) were recorded after scanning at 500nm.

The flavonoids profile of the methanolic extract of *Artemisia vulgaris* leaves was analysed using rutin as a standard. Yellow and yellow green fluorescence zone at UV 366nm was observed from the chromatogram, which confirmed the presence of flavonoids (Plate 24). There were four flavonoids identified in the methanolic extract of *Artemisia vulgaris* leaves. There were also four unspecified spots in the chromatogram developed with the flavonoid-specific spray reagent. The peak table (Table 31) and peak densitogram (Figure 18) were recorded.

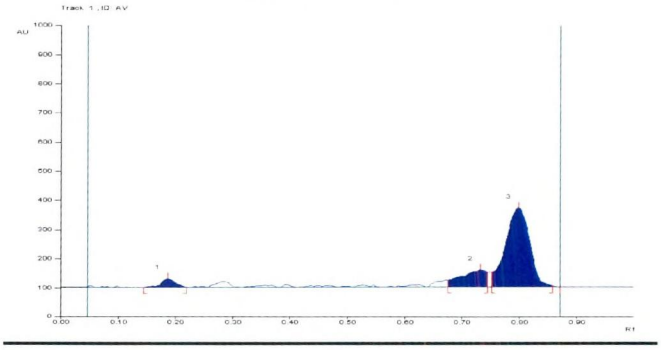
PLATE 22

Before derivatization **After derivatization**



CHROMATOGRAM OF ALKALOIDS

FIGURE 16



PEAK DENSITOGRAM OF ALKALOIDS IN THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES BY HPTLC

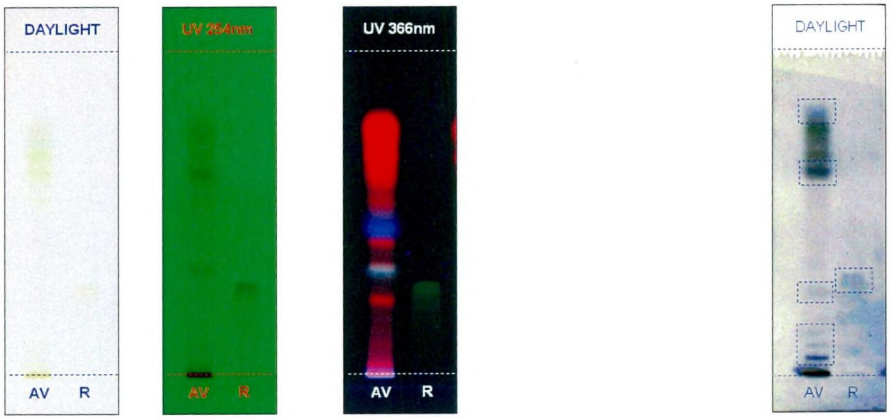
TABLE 29

PEAK TABLE FOR THE ALKALOIDS IN THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES BY HPTLC

Track	Peak no.	Rf	Height	Area	Assigned substance
AV	1	0.19	31	790.7	Unknown
AV	2	0.73	61.8	2588.2	Unknown
AV	3	0.80	273	10847.	Unknown
R	1	0.1	220.4	5591.8	Nicotine

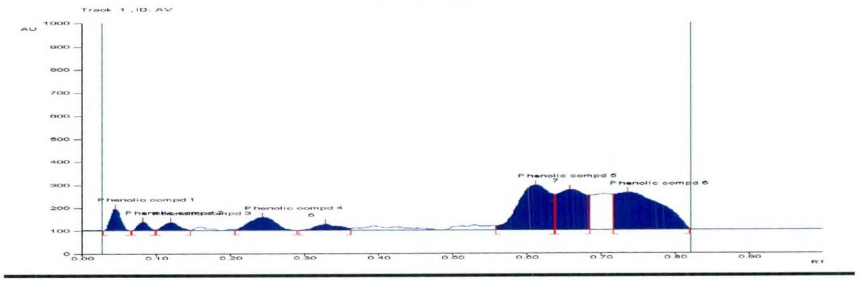
PLATE 23

Before derivatization **After derivatization**



CHROMATOGRAM OF PHENOLICS

FIGURE 17



PEAK DENSITOGRAM OF PHENOLICS IN THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES BY HPTLC

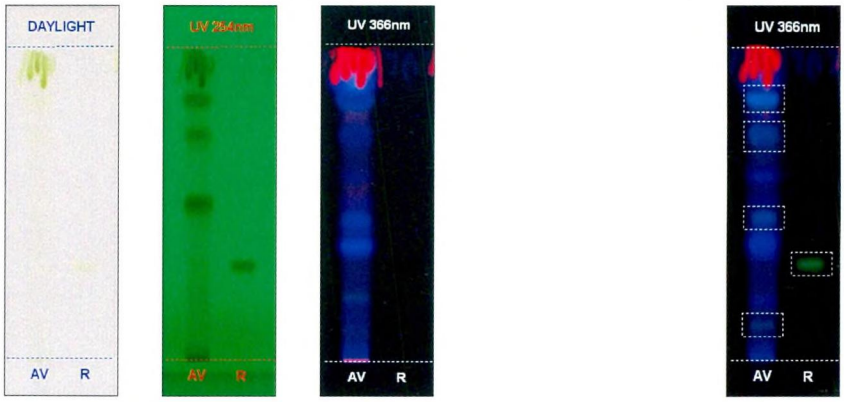
TABLE 30

PEAK TABLE FOR THE PHENOLICS IN THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES BY HPTLC

Track	Peak no.	Rf	Height	Area	Assigned substance
AV	1	0.04	95.8	1180.7	Phenolic 1
AV	2	0.08	37.9	481.6	Phenolic 2
AV	3	0.12	35.3	702.5	Phenolic 3
AV	4	0.24	57	1906	Phenolic 4
AV	5	0.33	27.7	810.8	Unknown
AV	6	0.61	195	7932.2	Phenolic 5
AV	7	0.66	174.7	6172.8	Unknown
AV	8	0.74	161.2	9556.2	Phenolic 6
R	1	0.27	202.8	9172.9	Quercetin

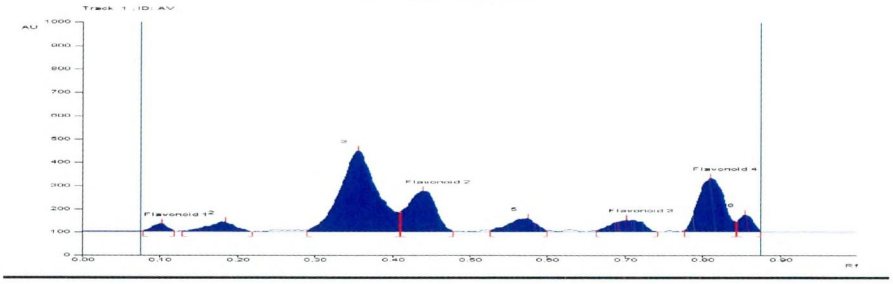
PLATE 24

Before derivatization **After derivatization**



CHROMATOGRAM OF FLAVONOIDS

FIGURE 18



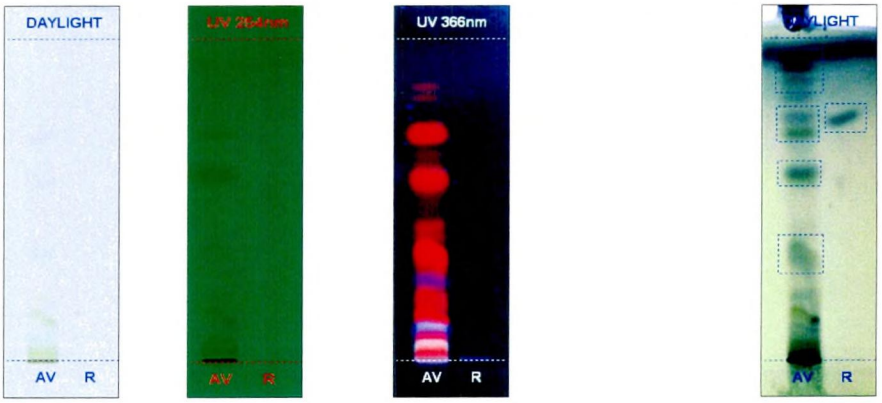
PEAK DENSITOGRAM OF FLAVONOIDS IN THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES BY HPTLC

TABLE 31
PEAK TABLE FOR THE FLAVONOIDS IN THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES BY HPTLC

Track	Peak no.	Rf	Height	Area	Assigned substance
AV	1	0.1	35.8	674.9	Flavonoid 1
AV	2	0.18	43	1483.5	Unknown
AV	3	0.36	349.5	15599.	Unknown
AV	4	0.44	176.4	5996.1	Flavonoid 2
AV	5	0.57	60.6	2061.9	Unknown
AV	6	0.7	53	2070.3	Flavonoid 3
AV	7	0.81	232.7	7269.9	Flavonoid 4
AV	8	0.85	76	1279.1	Unknown
R	1	0.29	81.3	1763	Rutin

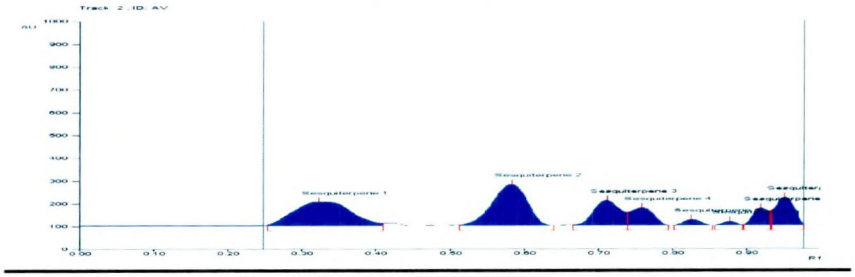
PLATE 25

Before derivatization **After derivatization**



CHROMATOGRAM OF SESQUITERPENOIDS

FIGURE 19



PEAK DENSITOGRAM OF SESQUITERPENOIDS IN THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES BY HPTLC

TABLE 32

PEAK TABLE FOR THE SESQUITERPENOIDS IN THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES BY HPTLC

Track	Peak no.	Rf	Height	Area	Assigned substance
AV	1	0.32	106.2	7869.4	Sesquiterpenoid 1
AV	2	0.58	182.9	8002.5	Sesquiterpenoid 2
AV	3	0.71	111.7	3691.9	Sesquiterpenoid 3
AV	4	0.76	78.9	2243	Sesquiterpenoid 4
AV	5	0.82	26.7	571.7	Sesquiterpenoid 5
AV	6	0.88	18	294.1	Sesquiterpenoid 6
AV	7	0.92	76.3	1528.1	Sesquiterpenoid 7
AV	8	0.95	125	2898.5	Sesquiterpenoid 8
R	1	0.76	148.1	4793.2	Solanesol

The sesquiterpenoids were separated and detected as mentioned in the methodology chapter. The chromatogram showed distinct bands of sesquiterpenoids (Plate 25) separated in the extract of *Artemisia vulgaris* leaves. The Rf values obtained for all the sesquiterpenoids (marked from 1 to 8) are depicted in Table 32. The table shows the presence of eight sesquiterpenoids in the plant extract. The peak table (Table 32) and peak densitogram (Figure 19) were recorded.

HPLC ANALYSIS OF THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES

The HPLC analysis of the methanolic extract of *Artemisia vulgaris* leaves was carried out using C18 RP column (Shimadzu equipped with UV detector). The results obtained are presented in Figure 20.

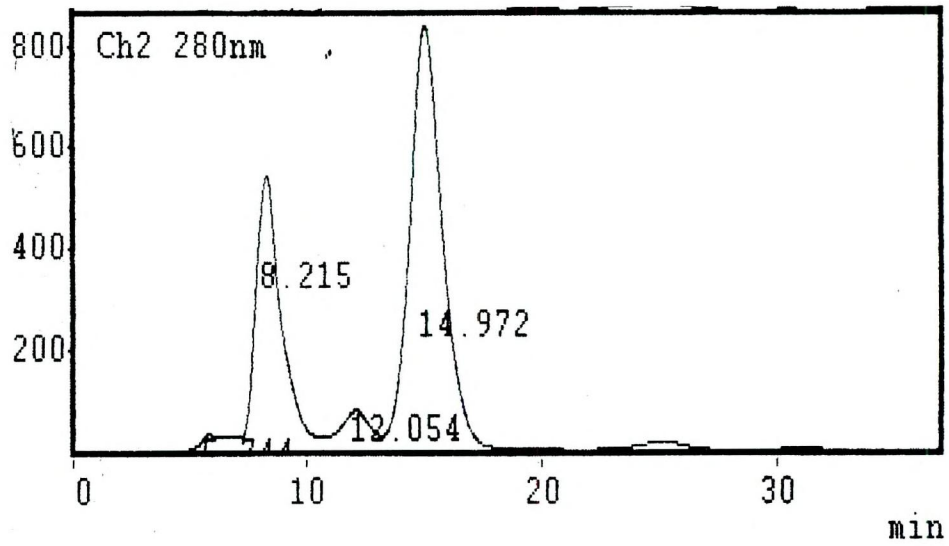
The HPLC spectrum showed 3 peaks (2 major and 1 minor), indicating the presence of three principle components in the methanolic extract of *Artemisia vulgaris* leaves. The retention times of the major and minor peaks were 8.215, 14.972 and 12.054 minutes respectively. The retention time, height and peak area of all the three peaks are presented in Table 33.

TABLE 33

PEAK TABLE OF THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES SUBJECTED TO HPLC

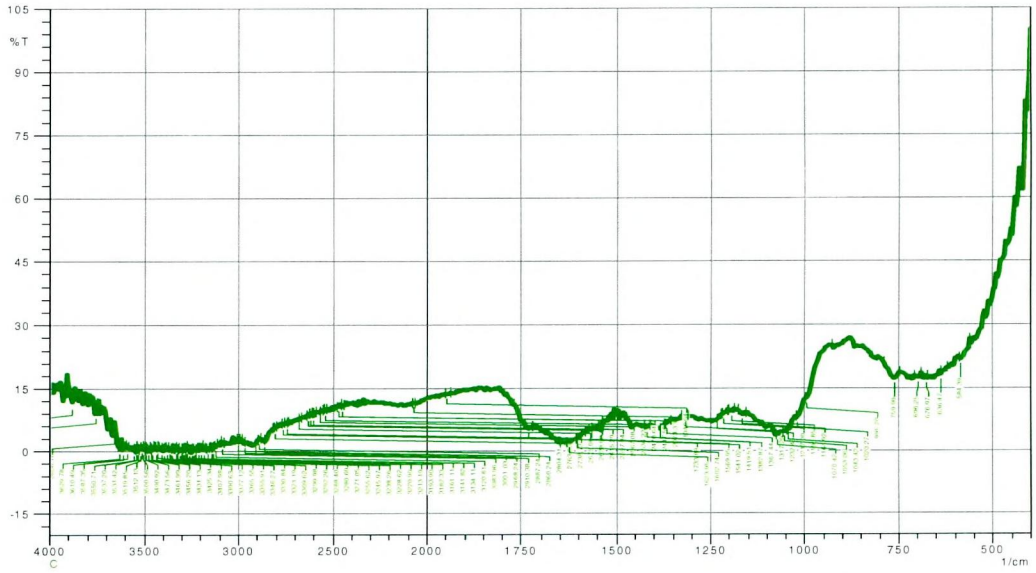
Peak no.	Time (min)	Area	Height
1	8.215	36062145	513186
2	14.972	77832494	823738
3	12.054	4077992	56628

FIGURE 20



HPLC PROFILE OF THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES

FIGURE 21



IR SPECTRUM OF THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES

IR ANALYSIS OF THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES

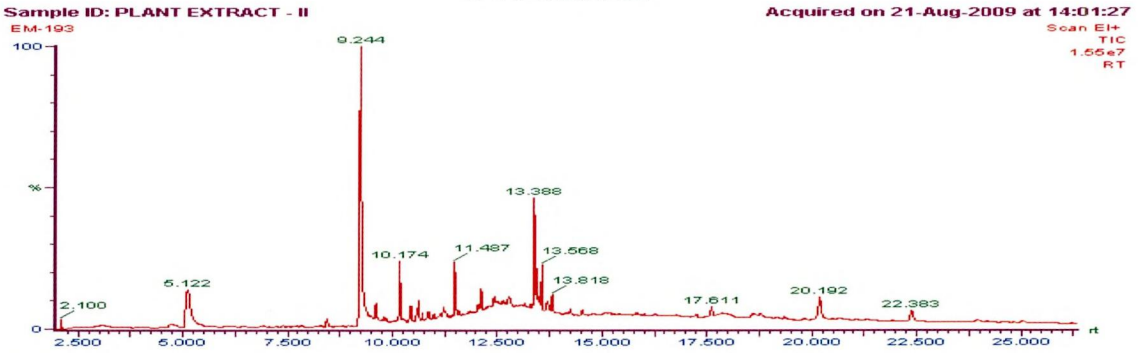
The methanolic extract of *Artemisia vulgaris* leaves were analysed for the IR spectrum using FT-IR spectrophotometer using KBr pellet method (Figure 21). The results of the IR spectrum showed major peaks at 3400-3500cm^{-1} (broad and strong indication of $^{-}$OH) indicating the presence of polyphenolics/flavonoids and a peak at 1700cm^{-1} (indicative of carbonyl group/lactone), which concludes the presence of flavonoids. The peak at 1053 cm^{-1} indicated the presence of C-O linkage.

GC-MS ANALYSIS OF THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES

The GC-MS analysis of the methanolic extract of *Artemisia vulgaris* leaves was carried out to identify the nature of the components present. The GC results showed the presence of five major components at retention times 9.244, 10.174, 11.487, 13.388 and 13.568 respectively (Figure 22).

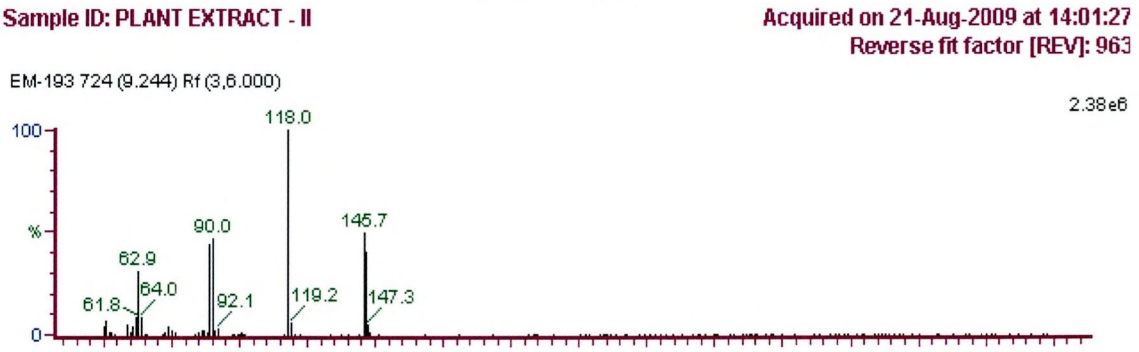
In the mass spectrum of GC peak at retention time 9.244, molecular ion peak was observed at m/z 145.7 and the base peak was observed m/z at 118. The other significant m/z peaks are at 145.7, 118.0, 90, and 62.9 respectively (Figure 23). The fragmentation pattern of this compound exactly matched to that of coumarin from the WILEY database.

FIGURE 22



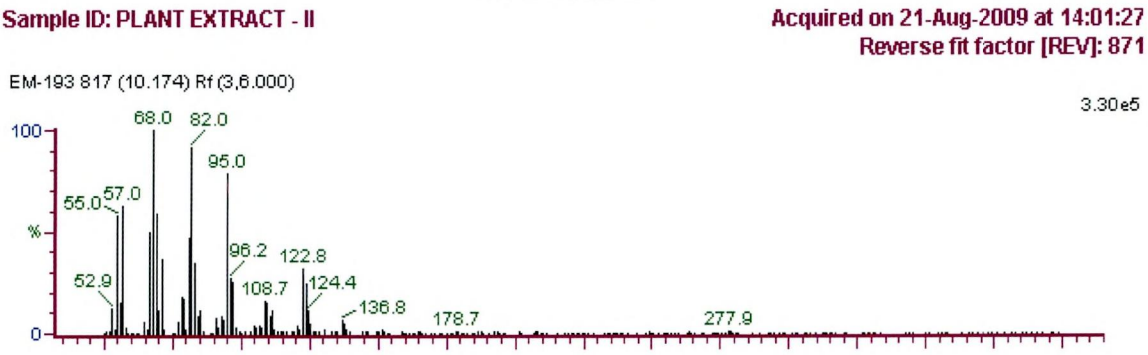
GC-MS PROFILE OF THE METHANOLIC EXTRACT OF *Artemisia vulgaris* LEAVES

FIGURE 23



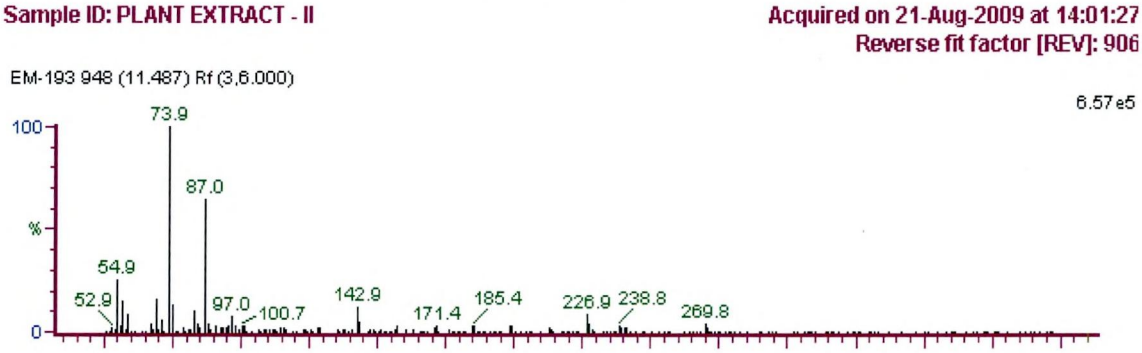
PEAK FRAGMENTATION OF GC-MS SPECTRUM (9.244)

FIGURE 24



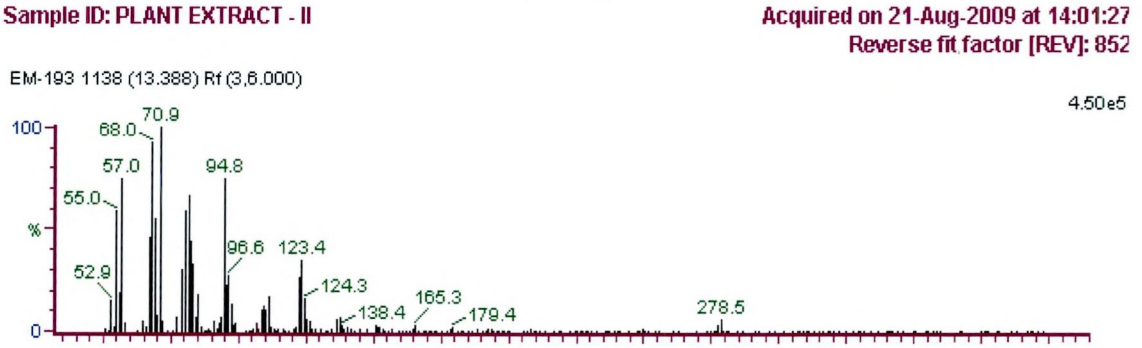
PEAK FRAGMENTATION OF GC-MS SPECTRUM (10.174)

FIGURE 25



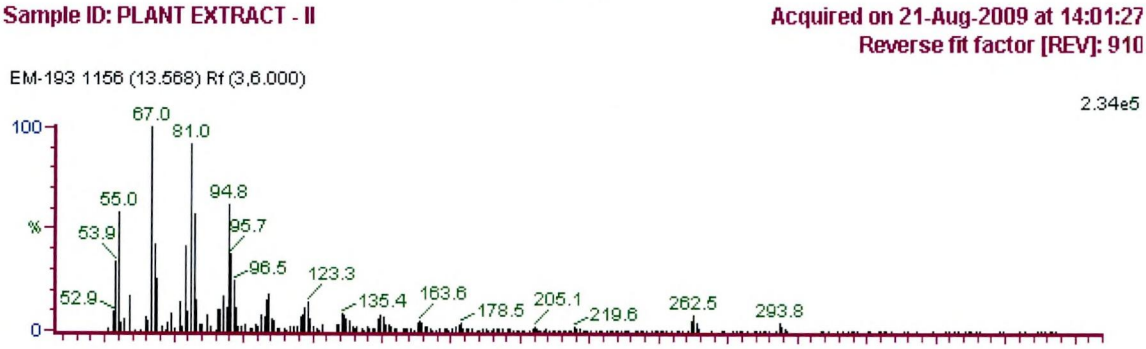
PEAK FRAGMENTATION OF GC-MS SPECTRUM (11.487)

FIGURE 26



PEAK FRAGMENTATION OF GC-MS SPECTRUM (13.388)

FIGURE 27



PEAK FRAGMENTATION OF GC-MS SPECTRUM (13.568)

The mass spectrum of the compound with the retention time 10.174 gave five major peaks at m/z 124, 95, 82, 68 and 57 (Figure 24). The fragmentation pattern showed the presence of (M-28) peak at m/z 96.2 which is characteristic of (M-CO) peak. Hence the compound may contain 'CO' group. Three (M-14) peaks were also indicating the presence of CH_2 groups. This indicates that the fraction may contain an aliphatic compound with 'CO' function.

The mass spectrum of the fraction at 13.388 gave peaks at m/z 138.4, 123.4, 96.6, 94.8, 81, 70.9, 55 and 52.9 (Figure 26). The fragmentation pattern of the compound eluted at retention time 13.388 showed (M-28) peak at m/z 96 and (M-44) peak at m/z 94, which is characteristic of M-CO and M-CO_2 groups. Hence the compound may contain 'CO' group and a carboxylic acid group.

The mass spectrum of the compound with retention time 13.568 gave peaks at m/z 293.8, 262.5, 178.5, 163.3, 135.4, 123.3, 96.5, 94.8, 81, 67, 55, 53.9 and 52.9 (Figure 27). The fragmentation pattern of the compound eluted at retention time 13.568 showed (M-28) peak at m/z 219 and (M-44) peak at m/z 178, which is characteristic of M-CO and (M-CO_2) groups. Hence the compound may be a flavonoid type compound.

Thus, the phytochemical analyses of methanolic extract of *Artemisia vulgaris* leaves revealed the presence of phenolics, flavonoids and sesquiterpenoid type compounds. These compounds may be responsible for the antioxidant and apoptosis-modulating effects of *Artemisia vulgaris* leaves.

The outcome of the research is discussed in the next chapter with reference to relevant published literature.