## 8. Antioxidant and DNA fragmentation assay of bioactives of *S. racemosa*

### 8.1. Instrument

Instruments used in the study were Gel electrophoresis assembly (Genei, India), UV spectrophotometer (Shimadzu, Japan) and Transilluminator (Genei, India).

### 8.2. Materials

DNA lysis buffer, RNase A and ethidium bromide dye used in the experiment were procured from MP Biomedicals India Pvt. Ltd., India. Other chemicals used in the experiment were of analytical grade.

### 8.3. Antioxidants

Antioxidants are substances, which are able to inhibit or greatly retard the oxidation of easily oxidizable materials. Antioxidants exert their activity by varied mechanisms such as suppressing the formation of active species by reducing hyper oxides (RHO) and $H_2O_2$ and also by sequestration of metal ions, active free radicals scavenging, repairing or clearing injury. In last few years, the probable toxicity of synthetic chemical antioxidants has been critiqued. Therefore, much attention has been focused on natural antioxidants which inhibit the lipid peroxidation and protect the tissue from damage due to free radicals. Plant products, bioactives or secondary metabolites including phenolics, tannins, steroids, triterpenoids and various plants or herbal extracts have been reported to be radical scavengers and inhibitors of lipid peroxidation (Patil et al., 2012).

#### 8.3.1. Evaluation of antioxidant activity

##### 8.3.1.1. DPPH free radical scavenging activity

DPPH radical is commonly used as the substrate to evaluate the antioxidant activity. It is a stable free radical that can accept an electron to become a stable molecule. The reduction of DPPH was determined by the decrease in its absorbance at 516 nm induced by antioxidants (Pawar and Surana, 2010, Tirzitis and Bartosz, 2010). The Principle reaction behind DPPH and its reduction by an antioxidant is shown in Figure 8.1.

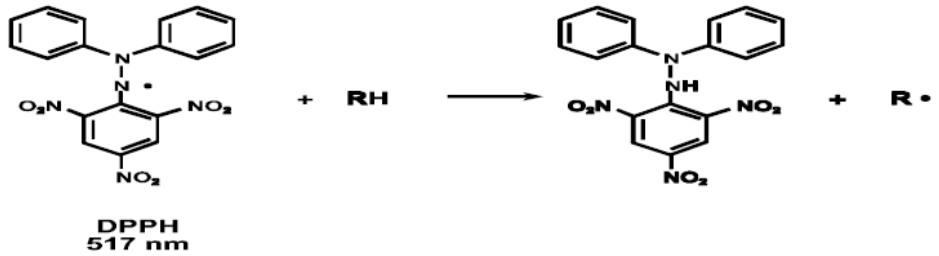


**Figure 8.1: Principle reaction behind DPPH assay**

0.1 mM DPPH solution was prepared in ethanol. 1 mL of this solution mixed with 3 mL of test solutions (5-25 µg/mL). The mixture was vigorously shaken and allowed to keep for 30 min at room temperature. Absorbance was measured at 516 nm (Patil et al., 2012). %Scavenging activity=$(A_0-A_t/A_0) \times 100$; Where, $A_0$=Abs. of control; $A_t$=Abs. of test

### 8.3.2. $H_2O_2$ radical scavenging activity

$H_2O_2$ is a weak oxidizing agent and can inactivate few enzymes directly, usually by oxidation of essential thiol (-SH) group. $H_2O_2$ can cross rapidly cell membrane, which reacts with $Fe^{+2}$ and $Cu^{+2}$ forms the hydroxyl radicals, may give the toxic effects.

20 mM $H_2O_2$ solution was prepared in phosphate buffer saline (pH 7.4). 2 mL of this solution mixed with 1 mL of test solutions (5-25µg/mL). The mixture was vigorously shaken and allowed to keep for 10 min at room temperature. Absorbance was measured at 230 nm (Saumya and Mahaboob, 2011). %Scavenging activity=$(A_0-A_t/A_0)\times$ 100; Where, $A_0$=Abs. of control; $A_t$=Abs. of test

## 8.4. DNA fragmentation assay

Normal organ development is controlled by a balance between cell proliferation and apoptosis. DNA fragmentation assay is the hallmark of apoptosis. In cancer, the balance between proliferation and programmed cell death is disturbed. There is strong evidence that the tumor growth is a result of uncontrolled proliferation and reduced apoptosis. Cancer cells have an acquired capability to evade apoptosis through a variety of ways. Inducing tumor cell apoptosis is an ideal way to kill cancer cells. The DNA of rapidly multiplying, cancerous cells is more exposed as compared to normal cells. Thus, the cell DNA is one of the targets for the treatment of cancer. Breakdown of DNA molecule is one of the sign of inhibition of DNA replication, which may be due to inhibition of topoisomerase, key enzyme in DNA replication (Ibrahim et al., 2012).

Both normal and cancer cells ($3\text{-}5\times10^6$) were seeded in a 35 mm-cell cultured dishes and treated with test compounds (100 µl) for 24 h. Both the controlled and treated cells were harvested, washed with phosphate buffer saline (PBS) and pellets were lysed in a100 µl of DNA lysis buffer for 30 min. After centrifugation, the supernatants were brought to an equal volume of 1% sodium dodecyl sulphate (SDS) and incubated with 5 µg/mL RNase A at 56 °C overnight. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol: chloroform: isoamylalcohol (25:24:1). DNA fragments were separated by electrophoresis in 1% agarose gel

and visualized under UV light after staining with ethidium bromide. The gel was photographed directly on UV transilluminator (Senthilnathan et al., 2006).

### 8.5. Results and discussion

#### 8.5.1. Antioxidant activity of methanol extract and ESME of *S. racemosa* bark

The antioxidant activity of methanol extract and ethyl acetate soluble fraction of methanol extract of *S. racemosa* bark have been determined using DPPH and $H_2O_2$ free radical scavenging assay method as compared to standard drug ascorbic acid. Table 8.1 showed the $IC_{50}$ value of methanol extract and ESME on DPPH and $H_2O_2$ free radical scavenging assay method (Figure 8.2 and 8.3). ESME showed the potent antioxidant activity due to the free radical scavenging activity which gives evident of potent anticancer activity.

**Table 8.1: Antioxidant activity of methanol extract and ESME**

| Concentration (µg/ml) | % scavenging activity | | | | | |
|---|---|---|---|---|---|---|
| | DPPH free radical | | | $H_2O_2$ radical | | |
| | Methanol extract | ESME | Ascorbic acid | Methanol extract | ESME | Ascorbic acid |
| 5 | 30.26± 1.8 | 35.55± 2.8 | 45.23± 2.8 | 6.86± 1.6 | 36.27± 1.4 | 41.18± 2.1 |
| 10 | 36.53± 1.9 | 49.56± 2.6 | 55.30± 5.5 | 13.73± 3.5 | 46.08± 3.8 | 47.06± 3.3 |
| 15 | 46.62± 3.1 | 59.35± 3.1 | 64.01± 4.1 | 26.47± 3.8 | 55.88± 2.6 | 56.86± 2.4 |
| 20 | 57.49± 3.6 | 75.42± 3.5 | 74.71± 5.3 | 37.25± 2.9 | 65.69± 3.1 | 68.63± 1.5 |
| 25 | 70.81± 3.2 | 87.37± 2.9 | 88.25± 3.1 | 50.98± 2.4 | 78.43± 1.9 | 80.39± 3.9 |

*Values are expressed as mean±SEM, n=3

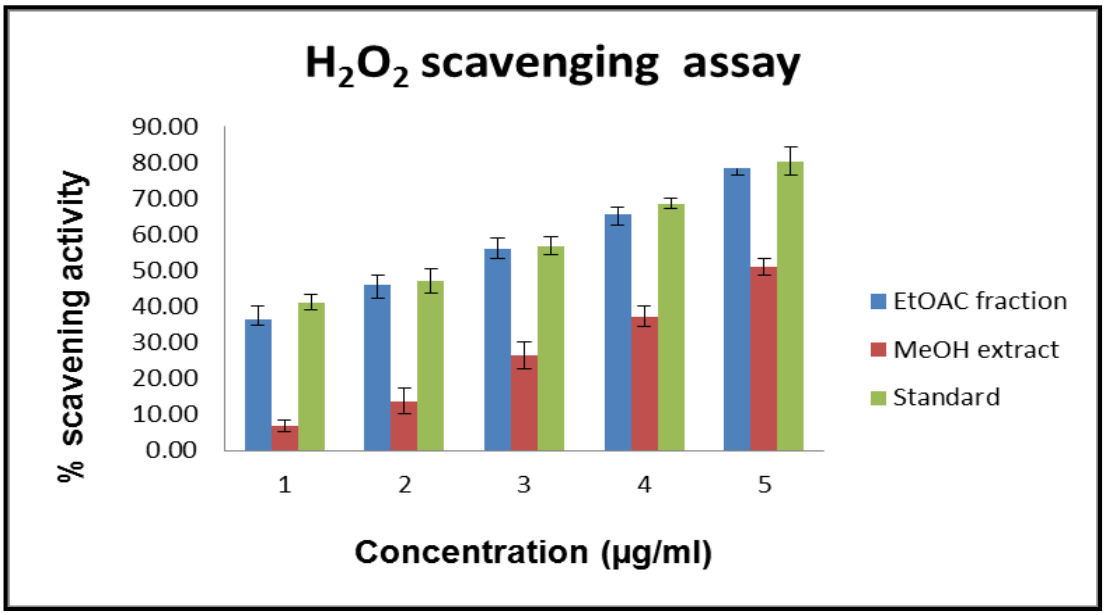


**Figure 8.2: $H_2O_2$ scavenging assay of ascorbic acid, methanol extract and ESME** (Values are expressed as mean±SEM, n=3)

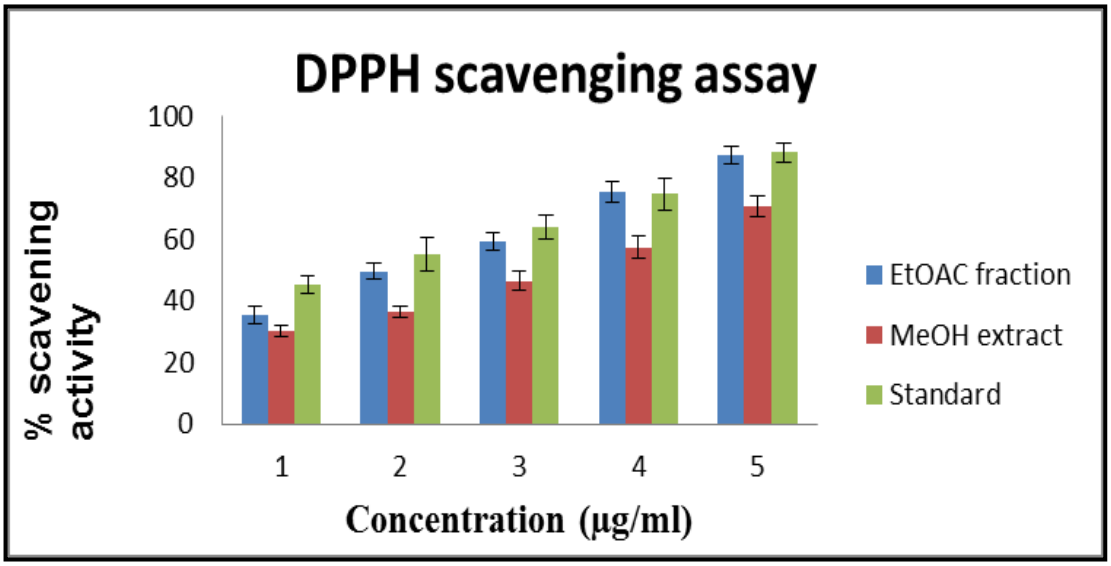


**Figure 8.3: DPPH scavenging assay of ascorbic acid, methanol extract and ESME** (Values are expressed as mean±SEM, n=3)

**Table 8.2: $IC_{50}$ value (µg/ml) of ascorbic acid, methanol extract and ESME**

| Name of extract | $IC_{50}$ value (µg/ml) | |
|---|---|---|
| | DPPH | $H_2O_2$ |
| **Methanol extract** | 16.03 | 25.26 |
| **ESME** | 10.58 | 11.89 |
| **Ascorbic acid** | 7.65 | 10.59 |

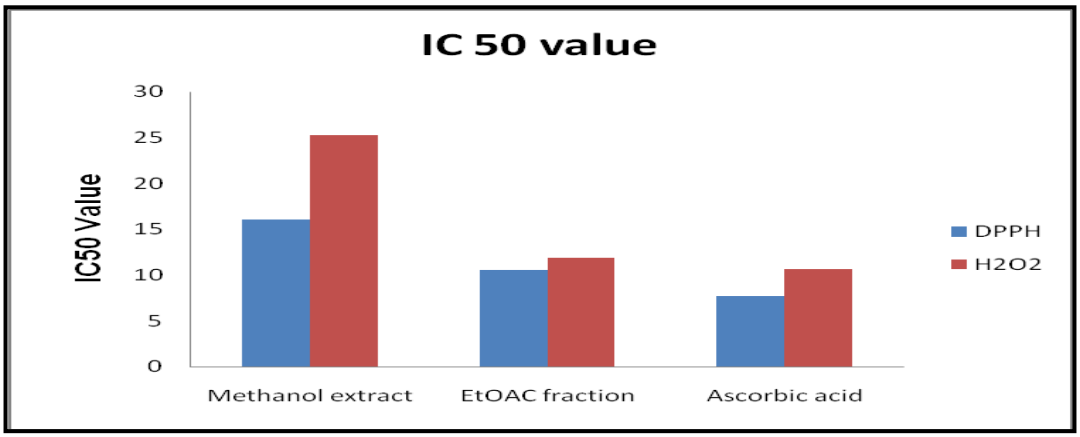


**Figure 8.4: Graphical representation of $IC_{50}$ value (µg/ml) of ascorbic acid, methanol extract and ESME**

### 8.5.2. Antioxidant activity of isolated compound 1 and 2

The antioxidant activity of isolated compounds from ethyl acetate soluble fraction of methanol extract of *S. racemosa* bark have been determined using DPPH and $H_2O_2$ free radical scavenging assay method as compared to standard drug ascorbic acid. Table 8.3 showed the $IC_{50}$ value of isolated compound 1,2 on DPPH and $H_2O_2$ free radical scavenging assay method (Figure 8.5 and 8.6). The isolated compounds from ethyl acetate soluble fraction of methanol extract of *S. racemosa* bark showed the potent antioxidant activity due to the free radical scavenging activity which gives evident of potent anticancer activity.

**Table 8.3: Antioxidant activity of ascorbic acid and isolated compound 1 and 2**

| Concentration (µg/ml) | % scavenging activity | | | | | |
|---|---|---|---|---|---|---|
| | DPPH free radical | | | $H_2O_2$ radical | | |
| | Isolated comp. 1 | Isolated comp. 2 | Ascorbic acid | Isolated comp. 1 | Isolated comp. 2 | Ascorbic acid |
| 5 | 30.95± 2.1 | 38.93± 1.5 | 45.23± 3.8 | 26.47± 2.1 | 22.55± 0.6 | 41.18± 1.5 |
| 10 | 45.96± 2.6 | 47.95± 3.3 | 55.30± 1.5 | 36.27± 3.1 | 33.33± 1.4 | 47.06± 2.8 |
| 15 | 56.45± 1.8 | 66.11± 1.9 | 64.01± 3.1 | 46.08± 0.9 | 47.06± 2.3 | 56.86± 2.8 |
| 20 | 73.66± 1.9 | 74.71± 2.7 | 74.71± 2.7 | 55.88± 1.8 | 58.82± 3.3 | 68.63± 3.4 |
| 25 | 86.46± 3.1 | 99.06± 3.4 | 88.25± 3.5 | 68.63± 2.7 | 76.47± 3.1 | 80.39± 2.4 |

*Values are expressed as mean±SEM, n=3

**Table 8.4: $IC_{50}$ value (µg/ml) of ascorbic acid and isolated compounds 1, 2**

| Name | $IC_{50}$ value (µg/ml) | |
|---|---|---|
| | DPPH | $H_2O_2$ |
| **Isolated compound 1** | 11.87 | 16.60 |
| **Isolated compound 2** | 9.77 | 15.92 |
| **Ascorbic acid** | 7.65 | 10.59 |

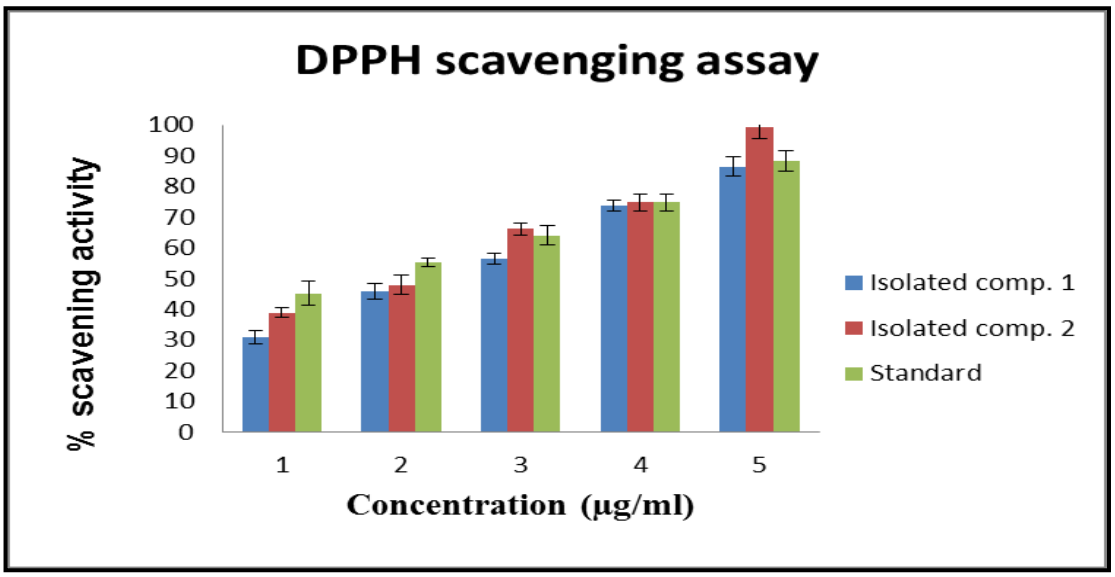


**Figure 8.5: DPPH scavenging assay of ascorbic acid and isolated compounds 1, 2 (**Values are expressed as mean±SEM, n=3**)**

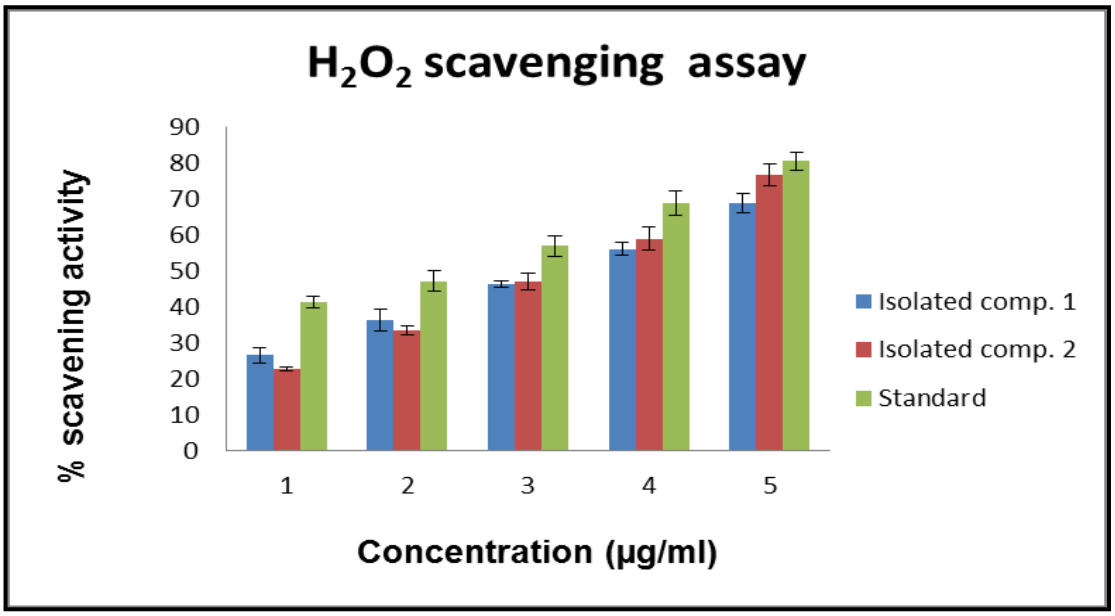


**Figure 8.6: $H_2O_2$ scavenging assay of ascorbic acid and isolated compounds 1, 2**

(Values are expressed as mean±SEM, n=3)

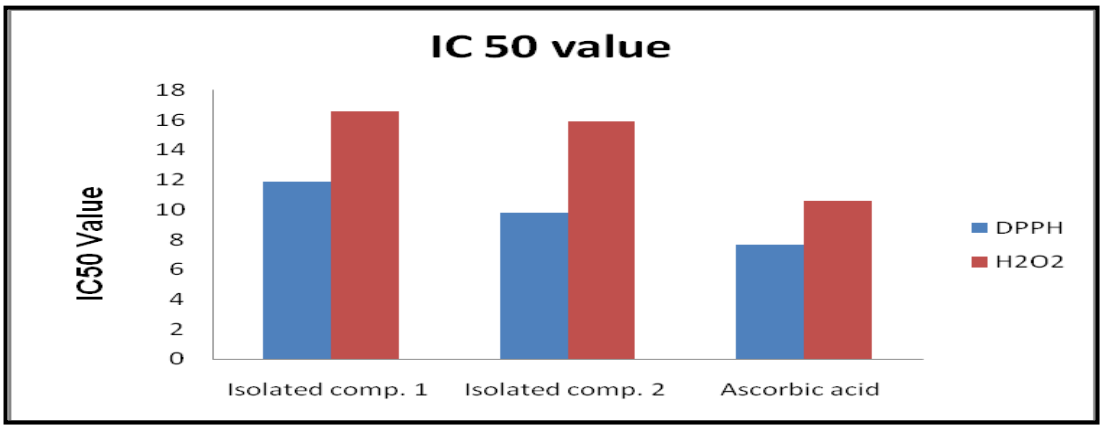


**Figure 8.7: Graphical representation of $IC_{50}$ value (µg/ml) of ascorbic acid and isolated compounds 1, 2**

Free radicals are highly reactive species produced in the body during normal metabolic function or introduced from the environment. These are the atoms or groups of atoms that have at least one unpaired electron, which makes them highly reactive. Reactive oxygen species (ROS) react with free radicals to become radical themselves. Antioxidants counteract these cellular byproducts called as free radicals, and bind them before they cause the damage. Free radicals believed to play role in different health conditions such as cancer, ageing process and atherosclerosis (Ames et al., 1993). Exogenous source of free radicals include smoking, ionizing radiation, certain pollutants, organic solvents and pesticides. As far as, ROS initiate the peroxidation of membrane lipids leading to accumulation of lipid peroxidation. Therefore, much attention has been focused on natural antioxidants which inhibit the lipid peroxidation and protect the tissue from damage due to free radicals (Gulcin et al., 2003). Ethyl acetate soluble fraction and isolated compound 1 and 2 showed the potent antioxidant activity by DPPH free radicals and hydrogen peroxide radical scavenging assay method.

### 8.5.3. DNA fragmentation study bioactives of *S. racemosa*

DNA fragmentation assay confirmed the antiproliferative effect of ESME. ESME showed the well-defined fragmentation pattern in the cell line Hep3B whereas no clear fragmentation pattern in the normal cell BRL-3A. So it indicated that ESME having very less or no effect on the normal cell. A cancer cell is a mutant human cell that differs from normal cell only in the rapid growth of cell. The DNA of rapidly multiplying, cancerous cells is more exposed as compared to normal cells. Thus, the cell DNA is one of the targets for the treatment of cancer. Breakdown of DNA molecule is one of the sign of inhibition of DNA replication, which may be due to inhibition of

topoisomerase, key enzyme in DNA replication. Figure 8.7 showed the cluster of fragmented DNA throughout the agarose gel matrix. It is well established that the susceptibility of tumor cells to apoptosis is a crucial determinant of chemotherapy efficacy (Stumm et al., 2004). Apoptosis and associated cellular events have profound effects on the progression of benign to malignant phenotype and could be an ideal target for the therapy of various cancer cells. The induction of apoptosis is a fundamental mechanism of antitumor agents, especially natural products (Kaufmann and Earnshaw, 2000; Ciucci, et al., 2006).

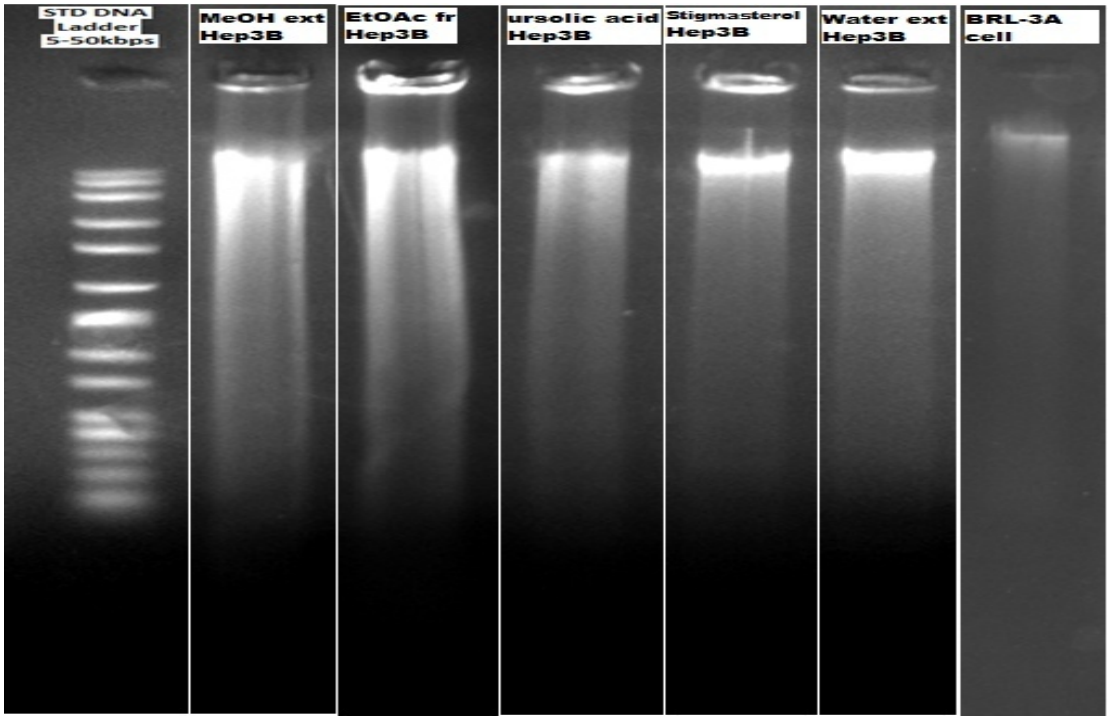


**Figure 8.8: DNA fragmentation assay bioactives of *S. racemosa***

**8.6. Conclusion**

Free radicals are the highly reactive species formed in the body during normal metabolic function or introduced from the environment. Antioxidants help to stabilize these cellular byproducts called as free radicals, by binding them before they cause the damage. Free radicals have been implicated to play a major role in various diseases as well as disorders like cancer, diabetes, arthritis, ageing process and atherosclerosis. Smoking, ionizing radiations, few pollutants, organic solvents, pesticides, etc. act as exogenous source of free radicals. ROS lead to the peroxidation of membrane lipids leading to accumulation of lipid peroxidation. Therefore, many of the recent studies are focused on natural antioxidants in order to inhibit the lipid peroxidation and protect the tissue from damage due to free radicals. ESME and isolated compounds showed

the potent antioxidant activity by DPPH free radical and hydrogen peroxide radical scavenging assay method.

Due to this evident, ESME, isolated compound 1 (stigmasterol) and 2 (ursolic acid) showed the prominent *in vitro* and *in vivo* anticancer activity against hepatocellular carcinoma (Hep3B cell) indicating the chemoprevention.

Apoptosis is a common mode of action of chemotherapeutics. Induction of apoptosis leads to identification of plant products as anticancer agents. DNA fragmentation assay confirmed the antiproliferative effect of ESME and isolated compounds. The DNA of rapidly multiplying, cancerous cells is more exposed as compared to normal cells. Thus, the cell DNA is one of the targets for the treatment of cancer. Breakdown of DNA molecule is one of the sign of inhibition of DNA replication, which may be due to inhibition of topoisomerase, key enzyme in DNA replication.

Taken together, the potential anticancer activity of ESME and isolated compounds (Stigmasterol and ursolic acid) against hepatic cancer *in vitro* and *in vivo* and its partial molecular mechanisms of activities were investigated in this experimental study first time. The results demonstrated that ESME and isolated compounds (stigmasterol and ursolic acid) have a powerful anticancer activity against liver cancer without significant effect on normal cells through the apoptotic effect and antioxidant activity. These bioactives can serve as novel therapeutic agents in the treatment or prevention of hepatocellular carcinoma.

## 8.7. REFERENCES

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