The *in vitro* studies performed were given below

- Anti-oxidant Study
- Cytotoxicity study
- Cyclooxygenase-2 inhibition assay

### ANTI-OXIDANT STUDY

*Materials and reagents required*

DPPH, distilled water and methanol were used in the analysis. Other reagents were of analytical grade. Gallic acid (Lot: 110831-200302), corilagin (Lot: 111623-200302) and rutin (Lot: 760706) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products.

*DPPH radical scavenging activity*

About 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of the different concentration (100-800 µg/ml) of ethanol extract and control (without the test compound, but with an equivalent amount of methanol) in different test tubes. The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm using a spectrophotometer.\(^{67,68}\)

\[
\text{% Inhibition} = \frac{(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}) \times 100}{\text{Abs}_{\text{Control}}}
\]
Where Abs\textsubscript{Control} is absorbance of control at time = 0 and Abs\textsubscript{Sample} is absorbance of test sample. The IC\textsubscript{50} Value for extracts was also calculated. The results are mentioned in table.

**Nitric oxide scavenging assay**

Nitric oxide scavenging assay was measured by spectrophotometric, in this method, different concentrations of extract and fraction were mixed and dissolved in methanol and incubated at 250\degree C for 30 min. After 30 min 1.5 ml of the incubation solution were removed and diluted with 1.5ml of modified Griess reagent (sulphanilic acid with naphylethylene diamine dichloride in acetic acid). The absorbance was measured at 546 nm.\textsuperscript{69,70,71,72}

\[
\% \text{ Inhibition} = \frac{[\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}]}{\text{Abs control}} \times 100
\]

**CYTOTOXICITY STUDY**

**Tumor Cell line and Culture conditions**

Human cancer cell lines, MCF-7, MDA MB-231, HeLa, HepG2, were procured from the National Centre for Cell Sciences (NCCS, Pune, India) and maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin (100 µg/mL at 37\degree C in 5% CO\textsubscript{2} environment according to standard recommended protocols.

**Assay procedure:**

The cellular toxicity of ethanolic extracts of *pleiospermium alatum* were investigated against different human cancer cell lines using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma) assay. Pale yellow MTT was converted to
purple formazan crystals by mitochondrial enzymes in viable cells. Cells at a density of 
(5x10^4 cells per well) were seeded in clear 96-well plates and incubated for 24 h. After 24  
h treatment the medium was replaced with fresh medium along with 10 µl of the MTT  
solution (5 mg ml\(^{-1}\) in PBS) was added to each well together with 200 µl of DMEM growth medium,.  
The plates were further incubated for 4 h at 37 C. Then the medium was  
removed and the resultant formazan crystals were dissolved in 200ml of dimethyl sulfoxide. The absorbance intensity of each well was read on a microplate reader at 570 nm  
with reference wavelength at 690 nm. Each extract variables were assessed through MTT  
assay in triplicate.

COX-2 INHIBITORY ACTIVITY

In vitro COX-2 inhibiting activities of the compounds have been evaluated using ‘COX (ovine) inhibitor screening assay’ kit with 96-well plates. Both ovine COX-1 and COX-2 enzymes were included. This screening assay directly measures PGF2 produced by SnCl2 reduction of COX-derived PGH2. COX-1, COX-2, initial activity tubes were prepared taking 950µl of reaction buffer, 10µl of heme and 10µl of COX-1 and COX-2 enzymes in respective tubes. Similarly, COX-1, COX-2 inhibitor tubes were prepared by adding 20µl of inhibitor.

_Pleiospermium alatum_ (ethanol extract) in each tube in addition to the above ingredients. The inhibitory assays were performed in the presence of extracts at different concentrations (10 to 80 µg/mL). The background tubes correspond to inactivated COX-1 and COX-2 enzymes obtained after keeping the tubes containing enzymes in boiling water for 3 min. along with vehicle control. Reactions were initiated by adding 10µl of
arachidonic acid in each tube and quenched with 50µl of 1M HCl. PGH2 thus formed was reduced to PGF2α by adding 100µl SnCl2. The prostaglandin produced in each well was quantified using broadly specific prostaglandin antiserum that binds with major prostaglandins and reading the 96-well plate at 405 nm. The wells of the 96-well plate showing low absorption at 405 nm indicated the low level of prostaglandins in these wells and hence the less activity of the enzyme. Therefore, the COX inhibitory activities of the compounds could be quantified from the absorption values of different wells of the 96-well plate.73,74 The indomethacin (selective COX-1 inhibitor) and celecoxib (selective COX-2 inhibitor) were used as positive controls in the study. The test extract concentration causing 50% inhibition of PGE2 release (IC50) was calculated from the concentration-inhibition response curve by regression analysis.

RESULTS AND DISCUSSION:

In vitro antioxidant study:

Effect of Pleiospermium alatum leaf in DPPH radical scavenging assay:

The following figure depicts the free radical scavenging capacity of EEPA using DPPH generated radical in in-vitro. It was observed that increase in % inhibition of free radicals has observed in increasing concentration of EEPA. The IC50 values of EEPA were found to be 510µg/ml. All the fractions was compared with the standard ascorbic acid IC50-486µg/ml.
**Effect of Pleiospermium alatum leaf in Nitric oxide scavenging assay:**

The following figure depicts the ability of EEPA to quench NO radicals was tested *in-vitro*. The results indicate that EEPA were exhibited IC$_{50}$ values of 16.5 µg/ml and the extract was compared with standard vitamin C value of 19.5µg/ml.

In *in-vitro* antioxidant studies the leaf ethanolic extract shows the significant free radical scavenging capacity. As the ethanolic extract of *Pleiospermium alatum* leaf extract showed the presence of dose dependent antioxidant activity comparable with standard antioxidant Ascorbic acid. Generally the phenolic compounds are responsible for free
radical scavenging activity, the *pleiospermium alatum* also shows the presence of phenolic compounds like Rutin and gallic acid.

**In vitro cytotoxicity study:**

*Effect of ethanolic extract of pleiospermium alatum in In vitro cytotoxicity of HeLa cells treated with different concentration*

The *in vitro* cytotoxicity of treated and non-treated cells was investigated using MTT cell viability assay against HeLa cell line. The cytotoxicity of a treated ethanolic extract of *pleiospermium alatum* with increasing concentrations was analysed. The killing effect of the ethanolic extract was more pronounced at the low concentrations and exhibited higher cytotoxicity compared. When the experimental cells were exposed to the highest concentration 7 for the period of 24 h, 60% of cells were killed by ethanolic extracts. The higher cytotoxic effect of ethanolic extracts could be attributed to the competent cellular uptake and intracellular distribution of extract compounds.

*Effect of ethanolic extract of pleiospermium alatum in In vitro cytotoxicity of HepG2 Cells treated with different concentration*

The in vitro cytotoxicity of treated and non-treated cells was investigated using MTT cell viability assay against HepG2 cell line. The cytotoxicity of a treated ethanolic extracts the increasing concentrations were analysed. The killing effect of the ethanolic extract was more pronounced at the low concentrations (5µg/ ml) and exhibited higher cytotoxicity when compared. When the experimental cells were exposed to the highest concentration 40 µg/ ml for the period of 24 h, 60% of cells were killed by ethanolic extract. The higher cytotoxic effect of ethanolic extracts could be attributed to the competent cellular uptake and intracellular distribution than ethanolic compounds.
Effect of ethanolic extract of pleiospermium alatum in In vitro cytotoxicity of Mcf-7 Cells treated with different concentration

The in vitro cytotoxicity of treated and non-treated cells was investigated using MTT cell viability assay against MCF-7 cell line. The cytotoxicity of a treated ethanolic extract with increasing concentrations was analysed. The killing effect of the ethanolic extract was less pronounced at the low concentrations (5 µg/ ml) and exhibited higher cytotoxicity at (15 µg/ ml) when compared to methanolic extract. However, the difference was still significant even at the higher concentrations (45 µg/ ml). When the experimental cells were exposed to the highest concentration 50 µg/ ml for the period of 24 h, 85% of cells were killed by ethanolic extract. The higher cytotoxic effect of ethanolic extracts could be attributed to the competent cellular uptake and intracellular distribution.

Effect of ethanolic extract of pleiospermium alatum in In vitro cytotoxicity of MDA MB-231 Cells treated with different concentration

The in vitro cytotoxicity of treated and non-treated cells was investigated using MTT cell viability assay against MDA MB-231 cell line. The cytotoxicity of a treated ethanolic extract with increasing concentrations was analysed. The killing effect of the ethanolic extract was more pronounced at the low concentrations (1 µg/ ml) and exhibited higher cytotoxicity at (10 µg/ ml) when compared to ethanolic extract. However, the difference was still significant even at the higher concentrations (10 µg/ ml). When the experimental cells were exposed to the highest concentration 10 µg/ ml for the period of 24 h, 65% of cells were killed by ethanolic extract. The higher cytotoxic effect of ethanolic extracts could be attributed to the competent cellular uptake and intracellular distribution.
IN VITRO CYTOTOXICITY STUDY OF DIFFERENT CONCENTRATIONS OF

ETHANOLIC EXTRACT OF *PLEIOSPERMIUM ALATUM*

Fig. 8.3 Effect of ethanolic extract of *pleiospermium alatum* in In vitro cytotoxicity of HeLa cells treated with different concentration

Fig. 8.4. Effect of ethanolic extract of *pleiospermium alatum* in In vitro cytotoxicity of HepG2 Cells treated with different concentration

Fig. 8.5 Effect of ethanolic extract of *pleiospermium alatum* in In vitro cytotoxicity of Mcf-7 Cells treated with different concentration

Fig. 8.6 Effect of ethanolic extract of *pleiospermium alatum* in In vitro cytotoxicity of MDA MB-231 Cells treated with different concentration

All experiments were repeated thrice and found highly reproducible.
In-vitro anticancer activity was performed on 4 different human cell lines i.e., lung, liver, breast cancer using MCF-7, MDAMB-231, Hela, HepG2 cell lines by using MTT assay. The ethanolic extracts of Pleiospermium alatum leaves were shown to possess significant cytotoxicity in different cancer cell lines. From the overall study it was cleared that Pleiospermium alatum has potential activity on MCF-7, MDAMB-231, Hela, HepG2 cell lines compared with normal cells. So this plant drug has considerable anticancer activity.

**Inhibition of COX-2 Enzymatic Activity**

Inhibition of enzymes of the arachidonic acid pathway may also contribute to the anti-inflammatory effects. The COX-2 is responsible for the biosynthesis of PGs under acute inflammatory conditions. So, the COX-2 plays an important role to be target enzyme on anti-inflammatory activity. The inhibitory effects of ethanolic extract of *Pleiospermium alatum* leaves on *in vitro* enzymatic activities were measured against COX-1/COX-2. Therefore, the ethanol extract were studied at a concentration of 10-80 μg/mL whether they inhibit cyclooxygenase-1 and -2. Interestingly, the ethanolic extract exhibited an inhibition of cyclooxygenase-1 and -2 of 6.70% and of 62.9%, respectively at highest concentration 80 μg/mL (Table 8.1).

Flavonoids are the well studied class of polyphenols as COX-2 inhibitors. Kaempferol and quercetin exhibited anti-inflammatory activities by inhibiting iNOS and COX-2 protein levels in cultured human umbilical vein endothelial cells. Luteolin and galangin, well known flavonoid molecules were studied as first dietary polyphenols as inhibitors of arachidonic acid peroxidation. After this Chrysin and luteolin were considered as potent anti-inflammatory agents as they effectively suppressed COX-2
activity. In 2008, Li and coworkers reported that a new molecule Malsudone along with known flavonoids luteolin, isoquercetin, 7-methoxyflavone and luteolin-7-O--glycoside possess potent inhibitory effect on COX-2 with moderate inhibition of COX-1. More recently, human clinical trials with COX-2 inhibitor drugs have shown similar anti-inflammatory and analgesic efficacy to traditional NSAIDs, yet with significantly less gastrotoxicity. The present investigation showed that Pleiospermium alatum leaves selectively inhibited COX-2 which may be due to the presence of flavonoid rutin and gallic acid.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (µg/ml)</th>
<th>% Inhibition</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>COX-1</td>
<td>COX-2</td>
</tr>
<tr>
<td>Ethanol extract</td>
<td>10</td>
<td>10.4±1.54**</td>
<td>4.50±0.60*</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>16.0±0.79**</td>
<td>14.3±1.06**</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>8.9±0.90**</td>
<td>39.37±2.04**</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>-</td>
<td>59.9±0.80**</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>30</td>
<td>96.8±1.08**</td>
<td>53.66±1.81**</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>30</td>
<td>13.90±0.38**</td>
<td>90.36±0.90**</td>
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</table>

The pharmacologically active extract was subjected to column chromatography for the lead molecule isolation and characterization of bioactive compound was carried out.