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In Vitro Anti Inflammatory, Anti-Oxidant and Anti-Cancer Cell Line Studies on *Mollugo Cerviana* (L.) Ser

Padmapriya. P

PG and Research Department of Biochemistry, Sengamala Thayaar Educational Trust Women's College (Autonomous), (Affiliated to Bharathidasan University), Sundarakkottai, Mannargudi, Thiruvarur (Dt), Tamil Nadu, India.

**Corresponding author's E-mail: padmapriyap724@gmail.com*

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Abstract

Our traditional treatises on medicine have shown references to number of rare medicinal herbs prescribed to treat various ailments in human. An herbal plant preferred to treat fever, inflammation, rheumatic pain and wounds prevalently in the villages of the delta districts of Tamilnadu by name Mollugo cerviana was chosen for this research. The Ethanol extract of Mollugo cerviana was analysed for its in vitro anti-inflammatory, antioxidant and anticancer activity through standard procedures. The anti-inflammatory activity of the ethanol ethanolic extracts of varying concentrations were evaluated under HRBC (Human Red Blood Cell) Membrane Stabilization and Protein denaturation studies. Diclofenac sodium a nonsterioidal anti-inflammatory drug was the reference standard. The results revealed a concentration dependent increase in the percentage of membrane stabilization activity with increase in concentration of the test extract and a concentration dependent inhibition of protein (albumin) denaturation activity. Antioxidant studies revealed a significant free radical scavenging activity by the extract upon evaluation by DPPH free radical scavenging assay, nitric oxide assay and hydrogen peroxide radical scavenging activity assay. The studies on liver cancer cell lines using MTT assay revealed the anticancer activity exhibited by the ethanol extract of Mollugo cerviana. The studies as a whole reveals the anti-inflammatory, antioxidant and anticancer activity possessed by the herbal extract of Mollugo cerviana which invites attention to proceed with further research towards development of novel herbal drugs from Mollugo cerviana to treat inflammation and cancer.

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1. Introduction

Medicinal plants are considered to be the "back bone" of traditional medicine, more than 3.3 billion people in the less developed countries utilize medicinal plants on a regular basis (Davidson-Hunt, 2000). Medicinal plants have now become the subject matter of research in most of the pharmaceutical and biotechnology industries. The factors such as easy availability, affordability, minimal side effects besides scientifically proven therapeutic activity through clinical trials have raised the demand for the drugs derived from plant resources. Pharmaceutical industries are now in active search of alternative sources of medicine such as antibiotics, anti-diabetics, antimicrobials, and antioxidants derived from plants. The significant therapeutic activity of herbal medicinal preparations with minimal side effects has been demonstrated in numerous clinical trials. It is pertinent here to point out, novel drug formulations arising out of inventive research in plant species deserves grant of patents at par with the modern synthetic drugs. Natural antioxidants occur in all parts of plants which includes carotenoids, vitamins, phenols, flavonoids, dietary glutathione, and endogenous metabolites (Khopde *et al.,* 2001). These are compounds with free radical scavenging capacity and they exert protection against free radical damage. Medicinal plants are commonly rich in phenolic compounds such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins. These compounds have multiple biological effects including antioxidant activity (Packer *et al*., 1999). Antioxidant based drug formulations are used for the prevention and treatment of diseases like atherosclerosis, stroke, diabetes, alzheimer's disease and cancer (Devasagayam *et al*., 2004).

Mollugo cerviana is a herbal plant belongs to the family Molluginaceae. This plant is known by the name “Parpadakam” in Tamil and called as ‘thirakkodi’ by the local community. In the villages of the district of Thanjavur in the state of Tamilnadu this plant is conventionally used in treating wounds and inflammation as a poultice. *Mollugo cerviana* is also used as stomachic, and antiseptic. The roots of this plant are also used in treating gout and rheumatism. It is also used as febrifuge in treating fever. Among dozens of existing species in Molluginaceae family, *Mollugo cerviana* (L) was selected for this research based on its wide medicinal usage and references. The biologically active constituents of this plant species are responsible for its medicinal activity. The literature reviews on *Mollugo cerviana* discloses the pharmacological importance of this plant species and also reveals the scope for further research. The present study was aimed to evaluate the antioxidant and anti-inflammatory activity of the plant extract of *Mollugo cerviana* through *in vitro* studies and evaluation of the anticancer activity through cell line studies.

2. Materials And Methods

Collection, authentication and processing

The healthy plants of *Mollugo cerviana* were collected from Orathanadu Taluk of Thanjavur district in the state of Tamilnadu and got authenticated by John Britto Rapinat Herbarium at St.Joseph’s college, Tiruchirapalli. The collected plants were examined carefully and the infected portions if any were removed. Healthy plants were spread out and shade dried at room temperature for about 10 days and ground in to fine powder.

Preparation of the Ethanol extract:

From the above prepared powdered plant material of *Mollugo cerviana,* 10 g was weighed in to a conical flask and added with 100 ml of ethanol, shaken well and kept for 24 Hours. After that period the supernatant was collected and evaporated to remove the solvent. The crude extract was collected and stored at 4°C. After making suitable dilution with the solvents, the required concentrations of the extracts were prepared for evaluating the anti-inflammatory and antioxidant activity through separate procedures.

Evaluation of anti-inflammatory activity

HRBC (Human Red Blood Cell) Membrane Stabilization Method (Gandhisan *et al*, 1991):

Blood sample was collected from healthy human volunteers (who has not taken any NSAIDS (Non-steroidal anti-inflammatory drugs) for two weeks prior to the experiment) and the collected blood sample was mixed with equal volume of sterilized Alsever’s solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid & 0.42% sodium chloride in water). The above solution was centrifuged at 3000 rpm and the packed cells were washed with isosaline and a 10% v/v suspension was made with isosaline. This HRBC suspension was used for the estimation of anti-inflammatory activity. Different concentrations of the extract (100 µg/ml, 200 µg/ml, 400 µg/ml and 600 µg/ml) and the standard drug (Diclofenac sodium) were prepared and added with 1ml of phosphate buffer, 2 ml of hyposaline and 0.5 ml of HRBC suspension. Control was prepared by omitting the extracts. The mixtures were incubated at 37°C for 30 minutes and then centrifuged at 3000 rpm for 20 minutes. Spectrophotometrically at 560 nm the hemoglobin content of the supernatant liquid was measured. The percentage of HRBC membrane stabilization or protection was calculated using the Formula,

Percentage of Protection = 100 – [(OD of Test / OD of Control) x 100]

Protein denaturation method (Mizushima *et al*., 1968 and Sakat *et al.,* 2010)

The reaction mixture consists of 0.2 ml of egg albumin (from fresh hen's egg), 2.8 ml of phosphate buffered saline (pH 6.4) and 2 ml of different concentrations of the plant extract (100 µg/ml, 200 µg/ml, 400 µg/ml and 600µg/ml). Double-distilled water was served as control. The mixtures were then incubated at 37°C ± 2°C in BOD (Biological Oxygen Demand) incubator for 15 minutes and then heated at 70°C for 5 minutes. The mixtures were then cooled and their absorbance was measured spectrophotometrically at 660 nm. Varying concentrations of Diclofenac sodium (100-600 µg/ml) was the reference standard. The percentage inhibition of protein denaturation was calculated by using the formula,

Percentage inhibition = (Absorbance of Control – Absorbance of Sample) x 100

Absorbance of Control

Evaluation of antioxidant activity

DPPH assay (Mensor *et al.,* 2001).

1 ml of 0.3 mM solution of (DPPH) 2, 2 -diphenyl-1- picryl hydrazyl hydrate in methanol was added to varying concentrations of 10µg/ml - 50 µg /ml of the plant extract and the mixtures were allowed to remain at room temperature for 30 minutes. Ascorbic acid was the reference standard. The DPPH solution with methanol (without plant extract) served as the control. After 30 minutes of incubation, the discolouration of the purple colour was measured spectrophotometrically at 518nm. The free radical scavenging activity was calculated as follows,

$$\text{Scavenging activity \%} = \frac{(\text{OD of sample} - \text{OD of control})}{\text{OD of control}} \times 100$$

Assay of Hydrogen peroxide scavenging activity (Ruch *et al.,* 1989)

The Hydrogen peroxide (40mM) solution was prepared in phosphate buffer. Various concentrations of 10µg/ml- 50 µg /ml of the plant extracts were added to the above prepared Hydrogen peroxide solution in phosphate buffer. After 10 minutes the absorbance of the reaction mixture was recorded spectrophotometrically at 230 nm against a blank solution containing phosphate buffer without Hydrogen peroxide. Ascorbic acid was used as standard. The Hydrogen peroxide scavenging activity of the plant extracts was calculated as,

$$\text{\% scavenging activity} = \frac{(Ac - At)}{Ac} \times 100$$

Ac - Absorbance of control

At - Absorbance of the test

Assay of Nitric oxide scavenging activity (Green *et al*., 1982).

Varying concentrations of 10µg/ml-50 µg /ml of the plant extracts were incubated at 25°C for 2 hours with 3.0 ml of 10 mM sodium nitroprusside in phosphate buffered saline (pH 7.4). To the incubated mixture 0.5 ml of Griess reagent was added. Ascrobic acid was used as standard. The absorbance of the chromophore formed was measured spectrophotometrically at 546 nm against blank. Percentage inhibition of the nitrite ions generated is calculated.

$$\text{\% scavenging activity} = \frac{(Ac - At)}{Ac} \times 100$$

Ac - Absorbance of control

At - Absorbance of the test

Anticancer activity

Preparation of extract:

The ethanol extract of *Mollugo cerviana* was prepared by soaking 20 grams of the powdered plant material in 100 ml of ethanol for 24 hrs. The extract was then filtered using Whatman no.1 filter paper (125 mm × 100 circles). The filtrate was air dried at 28ºC and reconstituted in 20% Dimethylsulphoxide (DMSO) solution) and used for further experiments.

Maintenance of cell culture: From the Cell repository of (NCCS) National Centre for Cell Sciences, Pune the Liver cancer (HepG-2) cell lines were procured. Dulbecco`s Modified Eagle Media (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) was used for maintaining the cell line. To prevent bacterial contamination Penicillin (100 U/ml) and streptomycin (100 µg/ml) were added to the medium. The medium with cell lines was maintained at 37°C in a humidified environment with 5% CO_2.

MTT assay: The cytotoxicity of *Mollugo cerviana* on HepG-2 cell lines was determined by the method of Mosmann, (1983).

Cell viability assay:

HepG-2 viable cells were counted using haemocytometer and then diluted in DMEM medium to a density of 1×10^4 cells/ml. The viable HepG-2 cells were then seeded in 96 well plates and incubated for 24 hours.

Then the seeded HepG-2 cells in the well were treated with control and the test containing varying concentration of 100 µg/ml to 500 µg/ml of the plant extract.

Then the HepG-2 cells were incubated at 37°C in a humidified 95% air and 5% CO_2 incubator for 24 hours. After incubation, the extract containing cells washed with fresh culture medium and the MTT (5 mg/ml in PBS) dye was added to each well, followed by incubation for another 4 hours at 37°C. The purple precipitated formazan formed was dissolved in 100µl of concentrated DMSO and the absorbance was measured at 540 nm using a multi-well plate reader. The results were expressed at percentage of stable cells with respect to the control. The half maximal inhibitory concentration (IC_{50}) values were calculated and the optimum doses were analysed at different time period.

$$\text{Inhibitory of cell proliferation (\%)} = \frac{\text{Mean absorbence of the control} - \text{Mean absorbence of the sample}}{\text{Mean absorbence of the control}} \text{ X } 10$$

The IC_{50} values were determined from the dose responsive curve where inhibition of 50% cytotoxicity was compared to control cells. All experiments were performed at triplicate.

Measurement of apoptotic induction using Acridine orange/Ethidium bromide (AO/EB) dual staining method (Baskic *et al*., 2006)

Viable HepG-2 cells were seeded at 5 x 10^4 cells/well in a 6 well plate and incubated for 24 hours. Then treated with the test extract at a concentration of 300 µg/ml for 24 hours, the cells were detached, washed with cold PBS and then stained with a mixture of AO (100µg ml−1)/ EB (100µg ml−1) ratio (1:1) at room temperature for 5 minutes. The stained cells were observed through fluorescence microscope at 20x magnification. At the end of treatment, the cells were collected and washed three times with PBS. The plates were then stained with acridine orange/ethidium bromide (AO/EB 1:1 ratio; 100µg/ml) for 5 minutes and examined immediately under fluorescent microscope at 40x magnification. The number of cells showing feature of apoptosis was counted as a function of the total number of cells present in the field.

3. Results and Discussion

Anti-inflammatory activity

HRBC Membrane Stabilization Method

The results in Table 1 revealed a concentration dependent increase in the percentage of membrane stabilizing activity with increase in concentration of the extract of *Mollugo cerviana*. The maximum activity is noticed at the concentration of 600 (µg/ml) of the plant extract.

Protein denaturation method

The results tabulated in Table 2 reveal a concentration dependent inhibition of protein (albumin) denaturation by *Mollugo cerviana*. Diclofenac sodium at the concentration range of 100 to 600 µg/ml was used as reference standard. The maximum denaturation activity is noticed at the concentration of 600 µg/ml of the plant extract.

Gandhisan *et al*., 1991 reported that the percentage inhibition of membrane stabilization of HRBCs is thus the direct measure of anti-inflammatory response due to the similarity in membranes of HRBC and lysosomes. Shenoy *et al,* 2010 reviewed that the HRBC method was selected for the *in vitro* evaluation of anti-inflammatory property because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may as well stabilize lysosomal membranes.

Most biological proteins lose their biological function when denatured and hence ability to denature proteins is considered as a well-documented cause of inflammation. The inflammatory drugs (salicylic acid, phenylbutazoneetc) have shown dose dependent ability to thermally induced protein denaturation by Mizushima, 1968.

Table 1: HRBC Membrane Stabilizing activity of the ethanol extract of *Mollugo cerviana*

Concentration in (µg/ml)	100	200	400	600
% of Membrane stabilisation of Ethanol extract (Mean ± SEM) (n=3)	12.85 ± 0.49	32.6 ± 0.98	66.33 ± 0.63	80.66 ± 1.17
% of Membrane stabilisation of Standard (Mean ± SEM) (n=3)	18.27±0.22	43.93 ± 1.28	76.33 ± 1.13	94.5 ± 1.20

Table 2: Protein denaturation activity of the ethanol extract of *Mollugo cerviana*

Concentration in (µg/ml)	100	200	400	600
% of Protein denaturation of Ethanol extract (Mean ± SEM) (n=3)	10.53 ± 0.49	29.23 ± 0.61	65.03 ± 0.97	75.76 ± 0.77
% of Protein denaturation of Standard (Mean ± SEM) (n=3)	16.56± 0.81	35.46± 0.58	71± 0.84	88.3± 0.88

In vitro Antioxidant activity

Table 3. DPPH scavenging activity of Ethanol extract of *Mollugo cerviana (L.)* (Values expressed as Mean ± SD) (n=3)

S. NO	Name of the Extract	DPPH scavenging activity (%)				
		10µg/ml	20µg/ml	30µg/ml	40µg/ml	50µg/ml
1	Ethanol	20.11 ± 1.41	39.36 ± 1.52	52.81 ± 1.69	70.27 ± 2.12	83.72 ± 2.21
2	Ascorbic acid	24.73 ± 1.84	48.64 ± 3.39	64.64 ± 3.80	77.23 ± 5.12	92.09 ± 4.36

Table 4. Hydrogen peroxides radical scavenging activity of Ethanol extract of *Mollugo cerviana (L.)* (Values expressed as Mean ± SD) (n=3)

S. NO	Name of the Extract	Hydrogen peroxides radical scavenging activity (%)				
		10µg/ml	20µg/ml	30µg/ml	40µg/ml	50µg/ml
1	Ethanol	24.18 ± 1.92	41.34 ± 2.03	53.23 ± 1.84	61.92 ± 2.91	68.81 ± 1.89
2	Ascorbic acid	38.73 ± 0.97	52.85 ± 1.85	64.19 ± 3.95	71.89 ± 2.98	78.18 ± 2.85

Table 5. Nitric Oxide radical scavenging activity of Ethanol extract of *Mollugo cerviana (L.)* (Values expressed as Mean ± SD) (n=3)

S. NO	Name of the Extract	Nitric Oxide radical scavenging activity (%)				
		10µg/ml	20µg/ml	30µg/ml	40µg/ml	50µg/ml
1	Ethanol	31.81 ± 0.84	42.61 ± 1.79	48.52 ± 1.93	53.46 ± 2.14	60.37 ± 1.46
2	Ascorbic acid	38.55 ± 0.94	48.06 ± 1.82	54.32 ± 1.93	59.42 ± 1.75	65.21 ± 3.04

The percentage of DPPH radical scavenging activity, hydrogen peroxide radical scavenging activity and NO (Nitric oxide) radical scavenging activity of varying concentrations (10µg, 20 µg, 30 µg, 40 µg and 50 µg per ml) of ethanol extract of *Mollugo cerviana* and the radical scavenging activity of Ascorbic acid as standard were tabulated in Table 3, 4 and 5.

Burns, J *et al*., 2001 and Diaz, M.N *et al*., 1997 stated that the antioxidants scavenge free radicals from the body cells, and prevent or reduce the damage caused by oxidation. Reactive oxygen species play important roles in degenerative or pathological processes, such as aging, cancer, coronary heart disease, alzheimer's disease. The human body has a complex system of natural enzymatic and non- enzymatic antioxidant defenses which counteract the harmful effects of free radicals and other oxidants by Badarinath *et al*., 2010.

Valko *et al.,* 2007 reported that the free radicals are atoms or groups of atoms with an odd number of electrons (highly reactive oxygen species) and can be formed through exogenous chemicals or endogenous metabolic processes in the human body. Free radicals are formed naturally in the body and

play an important role in many normal cellular processes. Free radicals are responsible for causing a large number of diseases including cancer, cardiovascular disease, neural disorders, Alzheimer's disease, mild cognitive impairment, Parkinson's disease, alcohol induced liver disease, ulcerative colitis, aging and atherosclerosis by Velavan,2011. Wei SD *et al*., 2010 stated that the phenolic compounds are important plant antioxidants which exhibited considerable scavenging activity against radicals. Thus, antioxidant capacity of a sample can be attributed mainly to its phenolic compounds. Antioxidants may be of great benefit in improving the quality of life by preventing or postponing the onset of degenerative diseases. In addition, they have a potential for substantial savings in the cost of health care delivery. Various methods are used to investigate the antioxidant property of samples like diets, plant extracts, commercial antioxidants etc.by Nur Alam *et al*., 2013.

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides by Mishra *et al*., 2010. Antioxidants protect cells against damage caused by molecules known as free radicals. The antioxidant effects of plant extracts are mainly due to the presence of phenolic compounds such as flavonoids, phenolic acids, tannins and phenolic diterpenes (Polterait, 1997). In the present work, the ethanol extract of *Mollugo cerviana* has shown significant free radical scavenging activity upon evaluation by DPPH free radical scavenging assay, nitric oxide assay and hydrogen peroxide radical scavenging activity. The evaluation reveals the presence of phenolic, alkaloidal and flavonoidal contents in *Mollugo cerviana* which are considered to be the sources of natural antioxidants.

In vitro Anticancer activity

Table 6. Effect of varying concentrations of Ethanol extract of *Mollugo cerviana* on cell viability of HepG-2 (Liver cancer) Cell lines.

S.No	Concentration (µg/ml)	Cell viability (24Hours)
1.	100	81.50 ± 5.406
2.	200	65.83 ± 5.925
3.	300	47.40 ± 6.828
4.	400	28.88 ± 4.605
5.	500	18.00 ± 2.391
6.	Control	100

Values expressed in mean ± SD

The percentage of cell viability was measured through MTT assay. HepG2 cell lines were treated with increasing concentrations of ethanol extract (100 μg/ml - 500 μg/ml) of *Mollugo cerviana* and cell viability was assayed after 24 hours of treatment and the values are tabulated in Table 6. The study revealed a dose dependent decrease in cell viability with increasing concentration of the extract. The sensitivity of the ethanol extract was more prominent in higher dosage. The graphical representation of the decrease in the percentage cell viability with increase in concentration of the extract is shown in Figure 1.

Figure 2 is the Photomicrograph (10X) which represents morphological changes in liver cancer cells such as shrinkage and detachment induced by *Mollugo cerviana* (300 µg/ml) for 24 hours compared with control. Control has revealed normal intact cell morphology and the images were capture by light microscope.

Figure 3 is the fluorescence microscopic images of the HepG2 cells treated with 300 µg/ml ethanol extract of *Mollugo cerviana* at 24 hours and stained with dual dye AO/EB. The control image shows green fluorescence that indicates live cells without apoptosis. The *Mollugo cerviana* treated cells shows yellow and orange fluorescence which indicates early and late apoptotic cell death respectively showing condensed or fragmented nuclei and necrotic cells.

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries as per World Health Organization (WHO) update. According to Newman and Cragg., (2016) about 60% of anticancer agents have been originated from the natural sources. The MTT/MTS *in vitro* cell proliferation assay is one of the most widely used assays for evaluating preliminary anticancer activity of both synthetic derivatives and natural products. The highly reliable, colorimetric based assay is readily performed on a wide range of cell lines. (McCauley *et al*., 2013).

Figure 1.

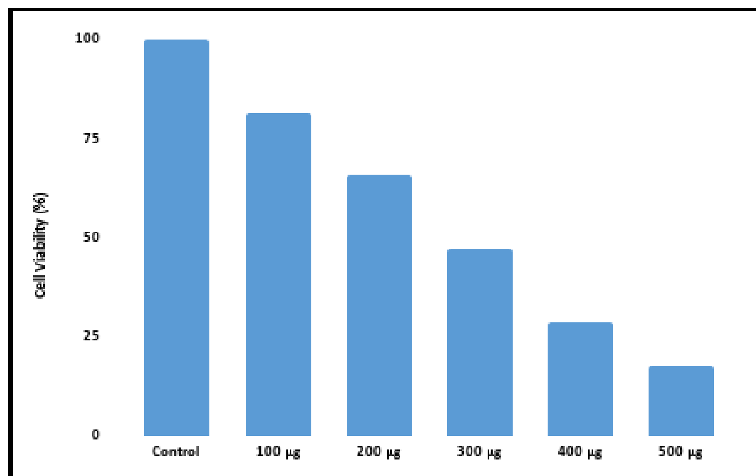
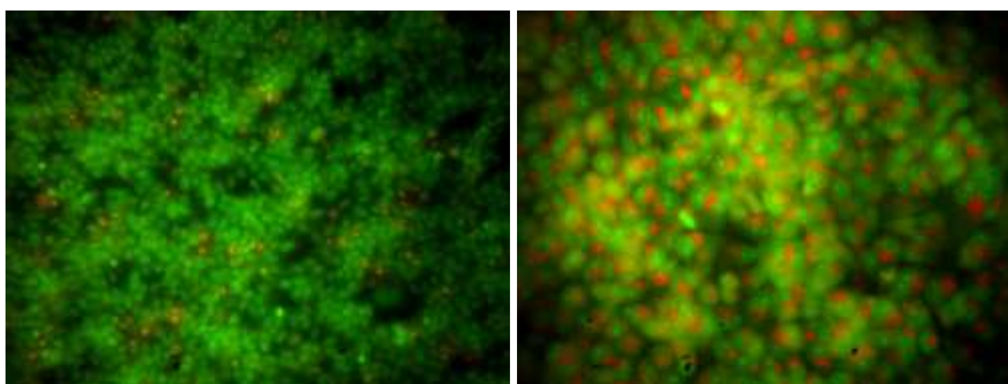


Fig 2. Effect of *Mollugo cerviana* on the apoptotic incidence in liver cancer (HepG -2) cells. Control (300 µg/ml) Test

Fig 3. Effect of *Mollugo cerviana* on the apoptotic incidence in liver cancer (HepG -2) cells (Fluorescence microscopy).

Control **Test (300 µg/ml)**



4. Conclusion

The *in vitro* anti-inflammatory activity studies by HRBC Membrane Stabilization Method and Protein denaturation method revealed a significant anti-inflammatory activity exhibited by the plant extracts of *Mollugo cerviana* and a concentration dependent increase in the percentage of activity with increase in concentration of the extract is also noticed. The *in vitro* antioxidant activity studies on the ethanol extract of *Mollugo cerviana* (L) revealed a significant free radical scavenging activity exhibited by the extract upon evaluation by DPPH free radical scavenging assay, nitric oxide assay and hydrogen peroxide radical scavenging activity assay. The *in vitro* anticancer activity of varying concentrations of ethanol extract of *Mollugo cerviana* (L) on liver cancer cell lines using MTT assay revealed a significant anticancer activity shown by the test extract. The Photomicrograph and fluorescence microscopic studies further confirm the morphological changes and apoptotic cell death of the cancer cells. The studies carried out and summarized above discloses the wide spectrum of medicinal activity exhibited by the whole plant extract of *Mollugo cerviana* and the presence of number of biologically active compounds contributing to its activity. The study as a whole reveals the preliminary anticancer,

antioxidant and anti-inflammatory activity exhibited by the herbal extract of *Mollugo cerviana* which invites further research towards development of novel herbal drugs to treat cancer and inflammation.

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