# Inhibition of inflammatory mediators by alcoholic extract of leaves of *Glycosmis pentaphylla* Retz.DC - An *in vitro* analysis.

Short running title:

**ANTI-INFLAMMATORY STUDIES ON *GLYCOSMIS PENTAPHYLLA* RETZ.DC.**

**Keywords**: *Glycosmis pentaphylla*, Anti-inflammatory, cyclooxygenase, lipoxygenase, myeloperoxidase, cellular nitrate, antioxidant.

**Authors: \*1Bindu AR, 2NA Aleykutty.**

1. Dept. of pharmaceutical sciences, Centre for professional and advanced studies, Cheruvandur campus, Kottayam, Kerala. Pin-686631. Ph.0481-2539494.

E-mail:- sindhuchandrasen@gmail.com

1. Caritas college of Pharmacy, Thellakom, Kottayam. Kerala. India.

**Abstract**

Mechanism of anti-inflammatory activity of the medicinal plants is of great importance. *Glycosmis pentaphylla* (F.Rutaceae) is an odorous herb found all over India. *G. pentaphylla* is used in indigenous medicine for cough, rheumatism, anaemia and jaundice. Juice of leaves is used in fever, liver complaints and as vermifuge. A paste of leaves with ginger is applied in eczema and skin infections. Total ethanolic extracts of the leaves of *G. pentaphylla* were subjected to preliminary phytochemical screening. Carotenoids present in the alcoholic extract of *G. pentaphylla* were estimated. Anti-inflammatory activity was screened by inhibition of protein denaturation, proteinase inhibition. Studies were carried out to assess the inhibition of cycloxygenase, 5-lipoxygenase, myeloperoxidase and cellular nitrite levels using lipopolysaccharide stimulated RAW 264.7 cell lines. Antioxidant studies on the extract were carried out by iron chelating, and total antioxidant assays. Results suggest that the anti-inflammatory activity shown by the extract was mainly by dual cycloxygenase and 5-lipoxygenase inhibition which may be due to the antioxidant components present. Dual cox/5-lox inhibition suggests the gastrointestinal safety of the extract as an anti-inflammatory agent. The result thus confirms the traditional claim and points further investigations for bioactivity guided isolation.

**Keywords**: *Glycosmis pentaphylla*, Anti-inflammatory, dual cox/5-lox, myeloperoxidase, cellular nitrate, antioxidant.

**Introduction**

Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemicals or microbiological agents. Inflammation is body’s response to inactivate or destroy the invading organisms, remove irritants and set stage for tissue repair. Conventional anti-inflammatory drugs such as steroidal and nonsteroidal anti-inflammatory drugs were used in the treatment of most of the acute and chronic pain and inflammatory disorders including rheumatoid arthritis. However, long term use of these agents may produce serious adverse effects. The leaves of *Glycosmis pentaphylla* was used by the local people for relieving from pain and inflammatory conditions, during the epidemic of chikungunya in kerala. Considering these points, anti-inflammatory screening of leaves of *Glycosmis pentaphylla*, were carried out.

*Glycosmis pentaphylla* (F.Rutaceae) is an odorous herb found all over India. *G. pentaphylla* is used in indigenous medicine for cough, rheumatism, anaemia and jaundice. Juice of leaves is used in fever, liver complaints and as vermifuge. A paste of leaves with ginger is applied in eczema and skin infections1. Leaves contain a glycoside, glycosmin-slightly bitter to taste. Literature review of the plant leaves contain tannin, phlobaphene and traces of salicin. Dried leaves were found to contain furoquinoline bases, kokusaginine and skimmianine. Other alkaloids isolated are glycosin, arborine, arborinine. Essential oil and phytoalexins were isolated from the leaves2,3. Leaves contain arborinol, isoarborinol, myricylalcohol, glycorine, glycosmicine, glycosmine, glycosminine, arborinone, faagarine, skimmianine and mupamine4.

**Methodology**

**Collection and extraction of the plant.**

Leaves of *Glycosmis pentaphylla* was collected from Ettumanoor, Kerala and identified by Mr. Rojimon.P.Thomas, CMS college, Kottayam and deposited voucher specimen (no.859) at their herbarium for future reference. The leaves were dried at room temperature, powdered and extracted with ethyl alcohol to get total ethanolic extract.

**Preliminary phytochemical studies**

Qualitative analysis of the total ethanolic extracts of *Glycosmis pentaphylla* were carried out according to the method described5 by Kokate, 2007.

**Estimation of carotenoids**

The experiment was carried out in the dark to avoid photolysis of carotenoids once the saponification was complete. 0.025g extract was homogenized and saponified with 2.5 ml of 12 % alcoholic potassium hydroxide in a water bath at 60o C for 30 minutes. The saponified extract was treated with petroleum ether to collect the carotenoids. Small amount of anhydrous sodium sulphate was added to the petroleum ether extract to remove excess moisture. The final volume of petroleum ether extract was noted. Absorbance was measured at 450 nm against blank6. $Amount of total carotenoids=\frac{A450 X volume of sample X 100 X 4}{ Weight of the sample}$. All results presented are mean ±SEM and were analysed in three replications.

**Anti-inflammatory activity-Protein Denaturation Method**

A solution of 0.2% Bovine Serum Albumin (BSA) was prepared in Tris buffer saline and pH was adjusted to 6.8 using glacial acetic acid. Stock solution of 10,000 µg/ml of extract was prepared by using methanol as a solvent. 50 µl of each extract was transferred using 1 ml micropipette. 5 ml of 0.2% BSA was added to 50 µl of extract in tube. The control consists of 5 ml of 0.2%W/V BSA solution with 50 µl methanol and standard is 10 mg/ml of Prednisolone. The tubes were heated at 72o C for 5 minutes and cooled for 10 minutes. The absorbance of the solution was determined by Spectrophotometer at a wavelength of 660 nm7. The percentage inhibition (denaturation of protein) was determined on a percentage basis relative to the control using the following formula. $Percentage inhibition=\frac{absorbance of control-absorbance of test}{absorbance of control} x100.$ All results presented are mean (±SEM) and were analysed in three replications.

**Proteinase inhibitory assay**

The reaction mixture contained 0.06 mg trypsin, 1 ml 25mM Tris Hydrochloride buffer (pH 7.4) and 1 ml of different concentration of the extract. The mixture were incubated at 37o C for 5 minutes. Then 1 ml of 0.8 % w/v Casein was added. Then the mixtures were incubated for an additional 20 minutes. 2 ml of 70 % w/v perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged8. Absorbance of supernatant was read at 280 nm. Percentage inhibition was calculated as in protein denaturation.

**Anti-inflammatory activity studies using RAW 264.7 cell lines**

RAW 264.7 cells were grown to 60 % confluence followed by activation with 1 µL lipopolysaccharide (LPS) (1µg/mL). LPS stimulated RAW cells were exposed with different concentration of extract solution. Diclofenac sodium, a standard anti-inflammatory drug in varying concentration corresponding to the sample was also added and incubated for 24 hrs.

**Cycloxygenase activity**

The COX activity was assayed by the method of Walker and Gierse9. The cell lysate was incubated in Tris-HCl buffer (pH 8), glutathione and hemoglobin (5 mM/L) for 1 minute at 25°C. The reaction was initiated by the addition of arachidonic acid 200 mM/L and terminated after 20 minutes incubation at 37°C, by the addition of 10% trichloroacetic acid in 1 N HCl. After the centrifugal separation and the addition of 1% thiobarbiturate, COX activity was determined by reading absorbance at 632 nm. % inhibition of the enzyme was calculated as, in protein denaturation.

**5- Lipoxygenase (5-LOX) activity**

The determination of 5-LOX activity was as per Axelrod et al.10. Briefly, the reaction mixture contained Tris-HCl buffer (pH 7.4), 50 µL of cell lysate, and sodium linoleate (200 µL). The 5-LOX activity was monitored as an increase of absorbance at 234 nm, which reflects the formation of 5-hydroxyeicosatetraenoic acid. % inhibition of the enzyme was calculated as, in protein denaturation.

**Myleoperoxidase (MPO) activity**

Cell lysate was homogenized in a solution containing 50 mM potassium phosphate buffer and 0.57% hexadecyltrimethyl ammonium bromide. Homogenized mixture was frozen in liquid nitrogen and thawed. After freeze thawing 3 times, the samples were centrifuged at 2000 g for 30 minutes at 4°C, and supernatant was assayed for MPO activity. MPO in the sample was activated by the addition of 50 mM phosphate buffer (pH 6) containing 1.67 mg/mL guaiacol and 0.0005 % H2O2 11. The change in absorbance at 460 nm was measured. MPO activity was presented as units per mL of cell lysate. One unit of MPO activity was defined as that degrading 1 µM of peroxide per minute at 25°C.

**Cellular nitrite levels**

Cell lysate (0.5 mL) was added to (0.1 mL) sulfosalicylic acid, vortexed for 30 minutes and centrifuged at 5000 rpm for 15 minutes. The protein-free supernatant was used for nitrite estimation. To 200 μL of the supernatant, 30 μL of 10% NaOH and 300 μL of Tris-HCl buffer was added. Contents were mixed well and 530 μL of Griess reagent was added, incubated in the dark for 10-15 minutes, and the absorbance was read at 540 nm against a Griess reagent12.

**Cytotoxicity studies**

1 mg extract was added to 1 mL of DMEM and dissolved completely by cyclomixer, filtered through 0.22 µm millipore syringe filter to ensure sterility. 100 µL of each concentration (6.25-100 µg/mL) was added to wells in triplicate and incubated at 37ºC in a humidified 5% CO2 incubator.

**Cytotoxicity study by direct microscopic observation:**

Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indications of cytotoxicity.

 **Cytotoxic study by MTT assay:**

15 mg of MTT (Sigma, M-5655) was reconstituted in 3 mL PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30 µL of reconstituted MTT solution was added to all test and control wells, then incubated at 37ºC in a humidified 5% CO2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100 µL of MTT solubilization solution (DMSO) was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured at a wavelength of 570 nm13. The percentage of growth inhibition was calculated using the formula:

Percentage$ viability=\frac{Mean OD of samples X 100}{ Mean OD of control group}$

**Antioxidant Activity Screening- Iron chelating assay**

Different concentrations of the extract, o-phenanthroline and 2M ferric chloride solution were mixed at ambient temperature for 10 minutes. After incubation, the absorbance of solution was measured at 510 nm against corresponding blank solution14. Percentage inhibition was calculated as, in protein denaturation.

**Total Antioxidant Assay**

0.3 ml of extract (1mg/ml) was mixed with 3 ml of reagent solution containing 1ml each of ammonium molybdate (4mM), Sodium Phosphate (28mM) and sulphuric acid (0.6M). The samples were incubated for 90 minutes at 90oC. Absorbance of the green phosphomolybdenum complex was measured at 695 nm. The reducing capacity of the extracts has been expressed on the ascorbic acid equivalent which is obtained from the standard graph15.

**Statistical Analysis**: Results were expressed as mean±SEM of three independent experiments. IC50 values were found out by using sigmaplot 12.2 version software.

**Result and Discussion**

Percentage yield of the extract of *G. pentaphylla* leaf was found to be 9.6 % w/w. Preliminary phytochemical studies showed the presence of alkaloids, steroids, phenolics, flavanoids, amino acids and cardiac glycosides in the alcoholic extract of *G. pentaphylla* leaves. Estimation of carotenoids resulted as 7.33 µg/100 gm of extract.

***In vitro* Anti-inflammatory screening by Protein denaturation method**

Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of proteins is a well-documented cause of inflammation16. The extract was effective in inhibiting heat induced albumin denaturation. IC 50 value of the extract was found to be 400.75µg/ml and that of standard was 72.11µg/ml (Table 1).

**Table 1: Inhibition of Protein denaturation and proteinase inhibition**

|  |  |  |  |
| --- | --- | --- | --- |
| **Extract** | **Concn.** | **Protein denaturation** | **Proteinase inhibition** |
| **Abs±SEM** | **%inhibition** | **Abs±SEM** | **%inhibition** |
| StandardPrednisolone | Control | 0.8884±0.0014 |  - | 0.5663±0.0012 |  **-** |
| 50 | 0.4816±0.0017 | 45.79 | 0.3207±0.0017 | 43.36 |
| 100 | 0.3910±0.0026 | 56.00 | 0.2550±0.0025 | 54.97 |
| 200 | 0.3517±0.0037 | 60.42 | 0.2247±0.0020 | 60.32 |
| 300 | 0.2630±0.0023 | 70.40 | 0.2190±0.0023 | 61.32 |
| 400 | 0.2220±0.0026 | 75.01 | 0.2000±0.0030 | 64.68 |
| *Glycosmis**pentaphylla*extract | Control | 0.1170±0.0017 |  - | 0.5663±0.0012 |  - |
| 50 |  - |  - | 0.3670±0.0032 | 35.23 |
| 100 | 0.101±0.0015 | 13.67 | 0.3260±0.0028 | 42.47 |
| 200 | 0.071±0.0024 | 39.31 | 0.2630±0.0023 | 53.59 |
| 300 | 0.063±0.0015 | 46.15 | 0.2256±0.0034 | 60.17 |
| 400 | 0.060±0.0011 | 48.71 | 0.1520±0.0034 | 73.17 |
| 500 | 0.052±0.0011 | 55.65 |  - |  - |

Values are mean absorbance (Abs.)±SEM. n=3

**Proteinase Inhibition method**

In proteinase inhibitory action neutrophils are known to be a rich source of serine proteinase and are localized at lysosomes. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors17. IC 50 value of the extract was found to be 182.23 µg/ml, and that of standard was 76.07 µg/ml (Table 1.).

**Cycloxygenase activity**

IC 50 value of the extract was found to be 7.36 µg/ml and that of standard was 2.84 µg/ml (Table 2). The inhibition of cycloxygenase pathway of arachidonic acid metabolism is a prime pharmacological target. Metabolism catalysed by COX enzymes gives rise to prostaglandins and thromboxanes that are responsible for the characteristic inflammatory symptoms18.

**5-Lipoxygenase activity**

IC 50 value of the extract was found to be 33.57 µg/ml and that of standard was 12.14 µg/ml (Table 2). 5-Lipoxygenase is the major isozyme of LOX pathway, which is involved in the biosynthesis of Leukotrienes (LTs), one of the potent inflammatory mediators19. Dual inhibition of COX/ 5-LOX inhibition is exhibited by the extract. 5-LOX inhibitory activity shown by the extract explains its use in skin disorders and asthma20.

**Table 2. Cycloxygenase and 5-lipoxygenase inhibitory activity studies**

|  |  |  |  |
| --- | --- | --- | --- |
| **Extracts** | **Concn.****(µg/mL)** |  **Cycloxygenase inhibition** | **5-Lipoxygenase inhibition** |
| **Abs.±SEM** | **%Inhibition** | **Abs.±SEM** | **%Inhibition** |
| Standard(Diclofenac sodium) | Control | 0.1937±0.0004 | **-** | 0.1500±0.0011 | **-** |
| 3.125 | 0.0993±0.0004 | 48.73 |  - | - |
| 6.25 | 0.0613±0.0009 | 68.35 | 0.0804±0.0009 | 46.4 |
| 12.5 | 0.0577±0.0006 | 70.21 | 0.0643±0.0020 | 57.13 |
| 25 | 0.0538±0.0002 | 72.22 | 0.0495±0.0002 | 67.00 |
| 50 | 0.0420±0.0002 | 78.32 | 0.0372±0.0008 | 75.20 |
| 100 | 0.0212±0.0006 | 89.06 | 0.0136±0.0006 | 90.93 |
| GP(*G.**pentaphylla*) | 6.25 | 0.0981±0.0078 | 49.35 | 0.0977±0.0013 | 34.87 |
| 12.5 | 0.0886±0.0004 | 54.25 | 0.0836±0.0011 | 44.27 |
| 25 | 0.0682±0.0005 | 64.79 | 0.0783±0.0007 | 47.80 |
| 50 | 0.0623±0.0008 | 67.83 | 0.0743±0.0006 | 50.47 |
| 100 | 0.0548±0.0010 | 71.70 | 0.0659±0.0001 | 56.07 |

 Values are mean aborbance (Abs.)±SEM. n=3.

**Myeloperoxidase estimation**

Accumulation of neutrophils is a prominent feature of a number of inflammatory diseases of the skin. Myeloperoxidase, a naturally occurring constituent of neutrophils, is used as a marker for tissue neutrophil content11. The extract showed decrease in enzyme level with increase in concentration (Table 3). This indicates dose dependent MPO inhibition by the extract and the standard.

**Cellular nitrite levels**

Capacity of cells to release NO in response to LPS was measured in terms of nitrites and results showed that the extracts inhibited nitrites in a dose dependent manner (Table 3). Nitrites and nitrates are products of nitric oxide. Cellular nitrite level is measured as a marker of NO. Excessive production of NO could potentially lead to tissue damage and activation of pro-inflammatory mediators. Decreasing NO production under inflammatory conditions is an important step in decreasing the threats of oxidative and nitrative stress as well as damage of inflammation21. Thus, the inhibition of COX, 5-LOX, MPO and decreased nitrite level exerted by the extracts is cellular mechanisms of their anti-inflammation.This indicates that the extract acts by multiple mode of mechanism as an anti-inflammatory agent.

**Table 3. Estimation of myeloperoxidase and cellular nitrite by *G.pentaphylla* leaves.**

|  |  |  |  |
| --- | --- | --- | --- |
| **Extracts** | **Concn.****(µg/mL)** |  **Myeloperoxidase estimation** | **Cellular nitrite level** |
| **Abs. ±SEM** | **Enzyme****U/mL** | **Abs. ±SEM** | **Nitrite con. (µg)**  |
| Standard(Diclofenac sodium) | control | 0.0595±0.0002 | **-** | 0.1537±0.0460 | **-** |
| 6.25 | 0.0340±0.0002 | 0.0452 | 0.0878±0.0012 | 434.94 |
| 12.5 | 0.0254±0.0006 | 0.0338 | 0.0650±0.0024 | 322.08 |
| 25 | 0.0216±0.0005 | 0.0287 | 0.0486±0.0005 | 240.57 |
| 50 | 0.0165±0.0006 | 0.0219 | 0.0337±0.0010 | 166.98 |
| 100 | 0.0081±0.0006 | 0.0107 | 0.0230±0.0010 | 114.18 |
| GP(*Glycosmis**pentaphylla*) | 6.25 | 0.0360±0.0014 | 0.0479 | 0.0950±0.0016 | 470.41 |
| 12.5 | 0.0317±0.0004 | 0.0422 | 0.0906±0.0007 | 448.80 |
| 25 | 0.0279±0.0002 | 0.0371 | 0.0817±0.0008 | 404.41 |
| 50 | 0.0223±0.0004 | 0.0297 | 0.0728±0.0011 | 360.52 |
| 100 | 0.0187±0.0006 | 0.0248 | 0.0623±0.0009 | 308.71 |

Values are mean absorbance (Abs.)±SEM. n=3

**Cytotoxicity studies by MTT assay on *G. pentaphylla* using RAW 264.7 cell lines**

The cytotoxic study showed that the extract of *G.pentaphylla* exhibited 89% viability at the concentration of 6.25 mcg/mL.

Photographs obtained from microscopic studies are given in figure 1. The photographs showed that there was no change in the morphology of cells indicating that the extracts are not cytotoxic.

**Figure 1.** Photographs of cytotoxic study of GP using RAW 264.7 cell lines. GP-ethanolic extract of leaves of *G.pentaphylla*. Concentrations used are 6.25, 12.5, 25, 50, 100 mcg.

**Antioxidant screening – Iron chelating Assay**

**Table 4. Percentage inhibition by Iron chelating Assay**

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **Concn.(µg/ml)** | **Absorbance±SEM** | **% Inhibition** |
| StdPrednisolone | Control | 0.022±0.002 | - |
| 5 | 0.030±0.002 | 26.66 |
| 10 | 0.041±0.002 | 46.34 |
| 15 | 0.074±0.002 | 70.27 |
| 20 | 0.140±0.003 | 84.28 |
| *Glycosmis* *pentaphylla* extract | 25 | 0.0480±0.0005 | 14.58 |
| 50 | 0.0596±0.0044 | 31.2 |
| 100 | 0.0956±0.0040 | 57.11 |
| 250 | 0.1163±0.0053 | 64.74 |
| 500 | 0.1333±0.0064 | 69.24 |

Values are mean absorbance (Abs.)±SEM. n=3

Iron is essential for life because it is required for oxygen transport, respiration and activity of many enzymes. However, iron is an extremely reactive metal and catalyzes oxidative changes in lipids, proteins and other cellular components22, 23. It causes lipid peroxidation through the Fenton and Haber-weiss reaction and decomposes the lipid hydroxide into peroxyl and Alkoxyl radicals that can perpetuate the chain reactions24.

**Total Antioxidant assay**

The study showed that the extract contains 7.34 mg/g extract of ascorbic acid equivalents. The method evaluates both water soluble and fat soluble antioxidants25. The potential of phosphomolybdenum reducing ability of the extracts may be due to the electron transfer or hydrogen ion transfer by the bioactive compounds26. As the extracts are able to prevent the ROS formation, which is obtained by antioxidant studies may be the reason behind the MPO inhibition.

**Conclusion**

The *in vitro* anti-inflammatory studies conducted on the ethanolic extract of *Glycosmis pentaphylla* indicates that it is a potential anti-inflammatory agent, thus confirming the traditional claim and explaining the mechanism of inflammation.

**Acknowledgement**

Authors acknowledge the Biogenix research laboratory, Trivandrum in providing the facilities for conducting the cell line studies.

**References**

1. Riyazuddin Ahmed, Sadananda Choudhary, Ingrid Vajczikova & piet A Leclercq. Analysis of essential oil from bark, leaves and seeds of *Glycosmis pentaphylla*, *J Essent Oil Res*, 12 (4) (2000) 471-474.
2. Pacher T, Bacher M, Hofer o & Greger H. Stress induced carbazole phytoalexins in *Glycosmis* species, *Phytochemistry,* 58 (1) (2001) 129-135.
3. HyuThai Tran, Laurent Severac, Nguyen & Xuan Phuong. Chemical components of the essential oil extracted from branches with leaves and flowers of *Glycosmis pentaphylla* (Retz), *Corr.Tap Duoc Hoc*, 10000 (2001)12-14.
4. Neeraj Tandon & Madhu Sharma. Reviews on Indian medicinal plants. (ICMR, New Delhi) 2009.
5. Kokate CK, Purohit AP & Gokhale. Pharmacognosy. (Nirali Prakashan, Pune) 2002.
6. Zakaria, H. et al. Use of reversed phase HPLC analysis for the determination of provitamin A Carotenes in tomatoes, *Journal of chromatography A*, 176 (1979)109–17.
7. R Ramalingam, B Bindu Madhavi, A RavinderNath, N Duganath, E Udaya Sri & David Banji. *In vitro* anti denaturation and antibacterial activities of *Zizyphus oenoplia*, *Der Pharmacia Lettre*, 2(1) (2010), 87-93.
8. Sachin S Sakat, Archana R Juvekar & Manoj N Gambhire. *In vitro* antioxidant and anti inflammatory activity of methanol extract of *Oxalis corniculata* Linn, *Int J of Pharm Pharm Sci*, 2(1) (2010) 146-155.
9. Walker, M.C.G. & Gierse, K. *In vitro* assays for cycloxygenase activity and inhibitor characterization, *Methods of molecular biology*. 644 (2010) 131–44.
10. Axelrod B. & TM Cheesebrough L.S. Lipoxygenase from soyabean, *Methods enzymol*,71 (1981) 441–5.
11. Peter P Bradley, Dennis APriebat, Robert D Christensen & Gerald Rothstein.P. Measurement of cutaneous inflammation: Estimation of neutrophil content with an enzyme marker. *J investig dermatol.* 78(3) (1982) 206–209.
12. Lepoivre M, Chenais B, Yapo A, Lemaire G, Thelander L, Tenu JP. Alterations of ribonucleotide reductase activity following induction of the nitrite-generating pathway in adenocarcinoma cells*, J. biol. Chem*.,265(24) (1990) 14143–14149.
13. Masoko, V.N. & Peter. *In vitro* assessment of cytotoxicity, antioxidant, and anti-inflammatory activities of *Ricinus communis* (Euphorbiaceae) leaf extracts, *Evid Based Complement Alternat Med*, 2014, Article ID 625961, 8 pages, doi:10.1155/2014/625961.
14. Rana, M.G. et al. *In vitro* antioxidant and free radical scavenging studies of alcoholic extract of *Medicago sativa* L, *Rom. J. biol. - Plant biol*, 55(1) (2010) 15–22.
15. Ujjwal Kaushik, Prachiti Lachake, C S Shreedhara & H N Aswana Ram. *In vitro* antioxidant activity of extracts of Avipattikarchurna, *Pharmacologyonline*. 3 (2009)581-589.
16. Leelaprakash, G. & Mohan Dass, S. *In vitro* anti-inflammatory activity of methanol extract of *Enicostemma axillare*, *Int J Drug Dev. Res.*, 3(3) (2011) 189–196.
17. Das SN & Chatterjee S. Long term toxicity study of ART-400, *Indian Indg Medicine*, 16(2) (1995)117-123.
18. Marnett, L. et al. Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition, *J Biol. Chem*, 274(33), (1999) 22903–22906.
19. Chandrasekharan, C. et al. Dual inhibitory effect of *Glycyrrhiza glabra* (Gutgard) on COX and LOX products, *Phytomedicine*, 18 (2011) 278–284.
20. Whitman S,Gezginci M, Timmermann BN & HolmanTR. Structure activity relationship studies of nordihydroguaiaretic acid inhibitors toward soybean, 12-human, 15-human lipoxygenase, *J Med Chem*, 45 (2002) 2659-2661.
21. Daycem, K. et al. Anti-inflammatory and acetylcholinesterase inhibition activities of *Globularia alypum, J Med Bioeng*, 2(4) (2013) 232–237.
22. Aliyu, A.B.I. et al. Free radical scavenging and total antioxidant capacity of root extracts of *Anchomanes difformis* ENGL. (Araceae), *Acta Poloniae Pharmaceutica ñ Drug Research,*, 70(1) (2013)115–121.
23. Kandhasamy, S. & Kang, S.C. Free radical scavenging activity from different extracts of leaves of *Bauhinia vahlii* Wight & Arn, *Saudi J Biol Sci*, 20 (2013) 319–325.
24. St. Angelo AJ. Lipids oxidation in food, ACS symposium series. In *American Chemical Society, Washington, DC*. American Chemical Society, Washington, DC 500 (1998) 54.
25. Smith, C., Halliwell, B. & Arouma, O. Protection by albumin against the pro oxidation actions of phenolic dietary components, *Food Chem Toxicol*, 30 (1992) 483–489.
26. Halliwell, B. & Gutteridge, J. Role of free radicals and catalytic metal ions in human diseases; an overview, *Methods enzymol*, 186 (1990) 1–185.