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RESEARCH ARTICLE

ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF BIOACTIVE EXTRACTS OF A POLYPORE MUSHROOM, *FOMITOPSIS PINICOLA* (SW.:FR.) P. KARST, FROM KASHMIR.

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Abstract

Fomitopsis pinicola, a polypore mushroom, was investigated for its medicinal properties. In this study, antioxidant and anti-inflammatory activities of the mushroom were examined. The fruiting bodies of the mushroom were defatted with petroleum ether, and then extracted with ethyl acetate (EA) and ethanol (ETH). Both the extracts were found to possess radical scavenging activities against $DPPH^{\cdot}$, $ABTS^{\cdot+}$, nitric oxide and $OH^{\cdot}$ radicals. The extracts also reduced Fe^{3+} ions and inhibited lipid peroxidation. The extracts were further evaluated for their anti-inflammatory activity against carrageenan and formalin induced acute and chronic edema respectively in mouse model. An oral dose of 500 mg/Kg body weight of EA extract inhibited both acute and chronic inflammation by 52.77 and 46.44% and the ETH extract at the same dose inhibited 33.33 and 30.44% respectively. EA extract also inhibited carrageenan induced leukocyte infiltration in to the peritoneal cavity of mice. Both EA and ETH extracts lowered ethanol induced liver lipid peroxidation in mice nearly by 60%. Anti-inflammatory activity against croton oil induced skin inflammation showed that topical application of 20 mg of EA reduced skin inflammation and leukocyte infiltration. The extract also lowered lipid peroxidation resulted from croton oil application by almost 60%. In Cylooxygenase (COX) activity assay, EA extract inhibited the activity by 55.85%. Major chemical constituents of the mushroom are steroids, alkaloids, coumarins, phenols, tannins, saponins and carbohydrates. The study thus demonstrated that EA and ETH extracts of *F. pinicola* possessed significant antioxidant and anti-inflammatory activities.

Introduction:-

Reactive oxygen species (ROS) are both beneficial and harmful to biological systems. They are useful as antimicrobial and signaling agents at levels at which their activity can be controlled by the antioxidant defense mechanism of the body. The level of ROS sometimes overshoots the buffering capacity of defensive antioxidant mechanism of the body resulting in oxidative stress and poising the system for potential damage. Oxidative stress,

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thus, forms the root cause of many diseases including various inflammatory conditions, diabetes, cardiovascular diseases, degenerative diseases and cancer (Pham-Huy et al., 2008). Since oxidative stress is an important etiologic factor for many diseases, the potential of antioxidants as prophylactic agents against a large number of diseases can be envisaged (Langseth, 1995). This realization has resulted in the use of antioxidants both as drugs and dietary supplements.

Antioxidant compounds are primarily of natural origin and plants, fungi, bacteria and animals are the main sources. A large number of mushrooms are known to possess excellent antioxidant properties (Ajith and Janardhanan, 2007). Species of *Ganoderma, Phellinus, Pleurotus, Morchella* etc. are excellent examples. Species of *Fomitopsis* are wood rotting polypore mushrooms widely distributed in temperate regions and have been considered valuable in traditional medicine. *Fomitopsis officinalis* had been mentioned by Dioscurides for its use against tuberculosis (Stamets, 2005). The most renowned member of the genus, *F. pinicola*, has been reported in various traditional treatments. Aqueous paste of the fruiting bodies was used as styptic and emetic for purification (Hobbs, 1986). In King's American dispensatory, *F. pinicola* is recommended for persistent and intermittent fevers, chronic diarrhea, dysentery, periodic neuralgia, nervous headache, excessive urination, jaundice, chills and fevers in consumptive patients. As homeopathic medicine *F. pinicola* is recommended for fevers with head-ache, yellow tongue coating, nausea, epigastric weakness, and constipation (Hobbs, 1986). Considering the importance in traditional medicine, we chose this mushroom for our ongoing studies on various medicinal properties and we report here the antioxidant and anti-inflammatory activities of this mushroom.

Materials and methods:-

Animals

Male Swiss albino mice weighing 25±3 g and 6-8 weeks old were purchased from Small Animal Breeding Station, Agricultural University, Mannuthy, Thrissur, Kerala, India. They were maintained under standard environmental conditions and fed with standard mice feed (Sai Durga Feeds & Food, Bangalore, India) and water *ad libitum*. Animal experiments were conducted as per the guidelines of Committee for the Purpose of Control and supervision of Experiments on Animals (Reg. No. 149/1999/ CPCSEA) and with the approval of the Institutional Animal Ethics Committee (IAEC).

Preparation of extract

Fruiting bodies of *F. pinicola* were dried at 50 °C and powdered. One hundred gram (100 g) samples of powdered material were extracted with petroleum ether and then with ethyl acetate and ethanol for 8-10 hours. The extracts were filtered through Whatman No.1 filter paper. The filtrate was concentrated by a rotary vacuum evaporator and solvents were completely evaporated at 40ºC. The yields of EA and ETH were 2.71 and 4.25 % respectively.

Estimation of In Vitro Antioxidant Activity

$DPPH^{\cdot}$ radical scavenging assay

A stable free radical $DPPH^{\cdot}$ (2,2-diphenyl-1-picryl hydrazyl) was used for the experiment. Various concentrations of the extracts were added to freshly prepared DPPH in methanol solution (150 µM) and kept under dim light. After 20 minutes, absorbance of the test samples was measured at 517 nm against methanol. The $DPPH^{\cdot}$ radical scavenging activity of the extracts was calculated by comparing the absorbance value of the test sample with that of control. The reduction in absorbance was expressed in percentage of radicals scavenged (Aquino et al, 2001).

Ferric reducing antioxidant power (FRAP) assay

Acetate buffer (300 mM, P^H- 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl and ferric chloride (20 mM) were mixed in a ratio of 10:1:1. The extracts were added to the reaction mixture at different concentrations and incubated in dark for 30 minutes. The blue color developed was measured at 593 nm. The reducing power of the extracts was calculated in terms of number of ferric ions reduced from a standard graph plotted using various concentrations of $FeSO_4$. $7H_2O$ (10-100 µM) (Benzie and Strain, 1996).

$ABTS^{\cdot+}$ radical scavenging assay

In this assay the extracts were tested for their $ABTS^{\cdot+}$ (2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging activity. Ammonium persulphate (2.45 mM, final concentration) was reacted with ABTS (7 mM) for more than 16 hrs in dark at room temperature. ABTS and persulphate reacted to form $ABTS^{\cdot+}$ radical. The OD of $ABTS^{\cdot+}$ radical solution was adjusted to an absorbance of 0.75 at 734 nm using ethanol. The extracts at various concentrations were added to 2 ml of $ABTS^{\cdot+}$ radical solution. The decrease in the absorbance was measured against

ethanol by a spectrophotometer after 6 minutes of initial mixing and expressed as percentage of reduction by comparing to the control. The reaction mixture without extracts served as control (Pino et al, 2005).

Nitric oxide scavenging activity

Freshly prepared 10 mM solution of sodium nitroprusside in PBS (pH 7.4) was incubated with and without the extracts in a final volume of 3 ml at 25ºC for 150 min. After incubation, 0.5 ml of the reaction solution was mixed with 0.5 ml of Griess reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride). The absorbance of the chromophore was read immediately at 546 nm against reagent blank. The nitric oxide scavenging activity was determined by comparing the absorbance of the reaction mixture with the extracts with that of control (Daniel, 1991).

Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by Fenton's reaction. Hydroxyl radical scavenging activity of extracts was determined by the method of Ohkawa et al (1979). The scavenging activity was expressed as percentage of inhibition by comparing absorbance of reaction mixture containing the extract with that of control (Elizabeth and Rao, 1990).

Inhibition of lipid peroxidation

Lipid peroxidation was induced by Fe^{2+} - ascorbate system in the rat brain homogenate in the presence and absence of various concentrations of the extracts in a final volume of 0.5ml. The thiobarbituric acid reducing substance (TBARS) formed was measured by the method of Ohkawa et al. 1979 (Hamid et al., 2009).

Activity against ethanol induced liver lipid peroxidation in vivo

Eight groups of male Swiss albino mice were used for the study. One group was kept as ethanol untreated control. The remaining seven groups were orally administered with absolute ethanol (0.1mL) twice with an interval of 24 hours between the two ethanol administrations. One hour before of each ethanol administration, EA and ETH extracts were orally administered in three different doses (500, 250 and 100 mg/Kg b.wt). The remaining one group was kept as ethanol control. After 24 hours of second ethanol treatment, the animals were sacrificed, liver was removed and assayed for lipid peroxidation inhibitory activity.

Determination of anti-inflammatory activity

Acute model

Eight groups of male Swiss albino mice (body weight - 25±3 g) with six animals in each group were used for the study. Group 1 was maintained as untreated control which received no drug and the standard drug diclofenac was orally administered (at 10 mg/Kg b. wt) to group 2. EA and ETH extracts were given orally to all the animals of the remaining groups at 500, 250 and 100 mg/Kg b. wt doses separately.

One hour after extract administration, edema was induced in the hind leg paw of all the animals of all the groups by subcutaneous injection of 20 µL of 1% carrageenan in 0.01% carboxymethyl cellulose. Paw volume of all the animals was measured before carrageenan injection using Plythismometer and it was taken as initial paw volume. After three hours of edema induction, paw volume was again measured and noted as final paw volume. The inhibition of paw edema was quantified by comparing the difference in paw volume of treated animals with that of untreated animals and expressed as percentage of reduction using the formula, $((UG - TG)/UG) \times 100$.

Where UG is mean value of net paw thickness of the untreated group, TG is mean value of net paw thickness of treated group.

Chronic model

The procedure was same as that of acute inflammation model except the inflammation was induced by injecting 2% formalin and the final paw thickness was measured on the seventh day after administration of drug/extract once daily for 6 consecutive days.

Inhibition of leukocyte migration induced by carrageenan

Animals were divided into six groups with six animals in each group. EA extract was administered orally to three groups at various doses (500, 250 and 100 mg/kg b. wt). Diclofinac (10 mg/Kg b. wt) was orally administered to the fourth group as standard drug. Fifth and sixth group were given saline and acted as carrageenan and normal controls

respectively. After one hour of drug/ extract administration, animals of five of six groups were injected with 0.25 mL of 0.75% carrageenan (i.p). After four hours of carrageenan administration, the mice were sacrificed and 10 mL of PBS was injected into the peritoneal cavity and the fluid was drawn back immediately from the peritoneal cavity and the leukocytes were counted (Al Amin et al., 2012).

Anti-inflammatory activity against croton oil induced inflammation

Hairs from the dorsal side of male Swiss albino mice were removed. The animals were grouped into seven groups, each group comprised six animals. After 48 hours of hair removal, 0.1 mL of 50% croton oil in acetone (v/v) was applied twice on the hair removed area of skin of all the animals of six groups with an interval of 24 hours between two applications. Of the croton oil applied groups, three groups received three different doses (20, 10 and 2 mg) of topical application of EA in 0.1 mL acetone thirty minutes before of each croton oil application. Fourth group received standard drug diclofenac (1 mg) instead of extract. The fifth group did not receive any kind of medication and served as control. The sixth and seventh groups were kept as acetone and croton untreated controls. One hour after the second application of croton oil, animals were sacrificed, the treated area of skin was removed and sections of equal area (9 mm diameter) were taken from the skin of all the groups. These sections were used to determine the mass, thickness and level of lipid peroxidation among the different groups.

In vitro COX inhibition assay

The COX activity was assayed by the method (Walker and Gierse, 2010). Briefly, RAW 264.7 cells were grown to 60% confluency followed by activation with 1 µL lipopolysaccharide (LPS; 1 µg/mL). LPS stimulated RAW cells were exposed to different concentrations of the extract (100, 50 and 25 µg/mL) and incubated for 24 hours. After incubation, 100 µL cell lysate was incubated in 1 mL reaction mixture containing Tris – HCl buffer (p^H-8), glutathione 5mM and hemoglobin 5mM for 1 minute at 25°C. The reaction was initiated by the addition of arachidonic acid 200 mM, incubated for 20 minutes at 37°C and terminated by the addition of 200 µL of 10% trichloroacetic acid in 1 N HCl. Centrifuged and the supernatant was treated with 200 µL of 1% thiobarbiturate and the tubes were boiled for 20 minutes. The reaction mixture was then cooled, centrifuged and absorbance was taken at 632 nm. Diclofenac was used as standard and the reaction mixture without any extract was served as control.
COX activity inhibition was expressed in percentage using the following relation

$$\% \text{ of inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Histopathological examination

Skin samples of all the groups were fixed in 10% formalin and then embedded in paraffin. Microtome sections were taken from the samples and stained with hematoxilin-oesin. The stained sections were observed under a light microscope.

Preliminary phytochemical analysis

Phytochemical analysis of the extracts was carried out by standard methods (Tiwari et al, 2010)

Statistical analysis

All the values were expressed as mean ± sd. Statistical significance compared to control was tested by One-way ANOVA. If theP value is <0.05, the result was considered significant.

Results:-

In vitro antioxidant activity

DPPH· radical scavenging activity

ETH and EA extracts were found highly effective to scavenge DPPH· radicals. EA at a concentration of 500 µg/mL scavenged 72.22% of the radicals and that for ETH extract was 63.89% (fig 1).

FRAP assay

EA extract showed weak ferric ion reducing power than ETH extract. EA extract reduced 60 nanomoles of ferric ions at a concentration of 300 µg/mL while that for ETH was 92.48 nanomoles at the same concentration (fig 2).

$ABTS^{\cdot+}$ radical scavenging activity
EA and ETH extracts scavenged nearly 90% of the $ABTS^{\cdot+}$ radicals at 250 µg/mL concentration (fig 3).

Nitric oxide radical scavenging activity
EA and ETH extracts were equally effective in scavenging nitric oxide radicals. At a concentration of 300 µg/mL both the extracts scavenged about 40% nitric oxide radicals (fig 4).

Hydroxyl radical scavenging activity
Hydroxyl radical scavenging activity of EA and ETH extracts was found to be less prominent compared to the scavenging activities against other radicals. The higher activity was shown by ETH extract, scavenging 47% of hydroxyl radicals while that of EA extract was 30.69% (fig 5).

Lipid peroxidation inhibition assay
EA and ETH were able to prevent or interfere with lipid peroxidation reaction. Both the extracts showed about 25% lipid peroxidation inhibition activity at 1000 µg/mL concentration (fig 6)

Inhibition of ethanol induced lipid peroxidation in liver
Ethanol administration caused extended lipid peroxidation in liver compared to control. Lipid peroxidation was found to be more than 80% compared to that of untreated group. Administration of EA and ETH extracts was very effective in lowering the extend of peroxidation. The groups treated with a dose of 500 mg/Kg b. wt of EA and ETH extracts lowered liver lipid peroxidation by 68 and 59 % respectively (fig 7).

Anti-inflammatory activity
Anti-inflammatory activity against acute inflammation
Carrageenan induced acute paw edema was significantly inhibited by the administration of both ETH and EA extracts. The inhibition was 52.77, 27.77 and 16.78% for EA extract and 33.33, 16.66 and 9.48% for ETH at doses of 500, 250 and 100 mg/Kg b.wt. respectively (fig 8).

Anti-inflammatory activity against formalin induced chronic inflammation
EA extract was more effective in reducing chronic inflammation, providing relief of 46.44 % of edema at a dose of 500 mg/kg b.wt while that for ETH extract was 30.44% for the same dose (fig 9).

Leukocyte migration inhibition assay
The induction with carrageenan caused infiltration of leukocytes in to the peritoneal cavity and the number of leukocytes in the peritoneal cavity was found to be around three times more than that of normal animals. In EA extract and diclofenac administered groups of animals, the number of infiltrated cells were comparatively less than that in carrageenan control group. The EA extract at a dose of 500 mg/Kg b.wt reduced infiltration of leukocytes by 43%. The inhibitory effect of EA extract was higher than that of diclofenac at 25 mg/Kg b.wt (fig 10).

Inhibition of croton oil induced skin inflammation
The effect of EA extract on skin inflammation caused by croton oil was assessed by monitoring skin thickness, mass of the skin sections of uniform area, level of lipid peroxidation and also histopathological examination.

Skin thickness: There was obvious change in skin thickness among the groups. The skin thickness of croton oil control group was nearly double compared to that of croton oil untreated control group. The topical application of EA extract in three different doses, 20, 10, and 2 mg, prevented inflation of skin thickness to varying extends. Diclofenac caused 70% decrease in thickness compared to the croton oil control group (fig 11).

Skin mass: Croton oil also caused increase in skin mass and the application of the extract caused a marked reduction in thickness (fig 12).

Lipid peroxidation: There was 100% increase in lipid peroxidation in croton oil control group. The application of 20 mg of EA extract prevented lipid peroxidation by 59%. (fig 13).

COX inhibition assay
The activity of COX, a group of related enzymes that are involved in inflammation, was found down regulated by 55.85% by the treatment of EA extract at a concentration of 100 µg/mL (fig 14).

Histopathology
Sections of skin from croton oil control group showed high infiltration of leukocytes whereas skin sections from extract and diclofenac treated groups exhibited considerably less infiltrates (fig 15)

Preliminary phytochemical analysis
The EA and ETH extracts responded to phytochemical tests for the presence of steroids, alkaloids, coumarins, phenols, tannins, saponins and carbohydrates.

Discussion:-

Chronic oxidative stress is a mediator and cause of many diseases. Hence antioxidant compounds are effective against a large number of diseases in which oxidative stress is crucially involved. In this study, aimed at examining antioxidant and anti-inflammatory properties of *F. pinicola*, it was found that EA and ETH extracts were effective in converting the radicals into nonradical form, showing the presence of compounds which can participate in electron transfer reactions. As a direct evidence of electron transfer reactions, the extracts converted ferric ions to ferrous ions in the FRAP assay. A general observation in ferric ion reducing assays is that the ferric ion reducing activity increases as the polarity of the extract used in the assay increases. The higher ferric reducing activity of ETH extract than that of EA extract found in this study supports this observation.

Since the EA and ETH extracts were found to possess strong antioxidant activity, these extracts were examined for their ability to interfere with lipid peroxidation. The current experimental results indicate that EA and ETH extracts of *F. pinicola* are capable of reducing peroxidation of lipid. The marked decrease in liver lipid peroxidation caused by these extracts in mice administered with ethanol can be attributed to their antioxidant activity since the mechanism of ethanol induced lipid peroxidation is known to be ROS mediated (Rouach et al., 2003).

Oxidative stress and inflammation are closely related physiological conditions. Free radicals produced by leukocytes and mast cells are important mediators and executioners of inflammation. The interdependence of oxidative stress and inflammation has been unequivocally proved. The high antioxidant activities of the EA and ETH extracts of *F. pinicola* suggested their possible potential as sources of anti-inflammatory compounds. So we studied anti-inflammatory activities of these extracts against acute as well as chronic inflammation induced by carrageenan and formalin, respectively in animal model.

Carrageenan induces an increase in TNF - ∞ and nitric oxide (Kataoka, 2012) thereby effecting acute inflammation. TNF - ∞ is involved in the upregulation of E-selectin, expression of which by endothelial cells is an essential part for the rolling step of leukocyte (Heemskerk et al., 2014), one of the three initial steps for transendothelial migration. In the two experiments, carrageenan induced paw edema and leukocyte migration assay, EA extract was able to reduce the edema formation and leukocyte transmigration respectively. Since TNF - ∞ is involved in both the events, this pro-inflammatory molecule is a possible target of some of the components of the EA extract.

The extracts also caused reduction of formalin and croton oil induced inflammations. One of the mechanisms by which formalin induces inflammation is by the activation of COX2 (Damas and Liégeois, 1999) and croton oil induces expression of COX, that increases the level of prostaglandin E2 which results in inflammation (Sunhee Shin et al., 2010). Since the various inflammation inducing agents cause edema by activating different proinflammatory agents, the EA and ETH extracts must be acting at different levels of inflammatory pathways. The experimental result of reduction of COX activity by EA extract indicates the possible involvement of the extract in these inflammatory pathways.

Results of our experiment using croton oil show that its application on skin resulted in oxidative stress as evident from the increased lipid peroxidation in the mouse skin and the application of extract reduced the level of lipid peroxidation. Since the proinflamatory effect of oxidative stress is well established, the antioxidant activity of the extract may be a possible mechanism by which it ameliorates inflammation. Our findings support a previous study by Jing-Jy Cheng and coworkers that ethanolic extract of *F. pinicola* suppresses interfon-ɤ induced inflammation marker, IP-10 (Cheng et al., 2008).

The observed antioxidant and anti-inflammatory activities of EA and ETH extracts of *F. pinicola* deserve further investigation to find out the bioactive compounds, their possible interaction and biological targets. This study also supports the age old belief that *F. pinicola* possesses medicinal properties and its use as a herbal medicine. The current experimental findings suggest the potential therapeutic use of *F. pinicola* as an anti-inflammatory agent.

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Conflict of interest
The authors declare that there is no conflict of interest in this study.

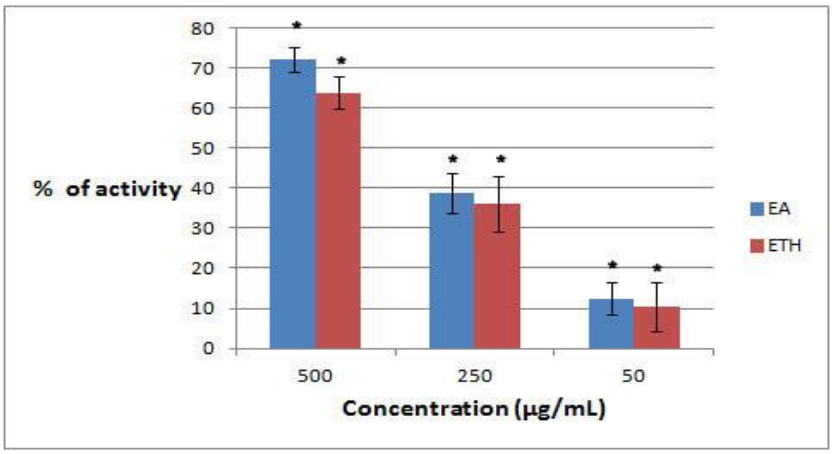

Fig 1:-DPPH· radical scavenging activity of *F. pinicola* extracts: The quantity of DPPH· radicals scavenged is shown in percentage. * $p < 0.05$

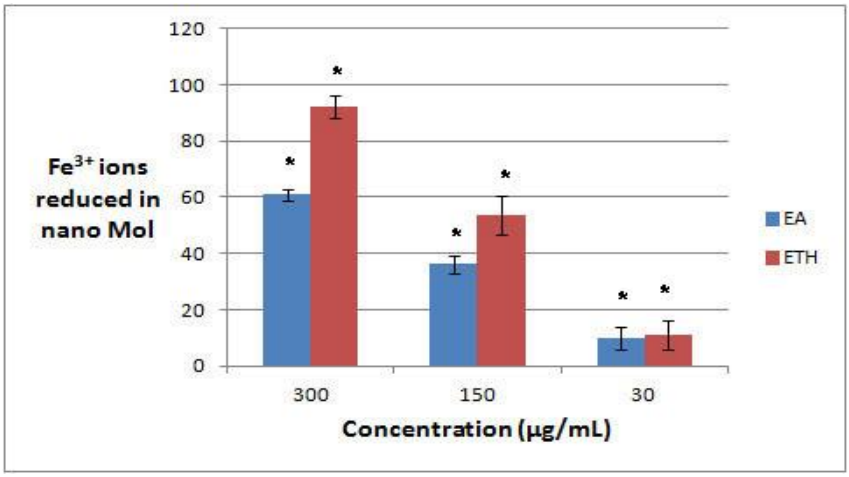

Fig 2:-Ferric reducing antioxidant power of *F. pinicola* extracts: The number of Ferric ions reduced at various concentration of the extracts is expressed in nanoMol. * $p < 0.05$

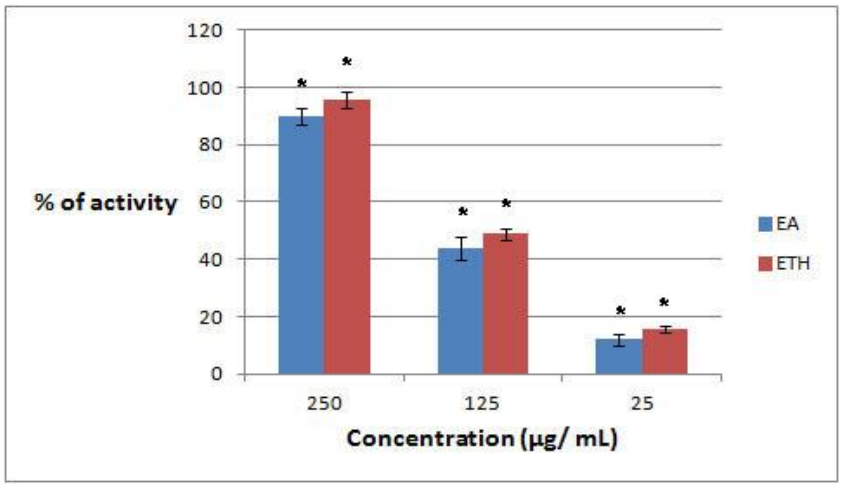

Fig 3:-ABTS$^{\cdot+}$ radical scavenging activity of *F. pinicola*: The amount of ABTS$^{\cdot+}$ radicalsscavenged by the extracts is expressed in percentage. * $p < 0.05$

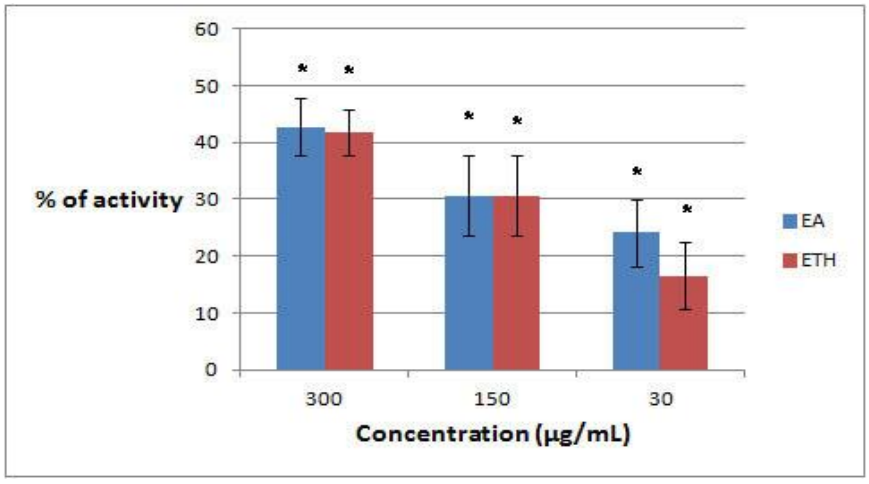

Fig 4:-Nitric oxide radical scavenging activity of *F. pinicola*: Nitric oxide radicals scavenged by the extracts is shown in percentage. * $p < 0.05$

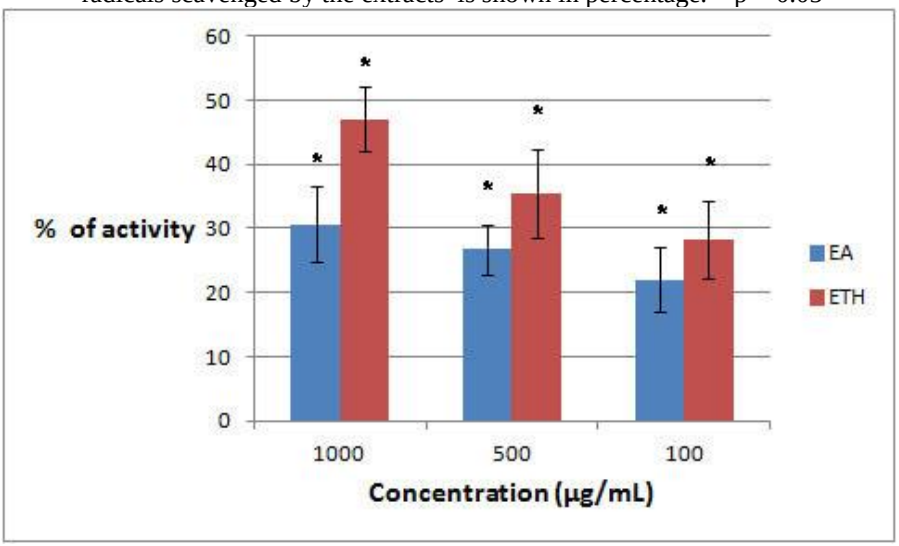

Fig 5:-Hydroxyl radical scavenging activity *F. pinicola*: Various concentrations of the extracts scavenged hydroxyl radicals to varying extends. * $p < 0.05$.

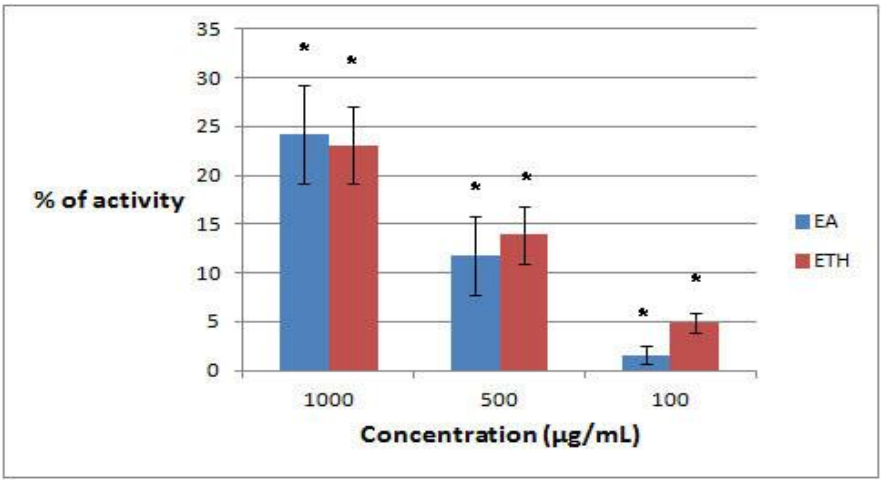

Fig 6:-Lipid peroxidation inhibition activity of EA and ETH extracts of *F. pinicola* is expressed in percentage. * $p < 0.05$

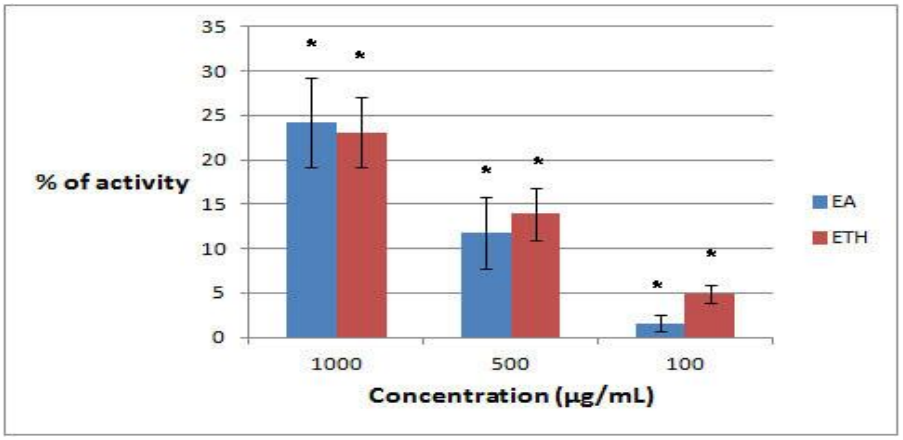

Fig 7:-Activity against ethanol induced liver lipid peroxidation by extacts of *F. pinicola* : The activity is expressed in percentage. * $p < 0.05$

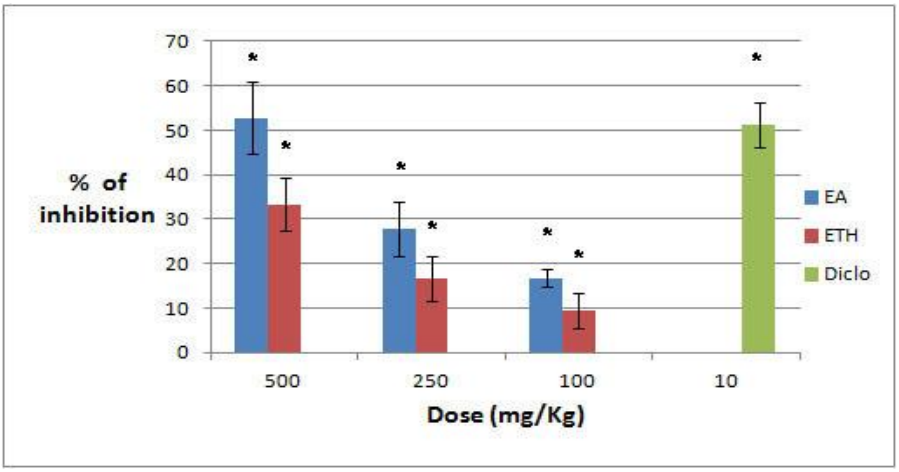

Fig 8:- Effect of *F. pinicola* extracts against acute inflammation. Dicolfenac was used as standard drug. * $p < 0.05$

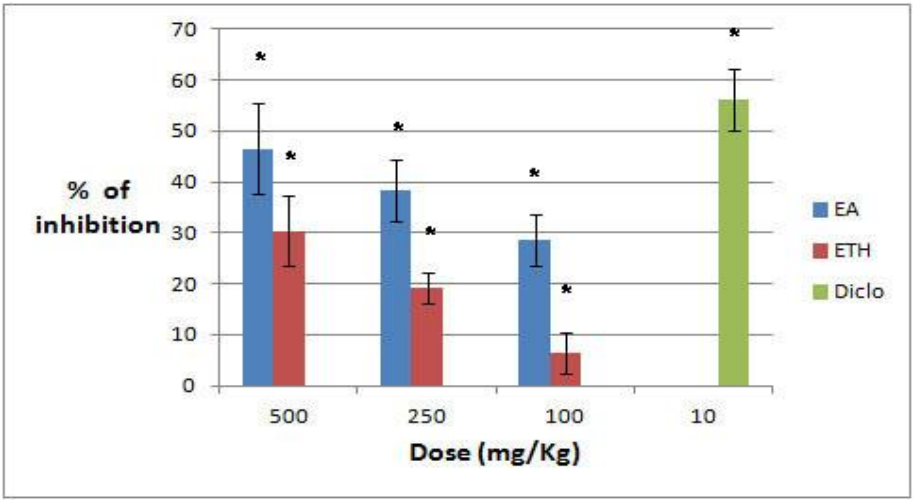

Fig 9:-Effect of *F. pinicola* extracts against chronic inflammation. * $p < 0.05$

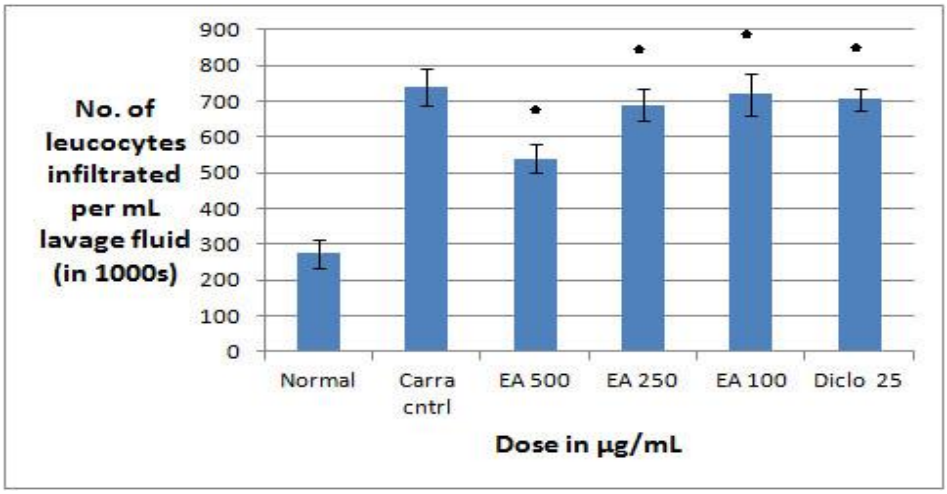

Fig 10:-Inhibition of leukocyte migration by *F. pinicola* extract. * $p < 0.05$

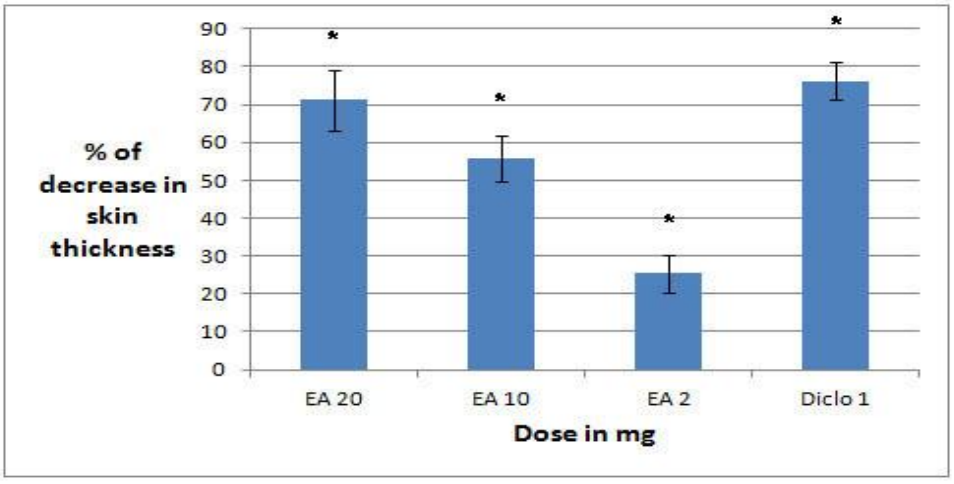

Fig 11:-Effect of *F. pinicola* extracts: Reduction in skin thickness. * $p < 0.05$

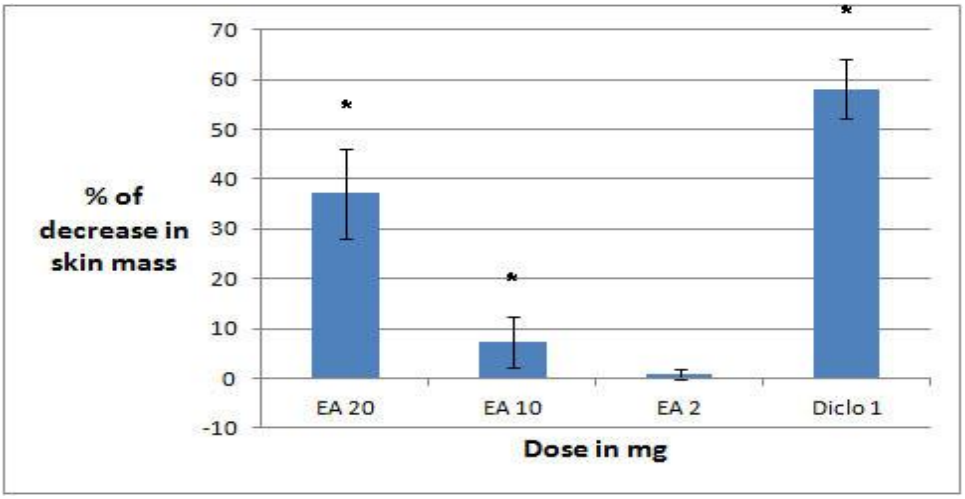

Fig 12:- *F. pinicola* extracts inhibited croton oil induced increase of skin mass: * $p < 0.05$

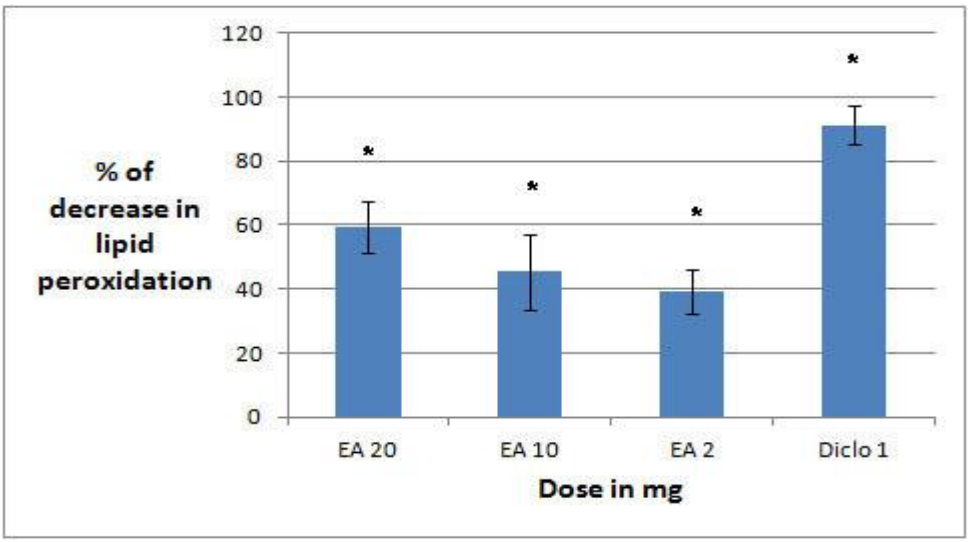

Fig 13:-Inhibition of croton oil induced skin lipid peroxidation by the extracts of *F. pinicola* in skin. * $p < 0.05$

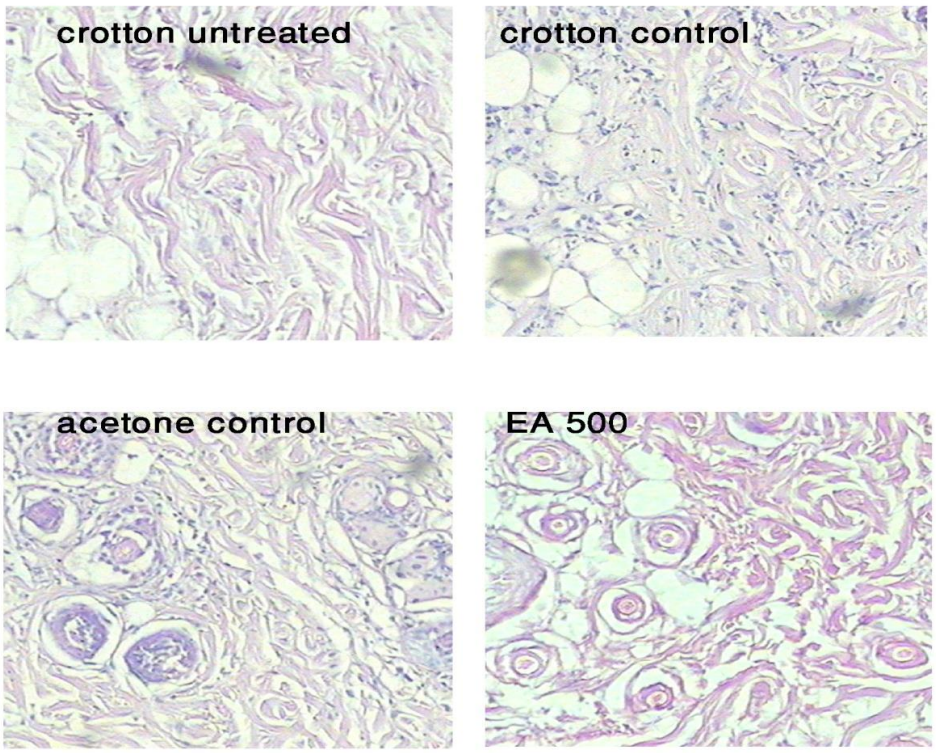

Fig 14:-Histopathology of skin : Croton oil induced skin inflammation and the effect of application of *F. pinicola* extracts. Sections of skin from various experimental groups show varying degrees of leukocyte infiltration

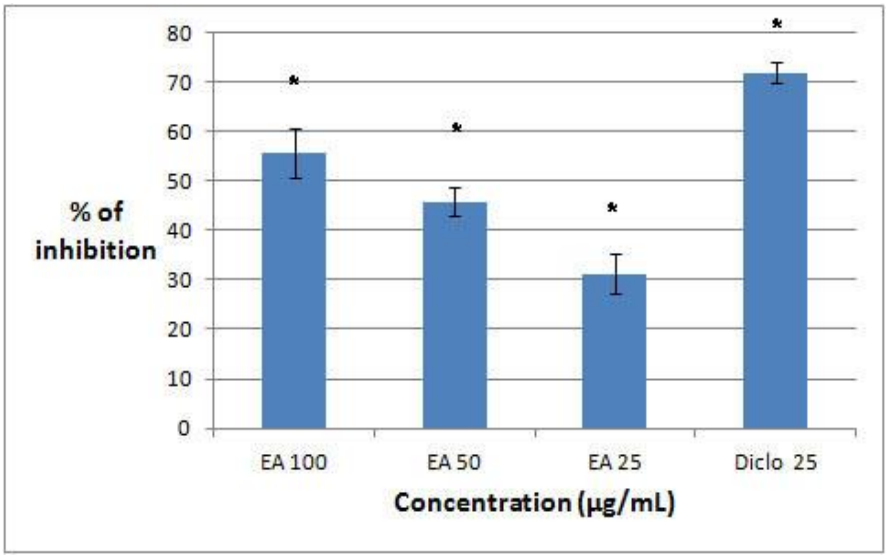

Fig 15:-COX inhibition assay : Inhibitory effect of various concentrations of EA extract on COX enzyme activity. * $p < 0.05$

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